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A diffusion-reaction kinetic model for the removal of aqueous 4-chlorophenol with immobilized peroxidase

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ABSTRACT

A diffusion-reaction kinetic model is presented for the kinetic analysis of the removal process of phenolic compounds using hydrogen peroxide and immobilized peroxidase. The good results obtained in the fitting of the experimental data to the model confirm its validity, in the experimental range considered, as well as the one of the extended version of the Dunford mechanism proposed in a previous paper.

The phenomenon of enzyme deactivation and/or sequestration by the precipitated oligomers/polymers, which has been widely described in the literature, is here modeled as the growth of a polymeric film over the external surface and inside the pores of the catalytic particles that contain the enzyme, thus determining the appearance of diffusional limitations and an increasing loss of activity. The deactivation phenomena, interpreted and modeled in terms of the effectiveness factor, are included in the kinetic model.

To confirm the validity of the model, several series of experiments were carried out in a discontinuous tank reactor. Some of these experimental series were used to obtain the values of the kinetic parameters by numerical calculation and using an error minimization algorithm. Since the model reproduces the behavior of the system for the series of experiments not used for the determination of the parameters, it can be affirmed that the model is suitable for the kinetic analysis of the system under study.

1. Introduction

Phenolic compounds are usually found in the wastewaters of numerous industries (pulp and paper, wood, steel and other metals, petroleum refining, resins and plastics) from which they may be released to the environment [1,2]. They are widely considered as priority pollutants and their persistence may cause severe environmental problems. Physical, chemical and biological methods have been used for the removal of these toxic compounds [3,4]. Among them, advanced oxidation processes, such as the use of Fenton's reagent [5–7] and photochemical degradation [8–10], are of particular interest and show great potential.

Biological degradation with different microorganisms has also been widely studied [11] and many efforts have been made to overcome substrate inhibition [12,13].

Some disadvantages of the conventional treatment methods, physicochemical and biological, can be overcome by adopting enzymatic methods, which tend to have a high degree of specifity and a minimal environmental impact [14]. Horseradish peroxidase catalyzes the oxidation of aqueous phenols by H_2O_2, to produce oligomers and polymers of low solubility. These products can be removed by filtration or sedimentation [15,16]. There are other peroxidases that catalyze this reaction, soybean peroxidase (SBP) being one of the most recently used [17–22] because it is readily obtained in quantity, is inexpensive, has a high thermal stability [18] and is more resistant to deactivation [22]. Enzyme immobilization has many advantages, including enzyme reusing and easy separation from the reaction medium [23]. Moreover, covalent immobilization greatly increases the operational stability, especially when porous supports and multipoint attachments are employed [24,25].

Usually, the oxidation of aromatic compounds with hydrogen peroxide, catalyzed by peroxidase, has been described by reference to the mechanism postulated by Chance-George, also known as Dunford mechanism [26,27], which is widely cited in the literature.

In a previous paper, [28], an extended version of the mentioned mechanism was proposed and applied to the kinetic analysis of the immobilized SBP/phenol/H_2O_2 system. According to this mechanism the reaction products (dimers, trimers, etc.) may interact with the enzyme, which must be taken into account when the kinetic equation is formulated. Also, in this work, an explanation for the deactivation of the enzyme is given as follows: When the immobilized enzyme is used, the reaction is pseudo-homogeneous at the beginning and takes place, mainly, on the surface of the catalytic particles. But quickly it becomes heterogeneous due to polymer formation and precipitation over the surface of the catalytic particles, which produces a polymeric film that, gradually, covers a fraction of

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the immobilized enzyme. This model showed good approximation to the deactivation phenomena that take place in the immobilized SBP/phenol/H_2O_2 system. Nevertheless, when the model is applied to the immobilized SBP/4-chlorophenol/H_2O_2 system, the approximation degree decreases and typical deviation of 9% or higher is obtained and, by this, it is necessary to make some modifications. This can be explained by the value of V_{max}, which is much higher for 4-chlorophenol than for phenol. Consequently, polymer formation is faster in the case of 4-chlorophenol and, in a few minutes, the polymeric film completely covers the surface and pores of the catalytic particles. Under these conditions, diffusional limitations appear and it is necessary to define a diffusion-reaction model for the process. In this paper, a new approximation, based on the extended version of the Dunford mechanism [28] and including, as novelty, an interpretation of the enzyme deactivation as a consequence of the diffusion-reaction phenomena, has been developed. The model was fitted and checked with experimental data obtained in a discontinuous tank reactor for the immobilized SBP/4-chlorophenol/H_2O_2 system.

2. Theory: the proposed diffusion-reaction kinetic model

2.1. Model hypothesis

Based on the experimental behavior of the system, and taking into account the mechanism proposed by Dunford for the peroxidase catalytic cycle, [26], a diffusion-reaction kinetic model has been proposed according to the following hypotheses:

The reaction between the phenolic compound and hydrogen peroxide is catalyzed by the peroxidase enzyme and follows the free radical mechanism proposed by Dunford:

1. Dunford cycle: 4-chlorophenol and peroxide consumption and dimer formation

$$\text{Steps 1 and 2:}\quad E + H_2O_2 \overset{K_1}{\rightleftarrows} EH_2O_2 \xrightarrow{k_2} EO + H_2O$$

$$\text{Steps 3 and 4:}\quad EO + AH_2 \overset{K_3}{\rightleftarrows} EOAH_2 \xrightarrow{k_4} *EOH + *AH$$

$$\text{Steps 5 and 6:}\quad *EOH + AH_2 \overset{K_5}{\rightleftarrows} *EOHAH_2 \xrightarrow{k_6} E + H_2O + *AH$$

$$\text{Step 7:}\quad *AH + *AH \xrightarrow{k_7} HA - AH$$

The reaction product (dimer HA-AH) may also interact with the enzyme in a second catalytic cycle, according to the expanded Dunford mechanism proposed by Gomez et al. [28]:

Gomez cycle: Dimer consumption and tetramer formation

$$\text{Steps 8 and 9:}\quad EO + HA - AH \overset{K_8}{\rightleftarrows} EOHA - AH \xrightarrow{k_9} *EOH + *A - AH$$

$$\text{Steps 10 and 11:}\quad *EOH + HA - AH \overset{K_{10}}{\rightleftarrows} *EOHHA - AH \xrightarrow{k_{11}} E + H_2O + *A - AH$$

$$\text{Step 12:}\quad *A - AH + *A - AH \xrightarrow{k_{12}} HA - A - A - AH$$

Compound I and Compound II, referenced in the literature as characteristic enzyme intermediates in the peroxidase cycle, have been described by the structures EO and *EOH, respectively, in both catalytic cycles.

Additional cycles can take place with products of high polymerization degree (tetramer, etc.), which determines small additional consumption of hydrogen peroxide. All these cycles must be taken into account when the kinetic equation is formulated. In this work, the kinetic equations derived from the Gomez expanded version of the Dunford mechanism [28] have been used.

2. The enzyme is immobilized and homogeneously distributed on the surface and inside the pores of the catalytic particles. At the beginning the diffusional limitations are negligible, and the reaction behaves pseudo-homogeneous, but quickly becomes heterogeneous due to polymeric products formation and precipitation over the surface and inside the pores of the catalytic particles. The growing polymeric film gradually covers the immobilized enzyme molecules, which finish by being entrapped inside it.
3. The catalytic particles are spherical and their radius and external surface area increases with the reaction time due to polymer precipitation, which is also proportional to the inverse of the initial enzyme concentration (more enzyme, more growing nucleus to precipitate the polymeric reaction products).
4. At $t > 0$, the enzymatic process takes place by substrate diffusion and reaction through the external polymeric film and inside the pores of the spherical particles and, as a result, it is necessary to include an effectiveness factor in the kinetic model. After a few minutes, the polymeric film almost totally covers the catalytic particles, so that the diffusional limitations become significant and the Thièle modulus is high. Under these conditions, and according to several authors [29–32], the result of the analytical solution for the first order kinetic can be considered as an adequate approximation to the current effectiveness factor. The effective reaction rate is the product of the effectiveness factor and the theoretical rate at the bulk solution concentrations.
5. In order to calculate the Thièle module, which is necessary for the estimation of the effectiveness factor, some hypothesis about the effective phenolic compound diffusivity inside the polymeric film must be made. Due to the progressive recovering of the catalytic surface and pore obstruction by the polymeric products, as more amount of reaction products we can expect less values for the phenolic compound diffusivity. As a consequence, a hypothesis about a direct dependence of this diffusivity on the phenolic compound concentration and inverse on the dimer concentration has been assumed. From preliminary fitting of experimental data, where for the inverse dependence on the dimer concentration values of a power n, with $n = 0$, for no dependence, and 1 and 2, for some dependence, were assayed, the smallest value of the typical deviation was obtained for $n = 1$ and, as a consequence and to improve the value of the typical deviation, it was established that phenolic compound diffusivity is proportional to the [phenolic compound]/[dimer]^n ratio, being n a power between 1 and 2 which must be obtained during the fitting of the model.

Basically, the hypothesis above formulated must be considered only as hypothesis of the model. But there is some previous experimental work which can support, partially, some of them, as follows:

- Concerning the hypothesis about homogeneous enzyme distribution on the surface and inside the pores of the catalytic particles, it must be pointed out that, as previous step to select the support, three immobilized derivatives were prepared on three different uncoated porous glass from Sigma (data not included here): PG 75-400, PG 75-120 and PG 350-80, with specific surface area of 182, 140 and 53.5 m^2/g, respectively. For the three immobilized derivatives, the activity yield was very similar, because the immobilization procedure was the same. But a linear relationship between the amount of protein attached to each support and its specific surface area was observed. This can be interpreted as a consequence of homogeneous distribution of the enzyme on the surface and inside the pores of the catalytic particles, in good agreement with the hypothesis of the model. PG 75-400 was

selected because it presented the higher amount of enzyme/g of support and the higher activity yield.

- Concerning the pseudo-homogeneous condition for the first minutes of reaction time, this is also a hypothesis. But some experimental results (see Section 4) show that this is a plausible hypothesis, as follows: (a) The activity yield of the immobilized derivative, estimated from values of the average reaction rate after 2.5 min of reaction time, is 82.4%. This high value can be interpreted in the sense that in the first minutes there are not important diffusional limitations or they are negligible. (b) If we consider the value of the intrinsic parameter k_{cat1} = 249 mmol of 4-chlorophenol/g enzyme min, obtained by extrapolation to time zero of the reaction rate, a comparison with the one obtained in a previous paper [33] for the free enzyme, k_{cat1} = 289 mmol of 4-chlorophenol/g enzyme min, gives a relationship of 0.86, close to the unity. We must take into account, also, that as a consequence of the immobilization some conformational modifications take place in the structure of the enzyme, with some decrease in its activity. All these considerations can be interpreted as an experimental confirmation that, at time zero and during the first 2 or 3 min of reaction time, there are not diffusional limitations or they are negligible.

2.2. Kinetics equations

From the expanded version of the Dunford mechanism [28], taking into account the influence of the effectiveness factor on the reaction rate, and by using the adopted nomenclature, the following rate laws can be formulated:

- Consumption rate for the phenolic compound:

$$r_{AH_2} = \eta_1 \frac{k_{cat1}[E]_0\,[AH_2]\,[H_2O_2]}{K_{M1}\,[AH_2] + K_{M2}\,[H_2O_2] + [AH_2]\,[H_2O_2] + K_{M3}\,[HA-AH] + K_{M4}\,[HA-AH]\,[H_2O_2]} \quad (1)$$

At time zero, Eq. (1) simplifies to:

$$r_0 = \frac{k_{cat1}[E]_0[AH_2]_0[H_2O_2]_0}{K_{M1}[AH_2]_0 + K_{M2}[H_2O_2]_0 + [AH_2]_0\,[H_2O_2]_0} \quad (2)$$

Eq. (2) is the kinetic law for the initial reaction rate of a ping-pong bisubstrate mechanism.

-Consumption rate and overall rate for the dimer:

The consumption rate for the dimer is given by:

$$r_{HAAH} = \eta_2 \frac{k_{cat2}[E]_0\,[HA-AH]\,[H_2O_2]}{K_{M1}\,[AH_2] + K_{M2}\,[H_2O_2] + [AH_2]\,[H_2O_2] + K_{M3}\,[HA-AH] + K_{M4}\,[HA-AH]\,[H_2O_2]} \quad (3)$$

and the overall reaction rate of the dimer is

$$r_{dimer} = r_{AH_2} - r_{HAAH} \quad (4)$$

- Effectiveness factor values:

From the analytical solution for a first order kinetic, the effectiveness factor values are given by the following equations:

$$\eta_1 = \frac{3}{m_1^2}\left(\frac{m_1}{th m_1} - 1\right) \quad \text{and} \quad \eta_2 = \frac{3}{m_2^2}\left(\frac{m_2}{th m_2} - 1\right) \quad (5)$$

where *th* is the hyperbolic tangent, and m_1 and m_2 are the Thièle modulus for phenolic compound and dimer, respectively, given by the equations:

$$m_1 = \sqrt{\frac{V_{max1}R^2}{K_{M1}D_1}} \quad (6)$$

$$m_2 = \sqrt{\frac{V_{max2}R^2}{K_{M3}D_2}} \quad (7)$$

- Estimation of R, D_1 and D_2:

From the hypotheses of the model, the polymer mass deposited over the catalytic particle is an unknown function of the time. As an approximation, the following expression for the variation of the polymer mass deposited over the surface of a catalytic particle is proposed:

$$\frac{dM_p}{dt} = \frac{1}{[E]_0}(a + bt) \quad (8)$$

with the initial condition: $t = 0$; $M_p = 0$

By integrating the former expression:

$$M_p = \frac{1}{[E]_0}\left(at + \frac{b}{2}t^2\right) = \frac{k_{Ro}}{[E]_0}(t + k_{R1}t^2) \quad (9)$$

where: $k_{Ro} = a$ and $2k_{Ro}k_{R1} = b$.

Since the mass of polymer can be expressed as a function of the current average radius at time t, R, and the average superficial density, ρ_p, as follows:

$$M_p = 4\pi R^2 \rho_p \quad (10)$$

and the value of R can be estimated as:

$$R = \sqrt{\frac{k_{Ro}(t + k_{R1}t^2)}{[E]_0 4\pi\rho_p}}. \quad (11)$$

According to the model hypothesis, the following expression for the diffusivity of the phenolic compound, D_1, is assumed:

$$D_1 = k_{D1}\frac{[AH_2]}{[HA-AH]^n} \quad (12)$$

and, for the dimer, D_2 is considered to be proportional to D_1:

$$D_2 = k_{D2}D_1. \quad (13)$$

From the above equations (Eqs. (6) and (7)), the values of m_1 and m_2 can be obtained:

$$m_1 = \sqrt{\frac{k_{cat1}k_{Ro}[HA-AH]^n\left(t + k_{R1}t^2\right)}{4\pi\rho_p K_{M1}k_{D1}\,[AH_2]}} \quad (14)$$

$$m_2 = \sqrt{\frac{k_{cat2}k_{Ro}[HA-AH]^n\left(t + k_{R1}t^2\right)}{4\pi\rho_p K_{M3}k_{D1}k_{D2}\,[AH_2]}} \quad (15)$$

and, by simplifying and defining two news parameters, k_1 and k_2, the modulus m_1 and m_2 can be expressed as follows:

$$m_1 = \sqrt{\frac{k_{cat1}k_1[HA-AH]^n\left(t + k_{R1}t^2\right)}{K_{M1}\,[AH_2]}} \quad (16)$$

$$m_2 = \sqrt{\frac{k_{cat2}k_2[HA-AH]^n\left(t + k_{R1}t^2\right)}{K_{M3}\,[AH_2]}} \quad (17)$$

where:

$$k_1 = \frac{k_{Ro}}{4\pi\rho_p k_{D1}} \quad (18)$$

and:

$$k_2 = \frac{k_{Ro}}{4\pi\rho_p k_{D1}k_{D2}}. \quad (19)$$

As a consequence, for further calculation, the individual values of the four parameters, k_{Ro}, k_{D1}, k_{D2} and ρ_p, are replaced by only two parameters k_1 and k_2. As it can be seen, m_1 and m_2 are doubly dependent on time: in their expression, the time variable appears as well as the current values of the phenolic compound and dimer concentrations, which are also dependent on time.

- Consumption rate for hydrogen peroxide:

According to the Dunford mechanism stoichiometry, hydrogen peroxide is consumed in the reaction with phenol, dimer and other high degree polymers, in a 0.5: 1 molar ratio.

The consumption rate in the reactions with phenol and dimer is given by the equation:

$$r_{H_2O_2} = 0.5\left(r_{AH_2} + r_{HAAH}\right). \quad (20)$$

For the other high degree polymer, hydrogen peroxide consumption is assumed to be proportional to one of the two first reaction cycles:

$$r_{H_2O_2} = k_n\left(r_{AH_2} + r_{HAAH}\right) \quad (21)$$

and, by adding the two above equations, the total rate for hydrogen peroxide consumption is

$$r_{H_2O_2} = k_{H_2O_2}\left(r_{AH_2} + r_{HAAH}\right) \quad (22)$$

where:

$$k_{H_2O_2} = 0.5 + k_n \quad (23)$$

2.3. Reactor design: mass balance equations

- Mass balance for phenolic compound:

For a discontinuous tank reactor with a constant reactor volume, V_R, the following terms must be considered:

$$\text{Accumulation} = V_R\frac{d[AH_2]}{dt} \quad (24)$$

$$\text{Generation} = -V_R r_{AH_2} \quad (25)$$

and the mass balance differential equation for phenolic compound, and its initial conditions are

$$\frac{d[AH_2]}{dt} = -r_{AH_2}$$
$$t = 0; [AH_2] = [AH_2]_0 \quad (26)$$

- Mass balance for the dimer:

In a similar way, the following mass balance differential equation is obtained:

$$\frac{d[HA-AH]}{dt} = r_{dimer}$$
$$t = 0; [HA-AH] = 0 \quad (27)$$

- Mass balance for hydrogen peroxide:

Now, the mass balance differential equation is:

$$\frac{d[H_2O_2]}{dt} = -r_{H_2O_2}$$
$$t = 0; [H_2O_2] = [H_2O_2]_0 \quad (28)$$

2.4. Solving procedure and calculation algorithm

The mass balance differential equations obtained above were solved simultaneously by numerical calculation using the Euler method. The following discrete equations were obtained:

$$[AH_2]_{t+\Delta t} = [AH_2]_t - \left(r_{AH_2}\right)_t \Delta t \quad (29)$$

$$[HA-AH]_{t+\Delta t} = [HA-AH]_t + (r_{dimer})_t \Delta t \quad (30)$$

$$[H_2O_2]_{t+\Delta t} = [H_2O_2]_t - \left(r_{H_2O_2}\right)_t \Delta t \quad (31)$$

with the above mentioned initial conditions.

Eqs. (29)–(31) are recurrence laws that permit the different substrate and product concentrations in the reactor to be calculated as time passes.

3. Materials and methods

3.1. Materials

Soybean peroxidase enzyme (SBP), (E.C. 1.11.1.7, 90 units mg^{-1}), hydrogen peroxide (35%, w/v), 4-chlorophenol (molecular mass 128.6, purity 99% or greater), 4-aminoantipyrine (AAP) and potassium ferricyanide were purchased from Sigma–Aldrich Fine Chemicals. Immobilization reagents and support: (3-aminopropyl) trietoxysilane (γ-APTES), glutaraldehyde (25%) and uncoated glass beads PG 75-400 (200–400 mesh particle size, 77 Å average pore diameter and $182 \times 10^4\ cm^2\ g^{-1}$ surface area) were also from Sigma. Catalase (E.C.1.11.1.6) from bovine liver (lyophilized powder, 2200 units mg^{-1}), acquired from Sigma, was used in the activity assays of free peroxidase. Other chemicals were of analytical grade and were used without further purification.

3.2. Immobilization

Immobilized derivatives were prepared by covalent coupling between the amine groups of the protein and the aldehyde groups of the porous glass treated with (3-aminopropyl) triethoxysilane and glutaraldehyde. The immobilization process was carried out according to the following steps [23]:

- *Preparation of the carrier*: glass beads were washed in 5% HNO_3 at 80–90 °C for 60 min and then rinsed with distilled water and dried in an oven for 24 h at 110 °C. If the PG support is represented by R-Si-OH to simplify, the process that takes place in this step can be described as follows:

$$\text{R-Si-OH} + \text{aqueous HNO}_3 \Rightarrow \text{clean R-Si-OH}$$

- *Support activation*: 18 ml of distilled water was added to 1 g of clean PG along with 2 ml of γ-APTES (10%, v/v) and the pH was adjusted to between pH 3 and 4 with 6N HCl. After adjustment, the mixture was placed in a 75 °C water bath for 2 h. The silanized glass was removed from the bath, washed with distilled water and dried overnight in an oven at 110 °C. The resulting product may be stored for later use. In this step, the initial –OH groups of the support are replaced by $-(CH_2)_3-NH_2$ groups as follows:

$$\text{R-Si-OH} + (C_2H_5O)_3Si(CH_2)_3NH_2 \Rightarrow \text{R-Si-}(CH_2)_3\text{-}NH_2$$

- *Immobilization on PG-glutaraldehyde:* 1 g of silanized glass was made to react in a jacketed column reactor (3 i.d. and 40 cm length) with 25 ml of glutaraldehyde 2.5% in 0.05 M phosphate buffer, pH 7. The reactor was equipped with a porous glass plate placed 4.5 cm from the bottom. The solution was recycled for 60 min with a peristaltic pump and the PG-glutaraldehyde was washed with 25 ml of the same buffer. In this step, glutaraldehyde is attached to the activated support according to the following reaction:

$$\text{R-Si-}(CH_2)_3\text{-}NH_2 + \text{CHO-}(CH_2)_3\text{-CHO} \Rightarrow \text{R-Si-}(CH_2)_3\text{-N} = \text{CH-}(CH_2)_3\text{-CHO}$$

Enzyme solution (40 ml of SBP 2 mg ml^{-1} solution) was then added to the reactor and recycled overnight at 4 °C. If the enzyme is represented by H_2N-E the reaction that takes place in this final step is

$$\text{R-Si-}(CH_2)_3\text{-N} = \text{CH-}(CH_2)_3\text{-CHO} + H_2\text{N-E} \Rightarrow \text{R-Si-}(CH_2)_3\text{-N} = CH\text{-}(CH_2)_3\text{-CH} = \text{N-E}$$

The derivative, where the enzyme is covalently attached to the support, was then washed three times with 0.1 M phosphate buffer, pH 7. The immobilized derivative was suspended in 50 ml of the same buffer and stored at 4 °C until use.

3.3. Protein determination

The amount of protein initially offered and in the wash-liquid after immobilization was determined by Lowry's procedure modified by Hartree [34] using bovine serum albumin as a standard. The amount of coupled peroxidase was the difference between the amount of the enzyme added initially and the amount of enzyme in the wash-liquid.

3.4. Activity measurements of free and immobilized enzyme

The initial reaction rates of both soluble and immobilized enzyme were measured in a jacketed batch reactor (50 ml total volume) at 30 °C and pH 7. Substrate concentrations (4-chlorophenol and hydrogen peroxide) were kept constant at 2 mM, while enzyme concentration was varied between 0.005 and 0.025 mg ml^{-1}. Samples were taken from the reactor every 2.5 min and 4-chlorophenol concentration was determined as described below. When the immobilized enzyme derivatives were tested for activity, the samples were passed through a nylon membrane (10 μm) to retain the solid biocatalyst and phenolic polymer particles in the reactor. When the enzymes were used in solution, 1 ml samples were poured over 1 ml of catalase solution (2200 units mg^{-1}) to stop the reaction by breaking down the hydrogen peroxide. Then, 0.1 ml of a coagulant ($AlK(SO_4)_2$ 40 g l^{-1}) was added to 1 ml of the former mixture, before centrifuging for 30 min at 10,000 × *g*. From the activity data of free and immobilized enzyme, the activity yield was calculated.

3.5. Analytical method

4-chlorophenol concentrations were measured by a colorimetric method [23], using solutions of potassium ferricyanide (83.4 mM in 0.25 M sodium bicarbonate solution) and 4-aminoantipyrine (20.8 mM in 0.25 M sodium bicarbonate solution). Aliquots (2.4 ml) of the treated sample (4-chlorophenol concentration up to 0.2 mM) were placed in a spectrophotometer cuvette (3 ml) together with 0.3 ml of ferricyanide solution and 0.3 ml AAP solution. After a few minutes to allow the color to develop fully, absorbance was measured at 505 nm against a blank (2.4 ml of water, 0.3 ml ferricyanide solution and 0.3 ml AAP solution). Absorbance values were transformed to 4-chlorophenol concentrations in the sample using a calibration curve ([4-chlorophenol] (mM) = $0.0986 \times Abs_{505}$, $R = 0.9999$).

3.6. Experimental system

Experiments were conducted in a jacketed batch reactor (30 °C) of 50 ml total volume. The substrates, 4-chlorophenol and hydrogen peroxide were aqueous solutions and immobilized enzyme derivatives were suspended in 0.1 M phosphate buffer pH 7. The same buffer was used to complete the reactor volume. First, 4-chlorophenol and the buffer solutions were placed in the reactor. When a temperature of 30 °C was reached the enzyme suspension was added followed by the hydrogen peroxide solution. The 4-chlorophenol, hydrogen peroxide and immobilized enzyme concentrations were varied between experiments. The reaction course was followed by taking 1 ml samples through a nylon membrane (10 μm), and analyzing its 4-chlorophenol concentration until the value remained constant.

3.7. Experimental planning

Four experimental series were carried out (see Table 1 for experimental design) varying the enzyme concentration, the substrate concentrations (in molar ratio of 1:1), the hydrogen peroxide concentration and the 4-chlorophenol concentration. In all experimental series, duplicate runs were made for each individual assay.

4. Results and discussion

4.1. Enzyme immobilization

The results obtained are shown in Table 2, where the most important characteristics (immobilized protein (%), enzyme loading (mgE (g support)$^{-1}$) and activity yield (%)) are reported. The high value of activity yield reached (82.4%) is of particular note.

4.2. Fitting the model: determination of parameters

To fit the model and determine the values of its parameters, we used the experimental conversion results for the series of variable enzyme concentration and fixed 1 mM concentration of both 4-chlorophenol and hydrogen peroxide, the series of variable substrate concentrations in 1:1 molar ratio and enzyme concentration of 0.017 mg ml^{-1}, and the series of variable 4-chlorophenol concentration and fixed concentration of both hydrogen peroxide (1 mM) and enzyme (0.017 mg ml^{-1}), leaving the rest of the series to check the model consistency.

The model includes a total of eleven parameters: k_{cat1}, k_{cat2}, K_{M1}, K_{M2}, K_{M3}, K_{M4}, k_1, k_2, k_{R1}, n and $k_{H_2O_2}$. Of all these parameters, Eq. (2), which represents the initial rate, only contains three, k_{cat1}, K_{M1} and K_{M2}, so that this equation can be used to determine them. To calculate the rest of the constants, Eqs. (1), (3), (16), (17) and (22), a numerical calculation method for their integration and an error minimization algorithm must be used. The method followed is described below.

4.2.1. Initial rates: ping-pong equation and intrinsic parameters

Since polymer precipitation over the external particle surface occurs from the very first moments of the reaction, it is not possible to obtain reliable values for the initial rate by extrapolation to time zero since it does not vary linearly with time at this moment. For this reason, instead of using the known linearization procedure of Eq. (2), which provides a family of parallel straight lines, from whose ordinates and slope the desired values of the constants can be obtained, we used our own parameter determination method [33,35].

Basically, this method is based on three linear relationships obtained from Eq. (2) in the conditions specified below and the mean values of the reaction rate during the first moments, in our case after 2.5, 5.0 and 10.0 min. From these mean reaction rates, considered as an approximation of the initial rate, three sets of parameters, which, unlike the rate, do vary linearly are obtained. From their extrapolation to time zero, we can obtain the values of the intrinsic parameters of Eq. (2). The equations used for the method and the corresponding conditions are detailed below.

4.2.2. Variable substrate concentrations in the same molar ratio

When the initial concentrations of both substrates are equal, Eq. (2) adopts the form of a simple Michaelis Menten kinetic, so that the Lineweaver–Burk linearization is valid:

$$\frac{1}{r_0} = \frac{1}{V_{max\,1}} + \frac{K_{M1} + K_{M2}}{V_{max\,1}} \cdot \frac{1}{[AH_2]_0}, \quad (32)$$

Table 1
Experimental planning.

[E] (mg ml^{-1})	[4-Chlorophenol] mM	[H_2O_2] (mM)
Varying the enzyme concentration		
0.004		
0.009		
0.013	1	1
0.017		
0.021		
Varying the substrate concentration		
	1.0	1.0
	1.5	1.5
0.017, 0.034 and 0.051	2.0	2.0
	2.5	2.5
	3.0	3.0
Varying the H_2O_2 concentration		
		0.5 (only for 1 mM)
		0.75
		1.0
		1.25
0.017	1 and 2	1.5
		1.75
		2.0
		2.25 (only for 2 mM)
		2.5 (only for 2 mM)
Varying the chlorophenol concentration		
	0.50 (for 1.0 and 1.5 mM)	
	0.75 (for 1.0, 1.5 and 2.0 mM)	
	1.0 (for 1.0, 1.5 and 2.0 mM)	
0.017	1.25	1.0, 1.5,1.75 and 2.0
	1.5	
	1.75 (for 1.5, 1.75 and 2.0 mM)	
	2.0 (for 1.5, 1.75 and 2.0 mM)	
	2.25 (for 1.5, 1.75 and 2.0 mM)	

Table 2
Immobilization results.

Immobilized protein (%)	50.90
Enzyme loading (mgE (g support)$^{-1}$)	42.25
Activity yield (%)	82.40

where

$$V_{max\,1} = k_{cat1}[E]_0. \quad (33)$$

Since the intercept of Eq. (32) only contains the parameter V_{max1}, this equation can be used to determine it. Therefore, with the value of the slope of Eq. (32) and the calculated value of V_{max1}, the value of ($K_{M1} + K_{M2}$), but not of the constants individually, can be obtained.

As indicated above, the system being studied does not permit reliable values to be obtained for the initial rate. For this reason and following the method of Gómez and co-workers [33,35], the mean values of the reaction rate in the five experiments of the series of variable substrate concentrations at 2.5, 5.0 and 10.0 min were calculated and used as an approximation of the initial rate. These values are represented in Fig. 1 in the form represented in Eq. (32),

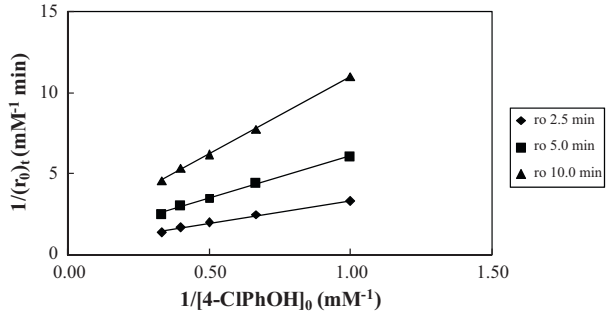

Fig. 1. Fit of r_0 (♦ 2.5 min, ■ 5 min and ▲ 10 min) to Eq. (32) for equal concentrations of hydrogen peroxide and 4-chlorophenol and 0.017 mg ml^{-1} of enzyme.

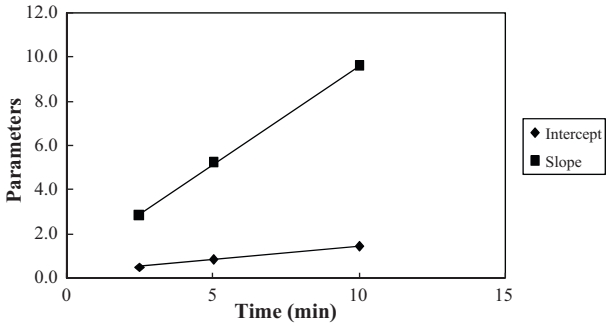

Fig. 2. Extrapolation of the apparent parameters to zero time.

and, by fitting the straight lines obtained, three sets of apparent values were obtained for the parameters included in this equation.

The apparent values are represented versus time in Fig. 2, where it can be seen that there is a good linear dependence with time. Using the Sigma Plot V 8.2 software, and by fitting these apparent values and extrapolating to time zero, the intrinsic values of the parameters have been calculated. The obtained values were

$$V_{max\,1} = 4.21 \pm 0.30\ \text{mM min}^{-1}; \quad K_{M1} + K_{M2} = 2.77 \pm 0.29\ \text{mM}.$$

From values of the V_{max} and the initial enzyme concentration, $[E]_0$, the specific activity of the enzyme in the initial catalytic cycle, k_{cat1}, was calculated:

$k_{cat1} = 249.25 \pm 17.76$ (mmol of 4-chlorophenol) (g enzyme)$^{-1}$ min^{-1}

In a previous work [33], where free enzyme was used, a value of $k_{cat1} = 289.20 \pm 41.87$ was obtained, which gives a high value (86%) for the activity yield of the immobilized derivative. Taken into account that immobilization involves some conformational modifications in the structure of the enzyme, with some loss in the enzyme activity, this high value of the activity yield, close to 100%, can be interpreted as an experimental confirmation that, at time zero and

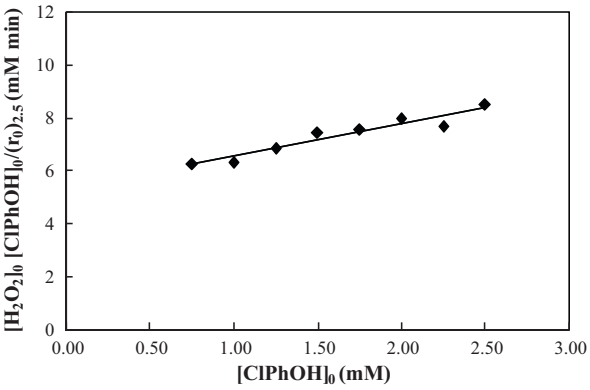

Fig. 3. Fit of r_0 (2.5 min) to Eq. (34) for varying 4-chlorophenol concentrations and a constant hydrogen peroxide of 1 mM.

during the first 2 or 3 min of reaction time, there are not diffusional limitations or they are negligible.

4.2.3. Varying the 4-chlorophenol concentration

For a fixed hydrogen peroxide and variable 4-chlorophenol concentration, the following form of linearization for Eq. (2) is used:

$$\frac{[AH_2]_0[H_2O_2]_0}{r_0} = \frac{K_{M1}[H_2O_2]_0}{V_{max\,1}} + \left(\frac{K_{M2} + [AH_2]_0}{V_{max\,1}}\right)[AH_2]_0, \quad (34)$$

which presents the advantages mentioned in the description of the method when it is compared with other representations of Eq. (2).

In agreement with Eq. (34), for a series of experiments involving a fixed hydrogen peroxide and variable 4-chlorophenol concentration, the value of K_{M1} and K_{M2} can be obtained from the slope and the ordinate on the origin of this equation for 2.5 min. In Fig. 3 it can be seen that the linear dependence expressed in Eq. (34) is accomplished with a correlation coefficient $r = 0.958$. From Fig. 3, we obtain the values: $(K_{M1})_{2.5} = 0.34$ mM; $(K_{M2})_{2.5} = 5.09$ mM.

Finally, the close linear dependence of the apparent kinetic parameters on time seen in Fig. 2 permits us to obtain the intrinsic values of K_{M1} and K_{M2} by the equations:

$$K_{M1} = \frac{K_{M1} + K_{M2}}{(K_{M1} + K_{M2})_{2.5}}(K_{M1})_{2.5} \quad (35)$$

$$K_{M2} = \frac{K_{M1} + K_{M2}}{(K_{M1} + K_{M2})_{2.5}}(K_{M2})_{2.5} \quad (36)$$

finally giving the following values:

$K_{M1} = 0.17 \pm 0.02$ mM; $K_{M2} = 2.60 \pm 0.27$ mM.

These values are very close to the ones obtained in a previous paper [33], working with the free enzyme:

$K_{M1} = 0.14 \pm 0.04$ mM; $K_{M2} = 1.52 \pm 0.13$ mM.

4.2.4. Integration of the overall model: determining the additional parameters

To determine the eight remaining parameters, k_{cat2}, K_{M3}, K_{M4}, k_1, k_2, k_{R1}, n and $k_{H_2O_2}$, the complete kinetic model needs to be integrated and an error minimization procedure followed.

In this work, the model equations were integrated using Euler's method, with a pass width of 0.05 min for the time, which is the independent variable. The numerical equations derived from the model equations were implemented in a program written in Visual Basic language. The program incorporated an error minimization routine based on the Simplex algorithm in the improved version proposed by Nelder and Mead [36]. This program seeks the best set of constants according to minimum squares criteria and supplies the standard deviation. In the calculation process, the constants determined by the linear fits mentioned above are not modified.

The quality of the initial guesses of the model parameters is decisive if Simplex is used. In this way, the following considerations have been taken into account by authors to choose the initial guesses of the model parameters:

- The values of k_{cat1}, K_{M1} and K_{M2}, obtained from initial reaction rate, were kept constant in the fitting procedure, which reduces the total number of parameters to be determined from 11 to 8.
- As initial value for k_{cat2}, 70% of k_{cat1} was assumed, and for K_{M3} and K_{M4}, the same values of K_{M1} and K_{M2} were taken.
- The maximum value for the sum $0.5 + k_n$, which appears in equation (23), is the unity, and it corresponds with the maximum consumption of hydrogen peroxide if all reaction products continue reacting. By this, an initial value of 0.5 was taken for k_n.
- And, finally, for the parameters, k_1, k_2 and k_{R1}, a common initial value of 0.1 was used, because not very large values for these parameters should be expected.

Concerning the minimization algorithm, other methods, as the Levenberg–Marquardt algorithm, can be used. We used the Nelder and Mead method, basically, because we have wide experience in using this procedure, and we have developed a highly optimized routine which has been used, successfully, in our research works.

On the other hand, the Nelder and Mead method is an enhanced version of the Simplex method, and it is recommended in the book of Himmelblau [37]. Also, this method has been used, successfully, in the Encora 1.2 computer program, software developed by J.J. Straathof to analyze the enzyme kinetics by fitting of the progress curve obtained in batch reactors [38], with very good results. This software is free, both the compiled program and the source code.

Taking this computer program as reference, our research group developed a customized fitting software to obtain the intrinsic kinetic parameters by fitting, simultaneously, a set of 15 progress curves obtained in a batch reactor under several experimental conditions [28]. The capacity that our software has to fit, simultaneously, several progress curves, from 1 to 50, is a very important difference with the Encora 1.2 program and with most of the commercial available software, and it allows to include, simultaneously, the influence of a lot of kinetic variables, such as enzyme concentration, substrate concentration, product concentration, and others, in the fitting procedure, which has positive influence to find the absolute minimum value of the objective function and, as a consequence, produces more reliable values for the kinetic parameters. On the other hand, if some parameters have been estimated by other way, they can be specified as constant in the fitting procedure, which diminishes the total number of parameters to be determined. This happens in this work where k_{cat1}, K_{M1} and K_{M2} were kept constant in the fitting procedure which, together with the high number of experimental conditions and conversion values used, simultaneously, in the fitting procedure, results in a high probability to find an absolute minimum error.

In the fitting process the experimental values corresponding to variable enzyme concentrations, variable substrate concentrations in 1:1 molar ratio (enzyme concentration of 0.017 mg ml^{-1}) and variable 4-chlorophenol concentrations (hydrogen peroxide 1 mM and enzyme concentration of 0.017 mg ml^{-1}) were used, leaving the rest of experiments for checking the goodness of the constants determined by minimization. The values obtained for these constants, together with those determined above from the initial rate equation, are shown in Table 3.

Fig. 4 (graphs A, B and C) show the experimental values (points) versus time for the first three series, while the continuous lines

Table 3
Values obtained for the 11 parameters of the model.

k_{cat1}	249.25 ± 17.76 mmol (g enzyme min)$^{-1}$	n	1.13 ± 0.03 (dimensionless)
K_{cat2}	102.71 ± 3.10 mmol (g enzyme min)$^{-1}$	k_{R1}	2.17 ± 0.06 min^{-1}
K_{M1}	0.17 ± 0.02 mM	K_{M3}	$(5 \pm 0.2) \times 10^{-3}$ mM
K_{M2}	2.60 ± 0.27 mM	K_{M4}	18.71 ± 0.56 (dimensionless)
k_1	$(1.7 \pm 0.2) \times 10^{-3}$ g l^{-1} (mM)$^{1-n}$	k_n	0.27 ± 0.01 (dimensionless)
k_2	$(3 \pm 0.1) \times 10^{-5}$ g l^{-1} (mM)$^{1-n}$		

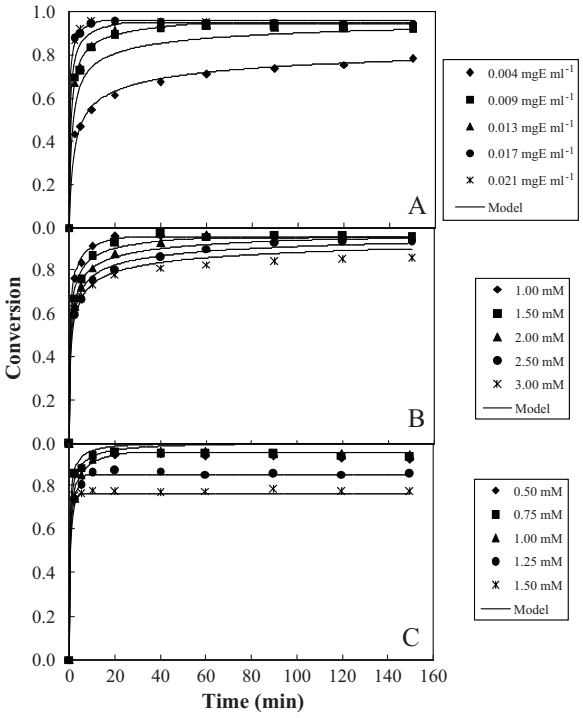

Fig. 4. Fitting the complete model: (A) variable enzyme concentrations, (B) variable substrate concentrations in 1:1 molar ratio and 0.017 mg ml^{-1} of enzyme, (C) variable 4-chlorophenol concentrations, fixed 1 mM hydrogen peroxide and 0.017 mg ml^{-1} of enzyme. Reactor volume: 50 ml, T^{a}: 30 °C and 0.1 M phosphate buffer pH 7.

represent the conversion values calculated by the model for these series. The typical deviation was 3.02%.

Fig. 5 is a comparison of the calculated and experimental values for these series and demonstrates the good fit obtained with the model, as seen from the value of the determination coefficient $R^2 = 0.988$.

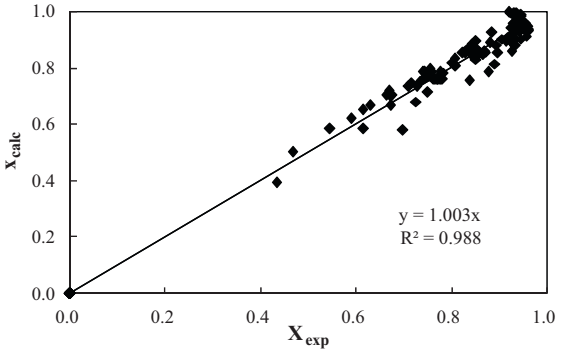

Fig. 5. Calculated and experimental conversion values. Experimental series of Fig. 4(A) B andC.

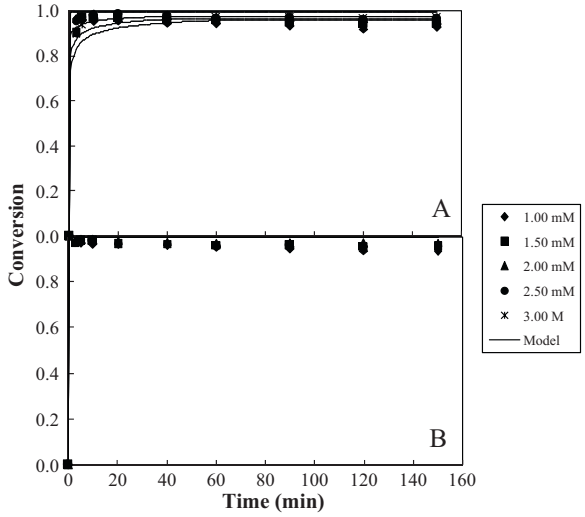

Fig. 6. Checking the model with variables substrate concentration in 1:1 molar ratio: (A) fixed 0.034 mg ml^{-1} enzyme concentration, (B) fixed 0.051 mg ml^{-1} enzyme concentration. Reactor volume: 50 ml, T^{a}: 30 °C and 0.1 M phosphate buffer pH 7.

4.3. Checking the model

Using the values found and with the minimization algorithm deactivated, the program was used to calculate the conversion values provided by the model for the rest of the series of variable substrate concentrations in 1:1 molar ratio (enzyme concentrations of 0.034 and 0.051 mg ml^{-1}), variable hydrogen peroxide (4-chlorophenol 1.0 and 2.0 mM, respectively, and enzyme concentrations of 0.017 mg ml^{-1}) and variable 4-chlorophenol concentrations (hydrogen peroxide 1.5, 1.75 and 2.0 mM, respectively, and enzyme concentrations of 0.017 mg ml^{-1}), which were not used for calculating the model constants.

The results obtained for the series of variable substrate concentrations are shown in Fig. 6 (graphs A and B). Fig. 7 (graphs A and B) shows the results for variable hydrogen peroxide concentrations and Fig. 8 (graphs A, B and C) for variable 4-chlorophenol concentration. As in Fig. 4, the points represent the experimental conversion values and the continuous lines the values calculated

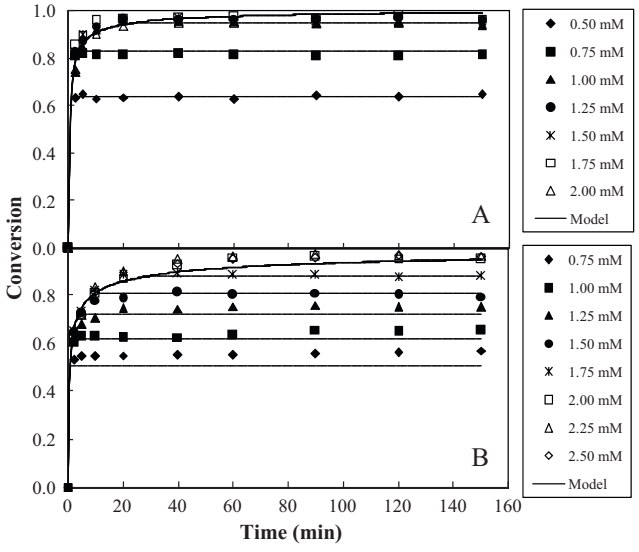

Fig. 7. Checking the model with variable hydrogen peroxide concentration: (A) fixed 1 mM 4-chlorophenol concentration and 0.017 mg ml^{-1} enzyme concentration, (B) fixed 2 mM 4-chlorophenol concentration and 0.017 mg ml^{-1} enzyme concentration. Reactor volume: 50 ml, T^{a}: 30 °C and 0.1 M phosphate buffer pH 7.

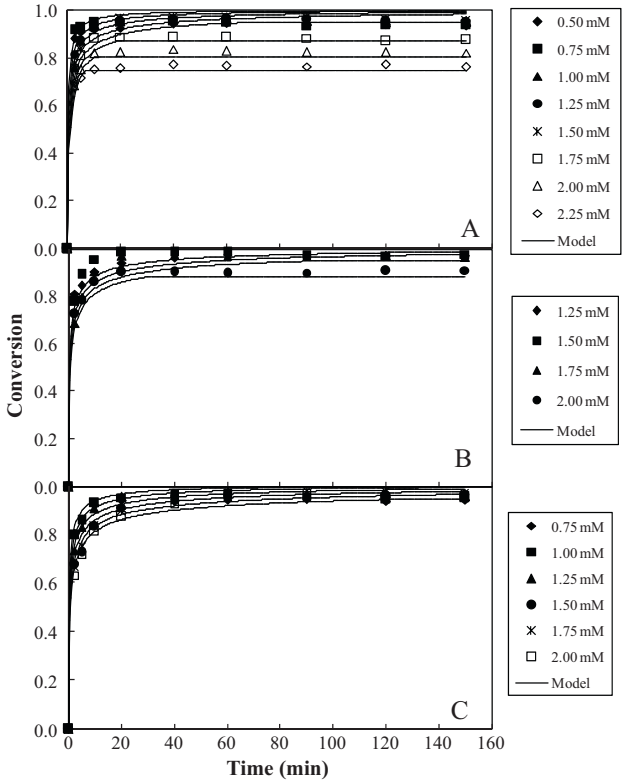

Fig. 8. Checking the model with variable 4-chlorophenol concentration: (A) fixed 1.50 mM hydrogen peroxide concentration and 0.017 mg ml^{-1} enzyme concentration, (B) fixed 1.75 mM hydrogen peroxide concentration and 0.017 mg ml^{-1} enzyme concentration, (C) fixed 2.00 mM hydrogen peroxide concentration and 0.017 mg ml^{-1} enzyme concentration. Reactor volume: 50 ml, T^{a}: 30 °C and 0.1 M phosphate buffer pH 7.

with the model. The fit obtained was as good as the fit reached when constants were being determined, since the standard deviation was 3.02, 3.36 and 2.51%, respectively. The plot of the calculated conversion values versus the experimental ones for all the experiments (Fig. 9) also provided a good determination coefficient, $R^2 = 0.988$, underlining the model's validity for predicting the behavior of the system under study. However, we cannot affirm that the model would be equally valid in conditions involving a great excess of peroxide since, according to the literature, this substance might give rise to inhibition phenomena not reflected in the model's kinetic equations, and even lead to a greater degree of enzyme deactivation.

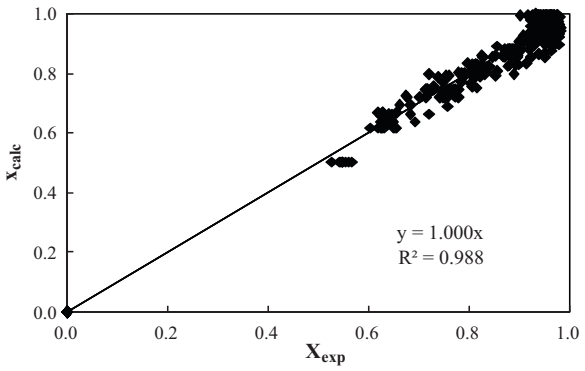

Fig. 9. Calculated and experimental conversion values. Experimental series of Fig. 6(A) and B, 7A and B and 8A, B and C.

4.4. Effectiveness factor: life-time of the enzyme

In previous research carried out with free enzyme, several authors have identified a loss of enzyme activity that was attributed to a deactivation caused by hydrogen peroxide. Some studies show this deactivation [39,40].

However, in the presence of phenolic substrates, the rapid consumption of hydrogen peroxide leads to a reduction in its concentration to levels where the deactivation is not so pronounced. In these conditions, the loss of activity observed in the oxidation reaction of the phenolic substrate has been attributed by other researchers [39,40] to enzyme adsorption to the reaction products (oligomers, polymers) that precipitate and remove the enzyme from the reaction media (enzyme sequestration). This hypothesis seems to be the most accurate and is the basis of the proposed model. However, with the immobilized enzyme, the adsorption of the free enzyme to the polymers formed in the reaction is replaced by the concept of the polymeric film that precipitates over the surface of the catalytic particles which are entrapped inside the polymeric film. This leads to internal diffusion resistances that can be characterized by the effectiveness factor.

In Fig. 10 the variation of effectiveness factor with time is represented for the experimental series used to check the model. It can be observed that, practically, there is no dependence of the effectiveness factor on substrate concentration, which is in good agreement with the hypothesis about the use of the analytical solution of the first order kinetic for its estimation. Also, it can be observed that in the first moments of the reaction the effectiveness factor is unity because the system is pseudo-homogeneous at that moment. However, it quickly decreases to 0.5 (in the first 3–5 min of the reaction), which means that at this time enzyme deactivation has reached 50%. The effectiveness factor is very close to zero after 20 min of

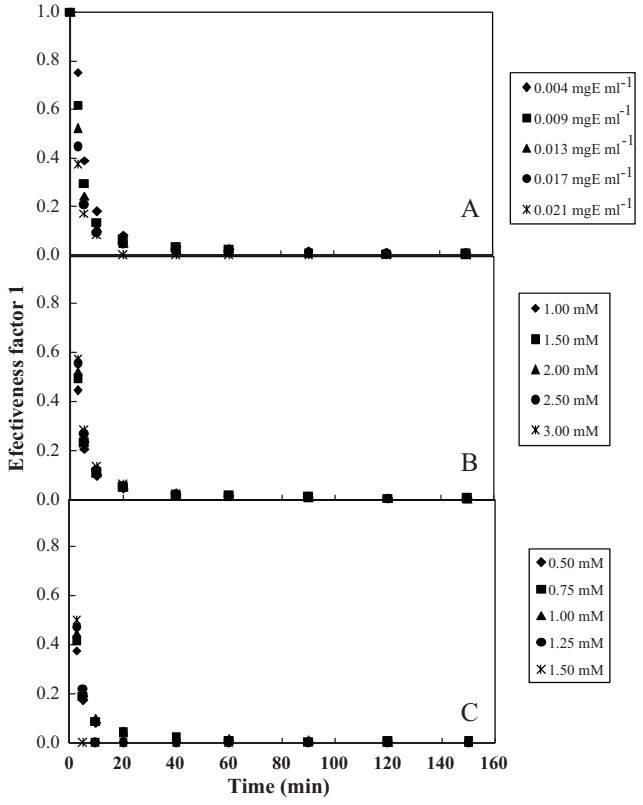

Fig. 10. Effectiveness factor 1: (A) variable enzyme concentration, (B) variables both substrate concentrations in molar ratio 1:1 and 0.017 mg ml^{-1} of enzyme, (C) variable 4-chlorophenol concentration and fixed 1 mM hydrogen peroxide and 0.017 mg ml^{-1} of enzyme.

reaction and is practically equal to zero after 40 min. This behavior is in good agreement with the loss of activity observed in other research using free enzyme. Therefore, the interpretation of a loss of activity in terms of the heterogeneous nature of the system and the appearance of limitations which increase with time does not contradict the results of previous research, but ratifies, in a quantitative way, the model of deactivation by enzyme adsorption and precipitation (enzyme sequestration) that has previously been formulated, in a qualitative way, by other researchers [41].

5. Conclusion

The model presented for the kinetic study of the immobilized SBP/4-chlorophenol/hydrogen peroxide system used the expanded version of the Dunford mechanism, which extends the peroxidase cycle to the reaction products and permits us to obtain a more general kinetic equation. The good results obtained in the fitting of the experimental data to the model confirm the validity of this expanded version of the Dunford mechanism.

At time zero, the extended kinetic equation is reduced to a ping-pong bisubstrate kinetics, which agrees with previous studies. Using an initial rate procedure, the three parameters of the initial rate equation can be determined.

In addition, the phenomenon of deactivation and/or enzyme-sequestration by the precipitated olygomers/polymers, widely described in the literature, has been modeled as the growth of a polymeric film covering the external surface and the pores of the catalytic particles that contain the enzyme, which determines the appearance of diffusional limitations and a growing loss of activity in the same. The deactivation phenomena, interpreted and modeled in terms of the effectiveness factor, are included in the kinetic model.

To confirm the validity of the model different series of experiments were carried out in a discontinuous tank reactor. Integration of the model by numerical calculation and the use of an error minimization algorithm, applied to some of these experimental series enabled us to obtain the values of the remaining parameters, with an excellent degree of agreement. Furthermore, since the model reproduces the behavior of the system for the series of experiments not used for the determination of the parameters, it can be affirmed that, in the experimental range considered, the model is suitable for the kinetic analysis of the system under study. The additional discussion on the effectiveness factor values permit us to establish that the mean life-time of the immobilized enzyme, defined as the time necessary to reduce the activity to 50% of the initial value ($\eta = 0.5$), is about 3–5 min.

Notation

a	First parameter of Eq. (8); $g^2\,l^{-1}\,min^{-1}$
b	Second parameter of Eq. (8); $g^2\,l^{-1}\,min^{-2}$
AH_2	Phenolic compound
AH*	Radical of phenolic compound
HA-AH	Dimer of phenolic compound
D_1	Diffusivity of phenolic compound in the polymeric film; $dm^2\,min^{-1}$
D_2	Diffusivity of dimer in the polymeric film; $dm^2\,min^{-1}$
E	Enzyme
EO	Compound I of enzyme
*EOH	Compound II of enzyme
H_2O_2	Hydrogen peroxide
k_1	Parameter defined in Eq. (18); $g\,l^{-1}\ (mM)^{1-n}$
k_2	Parameter defined in Eq. (19); $g\,l^{-1}\ (mM)^{1-n}$
k_{cat1}	Specific activity of the enzyme in the phenolic compound-oxidizing reaction; mmol of substrate (g of enzyme min)$^{-1}$
k_{cat2}	Specific activity of the enzyme in the dimer-oxidizing reaction; mmol of substrate (g of enzyme min)$^{-1}$
k_{D1}	Parameter defined in Eq. (12); $dm^2\ (mM)^{n-1}\,min^{-1}$
k_{D2}	Parameter defined in Eq. (13); dimensionless
$k_{H_2O_2}$	Proportionality constant for total peroxide consumption; dimensionless
K_{Mi}	Generic Michaelis constants in the kinetic equation ($i = 1$, 2, 3, 4), mM for $i = 1,2,3$ and dimensionless for $i = 4$.
K_{M1}	Intrinsic Michaelis constant for the hydrogen peroxide; mM
K_{M2}	Intrinsic Michaelis constant for the phenolic compound; mM
$(K_{M1})_{2.5}$	Apparent Michaelis constant for the hydrogen peroxide; mM
$(K_{M2})_{2.5}$	Apparent Michaelis constant for the phenolic compound; mM
k_n	Proportionality constant defined in Eq. (21); dimensionless
k_{R0}	Parameter defined in Eq. (9); $g^2\,l^{-1}\,min^{-1}$
k_{R1}	Parameter defined in Eq. (9); min^{-1}
m_1	Thièle modulus for phenolic compound; dimensionless
m_2	Thièle modulus for dimer; dimensionless
mM	milimolar; mmol l^{-1}
M_P	Total mass of precipitated polymer at time t; g
n	Power defined in Eq. (12); dimensionless
R	Average radius of external polymeric film; dm
r_{AH2}	Consumption rate of phenolic compound; mM min^{-1}
r_{HAAH}	Consumption rate of dimer; mM min^{-1}
$r_{H_2O_2}$	Consumption rate of hydrogen peroxide; mM min^{-1}
r_{dimer}	Overall reaction rate of dimer; mM min^{-1}
r_0	Initial reaction rate of phenolic compound; mM min^{-1}
SBP	Soybean peroxidase
t	Time; min
V_{max1}	Maximum reaction rate for phenolic compound ($=k_{cat1}[E]_0$); mM min^{-1}
V_{max2}	maximum reaction rate for dimer ($=k_{cat2}[E]_0$); mM min^{-1}
V_R	Reactor volume; L
Δt	Time increment; min
$[AH_2]_0$	Initial phenolic substrate concentration; mM
$[E]_0$	Initial enzyme concentration; g of enzyme l^{-1}
$[H_2O_2]_0$	Initial hydrogen peroxide concentration; mM
$[X]$	Concentration of a generic species X in the bulk reaction; mM
η_1	Effectiveness factor for phenolic compound reaction rate; dimensionless
η_2	Effectiveness factor for dimer reaction rate; dimensionless
ρ_p	Superficial density of polymeric film; g dm^{-2}

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