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DECOLORIZATION OF REACTIVE BLACK 5 BY IMMOBILIZED *Trametes versicolor*

DECOLORACIÓN DEL NEGRO REACTIVO 5 POR *Trametes versicolor* INMOVILIZADO

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Abstract

Azo dyes released in wastewaters of textile industry are an important factor of environmental pollution. The use of immobilized white rot fungi cells is considered one of the most adequate alternatives to decolorize these effluents. In this work, a 2^k factorial design was applied to increase the specific decolorization rate (r_d) of Reactive Black 5 (RB5) by *Trametes versicolor*, using free (FC) and immobilized cells by adsorption in polyurethane foam cubes (IC). The results indicated that IC system was more efficient than FC for decolorization of 200 ppm RB5. Immobilization modified fungal metabolism with a decrease in CO_2 production, while laccases increased in presence of RB5. Decolorization process at bioreactor showed that IC (removal of 84% in 24 h) was more efficient than FC (85 % in 144 h). The decolorization of RB5 analyzed using UV-vis spectrophotometry suggested that degradation was the main mechanism for dye removal by IC, whereas in FC both absorption and degradation contributed to color removal.

Keywords: fungi, azo dye, polyurethane foam, response surface, intrinsic parameters, effectiveness factor.

Resumen

Los colorantes azo liberados en las aguas residuales de origen textil son una fuente importante de contaminación ambiental. Para decolorar estos efluentes el uso de hongos de la pudrición blanca inmovilizados ha sido una alternativa adecuada. En este trabajo se empleó un diseño factorial 2^k para incrementar la velocidad de decoloración especifica (r_d) del Negro Reactivo 5 (RB5) por *Trametes versicolor*, utilizando células libres (FC) y células inmovilizadas por adsorción en espuma de poliuretano (IC). Los resultados obtenidos indicaron que IC fueron más eficientes que FC para decolorar el RB5 (200 ppm). Se demostró también que la inmovilización modifica el metabolismo fúngico provocando un incremento en la producción de CO_2, en tanto que la actividad de lacasas incrementó en presencia del RB5. En el proceso de decoloración llevado a cabo en el biorreactor se demostró que IC (remoción de 84% en 24 h) fueron más eficientes que FC (remoción de 85 % en 144 h). El análisis de la decoloración del RB5 por medio de espectrofotometría UV-vis sugirió que el principal mecanismo de remoción del RB5 por IC es la degradación, mientras que en FC tanto la absorción como la degradación contribuyen a la remoción del color.

Palabras clave: hongos filamentosos, espuma de poliuretano, superficie de respuesta, parámetros intrínsecos, factor de efectividad.

1 Introduction

White rot fungi immobilized on polyurethane foam (PUF) can be an important alternative for removing dyes generated by the textile industry; these compounds are discharged in large quantities causing environmental pollution. The most common used dyes are characterized by the presence of at least one azo bond (-N=N-) bearing aromatic rings, these are named azo dyes (Saratale *et al*, 2011). When azo dyes form a covalent binding with cellulose fibers are considered as reactive dyes; and because of the simple dyeing procedure they are used commonly in textile industries dominating the world-wide market of dyestuffs with a share of about 70%. However, during the dyeing process a high percentage of such dyes can no longer react with cellulose fibers, because of the hydrolysis side reaction. Thus, approximately 20% of

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the initial dye concentration is present in the reactive dye bath, giving rise to a highly colored effluent released into the water environment. This provokes serious pollution problems, due to many reactive dyes are toxic to human and biotic communities, affect the photosynthesis process and are highly recalcitrant (El-Zawahrya *et al*. 2016; Adnan *et al*. 2015). The environmental problems created by the textile industry have received increased attention for several decades, because this industry is one of the greatest producer of contaminated effluents: 50 L of water are consumed for processing about 1 kg of finished textile material (Chen *et al*, 2017). So, different methods have been used in order to remediate dye-containing effluents: electrochemical removal, adsorption and biological treatment (Vasconcelos *et al*. 2016). Even when electrochemical methods have generated good results, they are expensive; and referring to adsorption process, activated carbon is the most popular and widely used dye adsorbent, but it suffers from several drawbacks such as its high price of both manufacturing and regeneration, and it is ineffective against disperse and vat dyes (Osma *et al*. 2007).

With respect to the biological treatment of textile wastewater, the use of microorganisms for the decolorization of azo dyes is an attractive alternative to develop environmentally friendly bioremediation processes. These procedures can produce less sludge than physical and chemical systems; or sludge produced as the result of the cell growth has a low chemical content and amounts (Arslan *et al*, 2016). Besides, biological treatments are cost-effective, as they require less energy and chemicals compared to physicochemical treatment methods (Solís *et al*. 2012; Saratale *et al*, 2011). Dye decolorization processes catalyzed by fungal cultures have two simultaneous steps: the adsorption of dyes to fungal surface and the degradation of dyes by the enzymes produced by them. These enzymes are lignin peroxidases, manganese peroxidases, laccases, and some accessory enzymes, that are mostly oxidases generating H_2O_2 and dehydrogenases of lignin. The accessory enzymes provokes biological Fenton reactions, which potentiate the degradation of some compounds structurally similar to lignin (Peralta *et al*, 2017). So, these organisms are able to transform the structure of different dyes under aerobic conditions (Arslan *et al*, 2016; Ali, 2010). Among the enzymes involved in decolorization process, laccases are considered the most promising to be applied in wastewater treatment; however, the use of fungal cultures are preferred over purified enzyme due to the advantages that they offer (Majeau *et al*. 2010).

Dye decolorization with fungi can be achieved by free or immobilized cells cultures. The last one has several advantages over dispersed cells, such as simple reuse of the biomass, enhancement of its mechanical strength and easier liquid-solid separation. In addition, immobilized fungal cultures tend to have a faster growth, higher efficiency of metabolites and enzyme production (Sharari *et al*. 2013) and they are more resistant to stressful environmental conditions (Martínez-Trujillo and García-Rivero 2012). At this respect, several works have employed immobilized cells for the decolorization process of different dyes. These works have shown that environmental parameters affect microbial physiology during the process. pH is crucial because certain pH intervals can affect the solubility of the dye, which is retained mainly under acidic conditions; although in some cases a retention higher than 50% has been reported at basic pH values (Solís *et al*, 2012). Furthermore, the pH of the growth medium can affect the ligninolytic enzyme activity and the concomitant decolorization process (Adnan *et al*. 2015). On the other hand, it has become clear that ligninolytic enzymes are mainly expressed during the secondary phase of growth, when the limitation of carbon or nitrogen occurs in the medium (Hailei *et al*. 2009). Besides, the use of a carbon source that can be efficiently and rapidly utilized by the fungus, as glucose, help to increase growth and therefore produce high levels of laccase activity, which seems to be essential for decolorization (Elisashvili, and Kachlishvili, 2009). However, there is a lack of studies in which the joint effect of operational parameters as pH, glucose or dye concentration have been analyzed for the corresponding decolorization process by white rot fungus, as in the specific case of *Trametes* cultures.

On the other side, dye decolorization is a biochemically complex process for which is believed that dye is mineralized by the metabolic action of the microorganism. The release of CO_2 along the culture can be an indicative of metabolic activity of the fungus, mostly due to consume of carbon sources used during fungal growth (Soares *et al*. 2006, Zhao *et al*. 2015). So, the analysis of CO_2 production could help to understand the mineralization of different recalcitrant compounds. In fact, it has been suggested that a portion of CO_2 produced by *Trametes versicolor* comes from mineralization of recalcitrant compounds (Marco-Urrea *et al*. 2008). Even tough, the studies in which CO_2 productions are analyzed simultaneously

with dye decolorization along a microbial culture are scarce. In fact, both processes are studied from independent experiments (Zhao *et al*. 2015).

So, the aim of this work was to evaluate the effect of pH, glucose and dye concentration on the specific decolorization rate of RB5 by *Trametes versicolor* free or immobilized cells; as much as analyze CO_2 and laccases production along the culture. Afterward characterize the process at bioreactor level from a joint analysis of growth, substrate consumption and decolorization percentage in order to estimate the parameters of the immobilization efficiency.

2 Material and methods

2.1 Dye

The commercial product Reactive Black 5 (RB5) was provided by Química Mexibras, México. 100 mg L^{-1} were hydrolyzed in NaOH 0.1 M at 120 °C for 15 min to simulate dye house processes, followed by neutralization with sterilized hydrochloric acid 33 % (w/v) (Borchert and Libra 2001). Hydrolyzing process served to sterilize the dye solution.

2.2 Microorganism

The strain *Trametes versicolor* CDBB-H1051 belongs to the fungal collection of the Enzymatic Catalysis Laboratory of Tecnológico de Estudios Superiores de Ecatepec, México. This strain was grown on Sabouraud agar plates (DIBCO^{TM}) at 30 ± 3 °C for 10 days; and plugs of agar (diameter 1 cm) were taken from the periphery of the culture and used as inoculum.

2.3 Medium

The growth medium used for biomass immobilization and pellets production contained, in g L^{-1}: malt extract powder, 25; KH_2PO_4, 2; y K_2HPO_4, 0.4 (Borchert and Libra 2001). In the decolorization assays the medium used consisted of (g L^{-1}): D-glucose, 5.0; KH_2PO_4, 2.0; NH_4Cl, 0.05; $MgSO_4{\cdot}7H_2O$, 0.5; $CaCl_2{\cdot}2H_2O$, 0.1; trace element solution, 10 mL. Trace element solution consisting of (g L^{-1}): $MnSO_4$, 0.5; $FeSO_4{\cdot}7H_2O$, 0.1; $ZnSO_4{\cdot}7H_2O$, 0.1 was prepared separately (Radha *et al*. 2005); from now, this medium will be denominated as Radha medium. Both culture media were autoclaved at 15 psi for 15 min.

2.4 Pre-culturing of the fungus in Erlenmeyer flasks

2.4.1 Free cell culture

Five plugs of fungal mycelium were transferred into 500 mL Erlenmeyer flasks containing 150 mL of growth medium (Borchert and Libra 2001). The flasks were incubated for 10 days at 30 ± 3 °C and 200 rpm. Cell biomass were washed with saline solution 0.9% (w/v) and refrigerated at 4°C in saline solution until their use.

2.4.2 Cell fungi immobilization

Five plugs of fungal mycelium were transferred to 500 mL Erlenmeyer flasks containing 300 mL growth medium and 50 cubes of PUF of 1cm^3 (Saetang and Babel, 2010). Flasks were incubated for 10 days at 30 ± 3 °C and 200 rpm. The PUF cubes colonized with fungal biomass were washed with saline solution (NaCl 0.9%, w/v), and refrigerated at 4°C in saline solution until their use in decolorization assays.

2.5 Factorial design

The effects of pH, glucose and RB5 concentrations were investigated for biomass (BM, g L^{-1}), specific decolorization rate (r_d, mg$_{dye}$ g$^{-1}_{biomass}$ h^{-1}) of RB5 by *T. versicolor* and specific glucose consumption (q_s, g$_{\text{glucose consumed}}$ g$^{-1}_{biomass}$ h^{-1}) using a 2^3 factorial design with three replicates at the central point, for both free and immobilized cell cultures of *T. versicolor*. The independent variables with their coded and real values used for the design are shown on Table 1.

The responses obtained wit this design were related to the selected variables by a linear model, as described on equation 1:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{123} x_1 x_2 x_3 \quad (1)$$

Where y is the predicted response according to the model. The regression coefficients are: β_0 the intercept term; β_1, β_2 and β_3 the coefficients for linear effects; β_{12}, β_{13}, β_{23}, β_{123} are the interactions of the coefficients and x_1, x_2, x_3 are the coded values of the factors pH, glucose concentration and RB5 concentration, respectively.

All the assays were performed in 500 mL Erlenmeyer flasks containing 300 mL Radha medium with hydrolyzed RB5 solution at concentrations

according to the experimental design. For free cells experiments, the flasks were inoculated with cell suspension equivalent to 0.6 $g_{biomass}$ L^{-1}, while the immobilized cells assays started by adding 50 PUF cubes colonized with *T. versicolor* mycelium, equivalent to an initial biomass of 0.6 $g_{biomass}$ L^{-1}. Radha medium composition was modified for glucose concentration and pH, according to the experimental design. Assays were done in triplicate, incubating on a shaker for 10 d and 200 rpm at 30 ± 3 °C. Samples were taken at the beginning and at the end of each assay for quantifying microbial growth, dye and glucose concentrations. The results of the experimental design were analyzed and interpreted using the software SAS 9.1.3®.

2.6 Evaluation of CO_2 and enzymatic activity during decolorization assays

Taking into account the results obtained in the factorial design we defined culture conditions for decolorization of RB5 process. In a first assay the CO_2 production and laccase activity generated along the culture by using 250 mL Erlenmeyer flasks, assembled with teflon mininert valvs. The flasks contained 150 mL of Radha medium with the conditions of pH, glucose and RB5 concentration defined by the factorial design. These were inoculated with PUF cubes colonized with *T. versicolor* mycelium (immobilized cell assays, IC) or with cell suspension (free cell assays, FC), in both cases the initial biomass was equivalent to 0.25 g L^{-1}. Triplicated flasks were incubated on a shaker for 10 d and 200 rpm at room temperature. A non-dye control was carried out in the same conditions. Samples were taken to quantify biomass growth, glucose, RB5 concentrations, laccase activity and the CO_2 production was determined by gas chromatography (GC) throughout the study.

2.7 BR5 decolorization process in batch reactor

Decolorization RB5 assays were carried out in 1 L Applikon® reactor using free and immobilized cells. In both cases reactor contained 700 mL Radha medium in which pH, glucose and RB5 concentration were defined by the factorial design, and were inoculated with cell suspension or colonized PUF cubes equivalent to 0.33 ± 0.02 gbiomass L^{-1}. Cultures were maintained for 10 days and 200 rpm at room temperature. Control was carried out in the same conditions but without dye. Samples were taken to quantify biomass growth, pH, and glucose and RB5 concentrations.

2.8 Analytical methods

For both, free and immobilized cell cultures, residual glucose was determined as described by Miller *et al*. (1960) and biomass concentration was estimated by dry cell mass (Banerjee *et al*. 1993). For doing this, all samples of FC were filtered through a filter paper previously maintained at constant weight, and subsequently the weight of the filtrate after holding it at 60 ° C for 24 h was recorded. To express the concentration of biomass contained in each experimental unit, the total volume submitted to filtration divided the weight difference obtained from the papers after and before filtering. For those cultures of the immobilized cells, all samples were also filtered through the filter paper previously maintained at constant weight; and the sponges with immobilized cells were transferred to a petri dish, previously maintained at constant weight (A). The material retained in the filter paper corresponds to the cells released from the sponge during the culture, and their concentration was estimated as indicated for the FC. The sponges with the immobilized biomass were dried for 24 h at 60 ° C in the petri dish, recording the final weight (B). To express the concentration of biomass contained in the sponges of each experimental unit, we employed the equation 2:

$$\text{Immobilized biomass concentration}(gL^{-1}) = \frac{B - A - C}{V} \quad (2)$$

Where:

B corresponds to petri dish full of dried sponges with cells

A corresponds to empty petri dish

C refers to weight of the empty and dried sponges

V is volume of sample

Dye decolorization in the cultures was estimated spectrophotometrically, using the corresponding standard curve concentrations at 597 nm in a GENESYS^{TM} 10S UV-Vis Spectrophotometer. Percentage of decolorization (%D) was calculated by means of the equation 3:

$$\%D = \frac{Cd_0 - Cd}{Cd_0} \times 100 \quad (3)$$

Where Cd_0 is dye concentration (mg L^{-1}) at the beginning and Cd at a determined time (t).

The specific decolorization rate (r_d) was calculated by means of the equation:

$$r_d = \frac{\text{mg of RB5 decolorizated}}{dwb * t} \quad (4)$$

Where *dwb* corresponds to dry weight biomass (g L^{-1}) at the same time. Triplicates were used for each sample.

2.9 Quantification of CO_2 produced during decolorization assays

The CO_2 production was determined in order to know the metabolically active of *T. versicolor* throughout the study. For doing this, three replicates of each treatment were sampled, and 0.1 mL of headspace was injected into a gas chromatographer Gow-Mac 580 equipped with a packed column ZR-1 and a thermal conductivity detector. The GC settings were the following: column temperature, 140 °C; injector temperature, 170 °C; and detector temperature, 190 °C; carrier gas (helium) at a flow rate of 30 ml min^{-1}.

2.10 Laccase activity

Laccase activity was determined in order to know if it was related to RB5 decolorization. In control experiments (without dye), the activity was evaluated spectrophotometrically using guaiacol (2-metoxyphenol) as substrate (Bertrand, *et al*., 2015). The laccase reaction mixture contained 10 mM guaiacol in 100 mM acetate buffer (pH 5) and 50 μL of sample, in a total volume of 1 mL. Oxidation of guaiacol at room temperature was monitored through the increase in absorbance at 470 nm (ε_{470nm}= 26,600 M^{-1} cm^{-1}) for 10 min; the initial absorbance of each reaction mixture was taken as blank. One unit of enzymatic activity was defined as the amount of enzyme required to oxidize 1 μmol of guaiacol per minute, and laccase activity was expressed in U L^{-1}.

The coloration of the samples obtained from assays in which the RB5 was added did not allow the spectrophotometric determination of the laccase activity. So, this enzymatic activity was estimated using a modification of the methodology proposed by Plácido *et al*. (2016), for the evaluation of ligninolytic activity in solid media. In Petri dishes with bacteriological agar and 50 mM ABTS (2,2'-Azino-bis-3-Ethylbenzothiazoline-6-Sulfonic Acid), 12 wells with a diameter of 1 cm each were drilled. Nine of these wells were filled with 20 μL of the samples obtained from decolorization BR5 assays, and the other three with 20 μL of an enzymatic extract of known laccase activity (the standards). The plates were incubated at 30 ± 3°C during 15 minutes. The green coloration of each sample was compared against the standards to have an approximate estimation of the enzymatic activity, in a qualitative way.

3 Results and discussion

3.1 Factorial design for identifying the effect of variable process on specific rate of decolorization

The first step in this study was the identification of the variables that affected biomass concentration, specific substrate consumption and r_d of RB5 by *T. versicolor* cells (free and immobilized in polyurethane foam). In order to choose the best culture conditions for developing dye decolorization process, three factors (pH, glucose level and dye concentration) were tested. The levels of the factors and the results from the experiments are shown in Table 1.

In the immobilized cell system, it was observed a loss of BM from the sponges; although these values represented less than 5% of the total, so their values were neglected. In general, it was observed a greater growth in the IC than in the FC (0.31 gL^{-1} vs 0.22 gL^{-1}, respectively). In terms of q_S, their values depended on the experimental conditions of the design, and practically the average of both systems was the same, 0.5 $gg^{-1}h^{-1}$. As the purpose of the process was to study the degradation of RB5, the results of the factorial design were used to find the best conditions to increase the r_d value.

Specific decolorization rate of free cells cultures were slightly higher than the obtained by the immobilized cells in almost all the experimental conditions; nevertheless in free cells cultures dye decolorization was in average of 90.16 ± 1.4 %, compared to immobilized for which r_d value was around 97.97 ± 0.08 % (Table 1). Even tough, these results were higher than those reported for *T. gibbosa*, which reached barely 70% of decolorization of 50 mg L^{-1} of RB5 on a liquid culture containing 20 g L^{-1} of glucose at pH around 5, and 80 % in agar plates with the same medium after 15 days (Adnan *et al*. 2014).

Using the experimental data for first 8 runs, the linear model was fitted to decolorization results, except for biomass of free mycelium, as indicated by R^2 values shown on Table 2.

Table 1. Results obtained for biomass (BM), specific substrate consumption (q_S) and specific decolorization rate (r_d) during the 2^3 factorial design with central points, developed for Free Cells (FC) and Immobilized Cells (IC) cultures of *T. versicolor*.

Exp	Variables Coded (real values)			BM ($g L^{-1}$)		q_s ($g g^{-1} h^{-1}$)		r_d ($mg g^{-1}h^{-1}$)	
	X1 pH	X2 Glu (gL^{-1})	X3 RB5 ppm	FC	IC	FC	IC	FC	IC
1	-1 (3.5)	-1 (7)	-1 (50)	0.252 ± 0.007 (0.252)	0.610 ± 0.06 (0.624)	0.052 ± 0.001 (0.053)	0.028 ± 0.001 (0.028)	0.815 ± 0.001 (0.816)	0.287 ± 0.004 (0.295)
2	1 (5.5)	-1 (7)	-1 (50)	0.290 ± 0.037 (0.290)	0.285 ± 0.08 (0.249)	0.063 ± 0.001 (0.062)	0.069 ± 0.003 (0.073)	0.686 ± 0.002 (0.687)	0.541 ± 0.007 (0.535)
3	-1 (3.5)	1 (13)	-1 (50)	0.211 ± 0.009 (0.211)	0.490 ± 0.03 (0.496)	0.071 ± 0.010 (0.063)	0.027 ± 0.003 (0.024)	0.917 ± 0.001 (0.918)	0.358 ± 0.055 (0.334)
4	1 (5.5)	1 (13)	-1 (50)	0.255 ± 0.004 (0.255)	0.789 ± 0.04 (0.799)	0.066 ± 0.010 (0.076)	0.016 ± 0.001 (0.015)	0.717 ± 0.001 (0.717)	0.210 ± 0.016 (0.222)
5	-1 (3.5)	-1 (7)	1 (200)	0.335 ± 0.002 (0.335)	0.561 ± 0.31 (0.662)	0.028 ± 0.008 (0.030)	0.026 ± 0.001 (0.027)	1.821 ± 0.001 (1.820)	1.250 ± 0.076 (1.237)
6	1 (5.5)	-1 (7)	1 (200)	0.294 ± 0.105 (0.294)	0.203 ± 0.06 (0.220)	0.046 ± 0.006 (0.044)	0.088 ± 0.001 (0.087)	2.273 ± 0.001 (2.273)	3.309 ± 0.071 (3.249)
7	-1 (3.5)	1 (13)	1 (200)	0.296 ± 0.017 (0.296)	0.647 ± 0.04 (0.680)	0.036 ± 0.007 (0.029)	0.013 ± 0.002 (0.011)	2.244 ± 0.001 (2.244)	1.070 ± 0.074 (1.042)
8	1 (5.5)	1 (13)	1 (200)	0.310 ± 0.016 (0.309)	0.518 ± 0.17(0.394)	0.017 ± 0.005 (0.015)	0.021 ± 0.001 (0.021)	2.322 ± 0.001 (2.323)	1.297 ± 0.011 (1.305)
9-11	(4.5)	(10)	(125)	0.22 ± 0.02	0.312 ± 0.01	0.050 ± 0.001	0.050 ± 0.001	2.0 ± 0.002	1.624 ± 0.027

Predicted values are indicated in parenthesis.

Table 2. Coefficients of the model obtained after ANOVA test for biomass, Specific substrate consumption and specific decolorization rate in the 2^3 factorial designs for free (FC) and immobilized cells (IC).

Factor		β_0	β_1	β_2	β_3	β_{12}	β_{13}	β_{23}	β_{123}	R^2
Biomass	FC	0.281	0.007	-0.012	0.028	0.008	-0.014	0.007	0.006	0.1670
($g L^{-1}$)	IM	0.527	-0.068*	0.095*	0.001	0.119*	-0.040	0.001*	-0.041*	0.9622
q_S	FC	0.048	0.002	-0.003	-0.014*	-0.005	-0.002	-0.008*	-0.004	0.8738
($g g^{-1}h^{-1}$)	IM	0.036	0.013*	-0.017*	0.001	-0.013*	0.005*	-0.003*	-0.0004	0.9944
r_d	FC	1.475	0.025*	0.076*	0.691*	-0.055*	0.108*	0.042*	-0.038*	1.0000
($mg g^{-1} h^{-1}$)	IM	1.057	0.284*	-0.310*	0.695*	-0.290*	0.278*	-0.260*	-0.171*	0.9974

* Statistically significant ($p < 0.05$)

β values are the coefficients of the models that indicate the effect of the corresponding variable, x_1 (pH), x_2 (Glucose concentration) or x_3 (RB5 initial concentration); on BM, q_S or r_d. The interactions between variables are also shown as β_{12}, β_{13}, β_{23} and β_{123}.

Statistical analysis of the factorial design showed that both, the main effects and the interactions of two factors were significant on r_d values ($p < 0.05$), although dye concentration was the most significant for this response, as shown by the highest β values obtained for this factor (Table 2). Besides, the effects of pH and dye concentration were positive for both, free and immobilized cells; this means that r_d values will increase at higher pH values and dye concentrations.

Glucose concentration only was significant for biomass and r_d values of the immobilized cells. For r_d this effect was negative, contrary to that observed for free cells. It suggests that immobilized cells will have a higher r_d value as they have a lower glucose concentration on the culture. This can be explained considering that low glucose concentrations can be resembled low osmolarity conditions in the microenvironment generated into the support. This condition could facilitate the diffusion of laccases across the support to the bulk, favoring their performance on RB5 (Sharari *et al*, 2013).

On the other side, it has been reported that the production of ligninolytic enzyme can be affected by the limited availability of nutrient (Elisashvili and Kachlishvili, 2009; Villegas *et al*, 2016). However, the relationship among glucose concentration and dye decolorization have remained as a controversial issue, but more favorable conditions have found using glucose at low concentrations, as this is rapidly exhausted promoting dye degradation by microorganisms (Jasińska *et al*. 2015). In fact, this can explain the significance of the interaction among glucose and RB5 concentrations observed for immobilized cells.

Conversely, dye concentration had a significant negative effect on specific glucose consumption for free cells (Table 2). This fact suggests that dye

concentration can affect the behavior of free cells, lowering the specific substrate consumption, but immobilization can be protecting cells from the effect of dye. In fact, it has been reported that immobilized cultures tend to be more resilient to environmental perturbations such as toxic chemical concentrations because the diffusion of toxic substances into the immobilization matrix is reduced, generating a lower dye concentration in the microenvironment of the immobilized cells (Garzón-Jiménez and Barragán-Huerta, 2008; Ramsay *et al*,, 2005).

On the other hand, it is known that dye decolorization by white rot fungi is pH dependent in the acidic range between 4 and 6 (Kaur *et al*. 2015). The pH affects the color of the solution; also, the changes of solution pH alter the adsorption of fungal surface electrical charge (cell wall) that consists of several functional groups (thiol and phosphate) towards the ionic dye (Adnan el al, 2015). Additionally, the pH value may exert a strong influence on the activity of extracellular redox enzymes that are often involved in dye decolorization processes (Jasińska *et al*. 2015). This explains the positive significant effect of this factor for both, free and immobilized cells (Table 2).

The results obtained in the experimental design and the corresponding response surfaces showed that RB5 had the highest significance on r_d values; and that the best responses can be obtained by using 7 g L^{-1} of glucose and an initial pH or around 5. Based on the above, in the following experimental tests RB5 value was modified according to the analysis to be performed, using in all cases the glucose concentration in 7 g L^{-1} and the pH value in 5.0.

3.2 Evaluation of CO_2 and enzymatic activity

The statistical analysis of the first experimental phase showed that dye concentration had a significant effect on glucose consumption (q_s) by *T. versicolor* (Table 2), which suggests that there could be a direct effect of dye on fungal metabolism. On the other hand, decolorization ability of *T. versicolor* has been correlated to ligninolytic enzyme activity, and among the enzymes involved in that process the laccases has showed the greatest contribution (Singh *et al*., 2015).

So in this experimental phase we decided to analyze the effect of the presence of dye on fungal metabolism, estimated indirectly by quantifying CO_2 production, as well as the enzymatic activities patterns. For doing this, one assay was developed in duplicate at flasks level, using 50 ppm of RB5. The CO_2 and laccases activities produced along decolorization process were compared with a non-dye control, for both immobilized and free cells systems.

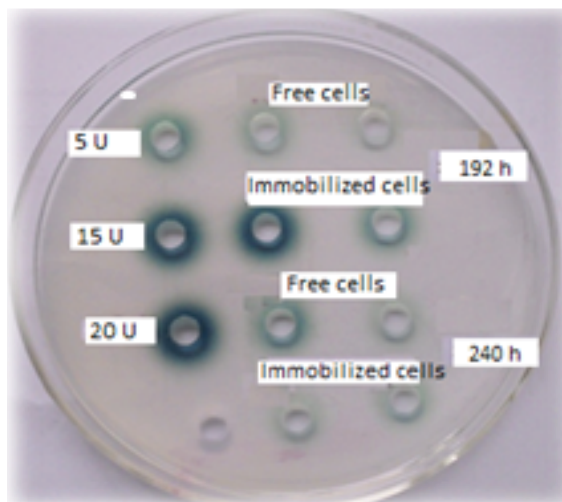

Fig. 1. Laccase activity (U L^{-1}) estimation of samples obtained from RB5 decolorization process by *T. versicolor* in Erlenmeyer flask.

In reference to enzymatic activity, laccases were obtained since the first 48 h, and increased as the culture time advanced, reaching its maximum values after 148 h. The higher laccase activity estimated in IC was about 20 U L^{-1}, at 192 h; while for free cells the highest activity (around 10 U L^{-1}) was observed at 240 h (Figure 1). In contrast, in the control experiment (without RB5) laccase activity was 2.3 ± 0.15 (192 h) and 3.3 ± 0.07 (144 h) U L^{-1} for FC and IC, respectively. The increase of laccases production in the presence of RB5 has been reported previously (Enayatzamir *et al*, 2009), and could be suggesting that this dye is an inducer of the enzyme. However, the studies have focused on the use of laccases on different biotechnological processes, as decolorization of RB5-containing effluents, while the reports about the role of dye molecule in the induction of laccases are scarce and not conclusive. At this respect, it is known that basidiomycetes produced small amounts of extracellular laccase constitutively that can be augmented by an inducer such as aromatic or phenolic compounds structurally related to lignin or lignin derivatives (Majeau *et al*, 2010).

The UV-Vis spectra recorded along the culture showed a significant change in the absorbance of the visible spectra between 250 and 700 nm, which was coincident with the laccases increase.

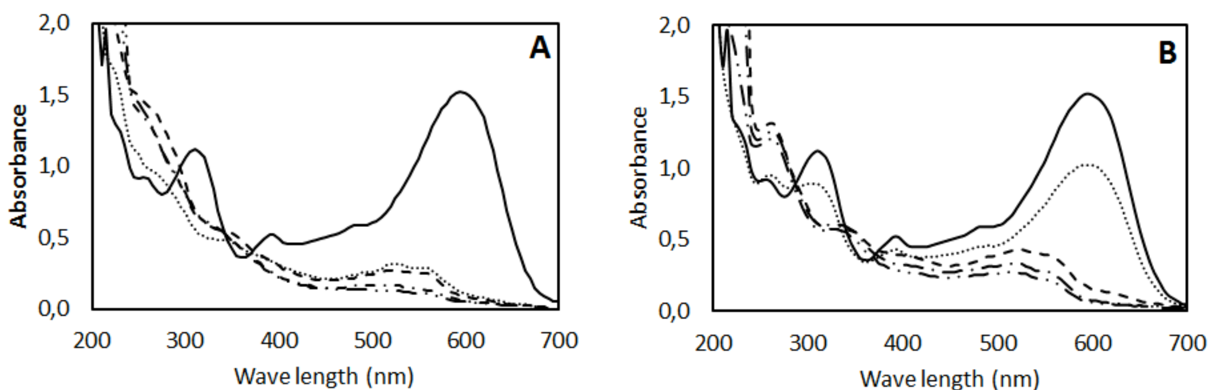

Fig. 2. Changes in the absorption spectra of RB5 by T. versicolor in process decolorization in (A) immobilized cell and (B) free cell culture in Erlenmeyer flask at different times: 0h (—), 48 h (●●●), 96 h (- - -), 144 h (-●-) and 240 h (-●●-).

Table 3. Carbon balance and metabolic rates of *T. versicolor* after 10 days of incubation with and without RB5 (50 ppm).

	BALANCES			Yields (g·g^{-1})			Rates		
	mmol CO_2 produced	BM formed, g L^{-1}	Glucose consumed, g L^{-1}	$Y^*_{X/S}$	$Y_{CO2/S}$	$Y_{CO2/X}$	Specific (r_{CO2}) mmol $g^{-1}h^{-1}$	Specific (q_S) g $g^{-1}h^{-1}$	Ref
FC									
Control	0.467± 0.005 ^{A}	0.447±0.035 ^{A}	3.74±0.41 ^{A}	0.12±0.003 ^{B}	0.039±4E-4 ^{A}	0.32±.003 ^{A}	0.0030±3.2E-5 ^{B}	0.035±0.001 ^{A}	This study
RB5	0.395±0.003 ^{B}	0.374±0.032 ^{B}	2.43±0.11 ^{B}	0.15±0.02 ^{A}	0.050±3e-4 ^{A}	0.33±.0025 ^{A}	0.0031±2.3e-5 ^{A}	0.027±.0035 ^{A}	This study
LSD	0.0139	0.1026	0.3644	0.0054	0.0181	0.0079	0.0001	0.0088	
IC									
Control	0.673±0.004^A	1.22±0.061 ^{A}	4.58±0.08 ^{A}	0.27±0.008 ^{A}	0.045±.0026 ^{A}	0.17±0.002 ^{A}	0.0016±9e-6 ^{B}	0.0156±5e-4 ^{A}	This study
RB5	0.581±0.008 ^{B}	1.185±0.003 ^{B}	4.35±0.06 ^{A}	0.27±0.004 ^{A}	0.041±5e-4 ^{A}	0.15±0.002 ^{A}	0.00141±9e-5 ^{B}	0.0153±2e-4 ^{A}	This study
LSD	0.0385	0.0396	0.3350	0.0257	0.0071	0.0132	0.0002	0.0011	
Agar plates									Calculated from
Control							0.188	0.121	Roy and
With recalcitrant							0.135	0.148	Archibald (1993)
Static FC									Calculated from
Control							0.0046 §	0.028§§	Soares *et al*
With reaclcitrant							0.0021	0.022	(2006)

*Yield is estimated based on the glucose consumed
§ average production rate (a_{CO2}, mmol h^{-1})
§§ average consumption rate (a_s, g L^{-1} h^{-1})
Those treatments with different capital letters have significant differences compared with the corresponding Least Significant Difference (LSD) value.

At this respect, there were two regions to highlight in the change of the specters: the first one was around 300 and 400 nm, for which the most important change was observed for FC system (Fig. 2B), that suggested the formation of more complex chemical structures (Castillo-Carvajal *et al*. 2012). The second region was around 550 and 650 nm, in which an important diminution in the intensity of the absorbance of the visible peak (597 nm) was registered since the first 48 h for IC (Fig 2A), although this decrease was less significant for FC (Fig 2B).

In addition to laccase activity, CO_2 production was measured along the 10 days of culture. At this respect, it was observed that CO_2 produced by IC was slightly higher than that obtained for FC. Besides, the presence of dye in the culture caused a significant decrease in CO_2 production, for both free and immobilized mycelium (Fig. 3). For being able to compare the behavior of mycelium under each experimental condition, carbon balances and metabolic rates were obtained, and the corresponding values are shown on Table 3.

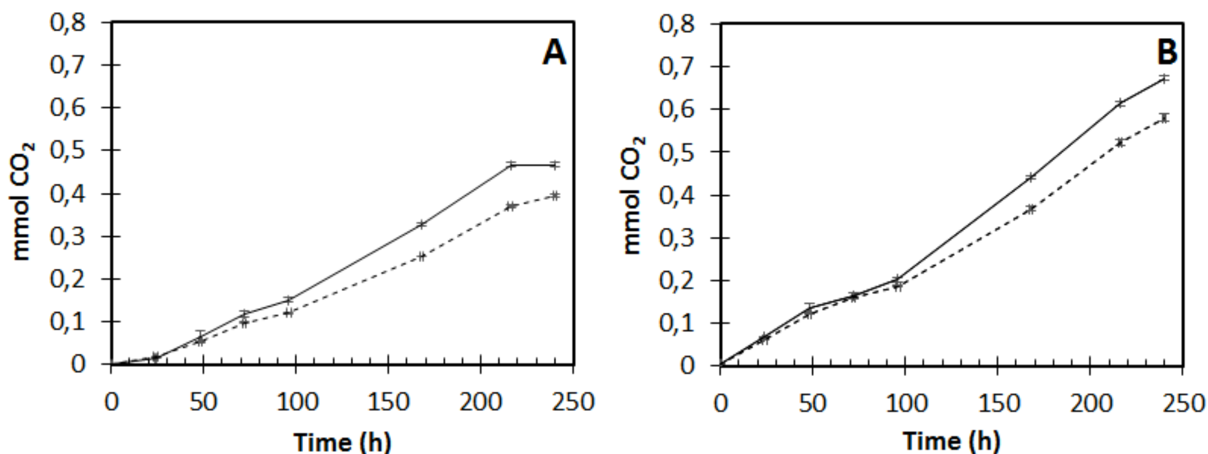

Fig. 3. CO_2 production by FC (A) and IC (B) at Erlenmeyer flasks. Error bars represent the SD of three replicates. Continuous lines correspond to non-dye experiments and dotted lines to experiments with dye.

Furthermore, with those data the average CO_2 production (a_{CO_2}) and average substrate consumption rates (a_s) were estimated dividing amounts of CO_2 produced and glucose consumed by 240 hours, respectively.

Although the BM formed in IC is about three times higher than the obtained in FC system, it is notable that a_{CO_2} in both systems decreased by about 15% in the presence of dye. In FC a_{CO_2} changed from 0.0019 mmol h^{-1} in the control to 0.0016 mmol h^{-1} in RB5 experiments, while in IC a_{CO_2} decreased from 0.0028 in non-dye control to 0.024 mmol h^{-1}, with the dye. In FC, the specific rates of CO_2 production (r_{CO_2}) were similar in RB5 and control experiments (Table 3). However, for IC the specific rate was lower with RB5. A similar behavior referred to a decrease in specific CO_2 production by *T. versicolor* also occurred in the presence of nonylphenol, another compound that prevails in the environment (Soares *et al*. 2006); and when the fungi is grown on hardwood kraft pulp and glucose (Roy and Archibald 1993). Values of these works are shown at the end of Table 3.

On other hand, average substrate consumption rates (as) were about 40% higher in the IC (0.019 g L^{-1} h^{-1}) than in FC (0.013 g L^{-1} h^{-1}), with or without RB5. This can be explained because in IC biomass was higher than in FC; these values are consistent in magnitude to those calculated from report for the degradation of nonylphenol by *T. versicolor* (Soares *et al*. 2006).

The decrease in CO_2 production can be explained taking into account the presence of dye and the laccase activity produced. At the end of Table 3, are the results of other works in which the production of CO_2 by *Trametes* growing simultaneously on glucose and other substrates was studied. They reported a decrease in the rate of CO_2 production associated with the removal of recalcitrant compounds and the presence of enzymatic activities as laccases and manganese peroxidases (Roy and Archibald, 1993) or only laccases (Soares *et al*. 2006). On the other hand, the presence of an alternative carbon source that has a different degree of reduction from that of glucose disrupts the normal metabolism of *Aspergillus niger* when used in conjunction therewith (Reyes-Ocampo *et al*., 2013). This can explain the significant negative effect that dye concentration had on q_s values (Table 2). Furthermore, a study performed with *Trichoderma atroviride* proved that RB5 biodegradation mechanism resulted in the production of phenolic and carboxylic acid (Adnan, *et al*, 2015), this suggests that CO_2 is not a compound expected to be the main product of the biotransformation.

In spite of the aforementioned changes, when we compared the yields CO_2-X of both systems (Table 3), the values obtained with RB5 are not significantly different from those of the control experiment (without dye); indicating that the production of CO_2 is mainly the result of fungal growth, and the dye was not mineralized. However, the YCO_2/X values of free cells were higher than those of the IC system, indicating that immobilization altered fungal metabolism.

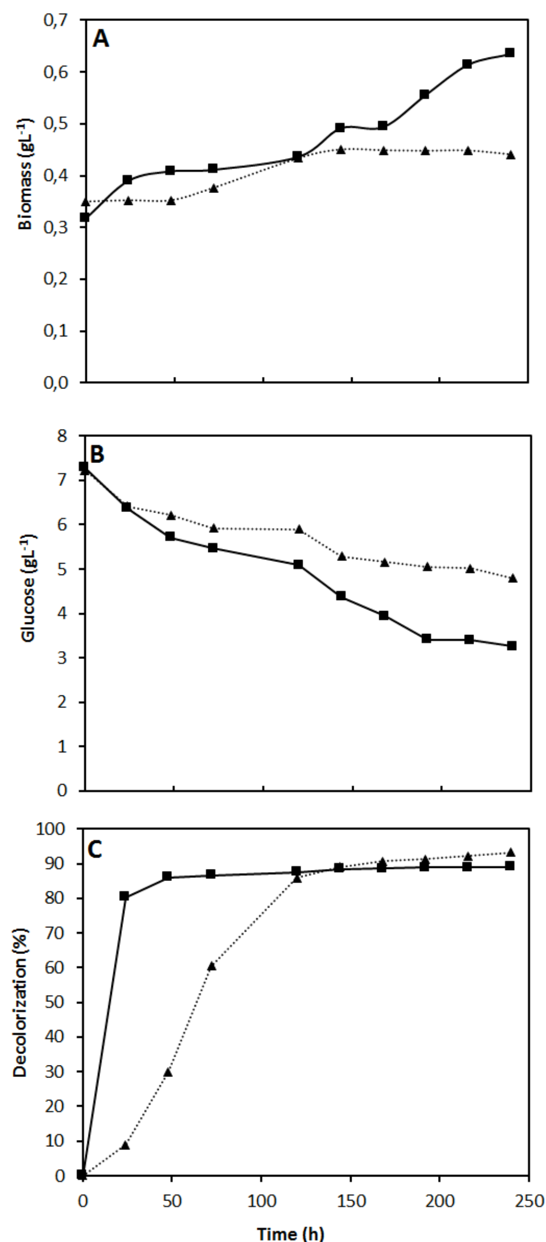

Fig. 4. Biomass production (A), glucose concentration (B) and RB decolorization (C) by free cells (▲) and immobilized cells culture (■). Initial concentration of RB5 equal to 200 ppm.

3.3 *Analysis of decolorization by spectrophotometry*

In order to characterize the RB5 decolorization process, in the third experimental phase a study with an initial dye concentration equal to 200 ppm, was performed to analyze the behavior of free and immobilized cells based on dye decolorization, glucose consumption and biomass production (Fig. 4). From the RB 5 decolorization data, it seems that almost all the RB5 was exhausted in both cultures, although the profiles of each of the measured variables showed different trends.

During the first 24 h, for IC culture 80% of RB5 was decolorized, with a glucose consumption of 1.1 g L^{-1} and slight biomass increase; while in FC culture it was observed the same glucose consumption, although in this case only 9 % of RB5 was decolorized and there was no biomass production. In this case, about 30% of the removal corresponds to adsorption in the biomass. At 24 dye decolorization started to decline in IC culture, and after 48 the change in decolorization percentage was barely 3%. Among 24 and 120 hours, the glucose consumption was of 1.4 g L^{-1} and a slight increase in the production of biomass was observed. In contrast, it was along of this period when the FC culture reached the highest RB5 decolorization, which increased from 9 to 76 %. However, there was a slight increase in free biomass production and glucose consumption. In the last phase (120 h to 240 h), the amount of glucose consumed increased and the biomass achieved the highest concentration (0.63 g L^{-1} at 240 h) in the IC culture. This biomass increase was approximately 143 % higher than that obtained from FC culture.

Conversely, according to literature the porous nature of PUF creates adequate conditions for growth of immobilized cells, resulting in an increase of microbial activity and more effective dye decolorization (Sharari *et al*. 2013). This could explain the fast decolorization obtained at the beginning of the IC culture and the high biomass concentration produced in this case.

The differences observed between both cultures can be explained based on the analysis of UV-Vis spectra. The decrease in absorbance at 597 nm and a change in the shape of the absorption spectrum occurred in IC culture during the first 24 h (Fig. 5a) corresponds to the high RB5 decolorization. This suggests that color removal was provoked by the transformation of dye molecule by *T. versicolor* producing intermediates that absorb energy in UV-range. In fact, Hailei *et al*. (2009) demonstrated that degradation of different kinds of azo dyes by a crude enzyme from *Phanerochaete* sp produced the appearance of absorption peaks in the UV-vis region due to the destruction of the structures of the dyes. Zille *et al*. (2005) postulated that direct azo dye degradation by laccase from *T. villosa* produces the

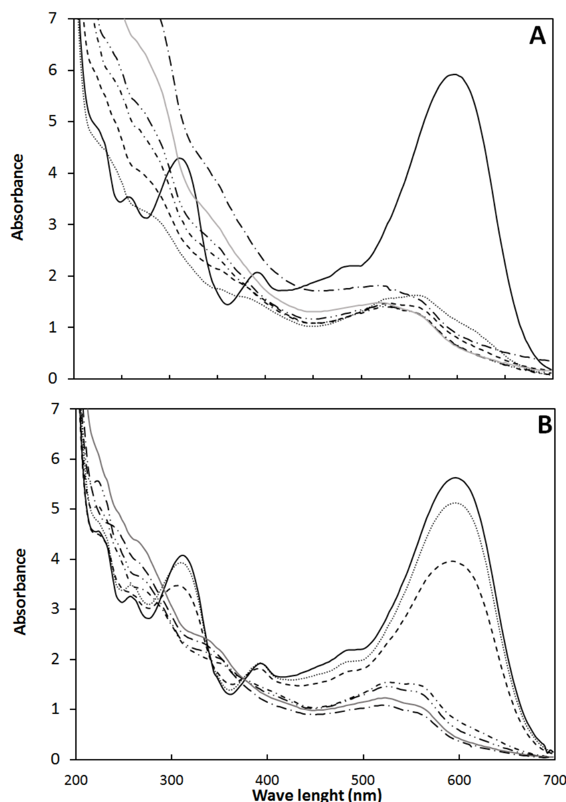

Fig. 5. UV-vis spectra during decolorization process of RB5 in batch reactor by Immobilized (A) and Free cells (B) at 0 (—), 24 (●●●), 48 (- - -), 96 (- ●-), 144 (-●●-), 216 (—) and 240 h (- ●-).

formation of peaks in the UV region. This could be attributed to the conversion of the degraded dye into different reaction products such as phenolic compounds and polymerized products.

It is important to note that during the first 24 h dye decolorization and biomass growth shown simultaneous trends, and taking into account that has been suggested both processes require glucose consumption (Saetang and Babel 2010). At the following two stages of dye decolorization by IC culture, the glucose consumed could be used mainly for biomass growth and color removal. The last one was indicated by a decrease in absorbance in the visible range of spectrum (520 nm to 700 nm) and an increase in the absorbance in the UV-vis spectra (200 nm to 500 nm), explained as a polymerization of compounds generated during azo dye degradation (Zille *et al*. 2005).

In the first 48 h of FC culture the UV-vis spectrum obtained only showed a decrease in absorbance at 597 nm, while the shape of the absorption spectrum remained unchanged (Fig. 5b) indicating the absorption of dye to mycelium (Parshetti *et al*. 2007). At this period, glucose consumption could be destined to produce the necessary enzymes for the subsequent decolorization observed; in fact, this work has discussed before that laccases production is associated with this process. After that time, occurred a significant increase in FC biomass and RB5 decolorization. The maximal wavelength for RB5 shifted from 597 nm to 527 while in the UV-espectra a general increase in absorbance was observed, indicating the transformation of the dye molecule (Zille *et al*. 2005). The glucose consumption was lower than that observed in IC culture, as were the changes in the UV-vis spectrum. In the last phase (120 h to 240 h) the FC biomass barely increased, so glucose consumed should have been used in the RB5 decolorization, which is consistent with the changes observed in the UV-vis spectrum.

It is known that dye decolorization may take place by adsorption on the microbial biomass or degradation by the cells (Enayatzamir *et al*. 2009). According with our results, dye degradation seemed to be the principal mechanism for dye decolorization by immobilized cells. This process was carried out during the first 24 h, with a change in the color medium from blue to a pink tone; although an almost colorless medium was obtained at 240 h. On the other hand, it was assumed that in the first 96 h of FC culture the dye biomass absorption was the principal mechanism to remove RB5, in which the color of the medium remained blue. In a second stage, dye concentration decreased slowly and the medium remained with pink tone.

3.4 *Kinetic aspects of decolorization assay*

The development and application of immobilized cell technology requires the understanding of cells behavior within support, from the physiological and kinetic points of view. Both aspects have a relevant influence on the design and operation of a bioreactor. Immobilization increases the cells/enzyme stability and allows easy separation and reuse of the catalyst, favoring continuous reactor operation. The main limitation of the utilization of immobilized cells/enzymes lies in the existence of both external and internal mass transfer limitations. External mass transfer can be reduced by the manipulation of the reactors hydraulic conditions, for example by increasing the level of agitation. However, intra-particle diffusional restrictions are generally more severe and much more difficult to overcome. In

order to maximize the advantage of immobilized enzyme/cells, both the effective diffusion coefficient of a substrate and the intrinsic kinetic parameters of immobilized cells are required (Jeison *et al*. 2003). Nevertheless, intrinsic kinetic parameters of the IC are difficult to evaluate; so the apparent kinetics of immobilized cells are used instead, to compare the kinetic behavior of immobilized cells with free cells (Chen *et al*, 2003).

To do this it is necessary to define the effectiveness factor (η), defined as:

$$\eta = \frac{V_{app}}{V_{int}} \quad (5)$$

Where V_{app} is the apparent reaction rate in immobilized cultures and V_{int} is the intrinsic reaction rate (this is, the reaction rate in the absence of internal mass transfer limitations, which corresponds to the rate evaluated at the concentration of substrate outside the support) (Illanes *et al*. 2010).

On the other hand, for completing the kinetic study is necessary to analyze the microbial growth, which is usually characterized by the exponential kinetics:

$$\frac{dX}{dt} = \mu X \quad (6)$$

where X is the biomass and μ is the specific growth rate (Lareo *et al*. 2006). Besides, we suppose that the rate of glucose consumption is proportional to the mass concentration of living cells until the stationary phase is reached:

$$\frac{dS}{dt} = q_s X \quad (7)$$

where q_s is the specific glucose consumption.

In this study, several effectiveness factors were calculated for decolorization assays in bioreactor with an initial dye concentration equal to 200 ppm, considering the reaction rates of free cells as the intrinsic parameters due to *T. versicolor* grows as disperse cells without pellets formation. The results of η for initial specific growth rate (0.0025 h^{-1} and 0.0023 h^{-1} for FC and IC, respectively), specific glucose consumption (0.044 g g^{-1} h^{-1} and 0.0101 g g^{-1} h^{-1} for FC and IC, respectively), and r_d (2.329 mg g^{-1} h^{-1} and 0.194 mg g^{-1} h^{-1}, for FC and IC, respectively) were 0.92, 0.23 and 0.08, respectively. Apparently, r_d is the most affected by diffusional effects. However, depending on the decolorization reaction mechanism, a particle size may exist at which the effectiveness factor will be a maximum and, therefore, the immobilized cell behavior will be optimum. This implies the need to develop further studies about the effect of the availability of PUF on the decolorization process and to know the mechanism of reaction that explains the consumption of the dye by the microorganism.

Conclusions

Specific decolorization increased at higher pH values and dye concentrations, and generally occurred faster in free-cell cultures than in immobilized cells. The $Y_{CO_2/X}$ values and the relation among laccases production and the changes in UV-Vis spectra suggested that *T. versicolor* provoked the transformation of dye structure and not its mineralization. Apparently, specific decolorization rate is the most affected by diffusional effects.

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