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Efficiency of enzymatic and non-enzymatic catalysts in the synthesis of insoluble polyphenol and conductive polyaniline in water

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Abstract

The present work analyzes the potential use of white-rot fungi (WRF) and hematin for phenol and aniline polymerization, as a low-cost alternative to horseradish peroxidase (HRPC). The objective is to evaluate the capability of these catalysts to produce tailor-made aniline as well as to eliminate phenols by precipitation from aqueous solution. 4-Aminoantypirine (4AAP) was used to test phenoxide formation by crude protein preparations of white-rot fungi at selected conditions. The crude extracts of *Pleurotus sajor-caju* (PSC) were selected because of the promising values obtained for the phenoxide formation rate. HRPC/H_2O_2 and *P. sajor-caju* derived enzymes/H_2O_2 (PSC/H_2O_2) systems produced soluble polyaniline in the presence of polystyrene sulphonated (PES), with high aniline conversions at 45 °C. For the case of insoluble polyphenol production, the PSC-derived enzymes, in absence of hydrogen peroxide, produced insoluble polyphenol with similar efficiencies as those found with HRPC or hematin in a one step phenol treatment (near 40% phenol conversion). For the aniline process, at least 75% aniline conversion was obtained when using PSC enzymes at room temperature. After long reaction times, the lignin-modifying enzymes derived from PSC only produced a conductive form of polyaniline (PANI) at lower temperatures than those required when employing HRPC. Fungal enzymes look promising for eliminating aniline/phenol from wastewaters since the obtained results demonstrated that they are able to polymerizate and precipitate them from aqueous solutions.

Keywords: Polyaniline; Polyphenol; White-rot fungi; Peroxidases

1. Introduction

Peroxidases from horseradish (HRPC) or soybean (*Glycine max*) (SBP) are typical oxidoreductases, which are able to catalyze different reactions using H_2O_2 as an H acceptor. They have been used in the oxidative polymerization of aromatic compounds to generate polyaromatics and as catalysts for phenol elimination as a clean alternative for detoxifying wastewater [1,2]. For example, the products from the HPRC/H_2O_2/phenol reaction are mainly polymers and phenol oligomers insoluble in water. Both polymers and insoluble phenol oligomers can be removed by sedimentation and filtration [3].

The enzymatic polymerization of anilines appears to be more efficient and promising than chemical or electrochemical technology [1,2,4]. HRPC, in the presence of H_2O_2, catalyzes the polymerization of aromatic amines to polymers with high molecular weight. When adding a polyelectrolite, such as sulphonated polystyrene (PES), aniline polymerizes and forms complexes with the polyanion, resulting in the conductive form of polyaniline [5]. PES, acting as a template, directs the para-link of the aniline radicals. The conductive, emeraldine form of polyaniline (PANI) has a para-link of the aniline moieties.

A methodology that involves "crude" enzymes to polymerize monomers is also an attractive and feasible proposal, especially because of the already known limitations in the use of HRPC. The so-called "white-rot fungi" (WRF) produces lignin-modifying extracellular enzymes (LME) that are able to degrade lignin: two glycosilated peroxidases (lignin peroxidase-LiP and Mn peroxidase-MnP), a phenoloxidase laccase (Lac) and an aryl alcohol oxidase (AAO). The latter produces hydrogen peroxide continuously. All these enzymes are able to synthesize aromatic polyalcohols and polyamines using substituted phenols

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Nomenclature

E	concentration of peroxidase in native form (mol/L)
E_1	Compound I concentration (mol/L)
E_2	Compound II concentration (mol/L)
E_3	Compound III concentration (mol/L)
k_1	reaction rate constant for native enzyme oxidation
k_2	reaction rate constant for phenol oxidation by intermediate active compound (E_1)
k_3	reaction rate constant for phenol oxidation by intermediate active compound (E_2)
k_{app}	reaction rate constant for E_2 oxidation to E_3
k_a	reaction rate for the decomposition of the intermediate compound E_3 to the native enzyme form
k_b	reaction rate for the decomposition of E_3 to E_1

and anilines as substrates [6]. Biomimetics are another alternative to the use of pure or crude enzymes.

This work presents the results for aniline and phenol polymerization using HRPC, the broth of the liquid culture from WRF obtained at the end of the tropophase (with emphasis on *Pleurotus sajor-caju* (PSC)), and hematin. Kinetic studies were also carried out using WRF and HRPC. Kobayashi et al. [7] and Tonami et al. [8] reported the quantitative production of soluble polyphenol by selecting of the reaction conditions appropriately. On the contrary, the aim of this work is to produce insoluble polyphenol (POFE) at high yields using peroxidase-like catalysts. These catalysts may be employed in decontamination of wastewaters, based on the ideas that were reported elsewhere [7–16] in terms of activity and polyphenol characterization. Besides, Fe-salen as biomimetic catalyst (*N*,*N*′ethylen-bis (salicylideneamine) has been developed by Kobayashi et al., as it was detailed in [7,8], achieving the efficient production of organic soluble functional phenolic polymers.

The goal of this manuscript is to present a comparative analysis of the capabilities of different enzymatic and non-enzymatic catalysts in specific reactions at the following selected conditions: aqueous soluble PANI synthesis with a template and insoluble POFE precipitation by phenol polymerization from aqueous solution. We demonstrate that low-cost crude enzymes from WRF (*P. sajor-caju*, mainly) can be used as catalysts for soluble PANI synthesis with a template and for insoluble POFE precipitation from wastewaters containing phenol. Firstly, a screening of the phenoxide radical generation rate was performed on different kinds of WRF. The best fungus (in terms of initial reaction rate of phenoxide formation) was selected to perform the PANI and POFE synthesis. This study also examines hematin (hydroxiferriprotoporfirine), both soluble and magnetite-supported, as a biomimetic catalyst and model for the active site of peroxidases. The effect of temperature was also addressed. Although synthesis of soluble polyaniline and insoluble polyphenol is not essentially new [3,17], the use of WRF is unusual and there are almost no reports on the use of magnetite-supported hematin.

2. Experimental

2.1. Materials and methods

Sulphonated polystyrene (PES, 99%) used for PANI synthesis was provided by Sigma and it was employed without further purification.

2.1.1. HRPC, hematin

Horseradish peroxidase was kindly provided by Amano Inc. and it was used without further purification. Hematin from Sigma Chemical Co. was employed as provided. Phenol (99%), aniline, 4-aminoantypirine (4AAP), and a pH 7 buffer were supplied by Merck. Other buffer solutions (pH 4 and 7) were provided by Anedra. Fe $(NO_3)_3{\cdot}9H_2O$ from Mallinckrodt was used.

2.1.1.1. Magnetite-supported hematin preparation. Magnetite was prepared by controlled oxidation in aqueous alkaline medium, using 36.8 g of $FeSO_4{\cdot}7H_2O$, 14 g of NaOH and 4.5 g of $NaNO_3$ in a total volume of 300 mL. The ferrous salt was added at a constant rate (ca. 2.8 mL/min), while it was vigorously stirred. A green-brownish gel was obtained, whose structure (or formula composition) was probably $Fe(OH)_2{\cdot}xH_2O$ $Fe_2O_3{\cdot}nH_2O$. This gel was kept at room temperature during 12 days to increase its oxidation. The final color was dark black. This product was carefully washed 10 times with twice-distilled water. Afterwards, it was centrifuged twice.

Magnetite and hematin were coupled using glutaraldehyde. One milliliter of 25% glutaraldehyde was put in contact with 160 mg of magnetite in 20 mL of phosphate buffer (pH 7) for 24 h. The recovered solid was reacted with 20-mg hematin in another buffer (pH 4). The latter was the optimum pH for the coupling reaction. After 2 h at 40 °C, the slurry was filtered through acetate filters. The product yield was ca. 86%, as tested with the 10-phenantroline method for Fe quantification. This yield implies that nearly 9.5% of the supported catalyst is hematin.

2.1.2. White-rot fungi

The following fungi were tested: *Lentinula edodes* (Shiitake) (Somycel 4055, Francia), *Ganoderma lucidum* (Reishi 34-D, Fungi Perfecti, Olympia WA USA), *P. sajor caju* (Fungi Perfecti Olympia WA USA), *Pleurotus florida* (ATCC 96997) and *Trametes versicolor* (Spanish Collection of Type Cultures, Universidad de Valencia CECT 20148). The different nutrient media for liquid cultivation of fungi were as follows: (1) *Lentinula edodes* (Shiitake): MYPA medium (20 g/L of malt extract, 10 g/L of yeast extract, 1 g/L of peptone) at pH 6.0, with 30 g/L of glucose and 25 g/L of milled sunflower hull; (2) *G. lucidum*: MYPA medium with 10 g/L of glucose and 65 g/L of sunflower seed; (3) *P. sajor caju* and *P. florida*: MYPA medium with 65 g/L of sunflower seed; and (4) *T. versicolor*: MYPA medium with 40 g/L of glucose and 65 g/L of sunflower seed. Two triangular sections of young mycelium from each strain, approximately 0.2 mg/mL, were inoculated in 200 mL of liquid culture medium in 1-L Erlenmeyer flasks to obtain an air-medium ratio of 5:1. The inoculated cultures were incubated in the dark at 25 ± 1 °C for

30 days on an orbital shaker at 90 rpm. The resulting broths from the liquid media were obtained by centrifuging each medium at 3000 rpm during 20 min.

Each broth corresponding to the media where fungi grew was divided in halves. One of them was freeze-dried. To precipitate the enzyme protein fraction, ammonium sulfate was added in the remaining half to obtain a 3M final concentration. After resting 15 min at 5 °C, the precipitates obtained by centrifugation were dissolved in water and dialyzed. Protein concentrations from each fraction were determined by the Bradford method [7,8] and were the following: 210 μg/mL for *G. lucidum*, 158 μg/mL for *P. florida*, 146 μg/mL for *P. sajor-caju*, 204 μg/mL for *L. edodes*, and 167 μg/mL for *T. versicolor*.

From this point on, WRF from broth (solid obtained after lyophilization) is considered different from WRF crude protein preparation, obtained after salting out and dialysis. They are respectively, labeled as WRF-broth (solid) and WRF-solution preparation (aqueous solution) in the sections bellow. If necessary, the specific fungus identification replaces WRF in the label.

Dyalized preparations coming from several WRF were tested to choose the best in terms of phenoxide generation, as detailed in Section 2.2. While growing, PSC fungus produces an aryl alcohol oxidase, a laccase, a Mn peroxidase and also some enzymes that produce hydrogen peroxide. To avoid the undesirable contribution of the laccase activity, the reactors were closed vials without O_2 purge. No Mn^{2+} was present.

2.2. Initial rate of phenoxide formation

In order to measure the initial rate of phenoxide formation, we used phenol, 4AAP ($AmNH_2$) and H_2O_2 as color-generating substrates [9–11]. The color-generation rate at 510 nm became proportional to the consumption rate of H_2O_2, as shown below: $(d[H_2O_2]/dt \propto (-dA_{510}/dt))$.

The color-generating reactions are:

$$PhO^{\bullet} + AmNH_2 \rightarrow PhOH + AmNH^{\bullet} \quad (1)$$

$$PhO^{\bullet} + AmNH^{\bullet} \rightarrow Am\text{-}NH\text{-}OPh \quad (2)$$

$$Am\text{-}NH\text{-}OPh + H_2O_2 \rightarrow Dye + 2H_2O \quad (3)$$

with the final reaction equation:

$$PhOH + AmNH_2 + 2H_2O_2 \rightarrow Dye + 4H_2O \quad (4)$$

We tested aliquots (3.45 mL) of a solution containing 60 mL of a pH 7 buffer with 4 mL 0.14 M phenol, 3 mL 2.4×10^{-3} M 4AAP, and 20 μL of an HRPC solution (6.4–10 mg HRPC/10 mL). Those aliquots were reacted with 10 to 100 μL of 0.091% H_2O_2, 0.91% H_2O_2, or 9.1% H_2O_2, to produce little changes in the volume. Reactions were performed at room temperature.

In order to obtain the initial rates of phenoxide formation with fungi enzymes, the fractions obtained by salting-out were used just after their recovery, and also after 3 months of storage at 0–2 °C. We selected different conditions, for example: (1) 1 mL of WRF solution (protein concentrations have been given above), 0.7 mL of phenol solution (656 mg/50 mL), 0.05 mL of 4 Aminoantipyrine (4AAP) (99 mg/10 mL), 1.3 mL of buffer (pH 7), and 50 μL of H_2O_2 (9.1%, w/v) and (2) 0.5 mL of 4AAP (99 mg/10 mL) and between 0.1 and 0.15 mL of WRF solution, keeping the other conditions the same as in (1).

The model proposed by Nicell et al. [10] and Buchanan and Nicell [11] was employed to describe the H_2O_2 decomposition rate at several enzyme concentrations during the colorimetric assay for both the HRPC/H_2O_2 and white-rot fungi/H_2O_2 assay systems.

The selected kinetic mechanism is shown in Eqs. (5)–(11):

Native enzyme (E) oxidation

$$E + H_2O_2 \xrightarrow{k_1} E_1 + H_2O. \quad (5)$$

Phenol oxidation by intermediate active compound (E_1)

$$E_1 + PhOH \xrightarrow{k_2} E_2 + PhO^{\bullet}. \quad (6)$$

Phenol oxidation by intermediate active compound (E_2)

$$E_2 + PhOH \xrightarrow{k_3} E + PhO^{\bullet} + H_2O. \quad (7)$$

E_2 oxidation

$$E_2 + H_2O_2 \xrightarrow{k_{app}} E_3 + H_2O. \quad (8)$$

Decomposition of intermediate compound E_3 to the native enzyme form

$$E_3 \xrightarrow{k_a} E_1O_2^{-\bullet} + H. \quad (9)$$

Decomposition of E_3 to E_1

$$E_3 + PhOH \xrightarrow{k_b} E_1 + PhO^{\bullet}. \quad (10)$$

Phenol reaction with 4AAP and dye formation (dye responsible for color)

$$PhOH + Am - NH_2 + 2H_2O_2 \rightarrow Dye + 4H_2O. \quad (11)$$

2.2.1. Mass balance equations

The mass balances for hydrogen peroxide, phenol, native enzyme, and all the intermediate compounds were established. The quasi-steady-state assumption was used for all the enzyme species. The stoichiometry of the color formation in the assay was useful to obtain the differential equation for phenol concentration:

$$\frac{d[H_2O_2]}{dt} = -\frac{2}{3}\{[H_2O_2](k_1E + k_{app}E_2) + [PhOH](k_2E_1 + k_3E_2 + k_bE_3)\} \quad (12)$$

$$\frac{d[PhOH]}{dt} = \frac{1}{2}\frac{d[H_2O_2]}{dt} \quad (13)$$

$$E_0 = E + E_1 + E_2 + E_3 \quad (14)$$

$$E_1 = \frac{k_1[H_2O_2]E + k_b[PhOH]}{k_2[PhOH]} \quad (15)$$

$$E_2 = \frac{k_1[H_2O_2]E + k_aE_3}{k_3[PhOH]} \quad (16)$$

$$E_3 = \frac{k_{app}[H_2O_2]E_2}{k_a + k_b[PhOH]} \quad (17)$$

A standard algorithm for least-squares estimation of nonlinear parameters was employed to obtain an appropriate set of kinetic parameters that minimizes the sum of the quadratic differences between our experimental hydrogen peroxide decomposition and the calculated H_2O_2 initial decomposition rate during the colorimetric assay.

2.3. Polymerization reactions

2.3.1. Phenol polymerization

The reaction was performed in 10-mL vials at room temperature. In order to cover the range of contamination found in wastewaters (up to 1500 ppm), three different phenol concentrations (from 0.00419 to 0.0168 M) were used, while employing a buffer in the range between pH 7 and 11. From 0.33 to 1 mg hematin or HRPC and hydrogen peroxide were also added. The H_2O_2/phenol molar ratios employed in our experiments were 1.5 according to Nicell et al. [10] for HRPC, and 11 for the case of hematin [3]. The stoichiometric consumption of hydrogen peroxide by phenol was reported to be nearly 1:1 [9,10,12]. One- or two-step pretreatment of phenol standard solutions with H_2O_2 was given. Once the reaction took place at each selected time, 1 mL of HCl (37%) was added to produce the polyphenol precipitation. With HRPC, the reaction time for the first step was 2 h, whereas the reaction time with hematin was 18 h. In the two-step reactions, once the first step took place, fresh catalyst and hydrogen peroxide were incorporated. Precipitation with HCl was performed once the second step ended. Afterwards, activated charcoal was added, and the mixture was stirred for an hour at room temperature. The solutions had a yellowish-brown color before the treatments with additives. After treatment with activated charcoal, the solutions appeared clear and transparent. The solids obtained by precipitation/filtration/evaporation presented colors in the range from clear brown to deep black. In the case of hematin we performed the reaction not only at room temperature but also at 50 °C, using non-supported and magnetite-supported hematin under the above-mentioned conditions.

We selected the freeze-dried preparation from the culture broth obtained from PSC, and tested its polymerization performance after three months of storage at 0–5 °C using cultures obtained after 10 and 30 days of fungi liquid cultivation. The reaction conditions were the following: 1–6 mg of solid PSC-broth per 10 mL of buffer (pH 7), 1.2 mL of phenol (695 mg/50 mL, 0.15–1 mL of H_2O_2 (9.1%, w/v). No hydrogen peroxide was added in order to test the non-peroxidatic basal activity. The PSC-broth (28 mg dry weight) was added to a solution containing 40 mg of phenol in 9 mL of buffer (pH 7). The reaction was performed at room temperature.

2.3.2. Aniline polymerization

The reaction mixture contained 15 μL of aniline, 5–10 mL of buffer (pH 4), 2.4 mg PSC-broth, and 8.5 mg of PES. Besides, 0.75 mL of H_2O_2 (9.1%, w/v) was also added at reaction times of 0, 10, and 20 min. The initial solution color was light brown.

When using HRPC as catalyst, the reaction mixture contained 1.2 mg of HRPC, 15 μL of aniline, 5–10 mL of buffer (pH 4), and 8.4 mg of PES. Besides, 0.75 mL of H_2O_2 (9.1%, w/v) was also added at reaction times of 0, 10, and 20 min. The initial color was green, in agreement with previous reports [17].

For the hematin experiments, reactions were performed at low and high hematin concentrations, whose compositions were 0.1 mL of hematin (9.6 mg/10 mL dimethylformamide-DMF) and 0.6 mL of hematin (12.2 mg/10 mL DMF), respectively. In both cases the mixture contained, 50 μL of aniline, and 5 mL of buffer (pH 4). Besides, 100 μL of H_2O_2 (9.1%, w/v) was also added at reaction times equals to 0, 10, 20, and 30 min. The amount of PES was 23.3 mg, when it was used. The molar ratio H_2O_2:aniline was 2, resulting in a 1:2 PES: aniline weight ratio.

Supported hematin, with or without PES, was also used as follows: the reaction mixture contained magnetite-supported hematin (17–19 mg), 50 μL of aniline, and 5 mL of buffer (pH 4). Besides, 100 μL of H_2O_2 (9.1%, w/v) was also added at reaction times of 0, 10, 20, and 30 min. The amount of PES was 23.3 mg, the molar ratio for H_2O_2:aniline was 2, and the resulting PES:aniline weight ratio was 1:2.

Aniline polymerization was performed for 18 h. For the first 3 h, temperature was set at 0–5 °C, at room temperature, or at 45 °C. Then, the reaction proceeded at room temperature (25 °C). After the polymerization reactions, 1–1.5 mL of HCl (37%, w/v) was added to the reaction media. At acidic pH, HRPC and PSC stop their catalytic action.

2.3.3. UV–vis measurements

In phenol polymerization, the phenol remaining in solutions having a pH lower than 2 was measured by UV spectrophotometry at 270 nm ($\varepsilon = 1766.9\ M^{-1}\ cm^{-1}$). The UV–vis studies were done using a double-beam spectrophotometer equipped with a computer-assisted system for data acquisition. This method considers a minimum value for the conversion, because soluble oligomers also absorb in this region. It is certain that the extinction coefficient value for phenol is different from those of the oligomers, but the procedure is still valid to obtain a minimum conversion for phenol. In consequence, by monitoring the absorbance of the test solutions at 270 nm we were able to estimate the minimum conversion for phenol. To confirm this, a treatment with activated charcoal was performed to remove remaining phenol and oligomers. In the case of polyaniline, the remaining solution was examined by UV spectrophotometry ($\varepsilon_{270\,nm} = 1421\ M^{-1}\ cm^{-1}$).

2.3.4. FTIR measurements

The recovered polyphenols were studied by FTIR. The FTIR spectra were obtained using a Nicolet 530 FTIR spectrometer.

The PANI samples for FTIR studies were those obtained with hematin. An aliquot of reaction media containing PANI was withdrawn and exposed to extreme pH (pH 1 or 13) to induce precipitation. The precipitated solids (green and blue, respectively) were recovered after liquid evaporation.

2.3.5. Conductivity measurements

The reaction media from aniline polymerization were allowed to rest until insoluble materials were decanted or were alternatively separated by centrifugation. The corresponding blanks were prepared using either 250 μg of HRPC enzyme or 480 μg of PSC broth, 0.15 mL of H_2O_2 (9.1%, w/v), 1 mL of buffer (pH 4), and 250 μL of HCl (37%, w/v). In the case of hematin, 250 μg of the compound was used. In all samples and blanks, a 1:200 dilution in twice-distilled water of the resulting mixtures was used for conductivity measurements at room temperature. An Oakton 35607-00 Basic Conductivity/TDS meter was used. Measurements were performed in the aqueous solutions.

2.3.6. Voltamperommetry conditions

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 to 0.80 V (SCE) at a scan rate amounting to 0.050 V s^{-1}.

The conditions for the analysis of PANI samples were as follows: Sample 1: HRCP at room temperature; Sample 2: HRCP at 45 °C; Sample 3: HRCP at 0 °C; Sample 4: PSC at room temperature; Sample 5: PSC at 0 °C; Sample 6: PSC at 45 °C; Sample 7: hematin at 45 °C; Sample 8: hematin supported at 45 °C; and Sample 9: hematin at room temperature.

3. Results and discussion

3.1. Initial rate of phenoxide formation with HRPC and PSC: kinetic analysis

Table 1 shows the average values of the initial rates of phenoxide formation obtained following the procedures described in Section 2.2, and using the fungal enzymes (assay conditions 1 and 2) and HRPC. The order in the initial reaction rate for phenoxide radicals is as follows: PSC > *P. florida* > *G. lucidum* > *L. edodes* > *T. versicolor* or *G. lucidum* > *L. edodes* > *T. versicolor* > PSC > *P. florida*, depending on WRF's crude-extracts concentration. Whatever the assay conditions, PSC-solution showed an almost constant initial rate of phenoxide generation, but lower values in phenoxide formation rates than those for HRPC. Moreover, no maximum rate occurs and deactivation with H_2O_2 is not evident.

Kinetic parameters obtained by data fitting, were higher than those obtained for HRPC (see Table 2). A remarkable difference, of about three to four orders of magnitude, was found in the kinetic constants corresponding to the regeneration reactions of the native enzyme and to Compound I formation (k_1). These results demonstrate that PSC can be an attractive alternative to HRPC. Figs. 1 and 2 show the differences in sensitivity to H_2O_2 concentration. HRPC showed a maximum at 3–4 × 10^{-4} M and the activity decreased three to four times at higher concentrations. The PSC-solution shows a constant phenoxide rate in the 3–8 × 10^{-4} M range. The PSC-solution includes other enzymes

Table 1
Initial rate of peroxide decomposition in presence of phenol, using the 4AAP method

Enzyme source	Initial rate of peroxide decomposition (molar/((μg/mL) s) × 10^6)	
	Assay 1	Assay 2
HRPC	2.54	7.12
G. lucidum	0.56	1.29
P. florida	0.64	0.25
P. sajor caju	0.69	0.62
L. edodes	0.47	0.89
T. versicolor	0.41	0.65

Conditions: HRPC: 60 mL of solution in a pH 7 buffer with 4 mL of 0.1369 M phenol, 3 mL of 2.4 × 10^{-3} M 4 AAP, and 20 μL of HRP ((6.4–10) mg of HRP/10 mL). Initial concentration of H_2O_2: 4–6 × 10^{-4} M. Conditions: [fungal enzymes] similar for assays 1 and 2, see details in Section 2. Assay 1 [4AAP] = 2.4 × 10^{-3} M, Assay 2 [4AAP] = 7.2 × 10^{-3} M (ε = 7210 M^{-1} cm^{-1}).

Table 2
Kinetic parameters for H_2O_2 consumption in the presence of HRP and PSC protein preparation obtained using Nicell's model [10,11]

Parameters (L mol s)	Horseradish peroxidase C-HRPC/H_2O_2	Enzymatic preparation of white-rot fungi (PSC)/H_2O_2
k_1	5.36 × 10^5	1.77 × 10^8
k_2	7.15 × 10^5	2.59 × 10^6
k_3	2.31 × 10^6	3.00 × 10^7
k_{app}	8.28 × 10^6	1.67 × 10^7
k_a	9.14 × 10^0	1.03 × 10^4
k_b	7.60 × 10^2	3.62 × 10^5

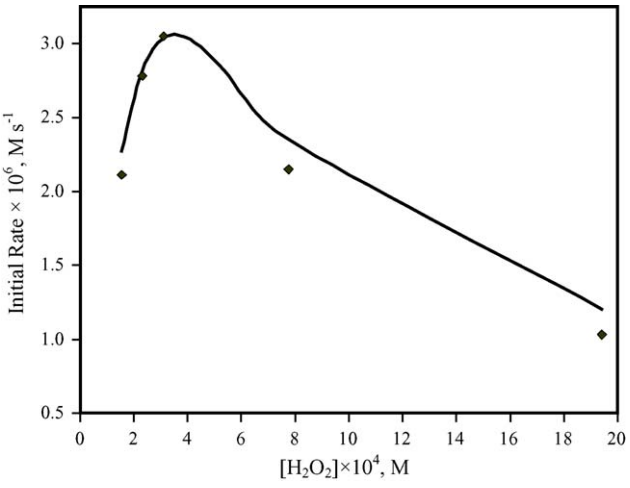

Fig. 1. Initial rate of H_2O_2 decomposition with 80 U L^{-1} of HRPC at pH 7 and r.t.: (♦) experimental and (—) fitted using Nicell's model [10,11].

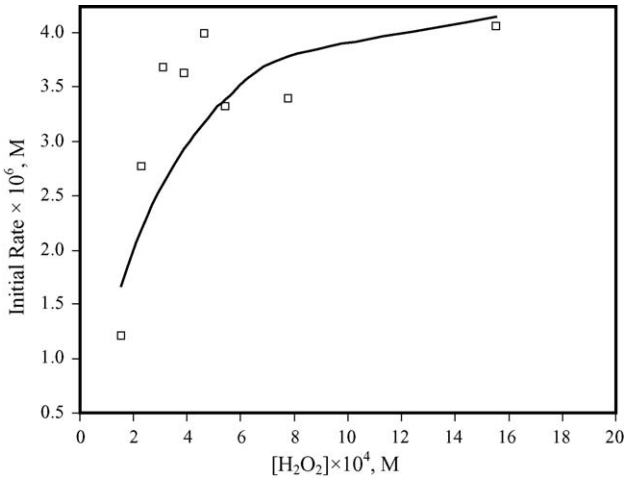

Fig. 2. Initial rate of H_2O_2 decomposition with PSC crude protein preparation (50 μg/mL of protein from PSC) at pH 7 and r.t.: (□) experimental and (—) fitted with Nicell's model [10,11].

that can contribute to the activity in phenoxide generation, especially laccase. However, the vials where the reaction was performed were closed and the amount of dissolved oxygen was low enough to avoid significant contribution of the phenoloxidase reaction. Besides, laccases are considered to have wide substrate specificity. Phenols and amines have been considered laccase substrates. Reported data demonstrated that phenolic compounds that were substituted with electron-donor groups (methoxy, methyl, amino and hydroxy) in orto or para positions were oxidized by laccase. Nevertheless, only those compounds with $E_{1/2}$ lower than 0.5 V were oxidized. In consequence, phenol did not suffer oxidation because its $E_{1/2}$ was 0.633 V. Similar results were obtained when using phenol as substrate and fungi *Pleurotus eriingy* as catalyst [18].

PSC may have another enzyme contributing to the reaction that is the aryl alcohol oxidase. This enzyme catalyzes the oxidation of an aromatic primary alcohol to aldehyde in the presence of O_2, generating H_2O_2. This screening of the abilities of PSC to polymerize phenol must include the consideration of this enzyme's contribution to the increase in the nominal H_2O_2 concentration because of the endogenously generated H_2O_2. However, the preferred substrate for this AAO is methoxy benzylalcohol rather than phenol. Previously published works reported that two-veratryl alcohol oxidase (VAOI and VAOII) enzymes were found in the culture medium when *P. sajor-caju* was grown in mycological broth. They oxidized a number of aromatic alcohols to aldehydes and reduced O_2 to H_2O_2. Both enzymes had the same Mr, approximately 71,000, but their isoelectric points slightly differ, being equal to 3.8 for VAOI and to 4.0 for VAO II. Their amino acid compositions were similar except for aspartic acid/asparagine and glycine. Both enzymes are glycoproteins that contain flavin prosthetic groups. 4-Methoxybenzyl alcohol was oxidized easily, followed by veratryl alcohol. The VAO enzymes thus represent a significantly different route for veratryl alcohol oxidation from the one catalyzed by the previously found lignin peroxidases from *Phanerochaete chrysosporium*. The experiments presented and fitted in Fig. 2 are related to hydrogen peroxide dependent enzymes. [19].

The contribution of radical generation due to laccase and AAO reactions cannot be ruled out. Intermediaries such as a benzaldehyde, catechol or quinones can be produced. The rate of phenoxide formation is affected in a positive way because there are other substrates to the PSC peroxidase (LiP), more reactive than the phenol itself and also producing the dye in the selected wavelength (see Fig. 2). Therefore, the fit obtained with Nicell's model for a peroxidase is not as good as in the case of HRPC. In the PSC case there are probably several enzymes working in parallel or series reactions.

3.2. Phenol polymerization

3.2.1. HRPC and PSC enzyme preparations

Phenoxide formation rate with HRPC/H_2O_2 was very high and a brown solid precipitated 2–3 s after the H_2O_2 addition. The total phenol conversion in a first step (from an UV–vis study) was 57–65% after 2-h reaction. The enzyme was inactivated because of the presence of H_2O_2 according to a known secondary reaction for this enzyme. The solution color changed from brown to dark yellow. In a second polymerization step, where fresh HRPC and H_2O_2 were added, an 84% phenol conversion was achieved. The best additive to be used at a final step was activated charcoal among several additives tested (chitosan, cellulose, activated charcoal) in the range of 2.0–4.0 mg/mL solution. The additives function as adsorbents for the soluble phenol oligomers [20]. After a 3-month storage, HRPC solutions presented a 7.5–8.0-fold decrease in activity while the corresponding decrease for PSC solutions was 20-fold. An increase in absorbance at 223–270 nm could be observed in the UV–vis spectra for the PSC solutions when the weight ratio for H_2O_2:PSC (g/g) decreased from 0.33 to 0.07. This fact can be related to the importance of the generation of H_2O_2 from AAO and to the appearance of benzaldehyde.

When using HRPC, a new UV–vis band near to 350 nm, which can be assigned to polyphenol species, increased its intensity, whereas no changes were found using crude preparations of PSC, in accordance to previously published studies [21]. A brown solid was obtained upon flocculation when PSC-broth was used and no H_2O_2 was added. An elimination of 50% of phenol from the solution was attained in this case. From this experimental finding it can be concluded that AAO may be an important contributor to phenol elimination when using PSC-broth. This contribution may take place by the reaction of AAO with phenol to generate H_2O_2 that is used afterwards by LiP to produce phenoxide radicals, when no H_2O_2 is added. This assumption is supported by kinetic data on phenoxide generation and by experimental data regarding phenol polymerization. Secondary reactions may be responsible of the formation of soluble oligomeric phenol compounds when using PSC in the presence of H_2O_2. The reaction with PSC-broth was not fast, and produced only soluble oligomers of phenol when H_2O_2 was added, probably because of inhibition by hydrogen peroxide in excess. Without H_2O_2, insoluble polyphenol was produced when using PSC. This can be related to aryl oxidase activity and peroxidase

that could be easily inhibited by H_2O_2. Phenol polymerization mediated by PSC-broth was demonstrated by important absorption changes in the 300–400 nm zone depending on the H_2O_2 concentration.

The best results for biomimetic catalysts were found at 50 °C, especially for magnetite-supported hematin at pH 11 in terms of aromatic compound conversion and production of polyphenol. With hematin, polyphenol needed addition of HCl to precipitate. The best conditions for HRPC and hematin-catalyzed phenol polymerization at room temperature were found to be at pH 7 and 11, respectively. Almost 90% of phenol conversion was achieved by adding the catalyst in two steps and the activated charcoal at a final step [21].

3.2.2. FTIR studies on polyphenol

In the electropolymerization of phenol on platinum electrodes, C–C bonds are mainly formed in acidic medium, whereas C–O bonds are found in alkaline medium [12,13]. Polyphenol obtained with enzymatic catalysts often has a structure consisting of a mixture of phenylene and oxyphenylene units. Generally, polyphenol presents bands at 3430 cm^{-1} (OH of phenolic group), 1219 and 1170 cm^{-1} (asymmetric vibrations of the C–O–C and C–OH linkages, respectively), and a peak at 1101 cm^{-1} due to the ether bond. The appearance of these peaks suggests the presence of phenylene and oxyphenylene units [13–16]. The spectrum of poly-phenylphenol obtained with HRPC shows peaks at 697, 781 834, 878, 1030, 1120, 1218, 1403, 1482, 1599, 1684, and 3028 cm^{-1}, and a broad band centered at 3200 cm^{-1} plus another peak at 3600 cm^{-1}.

The clear band at 1100 cm^{-1} is one of the main facts of the spectrum, apart from the absorption in the 1400–1800 cm^{-1} and 2800–3000 cm^{-1} and the strong absorption centered at 3400 cm^{-1}. The spectrum of the polyphenol obtained with hematin shows bands at 3200 cm^{-1}, 1720 and 1680 cm^{-1}, 1480, 1450 and 1400 cm^{-1}, 1200 and 1150 cm^{-1}, and 900 and 720 cm^{-1}.

Polyphenol obtained with hematin and with HRPC shows bands belonging to C–O–C coupling. This is closely related to the alkaline media used in the case of hematin (pH 11). Bands related to multisubstituted benzene are also present. FTIR of polyphenol obtained with hematin has neat bands, whereas the FTIR of the one obtained with HRPC is poorly resolved. This fact arises from the fast polymerization of phenol when HRPC was used as catalyst and from the amorphous character of polyphenol obtained in this case. From the FTIR results for the POFE obtained with HRPC and hematin, it is clear that HRPC produces an insoluble POFE with poor definition of several important bands, similar to those of electropolymerized POFE. POFE obtained with hematin is much more ordered in its structure. Therefore, it seems that in alkaline media, hematin produces a more ordered POFE than HRPC in pH 7. PSC produces POFE in higher amounts at pH 7 than at pH 4, without H_2O_2.

3.3. Polymerization of aniline

Table 3 presents the characterization of the aniline polymerization found for both enzymatic and biomimetic catalysts using a simple UV–vis method.

Table 3
Conversion results for aniline polymerization after flocculation (PANI obtained in presence of PES for HRPC and PSC from broth)

Catalyst	Conversion		
	r.t.	0–5 °C	45 °C
HRPC	95.1	94.1	86.7
PSC	75.0	90.3	89.0
Hematin without PES	99.0	–	89.2
Hematin with PES	98.0	–	74.5
Magnetite-supported hematin without PES	–	–	82.8
Magnetite-supported hematin With PES	–	–	95.3

$\varepsilon = 1425\ M^{-1}\ cm^{-1}$. Aniline conversion calculated as $(A_0 - A_f)/A_0 \times 100$. Conversion values were obtained using UV–vis measurements of absorbance at 280 nm.

3.3.1. Polymerization at room temperature

Fig. 3a and b shows the UV–vis spectra of the polyaniline/PES solution obtained with HRPC at room temperature. The UV–vis spectra are very similar for both the PANI obtained with HRPC and the one obtained with PSC-broth. The polaron transitions (414 and 850 nm) were observed and also the band due to the transition $\pi \rightarrow \pi^*$ of the bencenoid ring at 325 nm. When

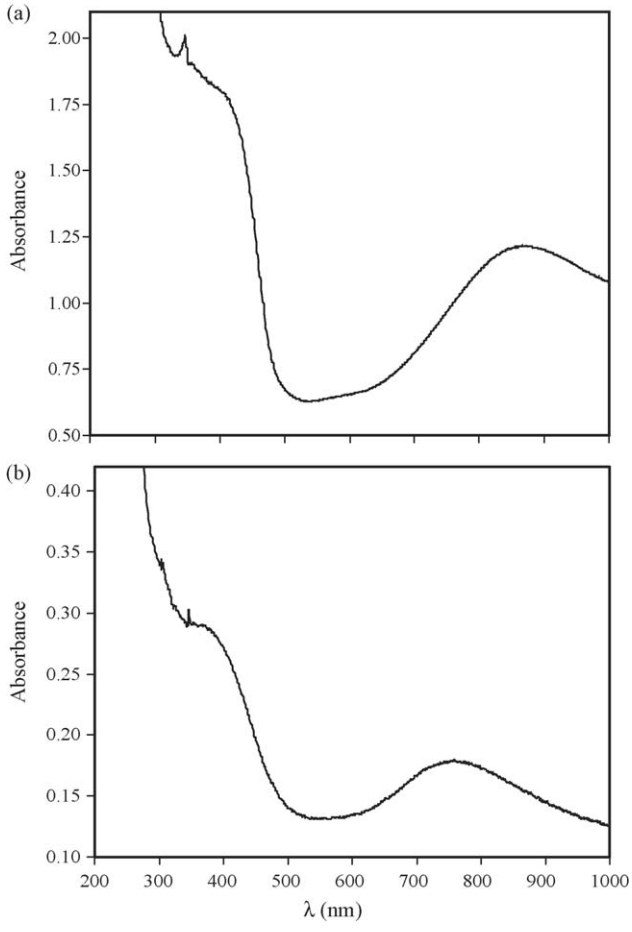

Fig. 3. (a) UV–vis spectrum for PANI obtained with HRPC-H_2O_2 at r.t., after flocculation. Initial [Aniline] = 0.0165 M. Initial [H_2O_2]:[Aniline] = 12. (b) UV–vis spectrum for PANI obtained with HRP-H_2O_2 at r.t., after a 4 day reaction (pH 1). Initial [Aniline] = 0.0165 M. [H_2O_2]:[Aniline] = 12.

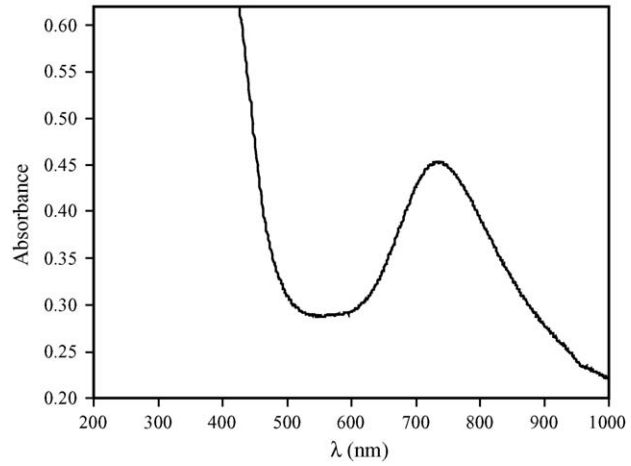

Fig. 4. UV–vis spectrum for PANI obtained with PSC-broth at r.t. after a 1 day reaction (pH 1). Initial $[H_2O_2]/[Aniline] = 12$.

PSC was used, an additional band appeared at 260 nm after a 4-day reaction (Figs. 4 and 5). The polaron transitions (414 and 850 nm) and also the band due to the transition $\pi \rightarrow \pi^*$ of the bencenoid ring at 325 nm were observed in this case. Polymerization of aniline at room temperature with HRPC and PSC-broth shows conversions of 95 and 75%, respectively, whereas with hematin the conversion achieves the same value as the one for HRPC (see Tables 3 and 4). It is clear from Table 4 that the conductivity of PANI obtained with stirring is nearly 15 times higher than the conductivity obtained without stirring. A micellar solution including conductive species is probably present when stirring is employed.

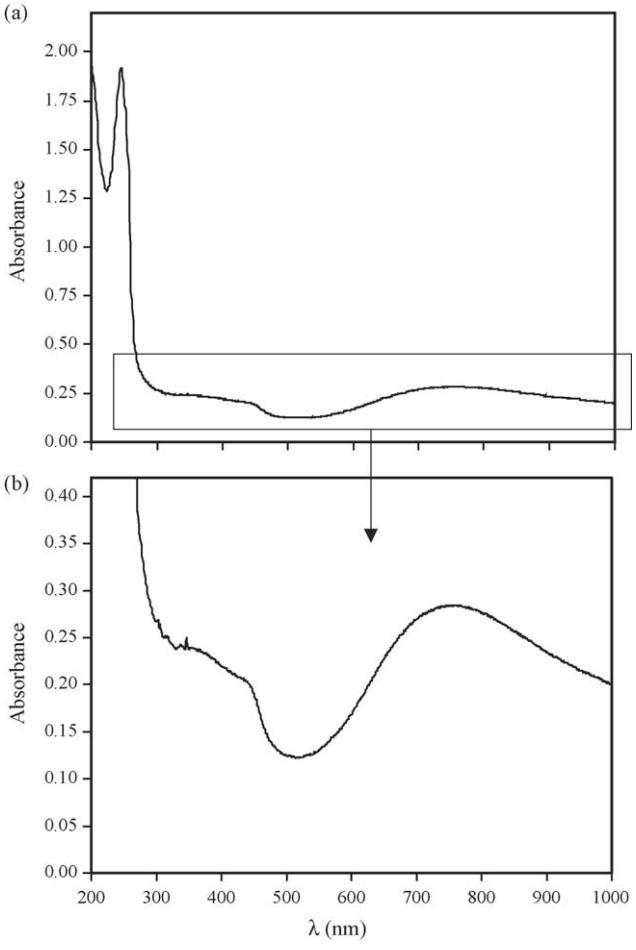

Fig. 5. UV–vis spectrum for PANI obtained with PSC-broth at r.t. after a 4 day reaction (pH 1). Initial $[H_2O_2]/[Aniline] = 12$.

Table 4
Conductivity measurements for soluble PANI*

Catalyst[a]	Conductivity (m S cm^{-1})		
	r.t.	0–5 °C	45 °C
HRPC-stirred	–	1.5	2.84
HRPC-non-stirred	–	1.28	2.54
PSC-stirred	16.83	10.30	2.47
HRPC-non-stirred	1.46	9.45	2.19
Hematin with PES	1.39	–	2.64
Hematin without PES	1.10		1.10
Magnetite-supported hematin-with PES	–	–	2.26

[a] Obtained with PES. Dilution 1:200. Reference: 4.87 mS/cm, $[HCl] = 6.336 \times 10^{-3}$ M, $[aniline] = 3.3 \times 10^{-3}$ M at 0–5 °C and 45 °C, $[aniline] = 1.6 \times 10^{-3}$ M at r.t.. $[hematin] = 4.9 \times 10^{-4}$ M (1:100 dilution).

3.3.2. Polymerization at 45 °C

When the reaction temperature was 45 °C, the initial solution with HRPC was pale green, but a brownish color developed quickly. The reaction media did not change color after 1.2 mL concentrated HCl (37%, w/v) was added. With PSC-broth as catalyst, the intensity of the above-mentioned bands was lower for the same reaction time. PSC broth contains metabolic residues and other hydrolytic enzymes distinct from LMEs. The UV–vis spectrum showed the formation of a non-conductive soluble form of PANI.

Bands of leucoemeraldine base and the corresponding to the salt of leucoemeraldine could be observed in the spectra before and after acidification, respectively. An increase in temperature decreased the formation of stable conductive complexes of PANI:PES. No redox reversibility was found when different amounts of NaOH were added. The spectra show bands of parasitic branching (in ortho instead of para position), and those of non-protonated species of PANI when using PSC.

After 24 h of reaction at room temperature (r.t.), a green precipitate was found in both systems. The one from HRPC was bright green whereas in the case of LMEs (PSC-broth) it was opaque green. After 48 h, both solutions presented similar UV–vis spectra, with the appearance of bands at 420 and 750 nm. No band at 850 nm could be found.

Total aniline conversion was lower at 45 °C for HRPC and hematin based catalysts (82–96%), but higher for PSC (89%) than those obtained at r.t. The conductivity of solutions was similar lying in the 2.2–2.8 mS/cm range. The only catalyst that behaves different was hematin without PES that produced a PANI solution with the same conductivity at r.t. and at 45 °C.

3.3.3. Polymerization at 0–5 °C

Several experiments were performed, where initial contact between phenol and peroxidases took place at 0–5 °C using an

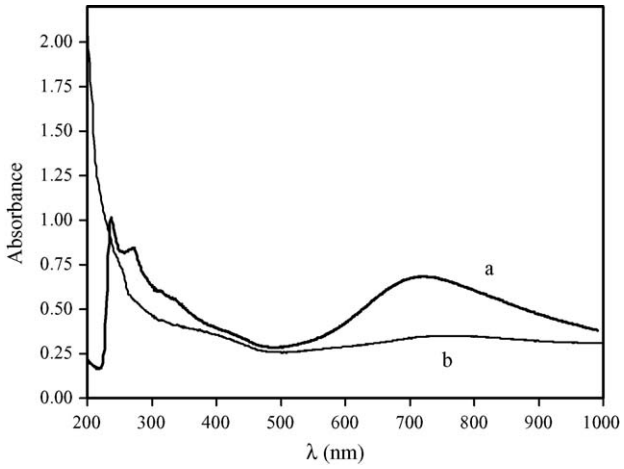

Fig. 6. (a) UV–vis spectrum for PANI obtained with HRPC at 0 °C after 30 min of reaction. (b) UV–vis spectrum for PANI obtained with HRPC at 0 °C after 3 h of reaction and at r.t after 15 h of reaction. Initial [Aniline] = 0.0165 M. Initial $[H_2O_2]$/[Aniline] = 12.

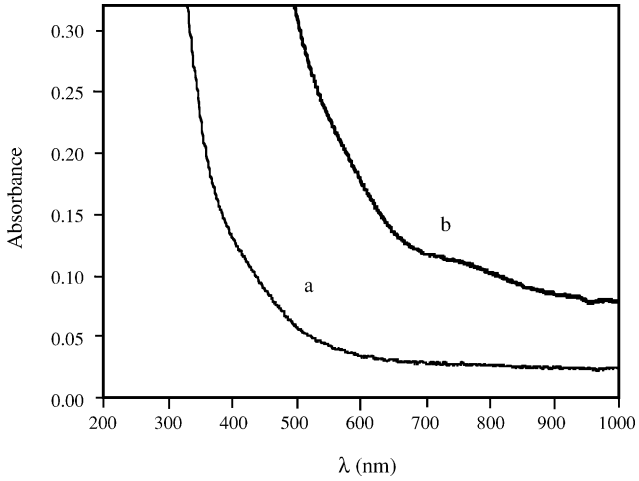

Fig. 7. (a) UV–vis spectrum for PANI obtained with PSC-broth at 0 °C after 30 min reaction. (b) UV–vis spectrum for PANI obtained with PSC-broth at 0 °C after 3 h of reaction and after 15 h of reaction at r.t. Initial [Aniline] = 0.0165 M. Initial $[H_2O_2]$/[Aniline] = 12.

ice bath. Samples were withdrawn at 30-min intervals during that period and also after 18 h. When using HRPC as catalyst, bands from PANI could be detected after 30 min at 237, 272, 310–320, 420, and 717–727 nm. These bands were assigned to diphenylamine (272 nm), the $\pi \rightarrow \pi^*$ transition of benzenoid ring (310–320 nm), polaron transitions (410–420 nm), and branched polymers (440 nm). After 2 h at 0–5 °C and 16 h at room temperature, bands at 750–790 and at 335 nm appeared. There is no evidence of parasitic branching. Polaron bands could be found at 414 and 750–797 nm (Fig. 6a and b).

In the case of LMEs (PSC-broth), the solution exhibited a brownish color after 18 h of reaction first at low temperature and then at room temperature. Addition of 0.2 mL HCl (37%) changed the color to green. The maximum was shifted from the 260 nm of aniline to higher wavelengths. After addition of 1 mL or more HCl (37%), the green color became more intense. The UV–vis spectrum showed no clear bands to be assigned after flocculation (see Fig. 7a and b). Aniline conversion was nearly 90% for the reaction catalyzed by PSC, and nearly 95% for the catalyzed by HRPC.

3.3.4. Aniline polymerization with non-supported hematin

After 18 h of reaction in the absence of PES (I) a brown solid appeared. When PES was present (II), the solution developed an intense green color. The UV–vis spectrum of solution (I) showed low absorbance values in the 400–1000 nm zone. After H_2O_2 addition, a peak at 420–430 nm appeared. The use of PES allowed the recovery of conductive emeraldine obtained at pH 4, partially soluble in distilled water. No redox reversibility was obtained without PES. The soluble fraction is obtained in the solution at pH 4, and the precipitates, when the pH is set at values lower than 1 (see Figs. 8 and 9).

3.3.5. Aniline polymerization using magnetite-supported hematin at 45 °C

The reaction was performed during 2 h at 45 °C at the highest aniline concentration. After flocculation, a dark green solid was

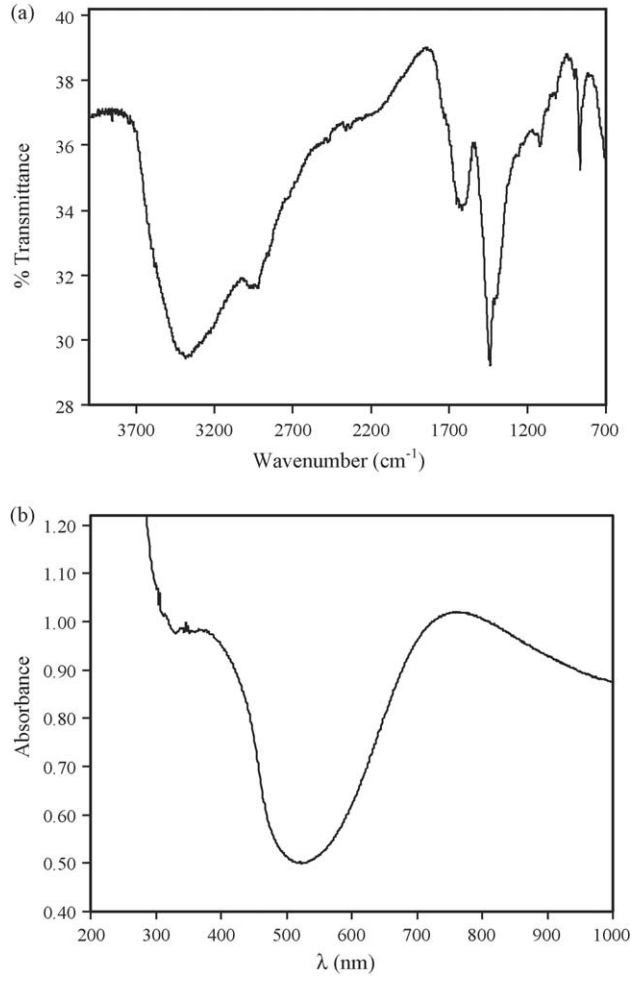

Fig. 8. (a) FTIR for nigraniline obtained with hematin/H_2O_2//PES at pH 13. Initial [Aniline] = 0.033 M. Initial $[H_2O_2]$/[Aniline] = 2. (b) UV–vis spectrum of a solution of PANI obtained with hematin at pH 1. Initial [Aniline] = 0.033 M. Initial $[H_2O_2]$/[Aniline] = 2.

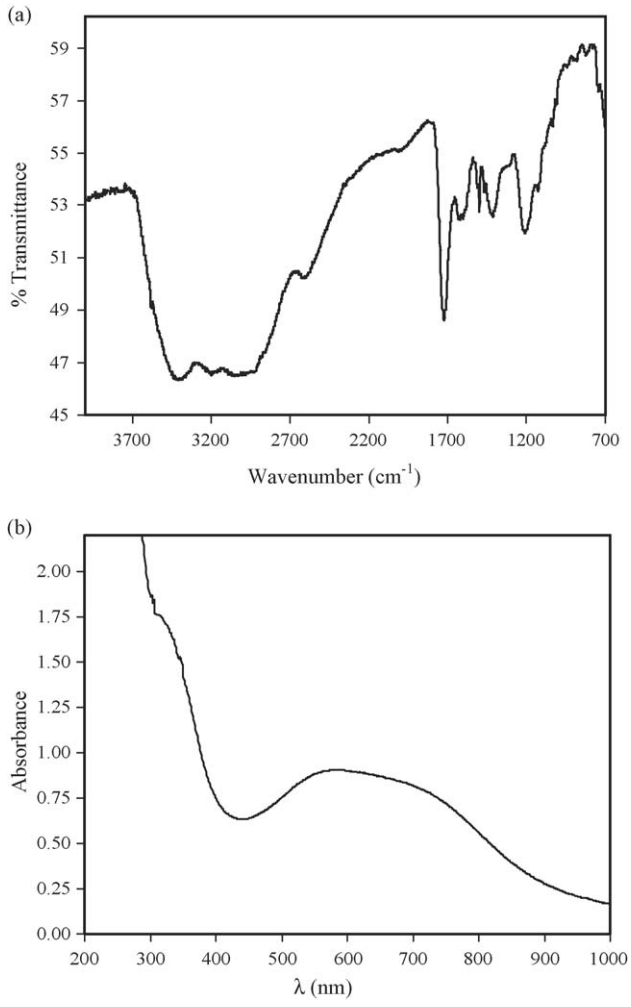

Fig. 9. (a) FTIR for emeraldine obtained with Hematin/H_2O_2//PES-pH 1. Initial [Aniline] = 0.033 M. Initial [H_2O_2]/[Aniline] = 2. (b) UV–vis spectrum for a solution of PANI obtained with hematin at pH 13. Initial [Aniline] = 0.033 M. Initial [H_2O_2]/[Aniline] = 2.

obtained with hematin either with or without PES. In the case of magnetite-supported hematin, the color of the filtrate was green with PES, or deep red in its absence.

At room temperature, conversion of aniline is higher than at 45 °C, with or without PES (see Table 3) using hematin, and it achieves a conversion of nearly 100%. However, although aniline is also polymerized, HRPC produces more soluble and higher amounts of conductive polyaniline than hematin.

The appearance of an absorption peak at 720 nm indicates that aniline has been polymerized. There is an increase of this band with reaction time, and an increase in the absorbance in the range of 500–600 nm. Absorption peaks in this range belong to soluble polyaniline, obtained with ferric sulfate [20,22]. The doping process involves the conversion of alternating benzenoid amine nitrogen (reduced unit) and quinoid imine nitrogen (oxidized unit) to a semiquinone nitrogen cation polaronic lattice. The fine spectrum structure at 250 nm is an indication of the existence of isolated benzene rings due to extensive protonation of amine nitrogens.

At room temperature, both enzymatic preparations produce emeraldine. In the case of PSC-broth, evidence of isolated benzene rings appeared after 4 days. We attributed this evidence to an extensive protonation of amine nitrogens. In the case of HRPC, the 850 nm absorption band was shifted to 750 nm. When PSC was used, the band at 750 nm appeared with higher intensity than the band at 425 nm, whereas the opposite was found to be the case for HRPC. Although the initial concentration of aniline was the same for both enzymatic catalysts, the resulting absorbance was much lower in the case of PSC-broth (compare Figs. 3a and 4). At 45 °C no band at 325 nm was found either for HRPC or for the PSC enzymatic preparation: hence no $\pi \rightarrow \pi^*$ transition of the bencenoid ring at 325 nm was present. At 0–5 °C, and after 30 min of reaction, polyaniline bands were found when PSC-broth was the catalyst, but no polyaniline band appeared when HRPC was the catalyst. However, a band at 750 nm just appeared after 30 min of reaction. Only a weak band nearly 700 nm and a broad band from 600 to 900 nm were found through catalysis mediated by PSC-broth. Magnetite-supported hematin at 45 °C produced nearly the same aniline conversion or an increment in it (see Table 3).

3.3.6. FTIR analysis of PANI obtained with hematin

Figs. 8a and 9a show the FTIR spectra of nigraniline and emeraldine salts obtained with hematin/H_2O_2/PES. The spectrum of nigraniline shows bands at 3397, 2977, 1616, 1440, 1120, and 864 cm^{-1}. In the case of emeraldine-salt obtained at acidic pH, we found bands at 3416, 3232, 3045, 2622, 1728–1724, 1633, 1509, 1498, 1465, 1413, 1209, 1126, and a minor band at 746 cm^{-1}. Minor bands were also found at 779, 827, and 877 cm^{-1}. The FTIR of the precipitate obtained with hematin/PES at 45 °C showed bands at 2620, 2545, 1257, 1392, and 945 cm^{-1}. Minor bands appeared at 850 cm^{-1} and also in the range from 800 to 650 cm^{-1}.

The PANI obtained with hematin and from a solution with pH 1 showed PANI protonation peaks that are characteristic of the emeraldine salt, with para-directed units. This observation became clear when the sample obtained at pH 13 was analyzed, where no protonation effects were present. In this case, nigraniline, which is non-protonation dependent, showed bands in the range of 1680–1620 cm^{-1} and also at 1440 cm^{-1}. The product obtained with hematin was mainly a para-directed PANI exhibiting the characteristic band at 864 cm^{-1} of 1,4 disubstituted aromatic rings in its non-protonated form.

The bands at 3450 and 3230 cm^{-1} could be assigned to the symmetric and antisymmetric -NH+ modes. Polyaniline undergoes protonation of the amine nitrogen on doping, leading to increased conjugation. Bands at 1617 and 1535 cm^{-1} have been assigned to stretching of quinoid and benzenoid rings, when PANI is obtained using benzoyl peroxide. The frequency of this band was shifted to a higher wavenumber (1725 cm^{-1}) when PANI was obtained from enzymatic or biomimetic systems [20–22]. Again, with hematin the structure of the bands is again well defined in the case of the produced PANI rather than in the case of HRPC.

3.4. Conductimmetry measurements

Table 4 shows the results of conductivity measurements of soluble PANI. Pure chemically synthesized PANI showed a conductivity of 1–10 S cm^{-1}, whereas sulfonated PANI showed 0.1 S cm^{-1} and poly-*N*-substituted aniline showed 10^{-3} to 10^{-7} S cm^{-1} using the method of cast and bulk films for conductivity measurements [18,23]. We used the soluble portion of PANI to test conductivity. Conductivity was high enough to be measured when low stirring was enough to assure a colloidal dispersion. In fact, upon resting, a solid precipitate was produced, showing different colors – green or blue – depending on the solution pH and the catalyst.

Conductivity of chemically synthesized PANI has been measured in bulk o cast film, not in solution. However, conductivity in materials obtained with hematin or PSC crude extracts is much lower than the one reported by chemically synthesized PANI. In this sense, these systems seem more suitable to eliminate aniline than to synthesize it with controlled properties. Even in the presence of a template low quality PANI was produced. However, it may be suitable for eliminating aniline by polymerization.

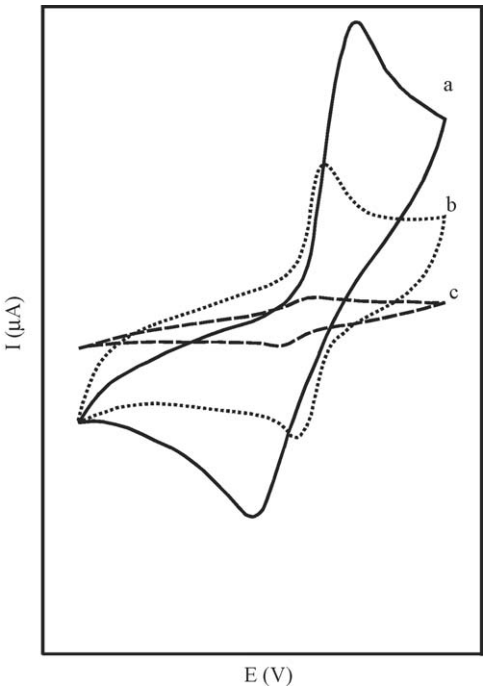

Fig. 10. Voltamperometric measurements of soluble PANI/PES: Sample 7 (curve a); Sample 5 (curve b); and Sample 2 (curve c).

3.5. Voltamperommetry measurements

The redox voltammogram of Sample 7 showed well-defined cyclic voltammetric peaks with $E_{1/2}$ at 0.41 V (Fig. 10, curve

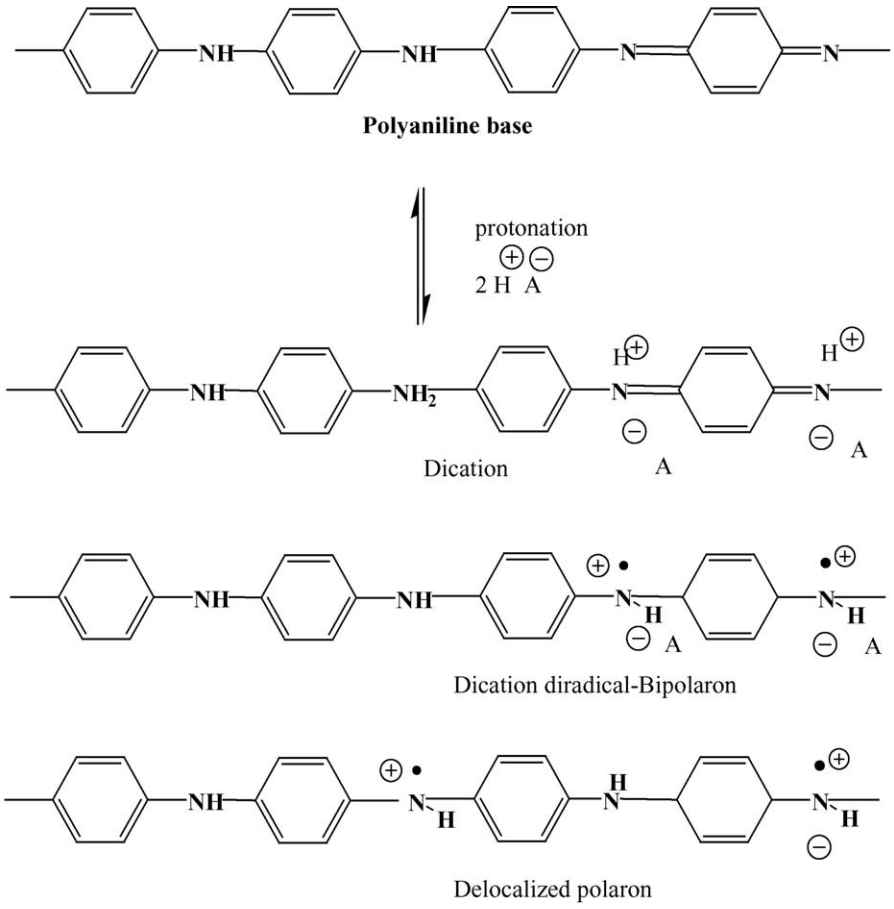

Fig. 11. Structures of non-protonated/protonated polyaniline.

a). The response obtained with Sample 2 (Fig. 10, curve c) is included in the same figure. It can be observed that the total charge substantially decreased, and that the curve was poorly defined. The current peaks practically remained in the same potential range, and the oxidation/reduction process was more reversible in this case. Samples 8 and 9 exhibited almost the same cyclic voltammetric behavior as that of Sample 2. Curve b in Fig. 10 corresponds to the stabilized voltammogram of Sample 5. The redox ability of the polymer considerably diminished when Samples 4 and 6 were employed. Reversibility (found in the UV–vis study) was also confirmed by voltamperommetry.

The voltammograms of Samples 7 and 5 showed only one set of well-defined redox peaks. The current peaks occurred by oxidation and reduction of the polymer between the leucoemeraldine and emeraldine redox states. This result is in agreement with the one obtained by Liu et al. [22] by casting the polymer onto the electrode. The authors argued about the absence of a second redox process due to the exceptional resistance of the polymer to be oxidized to pernigraniline (see Fig. 11). In our case, the best redox activity of the polymer was obtained in Sample 7. With regards to PANI production catalyzed by PSC-broth, formation of a more soluble PANI was found in Sample 5. In this case, the improvement of the polymer characteristics was probably due to a better contact between the polyanion and the polyaniline, especially at low temperature. The consequence was a more ordered structure and therefore improved conduction. Fig. 11 shows the different structures proposed for the different forms of PANI [24,25]. To the best of our knowledge, there are no reports concerning the voltammetric response of the polymer in solution/dispersion, which was the experimental approach followed in our study.

On the other hand, the lack of activity of PSC in presence of H_2O_2 can be related to the following secondary reactions: (1) phenol is converted at low reaction rates to phenoxide at the selected conditions, in presence of H_2O_2 at the concentrations we used. The lack of a high phenoxide concentration can produce other reactions, such as coupling, to give non-reactive species for the WRF peroxidase or a deactivating compound at the reaction conditions, in presence of H_2O_2. Perhaps a coupling compound, an aromatic peroxide, may be formed at these conditions that can be deleterious of enzymatic activity; (2) the enzyme was not inactivated in absence of H_2O_2, giving support to the idea that H_2O_2 plays a role in the lack of activity, in the presence of phenoxide radicals.

Some literature reports give support to the above assumptions. For example, oxygen was incorporated into some oxidation products when the lignin peroxidase from *P. chrysosporium* was used. The heme of Lignin peroxidase is exceptionally sensitive to degradation by hydrogen peroxide. This caused unsuccessfulness of the efforts to determine whether the heme edge was the site of electron-transfer reactions. In this way, faster deactivation for lignin peroxidase in the case of PSC is a plausible explanation, even when the enzyme is initially active in phenoxide radical formation [26].

4. Conclusions

The HRPC/H_2O_2 and PSC derived enzymes/H_2O_2 systems produced soluble polyaniline in the assayed conditions, in the presence of PES, with different properties depending on temperature. The best results were obtained at 0–5 °C and room temperature. The HRPC/H_2O_2 system also produced insoluble polyphenol at room temperature and pH 7. White-rot fungi enzymes, which were contained in lyophilizates of PSC, were active in the formation of phenoxide radicals at pH 7 in selected conditions. This activity decayed after 3 months of storage at 0–2 °C. In the absence of H_2O_2, the PSC broth produced insoluble polyphenol with similar efficiencies to the ones found with HRPC or hematin in the first step of treatment (nearly 50% conversion of phenol).

The soluble hematin and magnetite-supported-hematin in presence of H_2O_2 produced soluble polyaniline at pH 4 and insoluble polyphenol at pH 11. In the case of polyphenol, and using non-supported hematin, a flocculation step and a treatment with activated charcoal were needed. On the other hand, magnetite-supported hematin looked very promising, particularly at higher temperatures (50–60 °C). Partially soluble conductive polyaniline could be obtained depending on the pH reaction media, and on the addition of PES. With HRPC and PES, the emeraldine formation was faster at 0 °C than at 45 °C or room temperature. The PSC-broth only produced a conductive form of PANI after longer reaction times at lower temperatures than those needed when using HRPC. Although crude preparations of white-rot fungi are less efficient at phenol removal compared with hematin, fungal enzymes look promising for aniline elimination by polymerization.

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