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Coupling of chemical and biological processes in 17alfa-ethinylestradiol removal from aqueous solutions: a critical evaluation of adsorption and catalysis contribution

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ABSTRACT

The removal of 17alfa-ethinylestradiol (EE2) from synthetic aqueous solutions under adsorption process and enzyme catalysis mediated by immobilized laccase from *Trametes versicolor* was studied. Both processes were carried out in fluidized bed reactors filled with not activated or enzyme activated polyacrylonitrile (PAN) beads. Six EE2 concentrations (from 20 to 240 μM) were investigated and the enzyme contribution for each concentration was calculated by combining the time dependence of the two processes in the same experimental conditions. The results obtained after 90 min with laccase-activated PAN beads were compared with the ones due to adsorption alone. Results indicate that at concentrations lower than 145 μM the enzyme contribution is smaller than the one of adsorption, while at concentrations higher than 145 μM the enzyme contribution is greater.

Keywords: Endocrine disruptors; 17alfa-ethinylrstradiol; Enzyme remediation; Adsorption; Laccase; Bioreactors

1. Introduction

Surface waters contain several pollutants coming out from industry, hospitals, plants for wastewater treatment, domestic and rural drains. Some of these pollutants are harmful to fish and other aquatic species living there, so creating great concern for the equilibrium of ecosystems. These pollutants belong to the class of endocrine disrupting chemicals (EDCs), so called since 1993 [1] because they interfere with the endocrine system, inducing severe pathologies ranging from disorders in the male or female reproductive system to cancer or metabolic and neurobehavioral dysfunctions [2]. Due to their harmful effects, the fate of EDCs in the environment has become a social issue and consequently increased the urgency to remove them from the aquatic ecosystems, since the existing wastewater treatment plants were not designed to this aim.

For EDCs removal, three main classes of processes have been utilized: physical, physico-chemical and biological.

Physical processes are membrane filtration and adsorption. Physico-chemical processes are the advanced oxidation processes (AOPs) in which the oxidation of organic contaminants occurs primarily through reactions with hydroxyl

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radicals •OH. AOPs include photolysis, photocatalysis, sonolysis.

Biological processes, known as bioremediation, are carried out by means of microorganism from activated and anaerobic sludge, enzymes or whole plants.

Sometimes physical, physico-chemical and biological processes are simultaneously applied [3–5]. The present manuscript was aimed to the removal of the synthetic estrogen 17α-ethinylestradiol (EE2) using absorption on polyacrylonitrile (PAN) beads or bioremediation with laccase from *Trametes versicolor* immobilized on the same type of PAN beads. In the case of only adsorption PAN beads are identified as "bare" or "not activated", while in the case of bioremediation PAN beads are called "enzyme activated" beads. PAN beads were chosen because of our previous experience in their use either as enzyme carriers in bioremediation of aqueous solutions polluted by EDCs [6–8] or in adsorption experiments [9,10]. PAN beads have been used by other authors [11,12] in adsorption processes.

As endocrine disruptor model, we have chosen the synthetic estrogen 17alfa-ethinylestradiol (EE2), the active principle of the oral contraceptive pill. EE2 is excreted in urine and feces as active free form or inactive glucuronide or sulfate conjugate. Many studies have reported the presence of EE2 in surface waters, in concentrations ranging from ng L^{-1} to μg L^{-1} levels [13–16]. EE2 has been demonstrated in laboratory experiments to cause adverse reproductive effects in male and female fish [17–20]. More interesting are the experiments of Kidd et al. [21] who conducted a 7-year experimentation on a small lake in Ontario investigating the effects of EE2 on a population of *Fathead minnows*. The first 2 years were employed for control pre-exposure experiments. During the subsequent 3 years, they exposed the fish population to 5/6 ng/L contamination. The remaining 2 years for studying the post-exposure effects. Male fish produced vitellogenin, while females produced vitellogenin beyond their normal breeding season. This chronic exposure resulted in almost extinction of the fish population in the lake used for experimentation.

The main purpose of this manuscript is not the remediation of EE2 polluted waters by PAN beads, enzyme loaded or naked, but trying to understand the different roles played by the adsorption and enzymatic catalysis processes when both are simultaneously present. Given the delicacy of the investigation, we did not use the concentrations present in the environment which, due to their low value, could not be significantly detected, but we used concentrations of EE2 some orders of magnitude higher: mg/L instead of ng/L or μg/L.

Consequently, in this paper we separately studied the EE2 removal by adsorption on bare PAN beads and its removal by PAN beads activated with immobilized laccase from *Trametes versicolor*. Since laccase is less active on EE2 in respect to other phenol substrates, the enzyme yield has been improved by using the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) as mediator. The best experimental conditions for the enzyme activity, pH values and ratio ABTS/EE2 (mol/mol), have been investigated. The relative relevance of the adsorption process in respect to the catalytic one has been studied as a function of the initial EE2 concentration, finding that increasing the EE2 concentration the role of enzyme catalysis exceeds the adsorption process.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Acrylonitrile, sodium nitrate, ethanol, glycerin, dimethylformamide, glutaraldehyde and acetonitrile were purchased from Sigma-Aldrich (Milan, Italy) and used without further purification. Laccase (EC.1.10.3.2) from *Trametes versicolor* and 17α-ethinylestradiol were also bought from Sigma-Aldrich. Laccase was used in the case of activated PAN beads. Laccases are cupper proteins belonging to the group of blue oxidase enzymes. Laccase is a polyphenol oxidase catalyzing the reaction of several inorganic substances and aromatic compounds with concomitant reduction of oxygen to water. The reduction of oxygen to water is accompanied by the oxidation of the phenol substrate.

Since 17alfa-ethinylestradiol is weakly soluble in water, our aqueous solutions were prepared by previously dissolving EE2 in ethyl alcohol stock solutions. The effect of the ethanol solvent on the enzymatic activity of *Trametes versicolor* was not considered in this manuscript because this effect, however negligible to the percentages of the aqueous solutions used by us, had already been considered and published by us in our previous papers indicated in the text. Double distilled water was produced in-house. Its conductivity was found to be 0.055 μS cm^{-1}at 25°C.

2.1.2. Apparatus

Fluidized bed reactors, constituted by a glass pipe with an inner diameter of 1.7 cm and length of 20 cm, were used according to our previous papers [6–8]. The reactors were filled with 4 g (8 cm^3) of PAN beads. To uniformly suspend the PAN beads, the bioreactor was fed with 120 mL of EE2 in 0.1 M buffer citrate solution, duly thermostated, recirculating at a flow rate of 140 mL min^{-1} by means of a peristaltic pump. The reactor was equipped with an aerator operating with an air flow of 100 cm min^{-1} continuously supplying oxygen considering that the enzyme reaction consumes oxygen. The hydraulic circuits were made with silicone tubes. During the experiment, 200 μL of solution were extracted at regular time intervals and the EE2 concentration determined by using liquid chromatography (LC) coupled with ultraviolet/visible diode array (PDA). Between two subsequent experiments, the PAN beads, activated or not activated, were deeply rinsed by recirculation of 500 mL of buffer solution by means of the same peristaltic pump. Every hour the re-circulating buffer solution was replaced by another 500 mL of fresh buffer solution. These operations lasted from 4 to 5 h. When not operating, the reactors were stored, filled with the buffer solution and the beads, and kept in a refrigerator at 4°C.

2.2. Methods

2.2.1. Bare PAN beads preparation

Beads preparation was done, according to our previous papers [6–8], by dissolving acrylonitrile powder (54 g),

$LiNO_3$ (3 g) and glycerin (9 g) in 234 mL of dimethylformamide. The homogenized mixture was gently pipetted and precipitated in distilled water. Particle dimensions were regulated by the pipette tip. The obtained beads were water-washed and immersed for 24 h in a glycerin aqueous solution 30% (v/v). After this step, the beads were dried in an oven at 70°C until reaching a constant weight. The average diameter of beads was 3.5 ± 0.5 mm, as measured with a caliper.

2.2.2. Activated PAN beads preparation

The activation of PAN beads and laccase immobilization were carried out through diazotization of the phenol groups of tyrosine residues. This procedure was chosen since the catalytic sites of laccase do not expose tyrosine residues. The PAN beads were treated at room temperature for 1 h with a 2.5% (v/v) aqueous solution of glutaraldehyde used as a spacer arm and as a bifunctional reagent binding covalently the aminoaryl derivatives useful for enzyme immobilization. The aminoaryl derivatives on beads were generated by treating with a 2% phenylenediamine (PDA) solution for 90 min in 0.1 M sodium carbonate buffer (pH 9.0). After washing with water, the aminoaryl derivatives were treated for 40 min at 0°C with an aqueous solution containing 2 M HCl and 4% (w/v) $NaNO_2$. At the end of this treatment, the beads were washed at room temperature in a citrate buffer solution at pH 5.0 and then treated for 16 h at 4°C in enzyme solution (3 mg mL^{-1} of laccase) in buffer citrate at pH 5.0. At the end of this step, the beads were further washed with 0.1 M buffer citrate at pH 5.0 to remove the unbound enzyme. The amount of immobilized enzymes was calculated by subtracting the amount of laccase recovered in the solution at the end of the immobilization process and in the washing solutions from the amount of laccase initially used for the immobilization. The laccase concentration was measured using the Lowry method [22]. Under the experimental conditions reported above, the amount of immobilized laccase was 3.12 ± 0.4 mg, that is, about 0.78 mg of protein per gram of PAN beads.

2.2.3. Beads characterization

2.2.3.1. Scanning electronic microscopy (SEM) observations

SEM analysis was performed by means of a FEI Quanta 200 FEG SEM in high vacuum using a secondary electron detector and an acceleration voltage of 20 kV. Before analysis, samples were coated with a 15 nm thick Au/Pd layer with a sputter coating system.

2.2.3.2. FT-IR spectroscopy

A PerkinElmer Spectrum One FT-IR spectrometer equipped with an MIR TGS detector was used to record FT-IR spectra. Spectral acquisitions were performed with KBr pellets. All spectra were collected using four scans in the range from 4,000 to 500 cm^{-1} with a 4 cm^{-1} spectral resolution. Measurements were performed in triplicate. The spectra were analyzed using the application routines provided by the software package ("Spectrum" User Guide, PerkinElmer Inc. USA).

2.2.4. EE2 concentration determination

EE2 concentration was measured by LC, using an LC-20AT apparatus (Shimadzu, Kyoto) equipped with a PDA detector (SPDM20A, Germany). All analyzed samples were prefiltered through a 0.2 μm cellulose mixed esters syringe filter (Macherey–Nagel GmbH & Co. KG, Germany). The mobile phase was a mixture of acetonitrile (eluent A) and water (eluent B) with an elution gradient as follows: 0–3 min linear gradient from 50% to 90% eluent A, for 4 min 90% eluent A, in 1 min acetonitrile from 90% to 50% eluent A. During the chromatographic run, the flow rate was 1.3 mL/min. The EE2 retention time was found to be 4.0 min. By plotting as a function of concentration, the peak area at known concentrations, it is possible to obtain a calibration curve from which unknown EE2 concentrations can be determined. The method was able to assist in the quantification of EE2 up to 1 μM. The extension of the linear range was up to 100 μM. Repeatability in the same experiment was less than 1%, while the repeatability between different experiments at the same concentration was no more than 3.5 %. Experimental points in figures are the average value of three independent experiments. The percentage removal at time t is calculated by means of the equation:

$$\text{Percentage removal}\,(\%) = \left(\frac{(C_0 - C_t)}{C_0}\right) \times 100 \tag{1}$$

where C_0 and C_t are the initial and at time t EE2 concentration, respectively.

3. Results and discussion

3.1. Characterization of PAN beads

In Fig. 1a, a PAN bead picture, obtained by SEM observation, is shown. The SEM image confirms the spherical morphology and the size of the beads. The FT-IR spectra of free laccase (Fig. 1b), of a bare PAN bead (Fig. 1c) and of laccase-activated PAN bead (Fig. 1d) are reported in the other panels of Fig. 1. The FT-IR spectrum in Fig. 1b shows a large contribution from amide A and OH groups evident at 3,399 cm^{-1}, while the small peak at 2,931 cm^{-1} can be due to CH symmetric stretching. The peaks at 1,647 and 1,550 cm^{-1} can be assigned, respectively, to amide I and amide II contribution. The peaks at 1,409 cm^{-1} can be ascribed to C–H bending of CH_3 group; the peak at 1,260 cm^{-1} is due to amide III contribution, the band at 1,050 cm^{-1} is ascribed to C–O stretching and the small peak at 804 cm^{-1} can be due to amide V contribution. In Figs. 1c and 1d both the FT-IR spectra of PAN beads before (c) and after enzyme immobilization (d), have been normalized to the peak at 2,245 cm^{-1}, due to stretching of C≡N. The normalization is necessary because of this peak does not seem to be modified during the immobilization step, and since the C≡N group is probably not involved in the chemical activation of PAN beads. The main differences between the two spectra can be observed in the region around 3,300 cm^{-1}, where the contribution of the N–H stretch introduces a significant increase in the OH peak and in the region between 1,700 and 1,000 cm^{-1}.

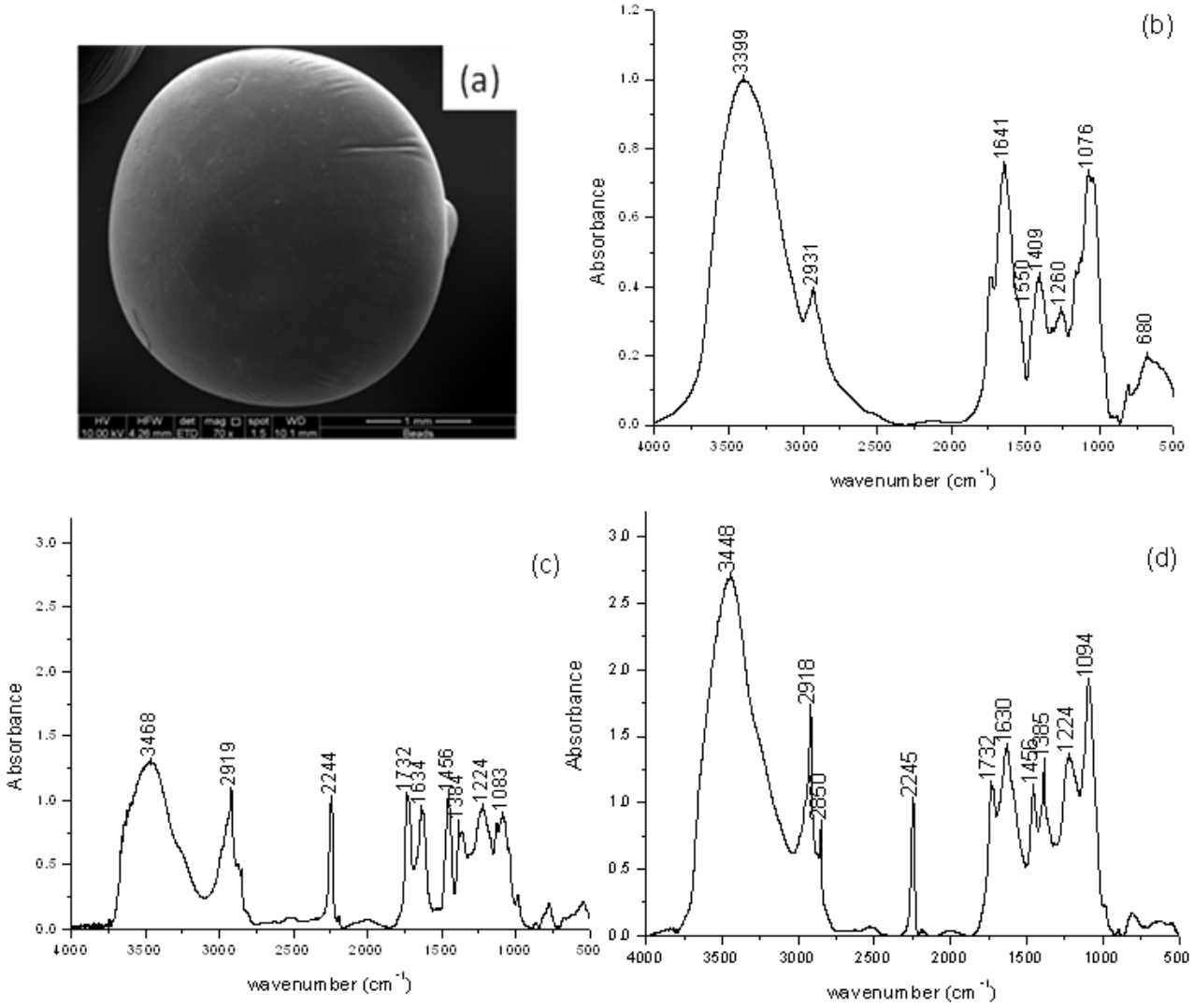

Fig. 1. Characterization of PAN beads: (a) a SEM image showing the shape and dimension of a PAN beads, (b) infrared spectrum of laccase, (c) infrared spectrum of a bare PAN bead and (d) infrared spectrum of a laccase-activated PAN bead.

From the comparison among the figures, it is evident that also the peak at 1,630 cm^{-1} is higher after enzyme immobilization due to the contribution of amide I. There is also an increase of peak at 1,260 cm^{-1}, due to amide III contribution, and an increase of the band at 1,094 cm^{-1}. All the differences in the spectra confirm that the enzyme was successfully immobilized onto the bead.

3.2. Preliminary experiments

In order to discriminate between the EE2 removal by adsorption from that operated by catalysis, parallel experiments have been performed on the same solution in two fluidized bed reactors: one filled with bare beads (for adsorption) and the other filled by laccase-activated PAN beads, where catalysis adds to adsorption. Being laccase not much reactive with EE2, we studied at first its removal as a function of a classical laccase mediator such as 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid): the ABTS.

Considering that the half-life of EE2 in the environment is of the order of 3–4 days, in the presence of photodegradation, and considering that we were not capable to "control" also the photodegradation effect in our synthetic solutions, we have limited the duration of our experiments to 90 min.

3.2.1. Dependence on ABTS concentration

Fig. 2a reports, as a function of contact time, the decrease of normalized values of EE2 concentration in experiments performed under different molar ratio C_0 (ABTS)/C_0 (EE2). C_0 (ABTS) and C_0 (EE2) are the initial molar concentration of ABTS and EE2, respectively. In all experiments, C_0 (EE2) was at concentration of 60 μM. The molar ratio is the curve parameter. Fig. 2b shows as the percentage EE2 removal after 60 min of treatment increases with the increase of the initial molar ratio ABTS/EE2. These results led us to use for the subsequent experiments the ABTS/EE2 molar ratio of 10.

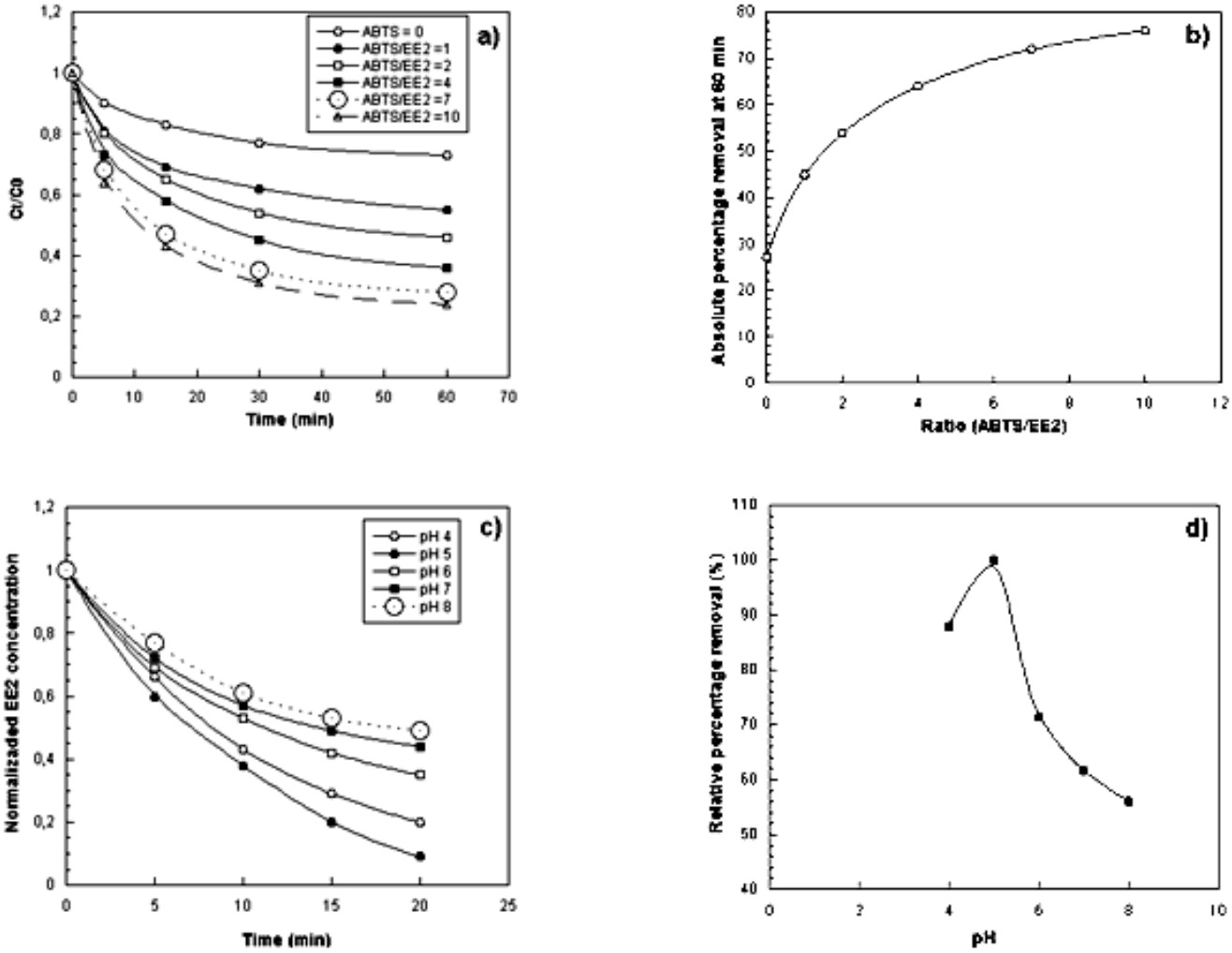

Fig. 2. (a) time decrease of normalized EE2 concentration as a function of contact time, (b) absolute values of percentage EE2 removal after 60 min of contact time as a function of molar ratio ABTS/EE2, (c) normalized EE2 concentration as a function of contact time, (d) relative percentage of EE2 removal as a function of pH values. In all experiments the initial EE2 concentration was 60 μM and the temperature of 25°C.

3.2.2. Dependence on pH

Furthermore, the effectiveness of the removal of the laccase-activated beads at various pH values of the solution has been scrutinized. Indeed, pH markedly affects the enzyme reaction rate by inducing modifications on the microenvironment around the catalytic site by the electric surface charge on the support. The optimum pH value at which the enzyme reaction is conducted varies according to the substrate nature and the support where the enzyme is immobilized. Fig. 2c reports the decrease of normalized values of the EE2 as a function of contact time. The curve parameter is the pH value. In all experiments, the initial EE2 concentration was 60 μM and the ABTS/EE2 molar ratio equal to 10. Fig. 2d shows the relative values of EE2 percentage removal as a function of pH value after 20 min of contact time. Clearly, it emerges that the optimum pH value for EE2 removal by laccase immobilized on PAN beads is pH 5.0 and for this reason the subsequent experiments were performed at this pH value.

3.3. Dependence of EE2 removal on the initial concentration

Six different EE2 concentrations have been used in the range between 20 and 240 μM. Two concentrations (20 and 40 μM) are representative of low pollution, two (75 and 105 μM) of average pollution and two (200 and 240 μM) for high pollution. The experimental results obtained with the two fluidized bed reactors are reported in Fig. 3. Open circles refer to results with enzyme activated beds, while open squares to adsorption experiments. Fig. 3a refers to the initial EE2 concentration of 20 μM, Fig. 3b to the initial EE2 concentration of 40 μM, Fig. 3c to the initial EE2 concentration of 75 μM, Fig. 3d to the initial EE2 concentration of 105 μM, Fig. 3e to the initial EE2 concentration of 200 μM and, finally, Fig. 3f to the initial EE2 concentration of 240 μM.

Results in Fig. 3 clearly indicate that at each time the EE2 removal by enzyme-activated PAN beads is greater than the removal by adsorption alone, indicating that in the former experimental conditions the catalysis effect adds to the adsorption process.

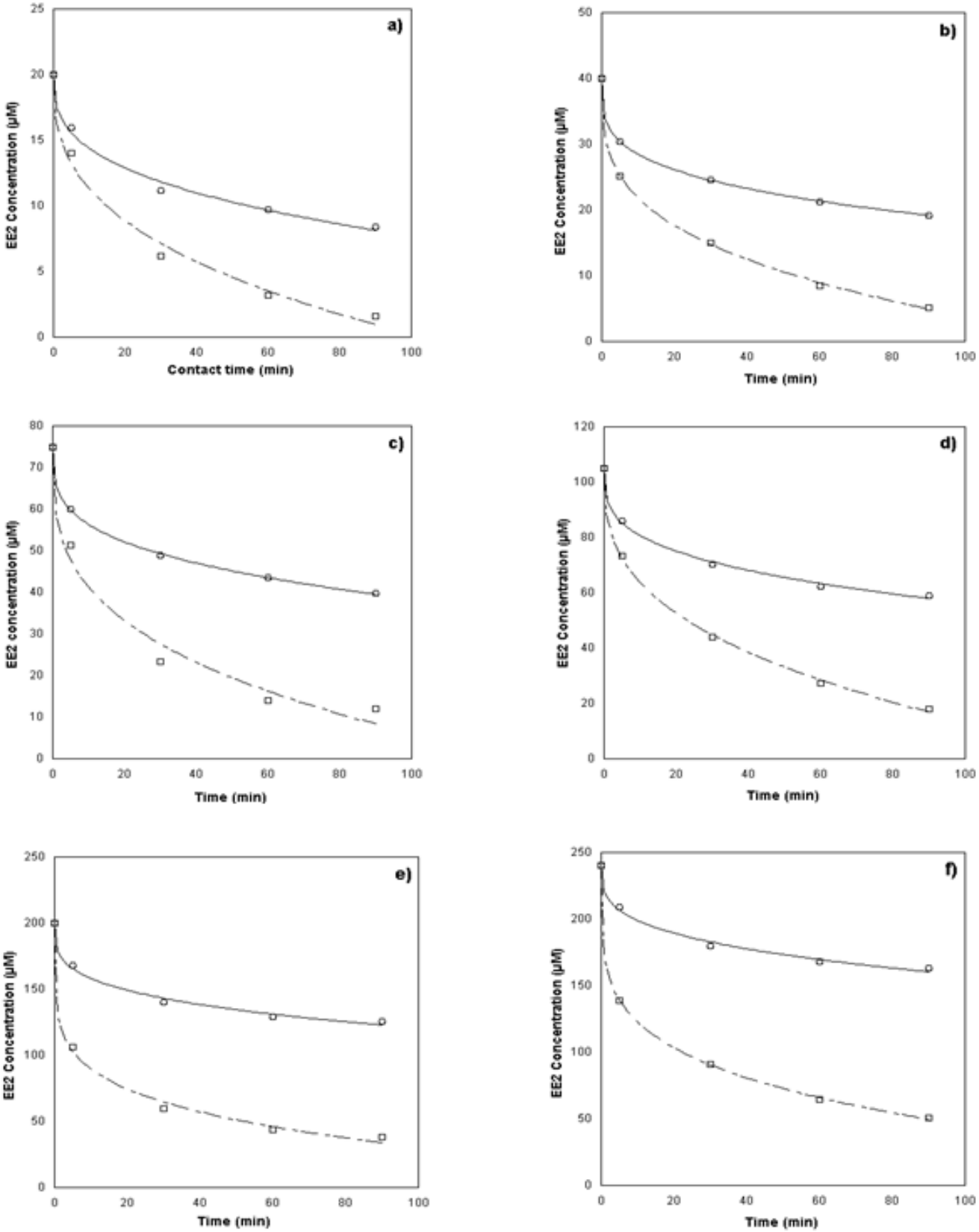

Fig. 3. Time decrease of EE2 concentration as a function of contact time: (a) 20 μM initial concentration, (b) 40 μM initial concentration, (c) 75 μM initial concentration, (d) 105 μM initial concentration, (e) 200 μM initial concentration, (f) 240 μM initial concentration. Symbols: (0) = bare beads; (□) = laccase-activated beads.

It is interesting to note that all experimental points in the different panels are fitted very well by the general equation:

$$y = a + bx^c \quad (2)$$

where a, b and c are constants, while x is the contact time. In Table 1 the analytical equations of all the experiments reported in Fig. 3 are listed.

Figs. 4a and b report the percentage of EE2 removal after 90 min of contact time with the bare beads (only adsorption)

Table 1
Analytical equations fitting the time dependence of EE2 removal at the six concentrations studied

EE2 concentration (μM)	Removal by adsorption	R^2	Total removal: adsorption + catalysis	R^2
20	$y = 20 - 2.62x^{0.336}$	0.992	$y = 20 - 3.9x^{0.352}$	0.990
40	$y = 40 - 6.20x^{0.267}$	0.998	$y = 40 - 9.14x^{0.299}$	0.998
75	$y = 75 - 9.62x^{0.290}$	0.998	$y = 75 - 16.89x^{0.307}$	0.984
105	$y = 105 - 12.26x^{0.299}$	0.998	$y = 105 - 18.68x^{0.345}$	0.998
200	$y = 200 - 22.40x^{0.276}$	0.992	$y = 200 - 72.04x^{0.186}$	0.996
240	$y = 240 - 20.81x^{0.299}$	0.994	$y = 240 - 70.70x^{0.220}$	0.998

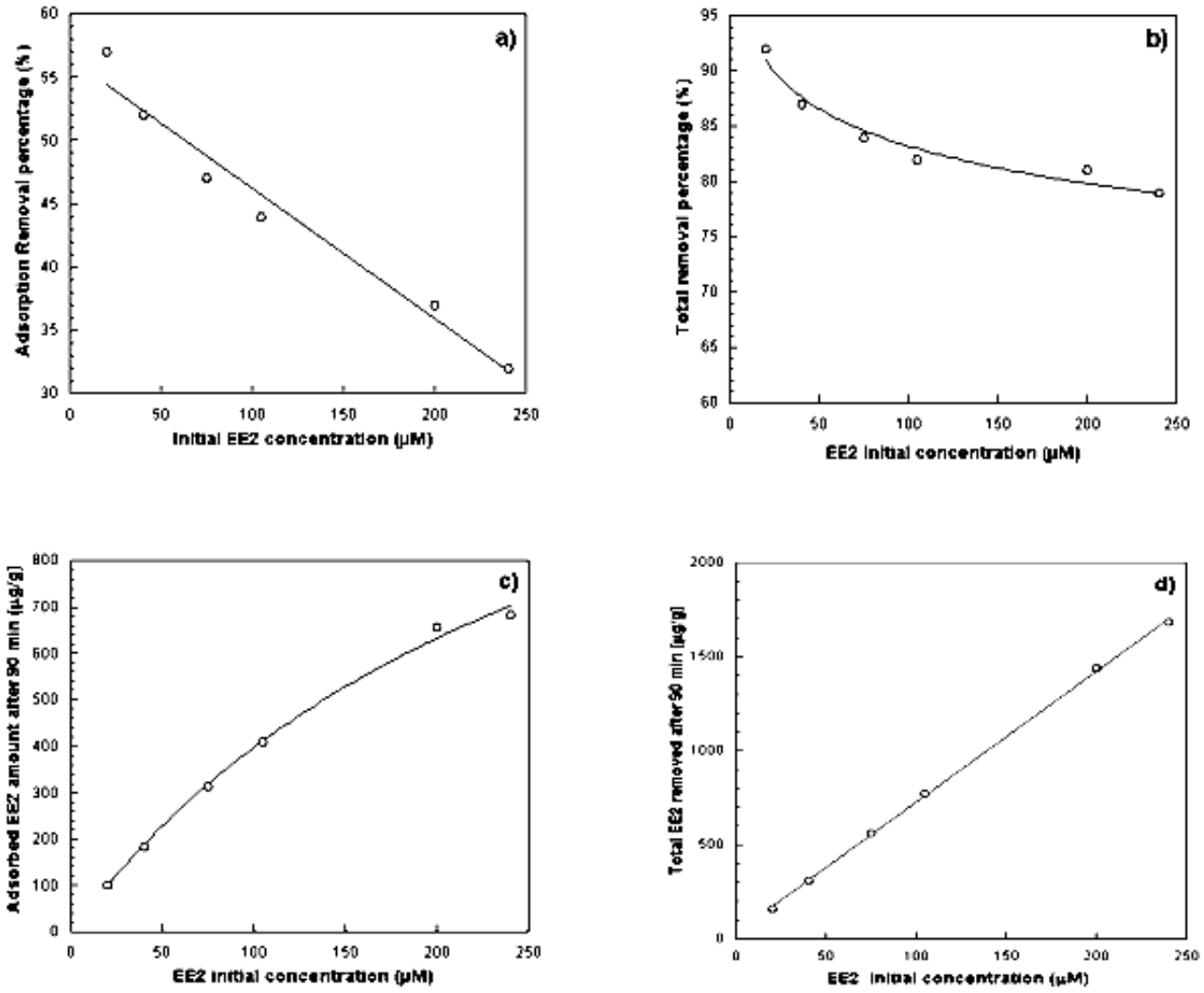

Fig. 4. Absolute removal percentages as a function of initial EE2 concentration in case of bare (a) or laccase activated (b) PAN beads. Amount of EE2 removal after 90 min of contact time to bare beads (c) or laccase-activated PAN beads (d). For all panels the contact time was 90 min.

or with laccase-activated beads (adsorption + catalysis). Both removal percentages decrease with the increase of the initial EE2 concentration, but while the adsorption percentage linearly decreases, the total EE2 removal by the coupled processes decreases in a logarithmic way. The average values obtained with the bare beads are twofolds smaller than those obtained with the laccase-activated beads.

The removed EE2 amount for gram of sorbent at time t both for adsorption and adsorption plus catalysis is calculated following Eq. (3):

$$Q_t\left(\mu g / g\right)=\left(\frac{\left(C_0-C_t\right)V}{W}\right) \tag{3}$$

where C_0 and C_t are EE2 concentrations (μg L^{-1}) at time zero and at time t, respectively, V is the volume of solution (L), and W is the mass (g) of PAN beads.

Figs. 4c and d show the values of Q_t for the bare beads and for laccase-activated beads, respectively. Results in Fig. 4c clearly show that the adsorption process linearly increases as a function of the initial EE2 concentration. This behavior agrees with the one obtained by Ra et al. [14] in the case of methylparaben absorption on bare PAN beads. Furthermore, the average values obtained with the bare beads are twofolds smaller than those obtained with the laccase-activated beads, as evidenced in Figs. 4c and d.

3.4. Evaluation of enzyme contribution

These results motivated us to estimate the single contribution of laccase activity for each studied EE2 concentration. This can be done by supposing that the amounts of EE2 adsorbed on the bare and laccase-activated PAN beads are equal. Under this hypothesis, it is possible to compare the values reported in the second and third column of Table 2 where the values of EE2 percentage removal after 90 min of contact time are listed. The second column refers to experiments performed with the bare beads, while the third column to experiments with the laccase-activated bead, in which the processes of adsorption and enzyme catalysis simultaneously occur.

Under our hypothesis, the enzyme contribution to the EE2 removal by the laccase-activated beads is obtained by subtracting the values of the second column by the ones in the third column. The results of this operation are reported in the fourth column of Table 2. The enzyme contribution increases with the EE2 initial concentration, but this is trivial, since it is well known that the enzyme activity follows the Michaelis–Menten law.

Very interesting are the results reported in Fig. 5, which shows that: (i) the total percentage removal decreases with the pollutant concentration; (ii) the percentage of the adsorption process decreases with the increase of EE2 concentration; (iii) the percentage of EE2 removal by immobilized laccase increases with the EE2 concentration; (iv) the existence of an EE2 concentration value in which the role of the single removal processes (enzyme vs. adsorption) are reversed.

The reversal point, under the experimental conditions used in this study, occurs at about 145 μM, as illustrated in Figs. 6a and b. In the last figure on the Y-axis the difference between the percentage adsorption removal (PAR) – percentage enzyme removal (PER) is reported t. It is possible to appreciate how this difference goes from positive values to negative values with the increase of EE2 concentration.

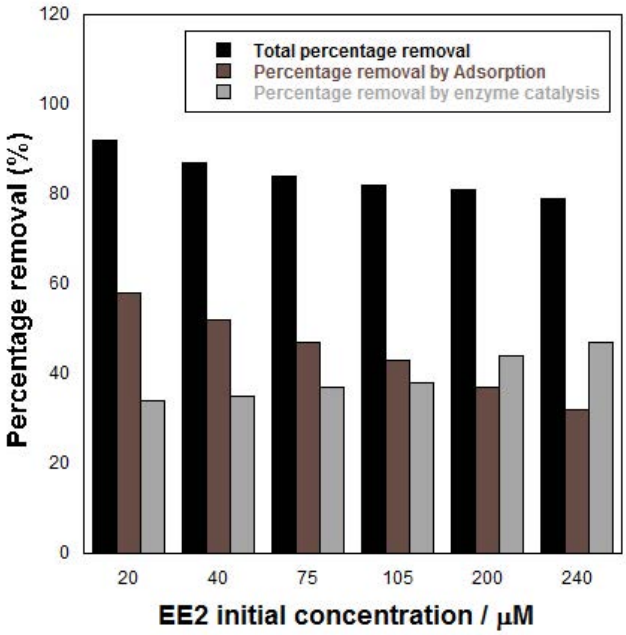

Fig. 5. Percentage EE2 removal as a function of initial EE2 concentration after 90 min of contact time.

Table 2
Percentage values of EE2 removal by bare and laccase-activated beads and estimation of enzyme contribution in removal

Initial EE2 concentration (μM)	Total percentage removal by laccase-activated PAN beads (8%)	Percentage removal by bare PAN beads alone (%)	Estimated percentage value of laccase contribution (%)
20	92	58	34
40	87	52	35
75	84	47	37
105	82	43	38
200	81	37	44
240	79	32	47

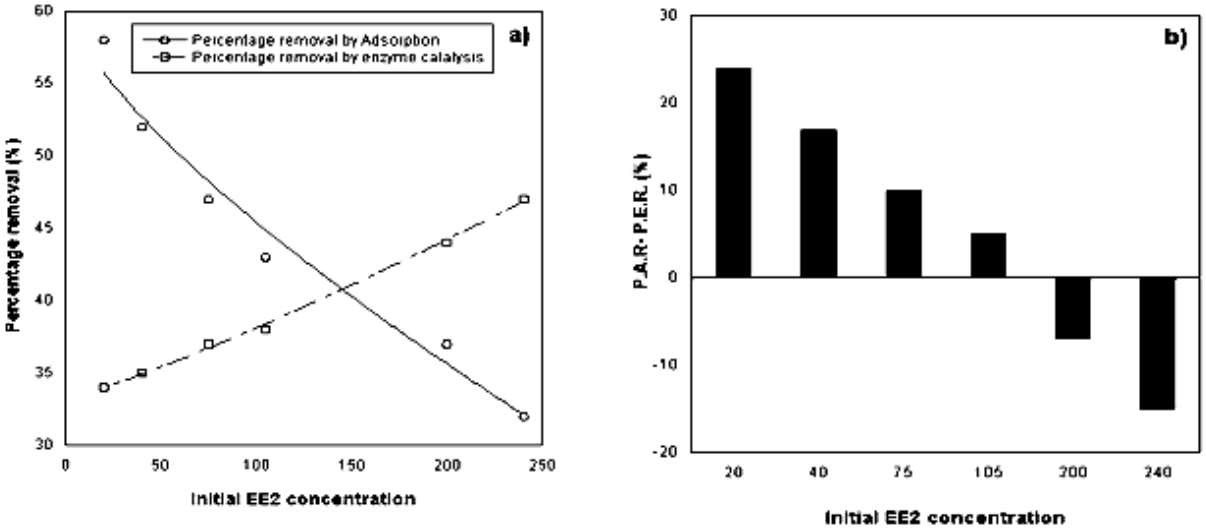

Fig. 6. (a) Percentage EE2 removal as a function of initial EE2 concentration for adsorption (o) and enzyme catalysis and (b) Percentage adsorption removal (PAR) – percentage enzyme removal (PER) as a function of initial EE2 concentration. The contact time was 90 min.

4. Conclusions

The results reported in this research show that the PAN beads, bare or laccase activated, are useful tools for EE2 removal from synthetic aqueous solutions. The yield of removal depends on: (i) the pollutant concentration; (ii) the solution pH; (iii) the molar ratio ABTS/EE2. The analysis of the removal mechanisms occurring when laccase-activated beads are used showed that the relative relevance of the two processes, adsorption and enzyme catalysis, depends on EE2 concentrations. At low concentrations the adsorption process is greater, while at higher concentrations the contrary occurs. The change of the significance of the two processes seems to occur at about 145 μM.

A last consideration. We are conscious that our experimental conditions are far from the environmental conditions, but considering that all the effects studied here are in percentage more significant at low concentrations, it is possible to hypothesize that the system used in the laboratory could be scaled to operational reality.

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