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Activity of free peroxidases, hematin, magnetite-supported peroxidases and magnetite-supported hematin in the aniline elimination from water-UV–vis analysis

S. Saidman [c], E.H. Rueda [a], M.L. Ferreira [a,b,*]

[a] *Department of Chemistry, UNS, Avda Alem 1253, 8000 Bahía Blanca, Argentina*
[b] *PLAPIQUI-UNS-CONICET, Camino La Carrindanga Km 7, CC 717, 8000 Bahía Blanca, Argentina*
[c] *INIEC, Department of Chemical Engineering, UNS, Avda Alem 1253, 8000 Bahía Blanca, Argentina*

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Abstract

This manuscript presents the UV–vis study of the aniline elimination by polymerization using peroxidases from different sources and hematin, free and supported. The use of magnetite-supported hematin has potential in the implementation of aniline elimination by polymerization in acidic media at pH 4. The temperature must be selected between 45 and 65 °C. Crude preparations of *Pleurotus sajor caju* (PSC) are an alternative to purified *Horseradish peroxidase* (HRP) as the catalyst of the reaction. Pernigraniline is the main species found in the reaction media after aniline elimination procedure. Supported oxidoreductases are active biocatalysts and conversion of aniline in the 30–90% range can be obtained, depending on the catalyst. The UV–vis spectra show bands corresponding to base and protoned (blue and purple-deep red) pernigraniline, a contribution of quinoid species at 480 nm and a minor contribution of base blue emeraldine and another oxidation intermediate states between pernigraniline and emeraldine. At selected conditions, the recovered solid showed greenish/reddish coloration. The goal is the precipitation of the aniline as polyaniline. Supported biocatalysts were less active than free enzymes.
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1. Introduction

With the increasingly stringent standards for the discharge of wastes into the environment, alternative processes for the treatment and disposals of wastes are more and more studied. The new technologies must meet the following objectives: (1) to recycle water-streams within a given facility to reduce the need of effluent disposal; (2) to reduce the quantity of effluent stream; (3) to convert wastes into marketable, non-toxic products. The methods used for carrying out the above objectives can be chemical or biological. Researchers have focused their attention on the environmental application of individual enzymes as an alternative route to the existing methods. With respect to anilines, the potential, scope and cost of enzymatic treatment using a crude enzyme preparation of soybean peroxidase was reported to be about 0.36 USD/m^3 for synthetic wastewater containing 1 mM aniline compared to an activated sludge process of 1.05 USD/m^3 and 1.31 USD/m^3 for activated carbon process. Thus, through choice of enzyme and its mode of operation, the treatment costs less than the conventional treatment strategies can be achieved [1]. Alternative chemical methods are being also explored. Recent work of Wang et al. demonstrated that the preparation of polyaniline using an acidic solution with H_2O_2 as oxidant and ferrous chloride as a catalyst is feasible [2]. This kind of systems, used for polyaniline synthesis, can be modified to eliminate aniline from wastewaters by polymerization.

Aromatic amines (AA) are used as intermediates in the manufacture of plastics, dyes, drugs and carbamate pesticides. These compounds are highly toxic, carcinogenic or mutagenic and impose serious health hazards to mankind and reach the environment from a variety of sources and routes.

The available conventional processes to eliminate AA include activated carbon adsorption, solvent extraction, microbial degradation and chemical oxidation. Most of them are not selective [1]. Such treatment strategies are more suitable for dilute wastewaters. They suffer from harsh conditions, high cost and

* Corresponding author. Tel.: +54 291 4861700; fax: +54 291 4861600.
E-mail address: mlferreira@plapiqui.edu.ar (M.L. Ferreira).

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concentration of oxidants, incomplete removal, formation of toxic by products and applicability to a limited concentration range. Use of Fenton's reagent to degrade aromatic amines showed that the oxidant (H_2O_2) to substrate ratio was still much too high. Biological treatments can be very effective, but may not remove specific chemicals at low levels consistently [3]. O'Neill et al. biodegraded aniline under aerobic condition [4].

Peroxidases, in presence of H_2O_2 and laccases, in presence of O_2, catalyze the oxidation of a wide variety of phenols, biphenyls, anilines, benzidines and other aromatic compounds. The mechanism of one electron oxidation of aromatic substrates by peroxidases is usually presented as:

$$E + H_2O_2 \rightarrow E_i + H_2O \quad (1)$$

$$E_i + AH_2 \rightarrow E_{ii} + AH^{\bullet} \quad (2)$$

$$E_{ii} + AH_2 \rightarrow E + AH^{\bullet} + H_2O \quad (3)$$

where AH_2 is the aromatic substrate, E is the native enzyme, which reacts to generate E_i (Compound I). E_i is oxidized by two electrons and carries the peroxidic O. Compound I carries out the one electron oxidation of AH_2 and the enzyme is reduced to E_{ii} (compound II). This compound II oxidizes a second aromatic molecule and regenerates the native enzyme. The global reaction is:

$$2AH_2 + H_2O_2 \xrightarrow{E} 2AH^{\bullet} + 2H_2O \quad (4)$$

The generated radicals combine to form dimers, trimers and further oligomers by a non-enzymatic reaction until they reach their solubility limits and precipitate. The Compound II can be oxidized by excess H_2O_2 to form catalytically inactive compound III. The formation of an inactivated intermediate P670 verdohaemochrome (inherently unstable and difficult to detect) can also take place. P670 is dominant at H_2O_2 concentration above 1 mM; however, the presence of a concentration of reducing substrate similar to that of peroxide inhibits the formation of P670. When produced by treatment of HRP with excess hydrogen peroxide (via Compound II), Compound II decay by two different pathways: formation of an inactive species (P670 and also P630) and with a smaller rate, decay to the HRP resting state (via Compound II). Compound III formation was shown to first involve a transient Fe(III)-superoxide complex, which reacts with a second hydrogen peroxide (or peracetic acid, but not *m*-chlorobenzoic acid) molecule to yield Compound III. Some aminoacid residues within the protein are also oxidized during these processes. Additives (polyethylenglycol, gelatin) have no effect in the peroxidase inactivation when the substrate is aniline [4]. A decreasing of the costs could be achieved from the enzyme immobilization on suitable supports, allowing the recuperation and reusing of the biocatalyst.

The white-rot fungus (WRF) produce multiplicity of enzymes, acting synergically such as celobiohydrolases (CBHs), celobiose oxidasa and quinone oxidoreductasa (CBQase). These fungus produce also unique combinations of peroxidases and oxidases [5–7]. *Pleurotus sajor caju* (PSC) produces a laccase and several peroxidases [8]. These WRF are sources of hydrogen peroxide [9]. There are several reports on the application of WRF in the polymerization of phenols [10–15]. The crude preparation of these fungus offers the possibility of reducing costs in the application of the enzymatic system to the industrial scale.

Another option instead of the enzymes is the use of biomimetic compounds. Peroxidases include a heme group, needed cofactor for the reaction to take place. Heme is the protoporphyrin IX complex of the ferrous ion (Fe^{2+}). Hemin is the protoporphyrin IX complex of the ferric ion (Fe^{3+}). Hematin is the hydroxilated hemin [16]. This biomimetic compound would avoid the problems associated to the lack of specificity found when using crude enzymatic preparations. Degradation of aniline using electrochemical and photocatalytic conditions has been recently reported. Besides, the use of electrochemical tools to degrade aniline is another application of Fe-based compounds [17–19]. Mineralization of aniline has been studied using anodic oxidation, photocatalysis, electro-Fenton and photoelectro-Fenton processes with success [19]. New methods, such as phytoremediation, have been recently reported for degradation of polyaromatic hydrocarbons, anilines and phenols [20].

There are a lot of published manuscripts about aniline polymerization using electrochemical, enzymatic and chemical methods, to obtain conductive polyaniline (cPANI) [17,2,21–23]. In this sense, the main goal of the published works is to generate cPANI, in the emeraldine form, soluble in several solvents with controlled molecular weight and structure. But this is not our goal with the present report.

This work presents the results of *aniline elimination* from artificially contaminated water by aniline oxidation using H_2O_2 as oxidant, HRP, crude extracts of *Pleurotus sajor caju* (PSC) and hematin, at selected conditions and at temperatures between 20 (room temperature- r.t.) to 65 °C. Magnetite (Fe_3O_4) was selected as support because it adds the interesting possibility of recovery the biocatalyst using the intrinsic magnetic properties of the support. Glutaraldehyde was selected as coupling agent between peroxidase and the oxide [24]. There is no need of surface modification to have reaction between an aldehyde and an oxide with Lewis acidic sites such as Fe_3O_4. Bare surfaces of oxides, such as Al_2O_3, are able to react with aldehydes with the formation of acetate and ethoxi groups at surface. These reactions have been supported by FT-IR studies [25].

It is important to point out that the intention is not to synthesize polyaniline, but to eliminate aniline from contaminated water. The main goal of the polymerization reaction is to eliminate aniline and to maximize the production of insoluble material, maximizing then the recovery of the effluent and minimizing the conductivity and redox species in solution. The aim of this manuscript is focused in the elimination of any residue of aniline (or oligomers) from solution. Oligomers could be subsequently adsorbed in a downstream step with activated carbon. A lower amount of adsorbent would be necessary than the required for the treatment of the initial aniline contaminated wastewater. Even in this case, we would have an oligomer of aniline adsorbed. In this sense, we are not interested on the properties of the obtained solid

of aniline polymerization, but in the properties of the remaining solution. The desirable properties are related to minimum conductivity and charge under potential, low degree of coloration and/or potential use of this stream in alternative uses.

2. Experimental

2.1. Materials and methods

Horseradish peroxidase (HRP) was generously provided by Amano Inc. (EEUU). Crude extracts from *Pleurotus sajor caju* (PSC) were provided by the Dr. Curvetto's group (CERZOS, CRIBABB, Argentine) using methods published elsewhere (Ferreira et al. [26]). Hematin was obtained from Sigma Inc. Buffers and reagents were provided by Sigma-Aldrich (HPLC grade).

The conversion of aniline was checked using UV–vis and gravimetry of the recovered solid. Chromatographic analysis was used to test the aniline concentration in aqueous solutions when the gravimetry gave different results than the UV–vis data.

2.2. Enzymatic catalysts-HRP and crude PS

The pH generally used in the chemical synthesis of aniline is off the normal pH scale ([HCl] = 5 M). The chlorhydric acid concentration must be extremely high and the environmental impact of the PANI industry is related to this. The pH was selected to allow the aniline to be protoned during the reaction. Since the p*K*a of aniline is 4.6, a pH of 4 is sufficient to provide the necessary cationic charges which promote alignment and polymer formation [21]. We used then pH 4 for HRPC and Hematin as catalysts. In the case of PSC, it is known that the lignolytic enzymes can be deactivated at pH 3, therefore, the selected pH was seven to assure optimal conditions [13].

The reaction was carried out using 50 μL aniline (0.05753 M aniline), 0.80 mL of hydrogen peroxide 30% (v/v)–9% (w/v)–(0.215 M H_2O_2)-added in aliquots of 0.2 mL in steps of 20 min- and 9 mL pH 4 buffer ($KHC_8H_4O_4$/HCl) with magnetic stirring. In the case of free HRP 1.1 mg (0.111 g/L) were used and 20 mg (2.03 g/L) of the immobilized HRP. The provider reported 240 U/mg and we confirmed this activity in our lab. After 2.5 h 1 mL of HCl 37% was added. UV–vis studies were carried out after HCl adding and after 24 h.

In the case of PSC, the reaction conditions were 50 μL aniline (0.0671 M) in 8 mL of pH 7 buffer (KH_2PO_4/NaOH) in continuous magnetic stirring. 53.1 mg of crude PSC and 0.40 mL of hydrogen peroxide 30% (v/v)–9% (w/v)–(0.125 M) were added or not. Also we studied another conditions: 34.0 mg crude PSC and 0.80 mL H_20_2 (0.25 M). After 2.5 h, 1 mL HCl 37% was added.

2.3. Hematin

The reaction was carried out using 50 μL aniline (0.05753 M aniline), 0.80 mL of hydrogen peroxide 30% (v/v)–9% (p/v)–(0.215 M H_2O_2) and 9 mL pH 4 buffer with magnetic stirring and 1.3 mg hematin. After 2.5 h 1 mL of HCl 37% was added. UV–vis studies were carried out after HCl adding and after 24 h. In the case of the supported hematin 20 mg were used in a first step, at 45 and 65 °C. In a second step, the temperature was fixed at 45 °C and 50 or 100 mg of supported hematin were used as catalyst.

2.4. Immobilized biocatalysts

2.4.1. Preparation of magnetite

0.4001 g of magnetite (Fe_3O_4) were added to 2 mL glutaraldehyde 25%, at 80 °C for 3.5 h. The resulting mixture was filtered, washed several times with bidestilled water and heated at 40 °C to eliminate residual water. 0.2998 g were recovered (33.3% yield).

2.4.2. Preparation of immobilized biocatalysts

0.0030 g HRP were contacted in 5 mL pH 4 buffer with 0.1385 g of glutaraldehyde treated-magnetite. Interaction between glutaraldehyde and magnetite has been described in a previous paper [24]. After 2 h at 40 °C, the solution was filtered, washed with distilled water and dried. 0.1030 g of supported HRP were recovered because of magnetite-attrition (72.8% yield). Taking into account the conversion of aniline using free HRP (corresponding to 1 mg HRP), we obtained the amount of fixed HRP considering the activity of the supported versus the free enzyme:

$$\frac{(\text{Conversion of aniline in converted mols of aniline/mass catalyst})_{\text{supported enzyme}}}{(\text{Conversion of aniline in converted mols of aniline/mass catalyst})_{\text{free enzyme}}}$$

The amount of HRP fixed on magnetite is nearly 2.9% in mass and this fact implies that almost 100% of HRP was fixed. Using 10 mg of supported HRPC we are using 0.3 mg of HRP. The enzyme concentration was 264 U HRP (1.1 mg) for free peroxidase and 144 U for supported peroxidase (20 mg of supported HRP). If the Units per mg of HRP are 240, then in 20 mg we have 0.6 mg of HRP and therefore this is 144 Units (240 Units *0.6 mg). These units (calculated with the one provided by the supplier) were rechecked with the 4-aminoantypirine method and confirmed [14]. The reaction with the free HRP was included in terms of comparison and to perform a quantitative method (at room temperature) to determine the amount of HRP fixed on glutaraldehyde treated-magnetite.

0.0202 g of hematin were added to 0.1606 g of glutaraldehyde-treated-magnetite at pH 4. After 2 h at 40 °C, solution was filtered and dried 2 days. 0.0666 g were recovered (36.8% yield). The amount of fixed hematin was near 9.5–10% of the recovered solid. This amount was determined by the 1–10 phenantroline method using a calibration curve (9159 M^{-1} cm^{-1}). Measurement of free hematin was included in terms of comparison.

2.5. Effect of temperature

Free HRP and crude PSC were tested at room temperature. Immobilized HRP was also tested at 45 and 65 °C. The same experiments as HRP were performed for unsupported and supported hematin.

2.6. Experimental methodology

2.6.1. UV–vis and gravimetric studies

UV–vis studies were performed using 0.2 mL of the reaction solution diluted at 5 mL with distilled water. Spectra were recorded after HCl adding, after 1 day of reaction and sometimes after 20–70 days after adding the HCl precipitating reagent, in closed vials. 1 mL of these solutions was centrifuged and the UV–vis spectra of 100 μL of these solutions diluted at 10 mL with distilled water were recorded. The concentration was changed to observe the peaks in the 200–400 nm range. The aniline conversion was obtained considering the changes in the UV–vis spectra after flocculation and the separation of the insoluble Polyaniline by filtration.The obtained solid was dried and weighed and the performance calculated as follows:

((mg solid after filtration and drying

−mg used immobilized catalyst) × 100)/50 mg

where 50 mg corresponds to the initial mass of aniline in solution.Aniline conversion was obtained using the same wavelength following:

$((A_0 - A_f)/A_0) \times 100$

being A_0 and A_f initial and final absorbance at a given wavelength (280 nm-E band of aniline). However, this is considered to be a minimum conversion. The contributions of oligomers and soluble polymers are expected to be located at longer wavelength because of a batochromic effect.

2.6.2. Chromatographic studies

A column (10% Carbowax 1540 on Chromosorb W HP) was used in a Varian 3700 with the oven at 180 °C, injector at 290 °C, detector at 90 or 60 °C with a flow of 20 mL/min of carrier. Experiments with water as solvent allowed us to perform a calibration curve with $R^2 = 1$ and an equation for the aniline quantification:

$$53\,928 \times 10^7 \text{ Area units per mol/L} \times [\text{aniline}](\text{mol/L}) = \text{Obtained area(Area units)} \quad (5)$$

Response was linear in the range of concentration considered. One milli litter from solution of polymerization test was centrifugated 10 min at 6000 rpm (or 2000 maximum relative centrifugal force, RCF). After that, the solid was separated by centrifugation and the solution was diluted at 5 mL. Several chromatographic injections demonstrated reproducibility in results. Dichloromethane was used as alternative solvent to avoid that the precision decays when water is solvent. To 1 mL of the acidic solution (obtained after flocculation with HCl), 100 mg KOH and 4 mL dichlorometane were added and shaken by 15 min. Then, between 5 and 8 uL of the organic phase were injected in the chromatograph to be analysed.

2.6.3. Conductimmetry measurements

A Oakton 35607-00 basic conductivity/TDS meter was used. Measurements were done in the aqueous solutions at 1:10 dilution with double distilled water of 1 mL from the flocculation step.

2.6.4. Voltamperometric studies

Vitreous carbon rods embedded in a Teflon holder with an exposed area of 0.070 cm^2 were used as working electrodes. Before each experiment, the electrode was polished with 200, 600 and 1000 emery paper, degreased with acetone and finally washed with twice distilled water.

Electric potentials were measured with a saturated calomel electrode (SCE/platinum electrode pair) inside a 20 cm^3 Metrohm cell coupled to a linear voltage sweep generator PAR model 175 and a potentiostat–galvanostat PAR model 173 with a HP 4007 B x–y recorder. Cyclic voltammetry was also used to qualitatively compare the redox behavior of soluble PANI obtained under different conditions. Data were recorded in the range of −0.20 and 0.80 V (SCE) at a scan rate of 0.050 V s^{-1}. Voltamperommetry was carried out of the remaining solutions after filtration and centrifugation. Dilution was 0.1 mL in 10 mL.

3. Results and discussion

3.1. Enzymatic catalysts

3.1.1. HRP

Fig. 1 shows the spectra of the initial solution after the adding of aniline. HRP, hematin or PSC do not show peaks in this range (results not shown). This figure shows also the spectra obtained initially and after flocculation, at room temperature. Using a higher dilution, after about 70 days, no polyaniline was found in the remaining solution (see Figs. 1–3). Fig. 2 shows the efficiency of the immobilized HRP. The conversion of ani-

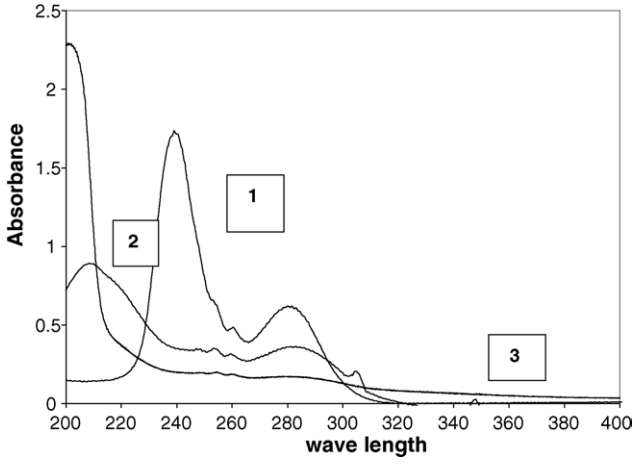

Fig. 1. UV–vis spectrum of sample obtained with free HRP at room temperature: initial (1); after flocculation (1 day) (2); after 70 days (3).

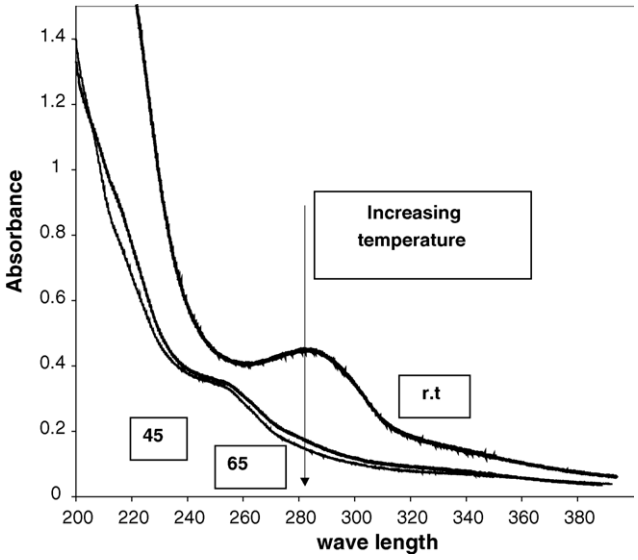

Fig. 2. Efficiency of immobilized HRP at different temperatures.

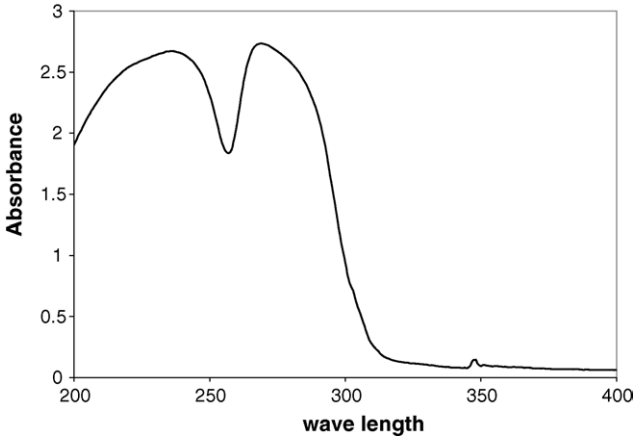

Fig. 3. UV–vis spectrum of the initial solution (using PSC).

line increases with the increase of temperature using supported HRP.

3.1.2. PSC

Fig. 3 shows the initial spectra of aniline in PSC solution. Fig. 4 shows the spectra after 36 h, without the added hydrogen peroxide and with the hydrogen peroxide added. Fig. 5 shows

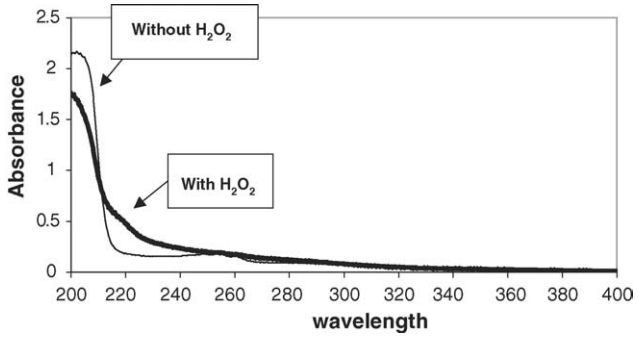

Fig. 4. UV–vis spectra after floculation using PSC, with or without H_2O_2.

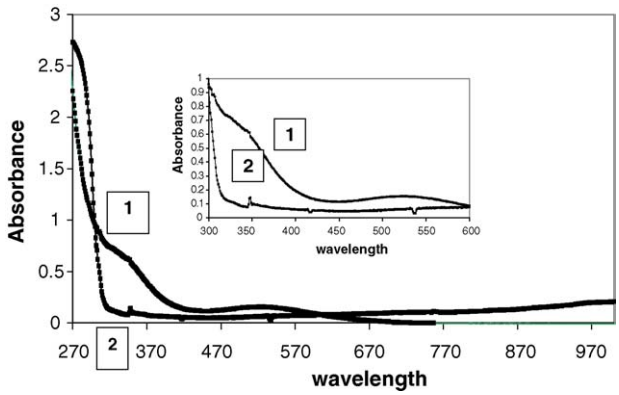

Fig. 5. UV–vis spectra of solution initial and after flocculation, using PSC and H_2O_2: initial (1); after flocculation (2).

the difference between the initial and final solution, using H_2O_2 in the reaction media.

The PSC solution is supposed to include some soluble species (probably with double bonds) with a different spectrum than in the case of HRP. This species do not flocculate when the pH is lowered. The solid obtained after flocculation is insoluble PANI. This fact is clear when Figs. 1 and 3 are compared. From Fig. 4 an increase in the absorbance by the presence of H_2O_2 is obvious. This behavior can be related to the presence of H_2O_2 producing enzymes in the PSC crude extract. Also, peroxidases from PSC can be inactivated by high level of H_2O_2 and the resulting amount of PANI soluble species is low because the generation of radicals decreases when H_2O_2 is present. Using H_2O_2, flocculation eliminated several soluble species and/or changed the spectra of remaining ones (see Fig. 5).

3.2. Biomimetic catalyst: hematin

Fig. 6 shows the difference found using free or immobilized hematin at room temperature. Fig. 7 shows the spectra obtained at different temperatures with supported hematin. With the increase of amount of supported catalysts the results are different (See Fig. 8a). The strong band in 240–250 nm can be assigned to some kind of reaction of the pernigraniline (in the solution) with the coupling agent (glutaraldehyde) or even the support (magnetite). Species in solution are found with higher concentration at low temperatures than at high temperatures (see Fig. 8b).

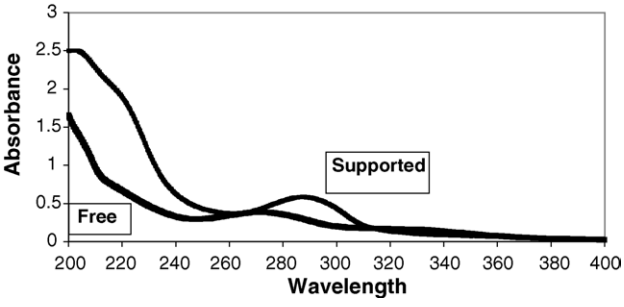

Fig. 6. UV–vis spectra of samples obtained with hematin free and supported, room temperature.

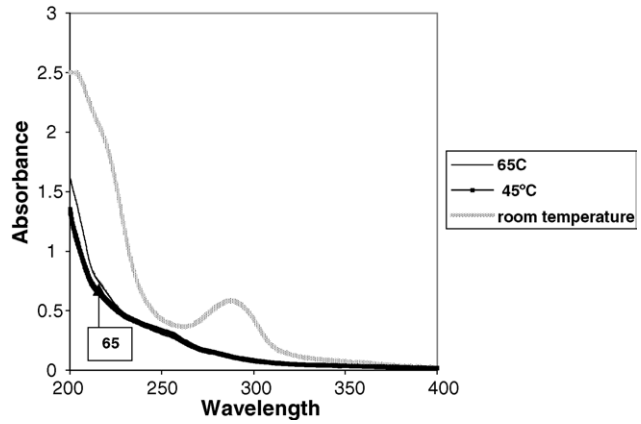

Fig. 7. Effect of temperature in the supported hematin efficiency.

3.3. Activity of enzymatic and no enzymatic biocatalysts

The conversion of aniline was evaluated after 1 day of reaction. Conversion to soluble and insoluble products was different because an important degree of polymerization must be achieved to obtain the insoluble material. The total conversion is expected to be higher than the conversion to insoluble material. Upon storage, there is a continuous increase of the amount of precipitate. Therefore, the amount of insoluble material reported is in fact a minimum amount of insoluble material obtained after 1 day of flocculation. Table 1 summarizes these results.

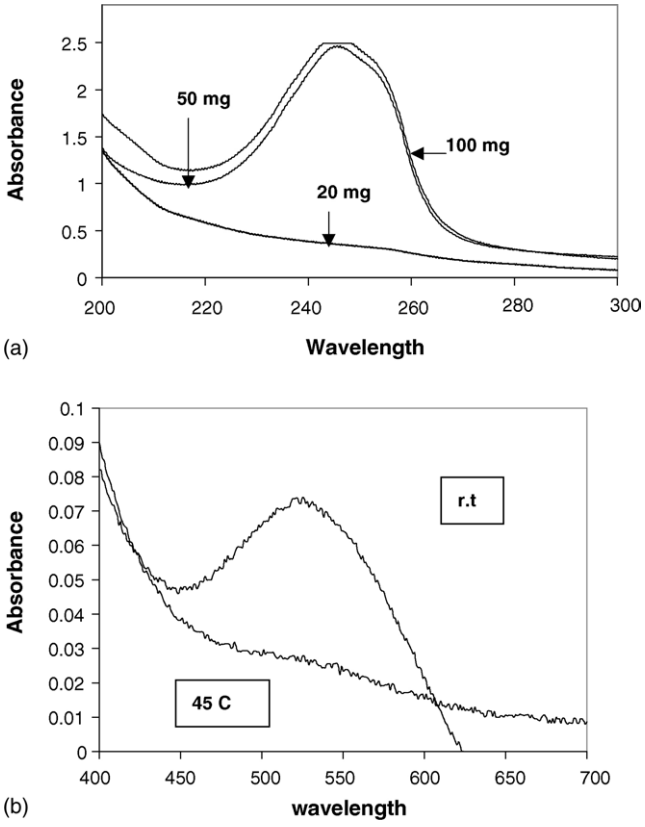

Fig. 8. (a) Effect of the amount of supported hematin in the UV–vis spectra of the solution after flocculation. (b) Remaining species in solution after floculation at different temperatures: room temperature and 45 °C using supported hematin.

Free HRP and supported HRP showed the highest level of conversion (from 79.1 to near 84%). The free HRP showed also a high level of conversion to insoluble material, only similar to supported hematin at 45 °C (about 62–65%). Supported HRP produced more soluble material than free HRP (near 25–33% versus 63%). Supported hematin at room temperature produced only near 30% total aniline conversion by UV, whereas at higher temperature the conversion achieved 82–83%. At 65 °C conversion to insoluble material using supported hematin decreased. The increase of the amount of the supported catalyst does not improve the conversion. The relative mass of recovered insoluble material is always higher for supported hematin than for supported HRP. To check the high aniline conversion, some chromatographic results are included and they correlate very well with the data obtained by UV–vis method. However, some problem arises in the aniline reaction with the supported hematin at room temperature because the chromatographic measures correlated with the gravimetric recovery (see Table 1).

3.4. Characterization of the solution after flocculation

3.4.1. UV–vis

The polyaniline solution is green in its emeraldine salt form, blue in its emeraldine base or nigraniline form, colorless as reduced polyaniline and purple as oxidized polyaniline. Figs. 9 and 10 shows the different interconversion between structures.

Fig. 1 shows the decrease of the absorptions of aniline at 230–240 and 285 nm. There are also peaks at 305 nm, where leucoemeraldine absorbs. After 70 days and with centrifugation, almost no polyaniline bands are present (see Fig. 1). Fig. 2 shows the efficiency of the supported HRP in the elimination of aniline. There are absorptions assignable to anilinium ion. The peak at 528 nm can be assignable to pernigraniline, coincident with the purple coloration of the solution. In the case of PSC, a peak at 532 nm (not shown) can be assignable to non-oxidised emeraldine and not to pernigraniline. In the case of using H_2O_2, with PSC, a peak at 298 nm can be assignable to protoned leucoemeraldine in solution. When hematin is the catalyst, the support seems to leave leucoemeraldine in solution plus pernigraniline, whereas in the case of free hematin, only pernigraniline is present (see Fig. 6). At a higher temperature, the conversion to insoluble material is higher and this is correlated with the decrease of the pernigraniline bands in solution (see Figs. 7 and 8).

It is known that the fully reduced tetramer is necessary to polymerization [2]. The octamer is not obtained through the oxidation of the middle-oxidized tetramer, but it could be obtained when tetramer is fully reduced [2]. The peroxidase/hematin reaction involves the reactions:

$$\text{CompoundI} + 1e^- + 1H^+ \rightarrow \text{CompoundII}$$

$$\text{CompoundII} + 1e^- + 1H^+ \rightarrow \text{Native}$$

Also, other reactions are known to generate $e^- + H^+$ (reaction of ferrous peroxidase from compound III to native enzyme [16]). Therefore, leucoemeraldine becomes emeraldine base blue and

Table 1
Total conversion of aniline and conversion to insoluble material

Sample	Percentage conversion (UV–vis after 1 day reaction)	Percentage Conversion to insoluble material	Color remaining solution after floculation with HCl
1-Hem. sup. 45 °C–100 mg HemS45-100	68.8	n.a.	Dark brown
2-Hem. sup. 45 °C–50 mg HemS45-50	78.1	n.a.	Dark purple
3-Hem. sup. 65 °C–20 mg HemS65-20	82.4 89 Water as solvent[a]	36.4	Dark purple
4-Hem. sup. 45 °C–20 mg HemS45-20	82.7 78.8-Dichloromethane[a]	64.8	Dark purple
5-Hem. sup. room temperature 20 mg HemSrt-20	29.8 Higher than 50%[a]	49	Dark purple
6-Hem. free with H_2O_2 HemFrt	54.5	46.2	Dark purple
7-HRP sup. 65 °C HRPS-65	83.5	33	Dark purple
8-HRP sup. 45 °C CHRPS-45	79.1	25.8	Dark purple
9-HRP sup. room temperature HRPS-room temperature	45.6	28.6	Dark purple
10-HRP free room temperature HRPFrt	81.2	62.2	Dark purple
11-PSC with peroxidev PSC-1	69.2	n.a.	Dark brown
12-PSC without peroxide PSC-2	87.2	n.a.	Brown

[a] Obtained with chromatography.

finally pernigraniline base (see Fig. 10). When HCl is added a mixture of pernigraniline blue and disordered emeraldine are formed. Intermediate states are possible too (disordered, not para directed).

The spectra show bands corresponding to base and protoned pernigraniline (blue and purple-deep red), contribution of quinoid species at 480 nm and minor contribution of emeraldine base blue and other oxidation intermediate states between pernigraniline and emeraldine, of deep red coloration. Leucoemeraldine contribution cannot be ruled out. Under selected conditions, the recovered solid showed greenish/reddish coloration.

3.4.2. Preliminary voltamperometric/conductimetric analysis

A sample with good cyclic behavior is a sample that demonstrated high charge and reversibility in the test of cyclic voltamperommetry. Sample 5 (HemSrt-20) is the best in terms of charge and cyclic behavior in cyclic voltamperometric measurements. Samples 1 (HemS45-100), 2 (HemS45-50), 9 (HRPS-rt), 10 (HRPFrt) and 12 (PSC-2) show high charges that grow during the cycling. Samples 3 (HemS65-20), 6 (HemFrt), 8 (HRPS-45) and 11 (PSC-1) give low charge that do not grow during the cycle. Samples 4 (HemS45-20) and 7 (HRPS-65) almost give no charge. Figs. 11 and 12 show the relationship between total/insoluble conversion of aniline and conductimetric measurements in the remaining solutions. Samples 5 (HemSrt-20), 6 (HemFrt) and 9 (HRPS-rt) showed the lower conversion. Samples "out of trend" 1, 10 and 12 were obtained with HRP free, PSC without peroxide and supported hematin at high catalyst concentration. The best results were obtained with supported hematin and HRP.

The solution from aniline elimination by polymerization after flocculation must present low conductivity, no charge in voltamperommetry studies and low level of coloration. This goal is very

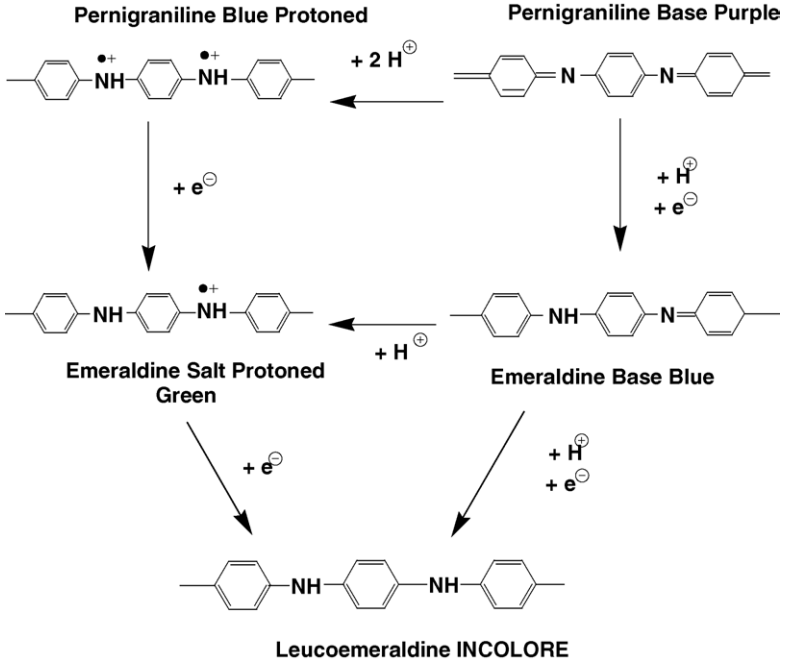

Fig. 9. Structures of oligomers of aniline in solution.

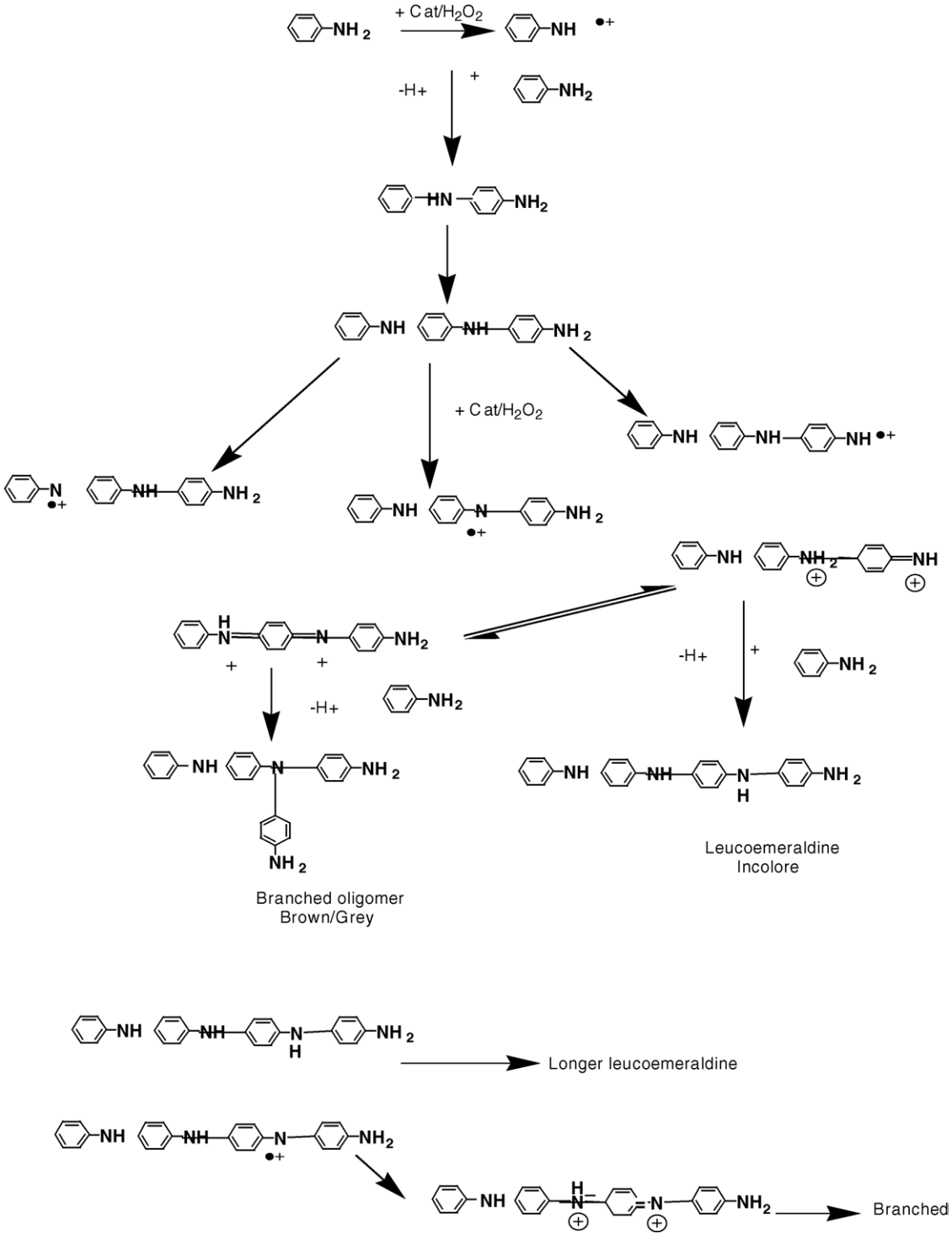

Fig. 10. Steps in aniline oligomerization.

difficult to obtain in only one step. However, we obtained the transformation of aniline in oligomers/polymers that, although partially soluble, are different from aniline.

In terms of insoluble material, free HRP (Sample 10) produces always more conversion than the supported (Samples 7–9). However, free Hematin (Sample 6) produces less insoluble material than the supported (Sample 4). Higher conversion to insoluble material is related to higher conductivity in the remaining solution, but Sample 4 does not follow this trend and it is especially useful in this sense. In case of supported hematin a higher amount of recovered solid is not correlated with higher conductivity in the remaining solid. Conductivity is very similar to Sample 8, obtained with supported HRP, which produces about 26 % conversion to insoluble material.

This kind of studies is very important for the application of new technologies in the aniline elimination from wastewaters.

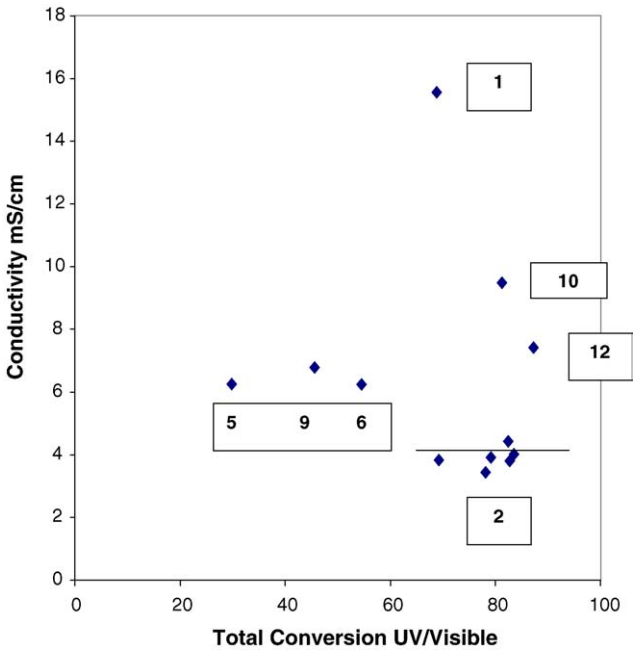

Fig. 11. Conductivity vs. total conversion of aniline: sample 1, HemS45-100; sample 2, HemS45-50; sample 3, HemS65-20; sample 4, HemS45-20; sample 5, HemSrt-20; sample 6, HemFrt; sample 7, HRPS-65; sample 8, HRPS-45; sample 9, HRPS-rt; sample 10, HRPFrt; sample 11, PSC-1; sample 12, PSC-2. Samples identified by numbers.

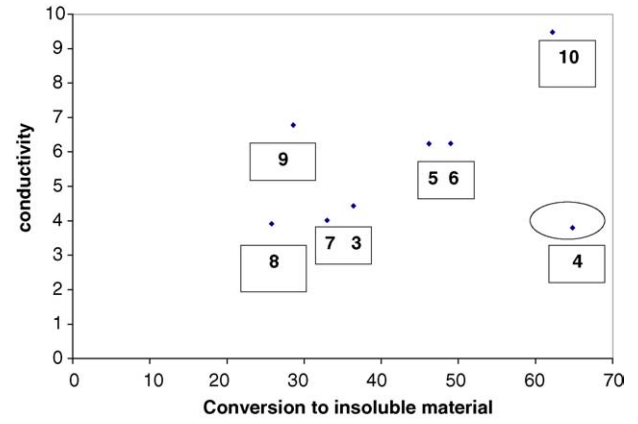

Fig. 12. Conductivity vs conversion to insoluble material: sample 1, HemS45-100; sample 2, HemS45-50; sample 3, HemS65-20; sample 4, HemS45-20; sample 5, HemSrt-20; sample 6, HemFrt; sample 7, HRPS-65; sample 8, HRPS-45; sample 9, HRPS-rt; sample 10, HRPFrt; sample 11, PSC-1; sample 12, PSC-2.

New methods such as the electro-Fenton and peroxi-coagulation have been developed recently [19]. Further, studies are necessary to address the complete potential of the approach presented in this manuscript.

4. Conclusion

The use of magnetite-supported hematin has potential in the implementation of aniline elimination by polymerization at concentrations of 20 mg supported hematin per 50 μL aniline in acidic media at pH 4. The temperature must be selected between 45 and 65 °C. PSC seems to be an alternative to HRP, but further studies are necessary to develop the supposed potential of this system, probably using a more purified extract. The solutions present pernigraniline as the majority species, after flocculation step. Supported hematin demonstrated also to generate high amounts of insoluble material. Pernigraniline remaining in solution could be treated in a second step. The studies of the minimization of the soluble remaining pernigraniline will be the focus of a forthcoming manuscript.

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References

[1] R. Mantha, N. Biswas, K.E. Taylor, J.K. Bewtra, Treatment of Wastewaters Containing Anilines Using Enzymes: an Overview CSCE/EWRI of ASCE Environmental Engineering Conference, Niagara, 2002.
[2] Z. Sun, Y. Geng, J. Li, X. Wang, X. Jing, F. Wang, Catalytic oxidation polymerization of aniline in a H_2O_2–Fe^{2+} system, J. Appl. Polym. Sci. 72 (1999) 1077–1084.
[3] J.W. Patterson, In Industrial Wastewater Treatment Technology, 2nd ed., Butterworths, Boston, MA, 1985 (pp. 101–113).
[4] F.J. O'Neill, Bromley-Challenor, R.J. Greenwoood, J.S. Knapp, Bacterial growth of aniline: implications for the biotreatment of industrial wastewater, Wat. Res. 34 (2000) 4397–4409.
[5] K.H. Perie, M.H. Gold, Manganese regulation of manganese peroxidase expression and lignin degradation by the white-rot fungus, Appl. Environ. Microbiol. 57 (1991) 2240–2245.
[6] G. Fahraeus, B. Reinhammar, Large-scale production and purification of laccase from cultures of Polyborus versicolor and some properties of laccase, Acta Chem. Scand. 21 (1967) 2367–2378.
[7] M.L. Niku-Paavola, E. Karhunen, P. Salola, V. Raunio, Lignolytic enzymes of the white-rot fungus Phlebia radicata, Biochem. J. 254 (1988) 877–884.
[8] E. Fukuzumi Odier (Ed.), Lignin: Enzymic and Microbial Degradation París, Institute National of la Recerche Agronomique, 1987, pp. 137–142.
[9] M.D. Aitken, R. Venkatadri, R.L. Irvine, Oxidation of phenolic pollutant by a lignin degrading enzyme from the white-rot fungus Phanerochaete chrysosporium, Wat. Res. 23 (4) (1989) 443–450.
[10] L. Al-Kassim, K.E. Taylor, J.K. Bewtra, N. Biswas, in: K.G. Welinder, S.K. Rasmussen, C. Penel, H. Greppin (Eds.), Aromatic Removal from Water by Arthromyces Ramosus Peroxidase Plant Peroxidases: Biochemistry and Physiology, 1993, pp. 197–200.
[11] L. Al-Kassim, K. Taylor, J.A. Nicell, J.K. Bewtra, N. Biswas, Enzymatic removal of selected aromatic contaminants from wastewater by a fungal peroxide from Coprinus macrorhizus in batch reactors, J. Chem. Technol. Biotechnol. 61 (1994) 179–182.
[12] M.S. Ibrahim, H.I. Ali, K.E. Taylor, N. Biswas, J.K. Bewtra, Optimization of the Removal of Phenol from Wastewater Catalyzed by Arthromyces Ramosus Peroxidase CSCE/ACSE Environmental Engineering Conference, vol. 2, Edmonton, Alberta, Canada, 1997.
[13] C. Kauffmann, B.R. Petersen, M.J. Bjerrum, Enzymatic removal of phenols from aqueous solutions by Coprinus cinereus peroxidase and hydrogen peroxide, J. Biotechnol. 73 (1999) 71–74.

[14] I.D. Buchanan, Y.S. Han, Assesment of the potencial of Arthromyces ramosus peroxidase to remove phenol from industrial wastewater, Environ. Technol. 21 (2000) 545–552.
[15] M. Masuda, A. Sakurai, M. Sakakibara, Effect of reaction conditions on phenol removal by polymerization and precipitation using Coprinus cinereus peroxidase, Enzym. Microb. Technol. 28 (2001) 295–300.
[16] H.B. Dunford, Heme Peroxidases, John Wiley, VCH, USA, 1999, p. 43.
[17] W.S. Huang, A.G. Mc Diarmid, Optical properties of polyaniline, Polymer 34 (9) (1993) 1833–1846.
[18] H.C. Lim, Y.J. Yoo, Synthesis of ortho directed polyaniline using *Horseradish peroxidase*, Process Biochem. 36 (2000) 233–241.
[19] (a) E. Brillas, J. Casado, Aniline degradation by electro-Fenton and peroxi-coagulation processes using a flow reactor for wastewater treatment, Chemosphere 47 (2000) 241–248;
(b) E. Brillas, E. Mur, R. Sauleda, L. Sánchez, J. Peral, X. Domènech, J. Casado, Aniline degradation under electrochemical and photocatalytic conditions, J. Adv. Ox. Tech. 4 (1) (1999) 109–114;
(c) E. Brillas, R. Sauleda, J. Casado, Use of an acidic Fe/O_2 cell for wastewater treatment: degradation of aniline, J. Electrochem. Soc. 146 (1999) 4539–4543;
(d) E. Brillas, E. Mur, R. Sauleda, L. Sánchez, J. Peral, X. Domènech, J. Casado, Aniline mineralization by AOP's: anodic oxidation, photocatalysis, electro-Fenton and photoelectro-Fenton processes, Appl. Catal. B: Environ. 16 (1998) 31–42.
[20] P. Harvey, B. Campanella, P. Castro, H. Harms, E. Lichtfouse, A. Schaffner, S. Smrcek, D. Werck-Reichhart, Phytoremediation of polyaromatic hydrocarbons, anilines and phenols, Environ. Sci. Poll. Res. 9 (1) (2002) 1–94.
[21] L. Samuelson, A.A. Anagnostopoulos, J. Kumar, K. Tripathy, Biologically derived conducting and water-soluble polyaniline, Macromolecules 31 (1998) 4376–4378.
[22] M. Shaolin, C. Chuanxiang, S. Yujun, Green synthesis of polyaniline, Chem. J. Int. 3 (6) (2001) 26.
[23] (a) L. Samuelson, W. Liu, R. Nagarajan, J. Kumar, F. Bruno, A. Cholli, S. Tripathy, Nanoreactors for the enzymatic synthesis of conducting polyanline, Synth. Met. 119 (2001) 271–272;
(b) W. Liu, J. Kumar, S. Tripathy, K. Senecal, L. Samuelson, Enzymatically synthesized conducting polyaniline, J. Am. Chem. Soc. 121 (1) (1999) 71–78.
[24] F. Horst, E. Rueda, M.L. Frerreira, Activity of magnetite-inmobilized catalase in hydrogen peroxide decomposition, Enz. Microb. Technol. (in press).
[25] J. Rasko, J. Kiss, Adsorption and surface reactions of acetaldehyde on TiO_2, CeO_2 and Al_2O_3, Appl. Catal. A: Gen. 287 (2005) 252–260.
[26] N. Curvetto, D. Figlas, A. Brandolín, S. Saidman, E. Rueda, M.L. Ferreira, UV–vis, FT-IR and electrochemical of characterization polyaniline and polyphenol obtained with enzymatic and biomimetic catalysts, Enzyme Microb. Tech. (in press).