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BIOKINETIC AND ZYMOGRAPHIC STUDY OF THE ACID BLUE 74 DYE BIODEGRADATION USING ACTIVATED SLUDGE ONTO ACTIVATED CARBON

ESTUDIO BIOCINÉTICO Y ZIMOGRÁFICO DE LA BIODEGRADACIÓN DEL COLORANTE AZUL ÁCIDO 74 MEDIANTE LODOS ACTIVADOS SOPORTADOS EN CARBÓN ACTIVADO

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Abstract

This study is aimed at developing a strategy for acid blue 74 dye (ab74) removal from synthetic water by activated sludge supported onto activated carbon. Low cost ecofriendly adsorbent has been investigated as alternative to expensive methods for removing dyes. Bacterial laccases have been found from activated sludge; however, their contribution has yet not been quantified. Activated sludge (AS), sludge with certain grade of permeability (PS) and sludge supported onto activated carbon (ACAS) were used to evaluate their capacity to discolor ab74. The zymogram and the oxidation of 2,2'-azino*bis*-(3-ethylbenzothiazoline-6-sulfonate) confirmed the presence of laccases either AS or PS. Inhibition assays also evidenced the contribution of laccases to discolor ab74; achieving discoloration of 23.2 % and 19.09 % for AS and PS, respectively. The discoloration of ab74 was found to be more effective in the ACAS cultures than AS and PS. ACAS displayed an interesting synergic effect for ab74 discoloration of 76.6±11 % of efficiency and specific discoloration rate of 5.9±0.4 mg ab74 /h $g_{treatment}$. A novel insight is that the activated sludge was able to regenerate *in situ* the adsorption capacity of the activated carbon; these results are promising to be applied in wastewater treatment technologies.

Keywords: laccases-activity, kinetics, activated sludge, pre-treated sludge, activated carbon, acid blue 74 dye.

Resumen

El objetivo de este estudio fue desarrollar una estrategia para decolorar azul ácido 74 (ab74) de agua sintética mediante lodos activados soportados en carbón activado. Se han investigado adsorbentes amigables al ambiente de bajo costo como alternativas a costosos tratamientos para la eliminación de colorantes. Se han encontrado laccasas bacterianas en lodos activados, sin embargo, su contribución aún no ha sido cuantificada. Lodos activados (AS), lodos con cierto grado de permeabilidad (PS), y lodos soportados en carbón activado (ACAS) se evaluaron en la decoloración del ab74. El zimograma y la oxidación de 2,2'-azino*bis*-(3-etilbenzotiazolin-6-sulfonato) confirmaron la presencia de laccasas en AS y PS. Ensayos de inhibición evidenciaron la contribución de laccasas para decolorar el ab74; decolorando 23.2 % y 19.09 % con AS y PS, respectivamente. La decoloración del ab74 más efectiva se observó en ACAS comparada con AS y PS. ACAS mostró un interesante efecto sinérgico en la decoloración del ab74; con una eficiencia de 76.6±11 % y una tasa específica de decoloración de 5.9±0.4 mg ab74 /h $g_{tratamiento}$. Como resultado novedoso, AS fue capaz de regenerar *in situ* la capacidad de adsorción del carbón activado; esos resultados son prometedores para ser aplicados en el tratamiento de aguas residuales.

Palabras clave: actividad lacasa, cinética, lodos activados, lodos pre-tratados, carbón activado, colorante azul ácido 74.

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1 Introduction

Effluents from carpet manufacturing, dyeing, textile, pulp and paper industries contain various types of dyes, which have to be removed before their discharge into rivers and lakes to avoid health hazards and destruction of these types of ecosystems (Aksu, 2003). Most dyes essentially used in the textile industry are toxic to plants, microbes and protozoa living in the water bodies and are recalcitrant to discoloration and degradation (Couto *et al*., 2006). Moreover, dye effluents discharged into water bodies without an appropriated treatment can present concentrations ranging from 10-50 mg/L, which might lead to reduce light penetration and destroy aerobic aquatic organisms by favoring anaerobic conditions (Chung and Stevens 1993; Banat *et al*., 1996; Zhang *et al*., 2012).

The methods for dye removal from wastewater fall into three categories: physical, chemical and biological. Several chemical methods have been proposed for treating this type of dyes from wastewaters, i.e. coagulation-flocculation, advanced oxidation, among others (Thomas *et al*., 2006); however, several drawbacks limit their application, namely most of them are relatively expensive to be applied at industry level (Mohan *et al*., 2009). Adsorption is yet a ubiquitous and effective process for the removal of synthetic dyes. In this scenery, adsorption based on the use of activate carbon has been cited by the US Environmental Protection Agency as one of the best available control technologies (Derbyshire *et al*., 2001). Enzymatic discoloration through laccases, and chemical coagulation also have been reported as methods to treat synthetic dyes, where even coagulation could be 1.4 times better than the enzymatic treatment (Solís-Oba *et al*., 2009). Nevertheless, although carbon activated can nowadays be produced from low-cost sources, its high-cost associated with its regeneration process has led to implement new technologies, but without an apparent success regenerating of the adsorbent (Lakshmi *et al*., 2009; Shi *et al*., 2010).

Biological technologies based on microbial discoloration are cost-effective processes for the treatment of dye-containing wastewater (Van der Zee and Villaverde, 2006). The activated-sludge process has been considered as the most versatile biological oxidation process for the removal of dyes from industrial wastewaters (Tomei *et al*., 2016). Microorganisms in this activated sludge have been attractive to decolorize dyes, because of their low production and operating costs (Sandhaya *et al*., 2005; Kornaros *et al*., 2006). Notwithstanding activated sludge contain different types of microorganisms, only a few bacteria seem to be the ones able to degrade azo dyes under aerobic operating conditions (Patil, 2013; Mahmood *et al*., 2016). These bacteria use oxygen as electron acceptor, then, breaking down dyes into mineralized byproducts. The RB4 dye was biologically eliminated due to sorption on activated sludge (Churchley *et al*., 2000), and RB2 dye was adsorbed onto pre-dried activated sludge (Aksu, 2003). It is worth stressing that only a few studies have evaluated the role of enzymes contained in activated sludge to mineralize azo dyes (Churchley *et al*., 2000; Patil, 2013). Cristóvao *et al*. (2008) evidenced the activity of a commercial laccase to discolor reactive dyes. Their results allowed the proposal of a kinetic model properly quantifying the activation of the laccase-mediator system. Besides, Patil (2013) discolored the reactive Orange HE2R using an activated sludge, in which certain types of enzymes were identified (Patil, 2013). In general, peroxidases, laccases and tyrosinases, contained in activated sludge, have been identified as the main oxidative enzymes carrying out the partial oxidation of dyes (Bagewadi *et al*., 2011; Mahmood *et al*., 2016; Ping *et al*., 2008); however, there are few evidences on their kinetic contribution during the discoloring process.

Hence, a proper quantification of their influence during treating wastewater from textile effluents could be insights on their importance for discoloring many types of dyes in addition to remove organic matter.

In this paper, we have explored the use of different treatments to remove acid blue 74 in aqueous solution by estimating the discoloration efficiency, specific discoloration rates, laccases participation on dye removal, regeneration of activated carbon, laccases identification by zymographic and scanning electron microscopy.

2 Materials and methods

2.1 *Inoculum*

Before its usage in the experimental part of this research, the AS was previously conditioned with organic pollutants from industrial effluents (3-10 g/L

of COD and 200 mg/L of total nitrogen). The activated sludge before its evaluation during the discoloration process of acid blue 74 has had an acclimation time of ca. 20 years. This activated sludge was pretreated by sonication for increasing its superficial area; this pretreated sludge will be hereafter named as PS. A sonicator (Cole Palmer 8893) was used to apply sound energy to agitate particles of sludge in a solution. Frequencies of ca. 50/60 Hz were applied in 50 mL of activated sludge into a bath with an ultrasonic probe during 10 minutes at a temperature of 20 ± 1 °C. Commercial laccase (LA) was provided by deni Lite II, Novozymes.

2.2 Sludge characterization

Scanning electron microscopy (SEM) was carried out to characterize superficial properties of both AS and PS. Images were obtained without sonication treatment (AS), and after the sonication treatment (PS). In all cases the sludges were taken after the discoloration process. Pellets of 2 mm of AS and PS were fixed and protected by glutaraldehyde (3.5 %) and osmium tetroxide 0.1 M. These samples were washed with Na_2HPO_4-NaH_2PO_4 -buffer- (0.1M), pH 5.8-8.0 and dehydrated with ethanol from 10 % to 100 %. Finally, these sludge pellets were encapsulated and dried to their critical point to be coated with gold and observed by SEM (Jeol JSM-5900), according to the procedure stated in Bozzola and Russell (1991).

2.3 Laccase identification

2.3.1 Zymography

An electrophoretic technique was applied for the detection of laccases contained in the inocula (AS, PS and LA) studied here. The zymography was based on a polyacrylamide gel electrophoresis (SDS-PAGE) at alkaline pH under non-denaturing conditions. The SDS-PAGE was carried out in a Bio-Rad Mini-PROTEAN system with 150 V during 60 minutes (Laemmli, 1970). To obtain the zymogram, the gel was stained for laccase activity using 2,2'-azine*bis*-(3-ethylbenzothiazoline-6-sulfonate acid), hereafter named ABTS, 25 mM in a solution 0.1 M of sodium acetate (pH 5) as substrate. The resolving and stacking gels were prepared with 12 and 5 % of acrylamide, corresponding to buffer solutions of 1.5 M Tris-HCl (pH 8.8) and 0.5 M Tris-HCl (pH 6.8), respectively.

2.3.2 Oxidation of ABTS

The activity of lacasses from the inocula was determined in batch stirred cultures by the oxidation of ABTS. Specifically, the system contained 0.27 mM of ABTS and 0.1 M of sodium acetate (pH 5) at 40 °C ± 1. Enzyme activity out of LA and sludges was expressed in units (U), one unit was defined as the amount of laccase necessary to partially oxidize 1 μmol of ABTS/L-min (Bourbonnais *et al*., 1995). The concentration of ABTS during its oxidation was measured by spectrophotometry (Shimadzu UV 1800) at 420 nm of wavelength. Besides, the protein concentration was measured by Lowry technique at 750 nm (Lowry *et al*., 1951).

2.4 Evaluation

Transient experiments were carried out in mini-reactors with nominal volume of 100 mL. The operating volume was of 75 mL. The following operating conditions were maintained as constant during these experiments: 30 °C ± 1, 300 ± 25 rpm, pH 6.4 and 2 mL/min of aeration. The dye molecule, ab74, was decolorized by AS, PS, ACAS and LA. LA and activated carbon (AC) were used as control assays. Initial concentrations of LA, AS and PS were 0.5 ± 0.1, 2.6 ± 0.05, and 4.8 ± 0.1 g (of protein)/L, respectively. A set of experiments was designed to evaluate the effect of initial concentrations of ab74 on its discoloration. In this sense, it was varied from 50 to 250 mg/L. An abiotic control experiment, using sterile AS and PS, was carried out to evaluate the presence of adsorption phenomena through the membrane of the evaluated sludge.

The contribution of laccases during the discoloration of ab74 on LA, AS or PS was indirectly determined inhibiting their enzymes with 50 mM of $HgCl_2$ (Petr and Jiri, 2002). The experiments were performed at the similar operating conditions than those experiments without inhibiting the activity of laccases. Initial concentrations of LA, AS and PS were 0.5 ± 0.1, 4.2 ± 0.2 and 4.6 ± 0.4 g protein/L, respectively. In experiments with and without inhibition of laccases, the concentration of ab74 was measured by spectrophotometry at 608 nm of wavelength (Shimadzu UV 1800). As commented earlier, the protein concentration was determined by Lowry technique at 750 nm.

2.4.1 *Response variables*

Evaluation of LA and sludge were analyzed in terms of intensive and extensive variables. These variables are presented as follows:

Total discoloration efficiency:

$$E_{\%} = \left(\frac{C_r}{C_i}\right) \times 100 \tag{1}$$

Laccase discoloration efficiency:

$$E_{LA\%} = (E_{\%}) - \left[\left(\frac{C_r}{C_i}\right) * (f_i) * 100\right] \tag{2}$$

Specific discoloration rate:

$$q = \left(\frac{C_r}{U * t}\right) \tag{3}$$

where f_i, is the factor of inhibition which accounts for the specific discoloration rate in presence and absence of the laccase inhibitor ($HgCl_2$), C_i is the initial concentration of ab74 (mg/L), Cr is the concentration of ab74 at a specific time, q is the specific discoloration rate which was normalized to compare rates from laccase contained in LA, AS and PS, and "t" is the reaction time (h).

2.4.2 *Biokinetic model*

The biokinetic parameters; affinity constant, K_m (mg/L), and maximum rate of discoloration, V_{max} (mg ab74/U-h), were estimated by the software program SigmaPlot 12.5, Systat Software Inc, 2011, using multi-response non-linear regression with the Levenberg-Marquardt algorithm at a 95% confidence interval. The mathematical model developed was based on Michaelis-Menten formalism and it is given in terms of the specific reaction rate as follows:

$$q = \frac{V_{\max} S}{K_m + S} \tag{4}$$

where S is the concentration of ab74 in mg/L.

3 Results and discussion

Firstly, a section on the characterization of AS and PS is presented. Images of the activated sludge obtained by SEM before and after of the sonication procedure has been analyzed. Secondly, a section presents the discussion on the activity of laccases in AS and PS. A zymography analysis is addressed to display images indicating the oxidation bands of ABTS for LA, AS and PS. Finally, a last section discusses the behavior of ab74 discoloration by the different treatments (LA, AS, PS, AC and ACAS), as well as the effect of HgCl2 as an inhibitor of laccases into biological treatments. The cost evaluation of enzymes from several inocula was also analyzed.

3.1 *Sludge characterization*

The bacterial morphology is observed in the images obtained from SEM, the presence of *Streptobacillus* was found in both AS and PS (Figure 1). Yu *et al*. (2001) showed the ability of *pseudomonas* from bacillary, isolated from activated sludge, to remove ab74 with efficiencies close to 80 %. Jadhav *et al*. (2010) also showed the ability of this type of *pseudomonas* to remove reactive orange dye with efficiencies close to 100%. Besides, images in Figure 1 suggested the presence of larger superficial areas for PS than for AS, after the sonication process. From a reactor engineering perspective, this larger superficial area on PS is beneficial to increase the conversion of ab74. Some works suggest that there are two ways to increase the microbial activity by sonication; increasing the superficial area or decreasing the particle size of the substrate (Yadvika *et al*., 2004).

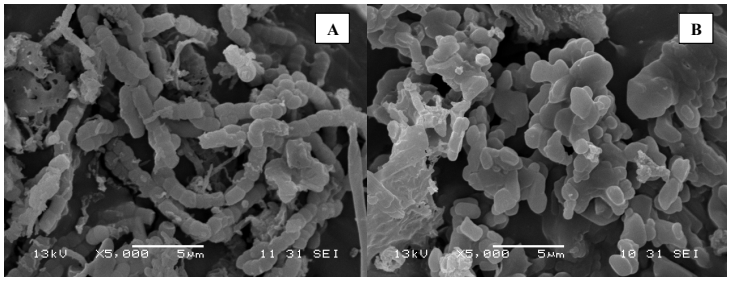

Fig. 1 Images of Scanning Electron Microscopy for AS (a) and PS (b).

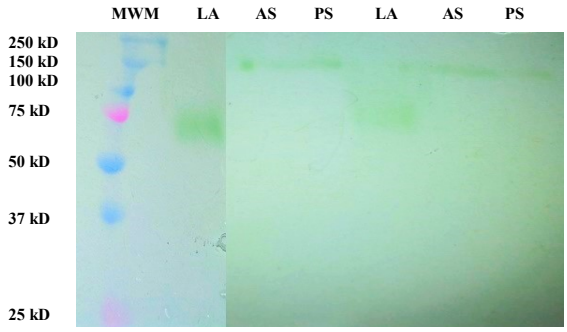

Fig. 2 Zymogram: Molecular weight marker (MWM), Laccase activity of commercial laccase (LA), activated sludge (AS) and pre-treated sludge (PS). Results presented by duplicate.

But at the same time the effect could be negative or positive, depending of the kind of inoculum. Foladori *et al*. (2007) observed that for a pure strains the sonication effect was negative showing damage and death of the free cells; but for activated sludge, the frequency needs to be high (more than 20 kHz) to damage the cells. But according to Covarrubias *et al*. (2015), sonication of sludge improved the contact between enzyme and substrate; in that study the substrate consumption enhanced 1.6 fold faster than whole cells. However, it is not clear whether kinetic properties from PS were also affected by the sonication treatment. Whereas that in the present work the sludge did not show statistical differences in the specific rates of discoloration either PS or AS, with 13.97 ± 0.16 and 13.57 ± 0.18 mg ab74/U-h, respectively.

3.2 Laccase identification

Figure 2 displays the zymogram obtained for AS, PS and LA. A molecular weight near to 75 kDa was observed for LA; bands around 150 kDa were observed for AS and PS, which were two fold larger than those from LA but indicating the presence of proteins with laccase activity. Activity bands from AS and PS were different than those of LA. In literature, there are studies identifying proteins with laccase activity with a molecular weight similar to those observed for LA, AS and PS, e.g. laccase from *Bacillus subtillis* of 52.7 kDa (Phelan *et al*., 2013) or laccase from *Streptomyces griseus* with a molecular weight of 209 kDa (Leigh, 1997). It is worth mentioning that laccase expression exhibits differential regulation, which has normally been influenced by environmental conditions such as pH, temperature, inductors, culture medium conditions, among others operating conditions, carbon and nitrogen source, presence of phenolic compounds (Bertrand *et al*., 2013; Giardina *et al*., 1999; Tellez-Tellez *et al*., 2008). On the another hand Villegas *et al*. (2016) have reported that for laccases from *Pycnoporus cinnabarinnus* could not express different isoenzymes during its evaluation under three different geometries of reactors. In this study, the difference in the type of laccases contained in AS and, PS and LA might be related to the type of microorganisms evaluated since all inocula were evaluated at identical operating conditions.

The oxidation of ABTS for AS, PS or LA indicated the presence of laccase activity. At earlier mentioned operating conditions, the specific activities of laccase were ca. 0.9, 1.53×10^{-5} and 2.89×10^{-6} U/mg protein for LA, AS and PS, respectively. The specific activity of LA was lower than those from other commercial laccases; for example, the activity in *Pseudomonas cepacia* and *Bacillus sp* is 7.11×10^{-10} and 8.13×10^{-10} U/mg protein, respectively (Ayed *et al*., 2012). The difference in activity among commercial laccases and the laccases from the studied sludge observed in Figure 2 might likely be due to the concentration of enzymes inside of the cells or their catalytic properties.

3.3 Acid blue 74 discoloration using LA, AS and PS

Figure 3 displays the concentration profiles as a function of time of the discoloration of ab74 on LA, AS and PS at different initial concentrations of ab74 (50-250 mg/L). All evaluated inocula were able to remove ab74 from the solution. At studied operating conditions LA led to the best discoloration results, which was associated to two properties recognized in literature, namely its high enzyme purity and its direct contact with the molecules of dye, furthermore in some cases the commercial enzyme by Novozymes includes an enzyme mediator which could help to improve the results (Tavares *et al*., 2009). Although PS seemed to have qualitative changes in its bacterial morphology (Figure 1) respect to AS, both sludges presented similar discoloration profiles, which were apparently affected by the initial concentration of ab74. In this regard, the treatment of AS by sonication was not able to favor lysis of cells since discoloration of ab74 on PS was no larger than that obtained on AS. Puvaneswari *et al*. (2006) reported how lysed cells release FAD cofactors in the extracellular environment that is reduced enzymatically by NADH, and $FADH_2$, increasing the discoloration of an azo dye.

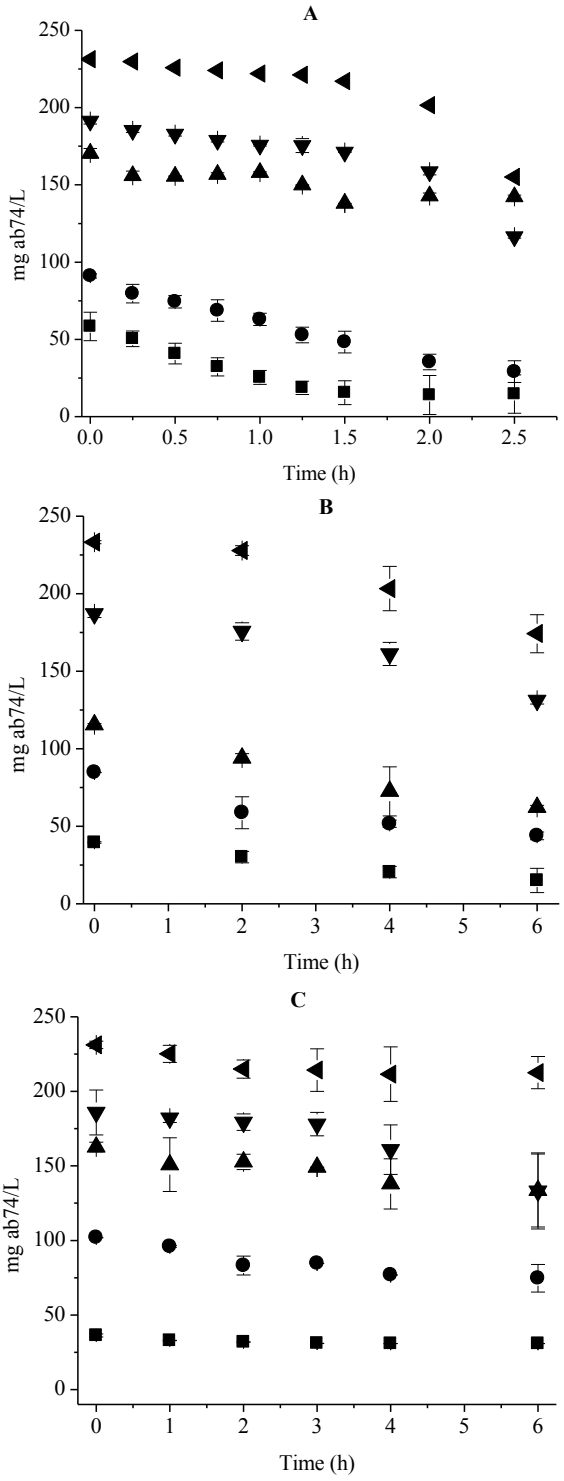

Fig. 3 Profiles of ab74 discoloration. A: Commercial Laccase (LA); B Activated Sludge (AS): C: Pretreated Sludge (PS). (■) 50 mg/L; (●) 100 mg/L; (▲) 150 mg/L; (▼) 200 mg/L; (◄) 250 mg/L.

The total discoloration efficiency ($E_{\%}$) is presented in Table 1. These response variables were different for each inoculum evaluated. AS and PS led to discoloration efficiencies from 2.2 to 23.8 % and from 2.6 to 18.4 %, respectively. So far, an increment of the initial concentration of ab74 affected negatively the discoloration efficiency. Membranes from AS and PS are barrier that protect cells, comprising lipids and protein and, hence, implicated in signaling, cell stability, and protein interactions (Rannikko *et al*., 2014). Thus, the intrinsic cellular capacity of either AS or PS was related to the relationship between external osmolarity and stress conditions produced between the cells and their environment (Machado *et al*., 2004). The abiotic control experiments evaluating AS and PS gave rise to a total discoloration efficiency of 2.8 ± 0.1 %, which was totally related to the adsorption of ab74 through the membrane of these sludges. For instance, Chen *et al*. (1999) showed studies where minimal amounts of dye are adsorbed on the cell surface, being the biological degradation the main mechanism limiting the process.

3.4 Laccases inhibition

Table 2 presents efficiencies and discoloration specific rates of ab74 by laccases contained in LA, AS and PS. These response variables were analyzed by one-way-analysis of variance (ANOVA) with the Tukey comparison test. It was statistically identified that the efficiencies and specific rates of discoloration of ab74 using LA, AS and PS were significantly different at studied operating conditions (P <0.001). In order to observe the role of laccase from the sludge on the ab74 discoloration process, an inhibitor of laccase activity ($HgCl_2$) was tested. This laccase inhibitor affected response variables, namely the efficiencies and specific rates of discoloration of ab74 on LA, AS and PS. First, $HgCl_2$ totally inhibited enzyme activity from LA obtaining null discoloration of ab74. Laccase activity from AS and PS was associated with discoloration of ab74 since the total discoloration efficiency decreased 23.2 and 19.09 % for AS and PS, respectively, and the specific discoloration rates decreased 23.8 and 20.2 % for AS and PS, respectively. These results showed the contribution by enzymatic activity due to laccases in the studied sludges; however, enzymes discoloring ab74, other than laccases might have also been contributed, and future studies need to be guided in this direction.

Table. 1 Total discoloration efficiency of Ab74 ($E_{\%}$), in batch cultures.

ab74 (mg/L)	Laccase (E%)	Activated sludge (E%)	Pre-treated Activated Sludge (E%)
50	57.6 ± 12.4	23.8 ± 8.4	12.2 ± 2.5
100	42.6 ± 7.0	22.1 ± 2.5	18.4 ± 6.1
150	19.0 ± 2.0	18.6 ± 2.1	6.1 ± 1.3
200	40.1 ± 11.7	6.1 ± 4.2	9.1 ± 2.3
250	38.9 ± 7.2	2.3 ± 0.9	2.6 ± 1.4

Table 2. Discoloration efficiencies and specific discoloration rates in presence of $HgCl_2$.

Inoculum	Efficiency (%) in absence of $HgCl_2$	Efficiency (%) in presence of $HgCl_2$	Specific discoloration rate* in absence of $HgCl_2$	Specific discoloration rate* in presence of $HgCl_2$
Comercial laccase	38.3 ± 2.7	0	0.279 ± 0.02	0.039 ± 0.006
Activated sludge	74.0 ± 13.4	56.8 ± 2.9	0.159 ± 0.001	0.125 ± 0.009
Pre-treated sludge	84.3 ± 5.2	68.2 ± 1.9	0.122 ± 0.003	0.094 ± 0.005

*Specific discoloration rate (mg ab74 decolorized/h $g_{inoculum}$)

Table 3. Discoloration efficiencies ($E_{LA\%}$) owing to Lacasses.

ab74 (mg/L)	Laccase ($E_{LA\%}$)	Activated sludge ($E_{LA\%}$)	Pre-treated Activated Sludge ($E_{LA\%}$)
50	57.6 ± 12.4	5.7 ± 0.4	2.9 ± 0.98
100	42.6 ± 7.0	5.3 ± 0.5	4.4 ± 0.38
150	19.0 ± 2.0	4.4 ± 0.5	1.5 ± 0.51
200	40.1 ± 11.7	1.5 ± 0.9	2.2 ± 0.9
250	38.9 ± 7.2	0.5 ± 0.2	0.6 ± 0.5

In fact, monooxygenases, peroxidases, and dioxygenases belong to other type of enzymes reported in a discoloration process and might have been contained in AS and PS (Kandelbauer *et al*., 2013; Méndez-Hernandez *et al*., 2013).

The extent of discoloration efficiency depends on several factors, such as the initial inoculum concentration, kind of substrate, reaction time, operating conditions, culture media, type of microorganism, and so forth. Table 3 shows the laccase discoloration efficiencies ($E_{LA\%}$), determined for all the studied inocula. Estimations are based on specific discoloration rates presented in Table 2 and account for activity of laccases contained in AS and PS. This variable, ELA% amounted from 0.5 to 5.7% for AS and from 0.6 to 4.4% for PS. Therefore, laccases contributed in ca. 20 % during the discoloration of ab74 at studied operating conditions. The specific discoloration rate (mg ab74/L-h) at 100 mg ab74/L for AS was ca. 6.6 times larger than *Pseudomonas* GM3 and the consortium of *Pseudomonas* strain reported by Yu *et al*. (2001).

3.5 Biokinetic of the acid blue 74 discoloration using AS and PS

As commented earlier, transient observations were described using the Michaelis-Menten model given in Eq. (4). Figure 4 presents the fit of rates of discoloration of ab74 as a function of initial concentration of ab74. Kinetic model was able to correlate coefficients presented in Table 4 as well. Specific reaction rates based on U (μmol of ABTS oxidized/L-min) were larger for AS and PS than for LA, corroborating afore mentioned findings.

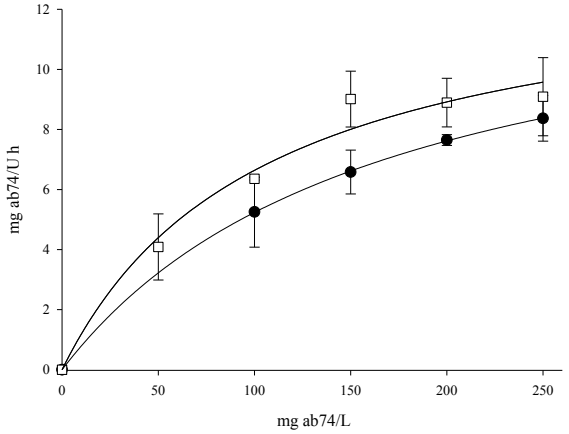

Fig. 4 Experimental models of Michaelis-Menten: (□) Activated sludge (AS), (●) Pre-treated Sludge (PS), Model: (—).

Table 4. Kinetic parameters by Michaelis-Menten model.

Inoculum	K_m (mg/L)	V_{max} (mg ab74/U h)
Commercial laccase	20.5 ± 3.0	0.23 ± 0.005
Activated sludge	104.4 ± 34.5	13.57 ± 1.8
Pre-treated sludge	166.7 ± 4.4	13.97 ± 0.16

Notwithstanding laccases from AS and PS led to lower values of U than LA during the oxidation of ABTS, these enzymes contributed in ca. 20 % to decolorize ab74. Biokinetic parameters are also presented in Table 4. Parameters were statistically significant obtaining narrow confidence intervals. Besides, biokinetic parameters involved in the model presented a negligible statistical correlation between them, i.e. across correlation coefficients of ± 0.4. Lower affinity constants (Km) were obtained for AS and PS (P <0.05). Affinity constant was of 20.5 mg/L for LA, being ca. 6 times larger than those from AS and PS. On the other hand, Vmax values out of AS and PS were ca. 60 times larger than that obtained for LA. On this basis, the smaller Km and the larger Vmax from the evaluated activated sludges, explain the greater specific reaction rates from the discoloration of ab74. Cristóvão *et al*. (2008) reported kinetic parameters for the discoloration of diverse reactive dyes by a commercial laccase, the value of Vmax from the AS or PS evaluated herein was

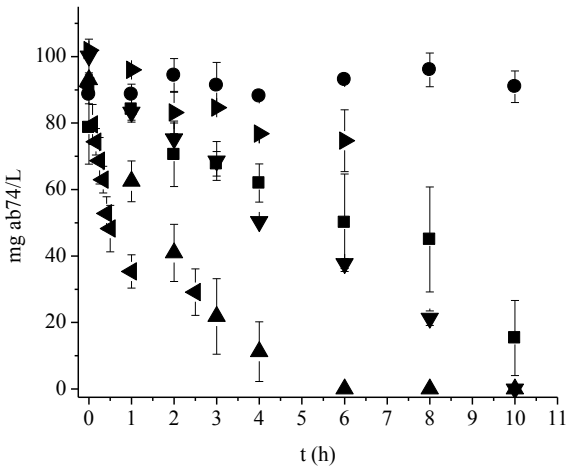

Fig. 5 Profiles of ab74 discoloration at initial concentration of 100 mg/L. (■) Activated Sludge, (●) Abiotic control, (▲) Activated Sludge + Activated Carbon, (▼) Activated Carbon, (◄) Commercial Laccase, (►) Pre-treated Sludge.

significantly superior to those values of Vmax obtained during the discoloration of Yellow reactive 15, Red reactive 239 and Black reactive 5 on a commercial laccase. These experimental results suggested that AS and PS are promising inocula for the discoloration of ab74; in addition, these laccases have presented other metabolic capabilities to decolorize dyes and even to mineralize the molecules derived from laccase catalysis (Méndez-Hernandez *et al*., 2013). Davison (1999) reported results from the evaluation of an activated sludge, stressing its capacity to eliminate more than one carbon source at the same experiment due to the horizontal gen transfer that occurred in the activated sludge as a mechanism of adaptation. So, many bacteria isolated from sludge presents metabolic versatility to discolor or remove different substrates. As, *Alishewanella* sp. is a Gram-negative bacteria isolated from dye contaminated soil (Kolekar *et al*., 2013) that has the capability to remove azo dyes. Additionally, *Alishewanella* sp. has also the capability to hydrolyze casein, form biofilms and produce pellicles (Jung *et al*., 2012). Mixed cultures or isolated bacterial from sludge could present many metabolic advantages to apply in biotechnological process like discoloration.

3.6 Biokinetic of the ab74 discoloration using ACAS

A biokinetic comparison in batch cultures was carried out in order to evaluate the discoloration of 100mg/L

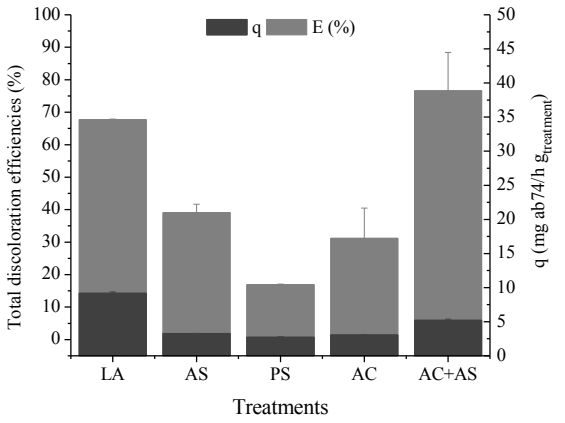

Fig. 6 Discoloration efficiencies and specific discoloration rates for all treatments evaluated.

of ab74. In this sense, Figures 5 displays concentration profiles as a function of time, and Figure 6 shows discoloration efficiencies and corresponding specific discoloration rates when evaluating all discoloration processes, namely ACAS, AS, PS, LA and AC. Discoloration efficiencies and specific rates values were treated statistically by ANOVA and Tukey test comparison, with significant difference (P <0.05). Although a commercial laccase (LA) was also evaluated, the best discoloration efficiency was obtained by the synergic effect of ACAS, namely a discoloration rate of 5.9±0.4 mg ab74/h-$g_{treatment}$, and discoloration efficiency 76.6±11.8 was obtained. This result can be also associated to the properties of activated carbon such as large porosity and superficial area (Navarro and Vargas, 2010). Discoloration results also suggest that LA is an attractive method for dye discoloration, however its cost to be industrially implemented is more expensive than all other technologies studied here. On the other side, ACAS, showing an efficiency 1.9 and 2.5 times higher than AC and AS, seems an interesting discoloration method to be implemented industrially, however before this, it is mandatory to evaluate the saturation and regeneration processes of activated carbon under the studied discoloration conditions since both are considered as the main bottlenecks in the development of an adsorption technology (Lakshmi *et al*., 2009).

Figure 7 displays an experiment evaluating the saturation process of activated carbon during the adsorption of ab74, and the capability of the activated sludge to regenerate the activated carbon in ACAS. The experiment accounted for different addressed stages (I-IV). The first and second stages (I-I) were aimed at saturating activated

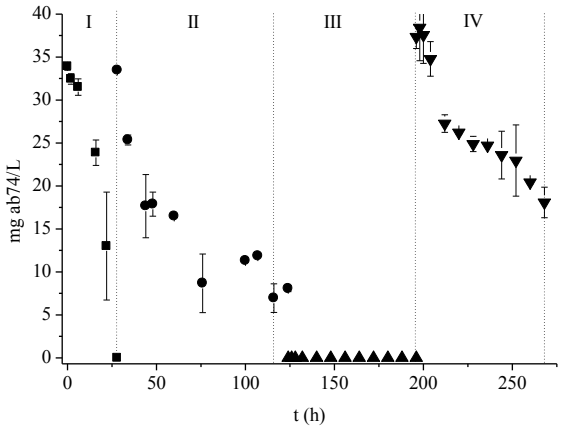

Fig. 7 Profiles of ab74 discoloration using activated carbon (AC). (I) ab74 adsorption into AC; (II) AC saturation by ab74; (III) AC regeneration by AS; (IV) Second ab74 adsorption into AC.

carbon. To achieve this, a solution with an initial dye concentration of 30 mg/L was fed into a batch system containing only activated carbon. At the first stage (I) activated carbon was able to adsorb all the dye, then, at the second stage the first-stage activated carbon was used to adsorb another batch of dye solution with an initial concentration 100 mg/L. Based on experimental error, at this stage, there was a time interval (75-125 h) where dye concentration did not vary as a function of time, ca. 7.5 mg/L; here, it was considered that the activated carbon was already saturated. The third stage (III) was addressed to regenerate the activated carbon. Namely from 125 h to 200 h, the activated sludge was fed into the second-stage activated carbon. As expected during this third stage, the dye concentration went to zero because of the discoloration activity of the sludge itself. At this stage, it was presumed that activated sludge was able to regenerate activated carbon *in situ*. Thus, to corroborate it, at the fourth stage (IV), the third-stage activated carbon is fed into another batch system only containing a dye solution with an initial concentration of 100 mg/L. As observed in Figure 7, activated carbon recovered its adsorption capability due to the biological activity of the sludge. This synergic finding seems an attractive result to be implemented industrially since biological discoloration activity can be increased by the use of activated carbon but also this adsorbent can be regenerated *in situ*. Thus, research on these findings needs to be addressed in future studies on dye discoloration of wastewater.

Conclusions

The sludge contained bacterial microorganisms able to remove ab74. These microorganisms with laccases activity significantly participated during the discoloration of ab74. It is worth stressing that this work evidenced for first time the quantitative contribution of laccases, ca. 20 %, in the evaluated activated sludge. On the other hand, the ACAS method showed the synergic effect of biological and physical processes to discolor ab74 from a synthetic influent. The discoloration efficiencies and specific rates but essentially the *in situ* regeneration of activated carbon make ACAS as an attractive system to be implemented for wastewater discoloration. In this sense, future studies aimed at evaluating certain engineering parameters to scale up this technology will be necessary.

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