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Biodegradation of acid dyes by an immobilized laccase: an ecotoxicological approach†

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Synthetic dyes in watercourses resulting from industrial effluent discharges cause serious ecological impacts, besides carcinogenic and mutagenic effects on human health. Thus, it is important to develop effective methods to remove the dyes from industrial wastewaters, and also to carry out adequate toxicity studies to establish their safety. Azo dyes are the main class of industrial dyes and important environmental contaminants. We have examined the decolorization of two azo dyes (Acid Red 88 and Acid Black 172) by a native *Trametes villosa* laccase immobilized on thiolsulfinate-agarose as well as the effect of different redox mediators in the reactions. The method was effective for the decolorization of both dyes. The immobilization method did not affect the capacity of the biocatalyst for dye degradation. Therefore, the insoluble enzyme removed 97% of the color of AR88 and 92% of AB172 in 24 hours at 22 °C in the presence of the selected redox mediators, vanillin (0.1 mM) and violuric acid (1.0 mM), respectively. In addition, the immobilized enzyme kept 78% of its initial capacity for decolorization of AR88 after three cycles of use. The ecotoxicological evaluation of the solutions showed a great variation depending on the biological systems used. In the phytotoxicity test, the decolorization products were not toxic to plants, whereas in *Daphnia magna* and Microtox® bioassays an acute residual toxicity was found. The last outcome shows the importance of using a battery of bioassays to determine the remaining ecotoxicity of the treated effluents before their discharge into the aquatic environment.

Water impact

Synthetic dyes coming from industrial effluents are recalcitrant compounds that impair the life of aquatic organisms due to their toxicity and because they prevent photosynthesis in watercourses. Methods used for wastewater treatment are not always eco-friendly, economical or totally effective. By the bioprocess described here decolorization was achieved whereas ecotoxicological assessment demonstrated the importance of warranting the safety of decolorized wastewater.

1. Introduction

The chemical industry has developed a great variety of synthetic dyes from which a wide range of different shades can be achieved.[1] There are different kinds of dyes according to the structure of the chromophore: azo, anthraquinone, indigoid, phthalocyanine, sulphurous, nitro and nitroso. It is estimated that over 10 000 different dyes and pigments are used industrially and over 7×10^5 tons of synthetic dyes are annually produced worldwide.[2] The most common environmental problems associated with the textile industry are those related to water pollution caused by the discharge of untreated effluents.

The azo dyes – aromatic compounds with one or more azo groups (–N=N–) – are the most important group of synthetic dyes used in commercial applications.[3] Industrial processes and in particular textile and papermaking produce large amounts of water with high dye contents, most of which belong to this azo group.[4] Eighty percent of all azo dyes synthesized are used only in the textile industry. It has been estimated that approximately 10 to 15% of the dyes used in dyeing processes do not bind to the textile fiber. Therefore approximately 280 000 tons of textile dyes are discharged into industrial effluents per year worldwide.[5] The introduction of

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these components in the environment produces various impacts, the main one is caused by their ability to absorb sunlight in water, which affects the photosynthetic activity of algae and other aquatic organisms and has a serious influence on the food chain.[6] On the other hand, the public perception of water quality is greatly influenced by the color of the water and since the presence of dyes is clearly visible even at very low concentrations (less than 1 mg L^{-1}), the elimination of wastewater color is often as or more important than the removal of colorless organic substances.[2] Depending on the exposure time and concentration of the dyes, they can have acute and/or chronic toxic effects on exposed organisms.[3] For this reason, the treatment of colored effluents prior to their discharge is essential. Moreover, the possibility of recycling the treated water represents a great advantage since large volumes of water are used in the textile industry.[7] The properties of dyes that determine their suitability for use (chemical stability against light, temperature, and pH) also determine the difficulty in their subsequent treatment.[8]

To date, diverse physicochemical treatment methodologies such as membrane filtration, activated carbon, coagulation and chemical flocculation have been applied to reduce the overall levels of dye pollution in aqueous environments. However, the efficacy of all these conventional approaches is limited due to high operating/energy costs, generation of sludge, production of damaging by-products, and requirements of large amounts of chemicals and energy.[9] More recently, technology based on biodegradation has attracted interest due to its economy, the reduction of sludge production and its ecologically benign nature.[3] Biodegradation methods can use isolated organisms, mixed cultures and isolated enzymes.[7,8]

Dyes are xenobiotics very recalcitrant in biodegradative processes, but despite this, it has been shown that several microorganisms are capable of transforming them into colorless products and even mineralizing them completely, as in the case of several ligninolytic fungi with their oxidase enzymes.[4] Among these, laccases seem very promising, proved to be efficient in decolorizing a wide range of industrial dyes without requiring cofactors for their activity, although in some cases the addition of redox mediators can serve to extend this range even further.[7] Laccases of diverse origin such as native fungi and bacteria as well as heterologous proteins expressed in different hosts have been efficient in the degradation of synthetic dyes.[10–16]

The use of isolated soluble enzymes presents some drawbacks such as the difficulty of their separation from the reaction products or the impossibility of their reuse, increasing the costs of the process in which they are applied. Variations in the conditions of the reaction medium can severely affect their activity. On the other hand, the heterogeneity of immobilized enzyme systems enables the easy recovery and multiple re-use of the biocatalyst, the design of continuous processes and the application in batches as in the use of soluble enzymes.[17] In addition to these advantages, with the reversible covalent method of immobilization, the regeneration and reuse of the solid support is possible, when the enzyme becomes inactive after several cycles of use.[18]

Biological toxicity tests allow estimating the effect of the effluent on the biota and assessing environmental risks as a complement to chemical analysis. In general, "bioassay batteries" are used for this evaluation. This methodology seeks to determine the effects of pollutants on a group of test organisms that represent different taxonomic groups of ecological importance and whose sensitivities are complementary. The battery can include animal species as well as vascular plants, microalgae and microorganisms.[19]

The purpose of this work was to study the decolorization of two azo dyes (Acid Red 88 and Acid Black 172) by an immobilized *Trametes villosa* laccase. These dyes present different complexity in their structures, AR88 is a mono-azo and AB172 is a diazo complexed with chrome. They are used in the dyeing of different materials such as silk, polyamide, wool and leather in the case of AR88[20] or wool, polyamide, leather and paper in the case of AB172.[21] Since in other works no precise correlation has been found between decolorization and detoxification of textile dyes,[22,23] different bioassays were carried out to evaluate the ecotoxicological effects of these dyes and their decolorization products. Three bioassays were used for the evaluation of the acute toxicity of the azo dyes and the products obtained from the enzymatic treatment. These included a plant species (*Lactuca sativa*), a freshwater microcrustacean representative of zooplankton (*Daphnia magna* Straus) and a bioluminescent bacterium (*Vibrio fischeri*).

2. Materials and methods

2.1. Chemicals

2,2′-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), acetosyringone (As), acetovanillone (Av), vanillin (Vn), violuric acid (VA), and the dye Acid Red 88 were purchased from Sigma-Aldrich. The dye Acid Black 172 was supplied by Huntsman (Argentina). The characteristics of the dyes are summarized in Table 1. All other chemicals were of analytical grade.

2.2. Determination of laccase activity

Laccase activity was measured using ABTS as a substrate.[24] For soluble laccase activity, the reaction mixture consisted of 1.0 mL ABTS (5 mM) in 100 mM acetate buffer, pH 4.8 and 0.1 mL of enzyme solution. Immobilized enzyme activity was assayed by incubating 2.0 mL of ABTS (5 mM) in 100 mM acetate buffer pH 4.8 with 0.2 mL of a suspension of gel derivative (containing 10 mg suction-dried gel in sodium phosphate 0.1 M pH 6.0) at 22 °C under magnetic stirring.

Oxidation of ABTS was monitored by the increase in absorbance at 436 nm (ε_{436} = 29 300 M^{-1} cm^{-1} [25]).

One enzyme unit (EU) was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute under the specified conditions and activity was expressed as UE L^{-1}.

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Table 1 Dyes used in this work

Dye	C.I. number[a]	$\lambda_{máx}$[b] (nm)	Class	Structure
Acid Red 88 (AR88)	15620	504	Monoazoic	
Acid Black 172 (AB172)	15711 : 1	572	Diazoic chrome-complex	

[a] CI = colour index. [b] Maximum absorption wavelength.

2.3. Laccase production and immobilization

Laccase was produced under semi-solid state fermentation using a native strain of the white-rot fungus *Trametes villosa* isolated from the Uruguayan commercial plantations of *Eucalyptus*. The strain (N° 1449) is deposited at the Department of Mycology from the Faculty of Sciences, Universidad de la República.

In this culture system *Eucalyptus grandis* bark (3.0 g) was used as a support-substrate and a liquid medium (20.0 mL) containing: copper sulfate 1.0 mM, KH_2PO_4 2.0 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.5 g L^{-1}, $CaCl_2 \cdot 2H_2O$ 0.1 g L^{-1}, yeast extract 0.1 g L^{-1}, thiamine 5.0 mg L^{-1}, glucose 9.5 g L^{-1} and peptone 15.3 g L^{-1} in citrate phosphate buffer 0.1 M, pH 2.5. After 14 days of static incubation at 28 °C, the enzyme was obtained in the supernatant and purified as previously described.[26] Covalent-reversible immobilization of laccase was conducted on thiolsulfinate-agarose (TSI-agarose) according to the procedure reported in the same publication.

2.4. Enzymatic dye degradation assays

2.4.1 Decolorization reactions. The reaction mixture consisted of an aliquot of *T. villosa* laccase (100 EU L^{-1}), the dye (100 mg L^{-1}) and the redox mediator (if appropriate), in acetate buffer 100 mM, pH 4.8 with a final reaction volume of 2.0 mL.

The concentration of the dye was selected according to the criteria established by the Globally Harmonized System of Classification and Labelling of Chemicals (GHS),[27] considering the subsequent ecotoxicological study.

Experiments were performed in triplicate at 22 °C under magnetic stirring for 24 h. Two controls were carried out in parallel with the redox mediator and the support used for immobilization (TSI-agarose), in the absence of the enzyme.

2.4.1.1 Decolorization quantification. The decrease in absorbance at the maximum absorption wavelength in the visible spectrum of the dye was monitored (see Table 1). Decolorization efficiency was calculated as a percentage, using the following formula:

$$\text{Decolorization}(\%) = (\text{Area}_{t=0} - \text{Area}_{t=24\text{h}}/\text{Area}_{t=0}) \times 100$$

where: $\text{Area}_{t=0}$: peak area of the initial dye; $\text{Area}_{t=24\text{h}}$: peak area of the product mixture (at the end of the reaction time).

2.4.2 Effect of the laccase–mediator system (LMS) on dye decolorization. First, to select the most effective mediator for each dye, the compounds violuric acid (VA), vanillin (Vn), acetosyringone (As) and acetovanillone (Av) were used at a concentration of 1.0 mM. Then, concentrations between 0.1 and 1.0 mM of the selected compound were tested.

2.4.3 Operational stability of the TSI-laccase derivative. Three successive decolorization reactions of AR88 and AB172 were performed, washing the insoluble biocatalyst with acetate buffer 100 mM, pH 4.8 after each cycle and recharging with fresh dye solution and the selected redox mediator.

2.4.4 HPLC analysis. The decolorization reaction was also monitored by analytical HPLC using a Shimadzu Prominence system with a diode array detector and a Zorbax Eclipse XDB-C18 column (Agilent Technologies, Santa Clara, CA, USA) of 250 mm, 4.6 mm i.d., and 5 μm particle size, with a guard cartridge Ultra-C18 of 10 mm and 4 mm i.d. The oven temperature of the column was maintained at 30 °C with a flow rate of 0.5 mL min^{-1} and an injection volume of 20 μL.

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The analysis was done using 30 mM ammonium acetate (pH 4.6) as solvent A and methanol as solvent B. The gradient elution program used for AB172, for its degradation products and for violuric acid was: 0–15 min, 40–80% B; 15–30 min, 80% B, 30–31 min, return to initial conditions. The program for AR88 was: 0–15 min, 70–90% B; 15–16 min, return to initial conditions. Meanwhile, for vanillin and products of AR88 degradation an isocratic elution was used: 20 min, 60% B.

2.5. Ecotoxicity assessment

Three acute-toxicity bioassays were performed for ecotoxicological evaluation of the biocatalytic degradation of dyes AB172 and AR88. Dye solutions, redox mediators and reaction mixtures obtained at 24 h were analyzed by the methods described below. Dye solutions and redox mediators were analyzed at the initial concentration used in the reaction: AR88 and AB172 100 mg L^{-1}; violuric acid 1.0 mM and vanillin 0.1 mM, and negative controls were made using buffer in all cases.

2.5.1 **Acute phytotoxicity assay.** Seeds of *Lactuca sativa* var. crispa (Lettuce) were used in the seedling emergence and early growth assay according to the method of Sobrero & Ronco with some modifications.[28] Lettuce seeds were provided by local organic producers (REDES Amigos de la Tierra, Uruguay). The germinative capacity and variability in radicle and hypocotyl elongation of the lot were verified before the test was carried out (germination ≥ 90%, coefficient of variation ≤ 30%).

In Petri dishes, twenty seeds were placed on filter paper soaked in 4.0 mL of each treatment and control solution. In order to calculate an endpoint to express quantitatively the toxicity result, five concentrations were tested performing two-fold serial dilutions of solutions. Concentrations between 100.0% and 6.3% were assessed. Moreover, five replicates per concentration were made.

The dishes were sealed with Parafilm® to avoid evaporation of water and incubated at 20 ± 2 °C for 120 h. At the end of the experiment, the number of germinated seeds was counted and the length of seedling hypocotyls and radicles was manually measured.

Then, data were processed with two-way ANOVA followed by a Tukey multiple comparison test and the confidence interval was set at 95% ($p < 0.05$).

2.5.2 **Microtox® acute toxicity assay.** Microtox analysis was carried out in a Microtox 500 analyzer (Modern Water) with the Microtox Omni TM Software by the Ambiental Laboratory (DINAMA – MVOTMA), according to a procedure based on standard protocols of the American Public Health Association (APHA) and the Canadian Environment Agency (EC). The effective concentration which causes an inhibition of 50% (EC50) was determined by measuring the decrease in light emission of a suspension of the marine bioluminescent bacterium *Vibrio fischeri* after 15 min of incubation, at 15 °C, for serial dilutions of the samples.

2.5.3 ***Daphnia magna* acute toxicity assay.** The study was implemented through an adaptation of Guide No. 202 of the Organization for Economic Cooperation and Development, "*Daphnia* sp., Acute Immobilisation Test",[29] and according to guidelines provided by the Department of Water and Chemical Products of the Technological Laboratory of Uruguay (LATU), where this test is performed under standardized conditions routinely. Neonates of the crustacean *Daphnia magna* Straus less than 24 hours old and originating from the same adult population were used. Culture conditions maintained adults in the parthenogenetic reproductive stage with the production of exclusively female offspring. Five concentrations were tested by two-fold serial dilutions, as described in 2.5.1. The test was performed in quadruplicate in glass tubes with 5.0 mL of each sample and 5 specimens for each one. Twenty animals were used in total for each sample concentration and control. The neonates were incubated 48 hours in a chamber with photoperiod and temperature control (20 ± 2 °C), without aeration or feeding. Dead individuals were identified from the observation of immobility for 15 seconds after gentle shaking of the sample. Data were analyzed by the method Probit using the statistical software ToxCalc™. Percentages of dead neonates were plotted against test concentrations and the median lethal dose (LD50) with 95% confidence limits ($p = 0.95$) was calculated. It was not possible to perform this test for Acid Red 88 as it was not soluble in the saline solution required for the organism.

3. Results and discussion

3.1. Dye degradation assays

Soluble *T. villosa* laccase was able to transform both dyes in the absence of redox mediators. It was observed that AR88 was decolorized to a greater extent than AB172 (85 and 75%, respectively). The effect of different redox mediators was studied in order to increase the decolorization achieved by the laccase (Fig. 1). A mediator of synthetic type (VA) and three of the so-called natural (Vn, As, Av) were selected for the test. With the four compounds used for AR88, similar

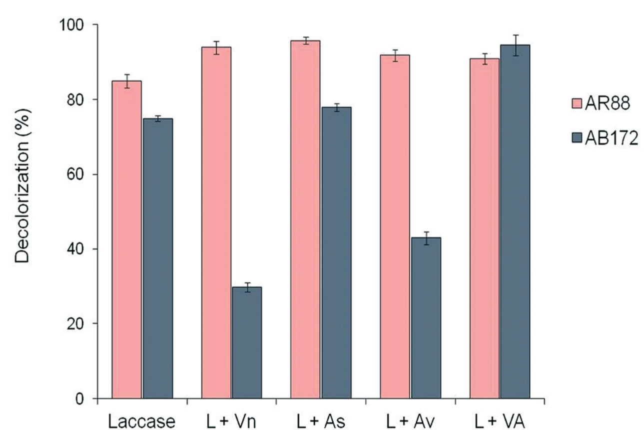

Fig. 1 Decolorization of dyes AR88 and AB172 (100 mg L^{-1}) by soluble laccase (100 EU L^{-1}) in the presence of different compounds acting as redox mediators, after 24 h at 22 °C. L: laccase, Vn: vanillin, As: acetosyringone, Av: acetovanillone, VA: violuric acid. Data represents means ± S.D.

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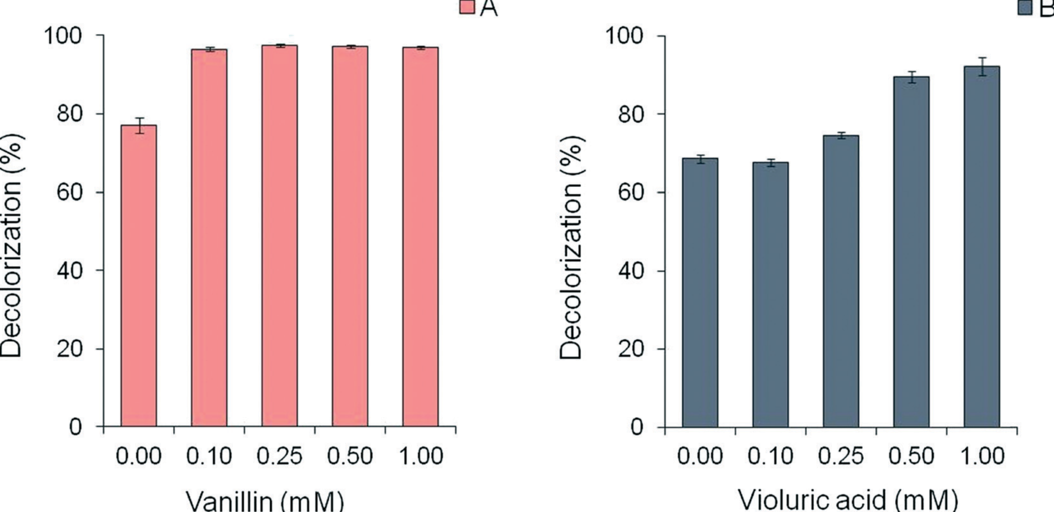

Fig. 2 Decolorization of AR88 (A) and AB172 (B) (100 mg L^{-1}) by TSI-laccase (100 EU L^{-1}) and variable concentrations of vanillin and violuric acid, respectively (0.0–1.0 mM), after 24 h at 22 °C. Data represents means ± S.D.

results were obtained, exceeding by about 10% the decolorization obtained without mediators (Fig. 1). Vanillin was selected for this dye since it is a "natural" mediator and due to its lower cost in relation to the others tested. Since mediators do not recover from the reaction medium, the main advantage of using phenolic compounds of natural origin compared to synthetic ones lies in their lower cost. They are obtained from a renewable source, for example as a by-product in the production of cellulose pulp.[30,31] In addition, the effectiveness of these compounds is in many cases comparable to synthetics, with some additional advantages related to a lower inhibitory effect on the enzymatic activity and the possibility of using a lower concentration.[32–34]

On the other hand, decolorization of AB172 by *T. villosa* laccase was lower without a redox mediator. This is consistent with its more complex structure and previous reports of its degradation by laccase without mediators.[35–37] Moreover, the results for this dye were variable for the different laccase–mediator systems employed. The combinations of laccase with acetovanillone and vanillin were less effective than the control without a mediator, whereas with acetosyringone a similar result to the control was obtained (around 80% decolorization). The highest percentage of decolorization was achieved with violuric acid (95%), in agreement with the usual results observed for this mediator.[11,38,39] Therefore, this "artificial" mediator was selected for the treatment of AB172 with the laccase–mediator system.

The immobilized laccase showed a great capacity to decolorize both azo dyes indicating that it retains the performance of the soluble enzyme (Fig. 2). After 24 hours of reaction, 77% decolorization was achieved for AR88 and 70% for AB172. These percentages increased in the presence of a redox mediator to 97 and 92%, respectively. This represents a remarkable result since immobilization of an enzyme onto a solid support often implies diffusional restrictions for the substrate or the compound to be transformed and the immobilization by means of covalent bonds could affect its catalytic capacity due to conformational changes in its structure.[40]

In addition to checking the modification of the chromophore responsible for the absorption in the visible spectrum (decolorization measurement), the removal of the dyes was verified by HPLC-DAD. The peaks observed in the dye solution chromatograms were not present in the chromatograms of samples obtained at 24 h of reaction (see the ESI†). In this analysis it was also observed that violuric acid remained in the reaction mixture after 24 hours when degradation of AB172 was analyzed, but vanillin disappeared in the degradation of AR88. This is frequently described in the literature for several redox mediators. Since one of the main characteristics attributed to a mediator—being cyclically reconverted—is not accomplished, some authors prefer to call them enhancers.[30,41]

The adjustment of the mediator concentration is an important issue taking into account the cost-efficiency of the process and the potential toxicity or contamination associated with the compound. Fig. 2 shows that an increase in the concentration of vanillin above 0.1 mM had no effect on the decolorization of AR88. For AB172, a lower percentage of decolorization was observed for violuric acid concentrations lower than 0.5 mM.

There are several studies on physicochemical treatments for Acid Red 88,[20,42–44] whereas only a few biodegradation studies are found[45–49] and only in one of these was an isolated enzyme applied.[46] The enzyme used in this case, a laccase from the basidiomycete EUC1, was efficient in the decolorization although the result was not quantified. Therefore, to the best of our knowledge this is the first time that degradation of AR88 by an immobilized laccase has been performed.

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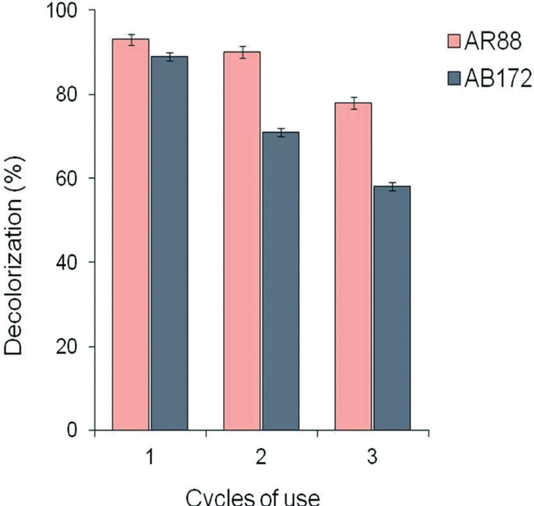


Fig. 3 Successive batchwise uses of immobilized laccase (100 EU L^{-1}) in decolorization of dyes AR88 and AB172 (100 mg L^{-1}), in the presence of vanillin and violuric acid 1.0 mM, respectively, after 24 h, 22 °C. Data represents means ± S.D.

With regard to AB172 its degradation by physicochemical methods[21,50,51] and by biodegradation[35,52–54] was also reported. In the work of Zheng *et al.*[36] a decolorization of 50.5% was achieved in 48 h with the laccase of *Trametes orientalis*. In other two studies, the immobilized laccase of *Trametes pubescens* was used reaching 69 and 77.5% decolorization in 48 and 96 h, respectively.[35,37] In the present work a decolorization similar to the largest reported in these studies was shown but using a reaction time four times shorter, 10 times less enzymatic activity, a temperature 30 degrees lower and twice the dye concentration. In addition, an increase in decolorization was achieved by using the laccase–mediator system. The reusability was verified for the immobilized biocatalyst (Fig. 3). It was observed that the decolorization capacity was gradually reduced for both dyes in the consecutive reactions carried out. The insoluble biocatalyst showed better stability for the degradation reaction of AR88, retaining a decolorization level of 78% in the third use.

3.2. Ecotoxicological evaluation of enzymatic treatments

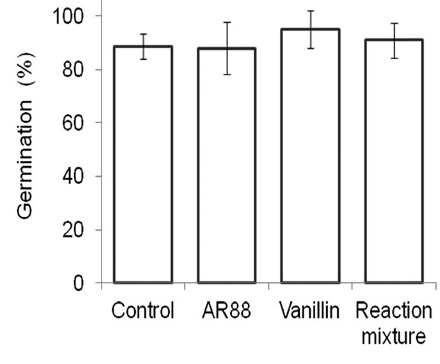
Results of germination for the three samples analyzed (AB172, violuric acid, products of AB172 degradation by LMS) were not statistically different relative to the negative control in the analysis of variance (ANOVA, α = 0.05). This means that no inhibitory effect was observed on the germination of the lettuce seeds for the samples in the concentrations analyzed (Fig. 4).

Regarding the radicle and hypocotyl elongation responses, no inhibitory effect was observed either for the dye and the biodegradation products (Fig. 5). For the redox mediator an inhibitory effect was detected for high concentrations. For the highest concentration the inhibition of radicle elongation was 47.1% and for hypocotyl 46.5%.

In the *Daphnia magna* bioassay, dye AB172 showed less acute toxicity than the reaction products, as observed in the dose–response curves and LD50 values in Fig. 6. An intermediate toxicity with respect to the other two samples was shown by violuric acid.

In the Microtox® assay (Table 2) the redox mediator did not present toxicity whereas both AB172 and the decolorization products were classified as "very toxic" with EC50 values below 25%.The dye Acid Red 88 presented a similar result to AB172 in the phytotoxicity assay since it did not cause inhibition of germination (Fig. 4). Moreover, the same result was seen for the redox mediator vanillin and the degradation products of the dye. In addition, inhibition of radicle and hypocotyl elongation was not observed for the dye or the decolorization products at the tested concentrations (Fig. 5). For vanillin, some inhibition occurred at the highest concentration tested (0.1 mM) on radicle and hypocotyl elongation. Nevertheless, this effect should not interfere with the response observed for the product mixture since vanillin was not detected in the reaction medium at 24 h (ESI†). This is in accordance with the result obtained for the sample of products for which no significant differences were seen at 100% with respect to the negative control in both measured responses.

In the Microtox® bioassay both the dye and the decolorization products showed EC50 values between 25 and



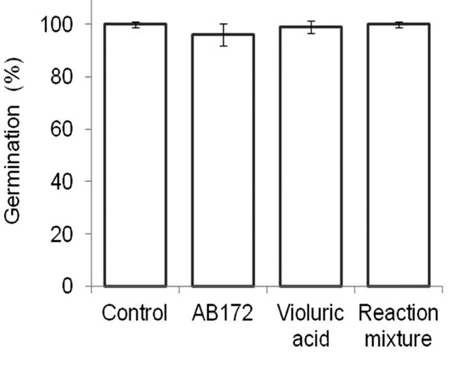


Fig. 4 Germination of *L. sativa* seeds in the presence of the negative control, the dye solution (100 mg L^{-1}), the redox mediator used in the degradation (1.0 mM) and the treated dye solution (reaction mixture), for AR88 (a) and AB172 (b). Data represents means ± S.D.

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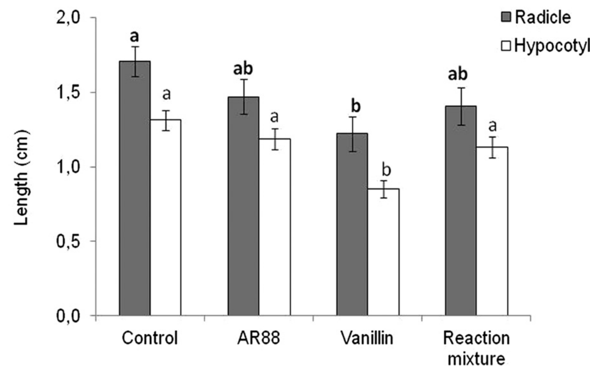


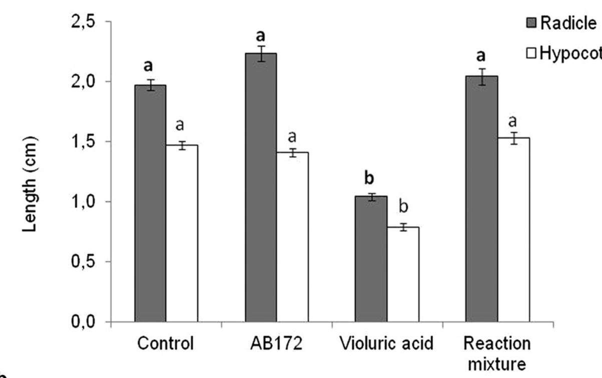


Fig. 5 Radicle and hypocotyl elongation of *L. sativa* seedlings in the presence of the dye (100 mg L^{-1}), the redox mediator used in its degradation by LMS (1.0 mM), the reaction mixture at 24 h (degradation products), and the negative control, for AR88 (a) and AB172 (b). Data represents mean length ± S.D. Means with the same letter are not statistically different according to Tukey's multiple comparison test (α = 0.05).

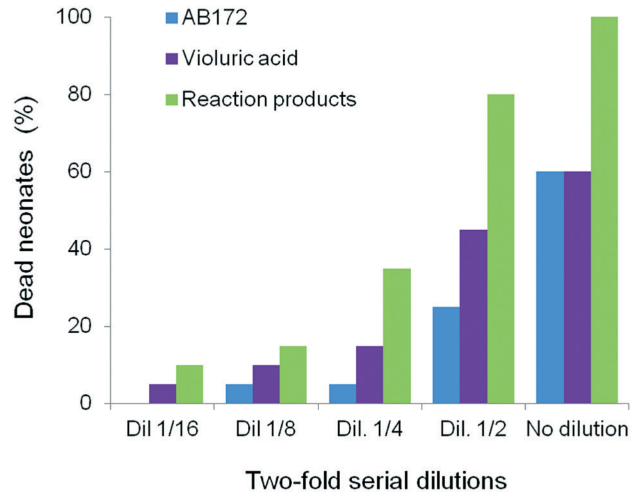
50% so they were categorized as "toxic" and the redox mediator showed no toxicity with EC50 greater than 100% (Table 2).

Table 2 Toxicity classification of samples analyzed in the Microtox® test according to the EC50 value

Sample	EC50 (%)	Classification
AB172	16	Very toxic
AB172 degradation products	11	Very toxic
Violuric acid	>100	Non-toxic
AR88	48	Toxic
AR88 degradation products	28	Toxic
Vanillin	>100	Non-toxic

EC50: effective concentration 50. Confidence interval: 95%.

The comparison of ecotoxicity results with other bibliography data is complex and could be inaccurate since they depend on several factors such as the chemical structure of the dyes as well as the methodology used in the selected bioassay, including the biological model, the endpoint and the expression of results. To evaluate the toxicity of dyes and the products obtained in different decolorization treatments, biological tests that involve very different procedures and organisms have been applied.[55] Regarding the test organisms, the species used belong to very distant taxonomic groups, for example crustaceans such as *D. magna* or *Artemia salina*, animals and animal cells, bacteria, bioluminescent bacteria (Microtox®), yeast, fungi and plant species (seed germination).[56–64] Moreover, in some of these studies a single bioassay was used whereas in others different assays were combined for a more comprehensive evaluation. The use of a battery of bioassays with organisms of different taxonomic ranges allows a better approximation to the environmental impact generated by a chemical compound or a mixture introduced in a given ecosystem.[28] In this study, the organism that showed the greatest sensitivity was the bacterium *V. fischeri*. This bioassay is not only highly sensitive but also shows a high correlation with the results obtained with fish



Sample	LD50 (%)
AB172	86,0
Violuric acid	70,6
Reaction products	31,3

b.

Fig. 6 *D. magna* bioassay for the dye AB172 (100 mg L^{-1}), the redox mediator violuric acid (1.0 mM) and the degradation products of AB172. (a) Percentage of dead organisms for two-fold serial dilutions of the three samples. (b) LD50 values (as percentages from initial concentrations) resulting from data analysis with the Probit model. A confidence interval of 95% was used.

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and *Daphnia*, being therefore adopted as a reference test for the assessment of acute toxicity in aquatic environments.[65] To classify a substance according to its toxicity, different concentrations must be analyzed to apply parameters such as LD50, EC50, NOEC or LOEC. According to the EC50 and LD50 results of Microtox® and *Daphnia magna* tests, respectively, and the criteria established by the Globally Harmonized System of Classification and Labeling of Chemicals (GHS), both dyes and their degradation products are classified as "harmful to aquatic life". This is category three and corresponds to the lowest toxicity for short-term (acute) aquatic hazards.[27]

The concentrations of chemical compounds in effluents for their discharge into watercourses are set by regulatory agencies taking into account the dilution produced in the receiving body.[19] In Uruguay, the current regulations establish that the parameters of toxicity quantification with fish, *daphniids* or *V. fischeri* must be greater than 75% of the concentration present in the effluent for the authorization of the discharge.[66] The estimated concentration of AB172 in the effluent of the textile industry would be approximately 30 mg L^{-1}.[67] Therefore, according to Microtox® results (the most sensitive test) and the established limit of toxicity (EC50 75%) corresponding in this case to 22.5 mg L^{-1}, the discharge of the effluent would not be accepted (EC50: 16 mg L^{-1}) but neither after the treatment applied to it (EC50: 11 mg L^{-1}).

Regarding the phytotoxicity bioassay, the results attained in this work were different from those found by Ma *et al.*[37] where AB172 was toxic to different plant species. It is worth mentioning that in the present work the degradation products also showed no toxicity on *L. sativa*, with EC50 values higher than 100 mg L^{-1}. Without considering other characteristics of the effluent that exceed the study this result suggests a possible destination for the effluent such as irrigation water for different crops, particularly those not intended for human consumption.[23]

Detoxification associated with decolorization by laccase or LMS has been reported in different studies[37,59,62–64,68] whereas in others the toxicity does not change or even increase for certain dyes.[22,60,61,69] In this work, as in the last mentioned, it was shown that there is no strict correlation between decolorization and detoxification.

4. Conclusions

The laccase produced by a Uruguayan strain of the basidiomycete *Trametes villosa* was able to degrade the azo dyes Acid Red 88 and Acid Black 172 without redox mediators, showing greater catalytic efficiency than that of other published works. The extent of decolorization was increased for both dyes by using a laccase–mediator system. Moreover, the viability of applying the immobilized laccase in decolorization reactions and the possibility of reusing the insoluble enzyme were demonstrated. The degradation of both azo dyes could decrease the environmental impact associated with the reduction of light input in the watercourses, and the negative visual effect caused by the discharge of colored effluents could be minimized. It was shown that the decolorization of a dye does not imply a decrease in toxicity. An acute toxic effect of the dyes or their degradation products on *Lactuca sativa* was not observed which suggests a potential application of the treated effluent in the irrigation of plants. However, the enzymatic treatment was not suitable for reducing the aquatic toxicity of both dyes when Microtox® and *Daphnia magna* tests were used. The variable results of ecotoxicological assessment show the importance of carrying out a battery of bioassays involving different biological systems to determine the quality of treated effluents. In future studies, a laccase treatment could be developed for simultaneously decolorizing and reducing the ecotoxicity of the final effluent, in order to promote water sustainability.

Conflicts of interest

There are no conflicts of interest to declare.

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