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A covered particle deactivation model and an expanded Dunford mechanism for the kinetic analysis of the immobilized SBP/phenol/hydrogen peroxide system

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Abstract

An expanded version of the Dunford mechanism, which extends the initial peroxidase cycle to the reaction products, was developed and applied to the kinetic analysis of the immobilized soybean peroxidase/phenol/hydrogen peroxide system. At the same time, an enzyme deactivation model, based on the gradual covering of the catalytic particles by the products originated during the reaction (radicals and end-products, oligomers/polymers), was proposed. From the reaction mechanism and deactivation model, the kinetic equations for phenol, dimeric compounds and hydrogen peroxide were obtained and applied to the design of a batch reactor.

In order to check the mechanism, an immobilized derivative of the enzyme on PG-glutaraldehyde, which retains 74% of the free enzyme activity and which was characterized in a previous work, was used. In a batch reactor, and without adding protective agents, several series of experiments were carried out, and the influence of operational variables on the conversion was studied. Phenol removal percentages of more than 90% were obtained in some of the tested situations.

Using a method for initial rate estimation, three of the model parameters were calculated. In order to determine the remaining parameters, half of the experimental data series and a program for error minimization, based on the Simplex algorithm of Nelder and Mead, were used. A good fitting between the data and the model was obtained, and the typical deviation was 3.27%. Using the data from the remaining series, which had not been used for determining the parameters, the model was checked and even better agreement, with 2.72% typical deviation, was obtained, which confirms the validity of the proposed model.

Keywords: Soybean peroxidase; Phenol; Hydrogen peroxide; Mechanism; Kinetic model; Deactivation model; Covered particle model

1. Introduction

1.1. Removal of phenolic compounds with peroxidases

Phenolic compounds are present in a wide range of concentrations in the wastewaters of oil refineries and numerous other industries, including the plastics, resins, textiles, iron, steel and forestry industries [1–5]. Most of these compounds are toxic, and cannot easily be removed by conventional physical–chemical or biological techniques.

Some disadvantages of conventional treatment methods can be avoided by adopting an enzymatic method. These methods generally have a high degree of specificity, low energy requirements, mild operation conditions, a high reaction rate which reduces processing costs and a catalytic ability over wide ranges of pH, temperature and substrate concentration. Also, they have a minimal environmental impact.

The application of free or immobilized oxidoreductive enzymes to catalyze the oxidation of aromatic compounds from wastewater has been widely investigated. Horseradish peroxidase (HRP) and soybean peroxidase (SBP) catalyzes the oxidation of aqueous phenols by hydrogen peroxide to produce free radicals that spontaneously interact to form oligomers and polymers of high molecular weight and low solubility. These

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Nomenclature

α_i	parameter defined by a group of constant ($i = 1, 2, 3,\ldots$)
E	enzyme
E_{active}	active enzyme
$E_{inactive}$	inactive enzyme
E_0	total enzyme
EO	enzyme Compound I
$EO\Phi H_2$	intermediate addition complex of Compound I and Phenol
EOHΦ-ΦH	intermediate addition complex of Compound I and dimer
*EOH	enzyme Compound II
$*EOH\Phi H_2$	intermediate addition complex of Compound II and Phenol
*EOHHΦ-ΦH	intermediate addition complex of Compound II and dimer
ΦH_2	phenol
* ΦH	free radical of phenol
*Φ-ΦH	free radical of dimer
HΦ-ΦH	dimer of phenol
HΦ-Φ-Φ-ΦH	tetramer of phenol
k_i	rate constant of an irreversible step i ($i = 1, 2, 3,\ldots$)
k_{cat1}	enzyme catalytic constant in the phenol-oxidizing reaction
k_{cat2}	enzyme catalytic constant in the dimer-oxidizing reaction
k_{CR}	constant defined in Eq. (46)
k_d	enzyme deactivation constant
$k_{H_2O_2}$	proportionality constant for total peroxide consumption
k_n	proportionality constant defined in Eq. (36)
k_R	proportionality constant defined in Eq. (41)
K_i	equilibrium constant of a reversible step i ($i = 1, 2, 3,\ldots$)
K_{Mi}	generic Michaelis constants in the kinetic equation ($i = 1, 2, 3, 4$)
N_{active}	total number of enzyme active centres at time t
N_0	total number (active and inactive) of enzyme catalytic centres
N_{R*}	total number of radical molecules at time t
$r_{\Phi H_2}$	consumption rate of phenol
$r_{H\Phi\text{-}\Phi H}$	consumption rate of dimer
r_{dimer}	overall reaction rate of dimer
r_0	initial reaction rate of phenol
$R*$	generic radical of a phenolic compound
t	time
Δt	time increment
V_{max}	maximum reaction rate
V_R	reactor volume
$[X]$	concentration of the species X in the bulk reaction

products are precipitated from the solution and can be removed by filtration or sedimentation [6–10].

Enzyme immobilization has many advantages, including enzyme reuse and stabilization, the control of product formation and easy separation from the reaction medium [11].

There are several methods for enzyme immobilization as well as support materials. The methods and supports used are chosen to ensure the highest retention of activity and their stability. Conventional methods include physical adsorption, covalent binding, crosslinking, inclusion or encapsulation [12].

In the literature, numerous studies have proposed the mechanism and kinetic equations for peroxidase/phenolic compounds/hydrogen peroxide systems [13–24]. Some of them are described below.

1.2. Kinetics of the enzymatic reaction

1.2.1. Mechanisms

Usually, the oxidation of aromatic compounds with hydrogen peroxide, catalyzed by peroxidase, has been described through the mechanism postulated by Chance–George, also known as the Dunford mechanism [25,26], which is referenced in most of the papers found in the literature.

The steps of this mechanism are the following:

$$E + H_2O_2 \rightarrow E_1$$

$$E_1 + AH_2 \rightarrow E_2 + AH^{\bullet}$$

$$E_2 + AH_2 \rightarrow E + AH^{\bullet}$$

$$AH^{\bullet} + AH^{\bullet} \rightarrow HA{-}AH$$

The native enzyme, E, in the presence of hydrogen peroxide, forms a compound, E_1, called Compound I that, in turn, accepts an aromatic compound, AH_2, oxidizing it and giving the free radical $AH^{\bullet}$. This radical is released to the reaction bulk and the enzyme changes to the E_2 state, Compound II, which is able to oxidize another molecule of AH_2, giving another free radical and returning to the native state, E, so that the cycle is closed. The overall reaction is: $H_2O_2 + 2AH_2 \xrightarrow{E} 2AH^{\bullet} + 2H_2O$

Stoichiometric studies [25] have shown that the species E_2 only contains one of the two oxidation equivalents of the hydrogen peroxide molecule and that it is a covalent compound. However, the definitive structure of this species has not been well established until now and, as indicated in one of the consulted papers [27], "the sequence of steps involved in this catalysis is not, really, very well known", and is usually described by the above mentioned Chance–George mechanism.

As has been described in the literature [25,28,29], in excess of hydrogen peroxide E_2 can be oxidized to E_3, which appears as an inactive form of the enzyme:

$$E_2 + H_2O_2 \rightarrow E_3 + H_2O$$

This is not an irreversible deactivation because E_3 breaks down spontaneously to the native form [30], although with a

low reaction rate:

$$E_3 \rightarrow E + O_2{}^{-}\cdot$$

Baynton et al. [31] observed that, for peroxide concentrations below 1 mM and in the absence of phenol, a reversible inactivation took place through formation of an intermediate compound of the enzyme, called Compound III, from which the native enzyme can be regenerated. On the other hand, in the presence of aromatic substrates, an irreversible inactivation that depends on the substrate concentrations, phenol in this case, was observed, due to the reaction of the native enzyme and the intermediates Compounds I and II with phenolic radicals.

Nicell et al. [32] used horseradish peroxidase in simple and sequential continuous tank reactors to oxidize phenol and 4-chlorophenol. These authors described three enzyme inactivation pathways. Besides the formation of E_3 in excess of peroxide, they believe that a permanent inactivation, by the union of a free radical to the active centre of the enzyme, can take place, as well as an adsorption in the formed polymer. In another work [33], also with HRP and phenol, Nicell et al. established that polyethyleneglycol has a protective effect on these two inactivation modes, and consequently the same authors determined the minimum amount of polyethyleneglycol necessary to achieve maximum protection of the enzyme, as a function of the initial phenol concentration.

Patel et al. [34] focused their research on the oxidation of substituted phenolic compounds by the HRP Compound II. According to these authors, the HRP in this compound is in the form $HRPFe^{IV} = O$, with an oxidation state above that of the native enzyme, which is a hemo-protein with a Fe^{III} nucleus. When reacting with the phenolic substrate an intermediate complex of very low stability is obtained. From the complex, the native enzyme is again released, as well as the oxidation products, according to the following scheme:

$$HRPFe^{IV} = O + S \overset{K_1}{\rightleftarrows} [HRPFe^{IV} = O - S] \xrightarrow{k_2} HRPFe^{III} + P$$

where S is the phenolic substrate and P the reaction products.

As regards to those reaction products, dimeric compounds can appear in the bulk reaction as a result of the binding of two of the radicals formed from the aromatic compound. Although the resulting dimer is less soluble, it remains in the aqueous solution where it can be oxidized again in successive steps, leading to the formation of a longer chain polymer whose precipitation is favoured. This requires additional consumption of hydrogen peroxide and influences the stoichiometry of the overall process, which moves toward gradually higher hydrogen peroxide/phenol molar ratios, depending on the degree of polymerization achieved.

Although many works based on possible mechanisms have been found in the literature, only a few have proposed kinetic models and tried to solve the corresponding equations, fitting the model predictions with the experimental data. In this sense, it can be said that almost all the kinetic models developed and solved until now have been proposed by Nicell et al.

Accepting the Chance–George mechanism and the deactivation phenomena previously described, Nicell proposed three kinetic models for the HRP/hydrogen peroxide/4-chlorophenol system, in steady-state, fully transient and pseudo-steady-state, respectively [14].

As Nicell pointed out, the steady-state model led to an equation similar to the one obtained in a ping-pong bisubstrate enzymatic kinetic.

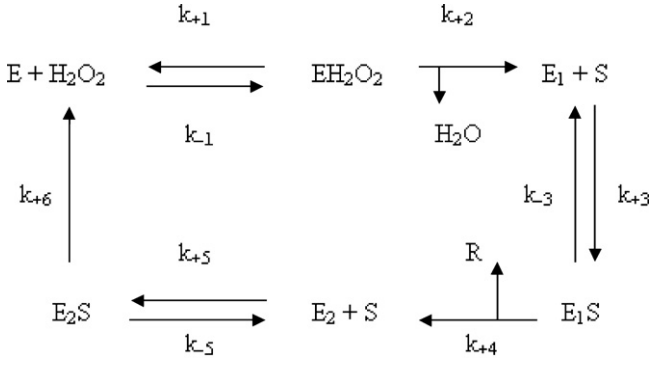
The transient-state model was formulated as a series of nine simultaneous differential equations, containing ten kinetic parameters to be determined by fitting. Due to its complexity, the model must be solved by the Runge-Kutta method and required long computing times, although there may be problems of convergence if the step size is not correctly chosen when applying the mentioned numerical method. Despite these drawbacks, the model provided a good fit of the experimental data.

The pseudo-steady-state model used the steady-state approximation only with some reaction intermediates and, despite certain simplifications, it remains complicated and it is still necessary to use numerical methods and a long computation time. The fitting of the model to the experimental data is 2% less precise than with the fully transient model, but in the authors' opinion, this loss of precision can be justified in order to achieve simplicity and to reduce the computation time.

Later, Buchanan and Nicell [15] and Buchanan et al. [13] applied the above model equations to obtaining the design equations for plug flow and continuous tank reactors in steady-state. Good fitting was achieved between the experimental data and the equations obtained for the HRP/phenol system, the enzyme inactivation rate in the continuous tank being lower than the one calculated in the plug flow reactor, working with PEG excess and keeping the complexity grade of the models as explained previously. Later, Buchanan and Nicell [35] produced a simplified model where the active species of the enzyme are gathered in the so-called "active enzyme", E_a, which reduces the number of constants and simplify the calculation.

Choi et al. [24] proposed a modification of the Chance–George mechanism, for the HRP/pentachlorophenol (PCP) system. According to these authors, intermediate enzyme-substrate complexes (EH_2O_2, E_1PCP and E_2PCP) are produced in the first three steps of the mechanism. These authors obtained the initial reaction rate by using the steady-state approximation, checking that oxidation of pentachlorophenol with HRP follows a bisubstrate ping-pong mechanism.

A phenol oxidation process by HRP involving intermediate complex formation has also been proposed by Gilabert et al. [36], according to the following scheme:



By using a quantitative initial rate analysis, these authors determined the values of the Michaelis–Menten constants for phenol and hydrogen peroxide, as well as the enzyme catalytic constant value.

Tong et al. [22] have also described the oxidation reaction of phenol, 4-chlorophenol and 3-chlorophenol, catalyzed by horseradish peroxidase, as a bisubstrate ping-pong mechanism, and calculated the kinetic constants for different hydrogen peroxide concentrations, demonstrating that an optimum peroxide concentration exists, above which peroxide becomes a reaction inhibitor.

On the other hand, Wu et al. [23] developed a kinetic model for the phenol/horseradish peroxidase system in the presence of polyethyleneglycol. The phenol oxidation was described by using a Michaelis–Menten bisubstrate equation.

Although most kinetic studies have been carried out with horseradish peroxidase, some of them apply the Chance–George mechanism to the oxidation of phenols with soybean peroxidase [16,19,21,37].

In this sense, Nicell and Wright [16] developed a model showing the dependence of the enzyme activity on the H_2O_2 concentration and they have also calculated the kinetic constants, which were lower than those obtained for HRP. They also noticed that soybean peroxidase, through the E_3 form, is more susceptible to permanent inactivation with hydrogen peroxide than HRP.

Nissum et al. [37] found that, in the presence of hydrogen peroxide, the mechanisms of both HRP and SBP are very similar, although Compound I of SBP is less stable at neutral pH.

According to the Chance–George mechanism, the stoichiometric peroxide/phenol ratio must be 1:2. However, some studies have been found in the literature that obtained different values for this relationship. For example, Al-Kassim et al. [38] obtained values within the range 0.5–0.83. A result approaching unity was obtained in other works [15,23]. Furthermore, Buchanan and Han [39], working with *Arthromyces ramosus* peroxidase, obtained an average value of 1.22 at high enzyme and phenol concentrations and a value of 1.01 at low concentrations. These different stoichiometries observed might be attributed to differences in the average number of monomers in the precipitated polymer.

As regards the nature of polymers, five dimers and a trimer were identified in the reaction solution by Yu et al. [40], working with the horseradish peroxidase/phenol system. They showed that three of these dimers are also peroxidase substrates and can be oxidized like phenol, following first-order kinetics obtained by simplifying a bisubstrate model. The dimers are *p*-phenoxyphenol, *p*,*p*-bisphenol and *o*,*o*-bisphenol, the oxidation rate of the first being higher than the phenol oxidation rate. According to these authors, the extra-consumption of hydrogen peroxide that takes place above the stoichiometric relationship can be explained by the oxidation of the mentioned compounds. Finally, a deactivation enzyme model, based on the attack of phenoxy radicals on the enzyme active centre following second-order kinetics and depending on the PEG concentration, is also proposed in [40].

2. Theory: the proposed new extended mechanism

According to the original Chance–George mechanism, and accepting the intermediate complex formation proposed by Choi et al. [24] and Gilabert et al. [36], an extended version of the mentioned mechanism is proposed, taking into account the following points:

- The reaction takes place on the surface of the catalytic particles where the enzyme is immobilized. There are no external diffusional limitations due to the good stirring of the reaction media.
- The reaction takes place under moderate values of the hydrogen peroxide/phenol ratio, and the formation of Compound III is negligible.
- The overall process consists of an indetermined number of catalytic cycles that depends on the operational conditions.
- The initial cycle corresponds to the Dunford mechanism, in the enhanced version of Gilabert et al. [36] which takes into account the formation of enzyme-substrate intermediate complexes and produces monomer radicals that, by coupling, can form dimers with several isomeric structures [40].
- The dimers formed in the first step remain soluble and react in the same cycle as phenol, leading to the formation of higher oligomers with higher peroxide consumption. Although the different dimers have different reactivities with the enzyme, only one average reaction rate for all of them is assumed.
- This cycle continues to run as long as there is hydrogen peroxide in the reaction medium, with the trimer, tetramer, etc. acting as the new substrate that takes part in the cycle, whose sequence remains the same as in the initial cycle.

The initial cycle for phenol and the one corresponding to the dimers are described below.

2.1. Initial cycle: phenol and peroxide consumption and dimer formation

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

$$\text{Steps 3 and 4 :} \quad \mathrm{EO} + \Phi\mathrm{H_2} \overset{K_3}{\rightleftarrows} \mathrm{EO}\Phi\mathrm{H_2} \xrightarrow{k_4} * \mathrm{EOH} + *\Phi\mathrm{H}$$

$$\text{Steps 5 and 6 :} \quad *\mathrm{EOH} + \Phi\mathrm{H_2} \overset{K_5}{\rightleftarrows} * \mathrm{EOH}\Phi\mathrm{H_2} \xrightarrow{k_6} \mathrm{E} + \mathrm{H_2O} + *\Phi\mathrm{H}$$

$$\text{Step 7 :} \quad * \Phi\mathrm{H} + *\Phi\mathrm{H} \xrightarrow{k_7} \mathrm{H}\Phi\text{-}\Phi\mathrm{H}$$

If only this cycle is considered, as in the previous works cited in the Introduction, the commonly accepted bisusbtrate ping-pong equation is obtained, and the initial reaction rate for phenol is given by the following equation:

$$(r_{\Phi\mathrm{H_2}})_0 = \frac{k_{\mathrm{cat1}}[\mathrm{E}]_0[\Phi\mathrm{H_2}]_0[\mathrm{H_2O_2}]_0}{K_{\mathrm{M1}}[\Phi\mathrm{H_2}]_0 + K_{\mathrm{M2}}[\mathrm{H_2O_2}]_0 + [\Phi\mathrm{H_2}]_0[\mathrm{H_2O_2}]_0} \quad (1)$$

where K_{M1} and K_{M2} are the Michaelis constants for hydrogen peroxide and phenol, respectively, k_{cat1} the specific activity of the enzyme and $V_{max} = k_{cat1}[E]_0$ the maximum reaction rate.

2.2. Cycle 2: additional peroxide consumption and tetramer formation

Steps 8 and 9 : $$EO + H\Phi\text{-}\Phi H \overset{K_8}{\rightleftarrows} EOH\Phi\text{-}\Phi H \xrightarrow{k_9} *EOH + *\Phi\text{-}\Phi H$$

Steps 10 and 11 : $$*EOH + H\Phi\text{-}\Phi H \overset{K_{10}}{\rightleftarrows} *EOHH\Phi\text{-}\Phi H \xrightarrow{k_{11}} E + H_2O + *\Phi\text{-}\Phi H$$

Step 12 : $$*\Phi\text{-}\Phi H + *\Phi\text{-}\Phi H \xrightarrow{k_{12}} H\Phi\text{-}\Phi\text{-}\Phi\text{-}\Phi H$$

Compounds I and II have been described with the structures EO and *EOH, respectively, in both catalytic cycles.

2.3. Additional hypotheses of the mechanism

Besides the initial hypotheses, the following are considered:

- The equilibrium approximation is applicable to the intermediate complexes formed in the steps 1, 3, 5, 8 and 10.
- For steps 4 and 6, in which phenol is consumed, although their real individual reaction rates might be different, an average rate is assumed with the same value for both steps. The same approximation is made for steps 9 and 11.

According to these additional hypotheses, the kinetic and equilibrium relationships can be formulated as follows.

2.4. Equilibrium relationships

For the different intermediate complexes and taking into account the equilibrium approximation for steps 1, 3, 5, 8 and 10, it can be verified that:

$$[EH_2O_2] = K_1[E][H_2O_2] \quad (2)$$

$$[EO\Phi H_2] = K_3[EO][\Phi H_2] \quad (3)$$

$$[*EOH\Phi H_2] = K_5[*EOH][\Phi H_2] \quad (4)$$

$$[EOH\Phi\text{-}\Phi H] = K_8[EO][H\Phi\text{-}\Phi H] \quad (5)$$

$$[*EOHH\Phi\text{-}\Phi H] = K_{10}[*EOH][H\Phi\text{-}\Phi H] \quad (6)$$

2.5. Relationships between the intermediate complexes

According to the proposed mechanism and taking into account the hypotheses mentioned above for steps 4 and 6 and for steps 9 and 11, the following equations can be obtained:

$$k_2[EH_2O_2] = k_4[EO\Phi H_2] + k_9[EOH\Phi\text{-}\Phi H] \quad (7)$$

$$k_4[EO\Phi H_2] = k_6[*EOH\Phi H_2] \quad (8)$$

$$k_9[EOH\Phi\text{-}\Phi H] = k_{11}[*EOHH\Phi\text{-}\Phi H] \quad (9)$$

From Eqs. (2)–(9), the concentration of the different forms of the enzyme can be expressed as functions of the intermediate $EO\Phi H_2$ complex and, after several operations, we obtain:

$$[E] = \frac{k_4}{K_1 k_2}\frac{[EO\Phi H_2]}{[H_2O_2]} + \frac{K_{10}k_{11}k_4}{K_1K_5k_2k_6}\frac{[H\Phi\text{-}\Phi H][EO\Phi H_2]}{[\Phi H_2][H_2O_2]} \quad (10)$$

$$[EH_2O_2] = \frac{k_4}{k_2}[EO\Phi H_2] + \frac{K_{10}k_{11}k_4}{K_5k_2k_6}\frac{[H\Phi\text{-}\Phi H]}{[\Phi H_2]}[EO\Phi H_2] \quad (11)$$

$$[EO] = \frac{[EO\Phi H_2]}{K_3[\Phi H_2]} \quad (12)$$

$$[*EOH] = \frac{k_4}{K_5k_6}\frac{[EO\Phi H_2]}{[\Phi H_2]} \quad (13)$$

$$[*EOH\Phi H_2] = \frac{k_4}{k_6}[EO\Phi H_2] \quad (14)$$

$$[EOH\Phi\text{-}\Phi H] = \frac{K_{10}k_{11}k_4}{K_5k_6k_9}\frac{[H\Phi\text{-}\Phi H]}{[\Phi H_2]}[EO\Phi H_2] \quad (15)$$

$$[*EOHH\Phi\text{-}\Phi H] = \frac{K_{10}k_4}{K_5k_6}\frac{[H\Phi\text{-}\Phi H]}{[\Phi H_2]}[EO\Phi H_2] \quad (16)$$

2.6. Total enzyme balance: active and inactive enzyme

Strong enzyme inactivation occurs during the reaction process. This inactivation has been attributed by several authors [41,42] to the ability of the different radicals, formed in the course of the reaction to bind to the enzyme active centres. The enzyme surface is covered by the end-products resulting for the coupling of radicals. These end-products (oligomers and polymers) are subsequently precipitated, dragging down the enzyme ("enzyme sequestration").

Later in this work we will propose a deactivation model for the enzyme but, before then, the following balance equation can be formulated by classifying the enzyme, as Nicell [35], into active and inactive forms:

$$[E]_0 = [E_{active}] + [E_{inactive}] \quad (17)$$

It can also be verified that:

$$[E_{active}] = [E] + [EH_2O_2] + [EO] + [EO\Phi H_2] + [*EOH] + [*EOH\Phi H_2] + [EOH\Phi\text{-}\Phi H] + [*EOHH\Phi\text{-}\Phi H] \quad (18)$$

By substituting in (18) the concentrations of the different active enzyme forms, given by Eqs. (10)–(16), and regrouping, becomes:

$$[E_{active}] = [EO\Phi H_2]\left(1 + \frac{k_4}{k_2} + \frac{k_4}{k_6} + \frac{k_4}{K_1k_2}\frac{1}{[H_2O_2]} + \frac{1}{K_3[\Phi H_2]} + \frac{k_4}{K_5k_6}\frac{1}{[\Phi H_2]} + \frac{K_{10}k_4}{K_5k_6}\frac{[H\Phi\text{-}\Phi H]}{[\Phi H_2]}\right.$$

$$+\frac{K_{10}k_{11}k_4}{K_5k_9k_6}\frac{[\text{H}\Phi\text{-}\Phi\text{H}]}{[\Phi\text{H}_2]}+\frac{K_{10}k_{11}k_4}{K_5k_6k_2}\frac{[\text{H}\Phi\text{-}\Phi\text{H}]}{[\Phi\text{H}_2]}+\frac{K_{10}k_{11}k_4}{K_1K_5k_6k_2}\frac{[\text{H}\Phi\text{-}\Phi\text{H}]}{[\Phi\text{H}_2][\text{H}_2\text{O}_2]}\Bigg) \quad (19)$$

By defining:

$$\alpha_1 = 1+\frac{k_4}{k_2}+\frac{k_4}{k_6}; \quad (20)$$

$$\alpha_2 = \frac{k_4}{K_1k_2}; \quad (21)$$

$$\alpha_3 = \frac{1}{K_3}+\frac{k_4}{K_5k_6}; \quad (22)$$

$$\alpha_4 = \frac{K_{10}k_4}{K_5k_6}+\frac{K_{10}k_{11}k_4}{K_5k_9k_6}+\frac{K_{10}k_{11}k_4}{K_5k_6k_2}; \quad (23)$$

$$\alpha_5 = \frac{K_{10}k_{11}k_4}{K_1K_5k_6k_2}; \quad (24)$$

Eq. (19) can be written as follows:

$$[\text{E}_{\text{active}}] = [\text{EO}\Phi\text{H}_2]\left(\alpha_1+\alpha_2\frac{1}{[\text{H}_2\text{O}_2]}+\alpha_3\frac{1}{[\Phi\text{H}_2]}+\alpha_4\frac{[\text{H}\Phi\text{-}\Phi\text{H}]}{[\Phi\text{H}_2]}+\alpha_5\frac{[\text{H}\Phi\text{-}\Phi\text{H}]}{[\Phi\text{H}_2][\text{H}_2\text{O}_2]}\right) \quad (25)$$

from which the following expression was obtained:

$$[\text{EO}\Phi\text{H}_2] = \frac{[\text{E}_{\text{active}}][\Phi\text{H}_2][\text{H}_2\text{O}_2]}{\alpha_1[\Phi\text{H}_2][\text{H}_2\text{O}_2]+\alpha_2[\Phi\text{H}_2]+\alpha_3[\text{H}_2\text{O}_2]+\alpha_4[\text{H}\Phi\text{-}\Phi\text{H}][\text{H}_2\text{O}_2]+\alpha_5[\text{H}\Phi\text{-}\Phi\text{H}]} \quad (26)$$

2.7. Reaction rates for phenol, dimer and hydrogen peroxide

2.7.1. Phenol consumption rate

According to the proposed mechanism, phenol consumption is given by summing steps 4 and 6, both of which have the same rate, so it can be verified that:

$$r_{\Phi\text{H}_2} = 2k_4[\text{EO}\Phi\text{H}_2] \quad (27)$$

where $r_{\Phi\text{H}_2}$ is the phenol consumption rate.

According to Eq. (26), and by defining:

$$k_{\text{cat1}} = \frac{2k_4}{\alpha_1}; \quad K_{\text{M1}} = \frac{\alpha_2}{\alpha_1}; \quad K_{\text{M2}} = \frac{\alpha_3}{\alpha_1}; \quad K_{\text{M3}} = \frac{\alpha_5}{\alpha_1}; \quad K_{\text{M4}} = \frac{\alpha_4}{\alpha_1} \quad (28)$$

the following equation for the phenol consumption rate is obtained:

$$r_{\Phi\text{H}_2} = \frac{k_{\text{cat1}}[\text{E}_{\text{active}}][\Phi\text{H}_2][\text{H}_2\text{O}_2]}{K_{\text{M1}}[\Phi\text{H}_2]+K_{\text{M2}}[\text{H}_2\text{O}_2]+[\Phi\text{H}_2][\text{H}_2\text{O}_2]+K_{\text{M3}}[\text{H}\Phi\text{-}\Phi\text{H}]+K_{\text{M4}}[\text{H}\Phi\text{-}\Phi\text{H}][\text{H}_2\text{O}_2]} \quad (29)$$

At zero time, Eq. (29) can be simplified to:

$$(r_{\Phi\text{H}_2})_0 = \frac{k_{\text{cat1}}[\text{E}]_0[\Phi\text{H}_2]_0[\text{H}_2\text{O}_2]_0}{K_{\text{M1}}[\Phi\text{H}_2]_0+K_{\text{M2}}[\text{H}_2\text{O}_2]_0+[\Phi\text{H}_2]_0[\text{H}_2\text{O}_2]_0} \quad (30)$$

which is identical to the above mentioned Eq. (1) for the initial reaction rate of a ping-pong bisubstrate kinetic.

2.7.2. Dimer formation rate

The dimer is formed by the binding of two phenoxy radicals and it is consumed in steps 9 and 11, so that both facts must be taken into consideration. Furthermore, the production of phenoxy radicals is proportional to the phenol removal rate, and each dimer molecule is produced at the expense of two phenoxy radicals, so the dimer formation rate is nearly half that of the phenol removal rate. According to the hypotheses corresponding to the proposed mechanism, steps 9 and 11 have the same reaction rate, and the following equation for the dimer reaction rate is obtained:

$$r_{\text{dimer}} = \frac{1}{2}r_{\Phi\text{H}_2} - r_{\text{H}\Phi\text{-}\Phi\text{H}} \quad (31)$$

where r_{dimer} is the overall dimer generation rate and $r_{\Phi\text{H}_2}$ and $r_{\text{H}\Phi\text{-}\Phi\text{H}}$ are the phenol and dimer removal rates, respectively. An additional equation for $r_{\text{H}\Phi\text{-}\Phi\text{H}}$ is necessary to evaluate r_{dimer}, since the expression corresponding to $r_{\Phi\text{H}_2}$ is given by Eq. (29).

Taking into account steps 9 and 11 of the mechanism, it can be deduced that:

$$r_{\text{H}\Phi\text{-}\Phi\text{H}} = 2k_9[\text{EOH}\Phi\text{-}\Phi\text{H}] \quad (32)$$

and, according to Eq. (15), it can be converted to:

$$r_{\text{H}\Phi\text{-}\Phi\text{H}} = 2\frac{K_{10}k_{11}k_4}{K_5k_6}\frac{[\text{H}\Phi\text{-}\Phi\text{H}]}{[\Phi\text{H}_2]}[\text{EO}\Phi\text{H}_2] \quad (33)$$

Substituting the value of $[\text{EO}\Phi\text{H}_2]$ given by Eq. (26) in the above expression, taking into consideration the auxiliary constants shown in Eq. (28) and defining a second catalytic constant as follows:

$$k_{\text{cat2}} = \frac{K_{10}k_{11}}{K_5k_6}k_{\text{cat1}} \quad (34)$$

the following expression for the dimer removal rate is finally obtained:

$$r_{\text{H}\Phi\text{-}\Phi\text{H}} = \frac{k_{\text{cat2}}[\text{E}_{\text{activa}}][\text{H}\Phi\text{-}\Phi\text{H}][\text{H}_2\text{O}_2]}{K_{\text{M1}}[\Phi\text{H}_2]+K_{\text{M2}}[\text{H}_2\text{O}_2]+[\Phi\text{H}_2][\text{H}_2\text{O}_2]+K_{\text{M3}}[\text{H}\Phi\text{-}\Phi\text{H}]+K_{\text{M4}}[\text{H}\Phi\text{-}\Phi\text{H}][\text{H}_2\text{O}_2]} \quad (35)$$

so the value of r_{dimer} can be calculated.

2.7.3. Hydrogen peroxide consumption rate

As regards the consumption of hydrogen peroxide, the following must be borne in mind:

- In each cycle, the peroxide consumed is half the consumption of the phenolic compounds involved in the cycle (phenol, dimer, trimer, etc.). If there is no large excess of hydrogen peroxide, as has been assumed in this work, any additional hydrogen peroxide consumption for Compound III formation can be considered negligible.
- Since only the phenol and dimer cycles are taken into account in the amplified mechanism proposed, we consider the additional consumption of peroxide in the remaining cycles in which other polymeric substances intervene to be proportional to the amount consumed in the two previous cycles. Therefore, the total peroxide consumption can be expressed as:

$$r_{H_2O_2} = k_n\,(k_4[\text{EO}\Phi\text{H2}] + k_9[\text{EOH}\Phi\text{-}\Phi\text{H}]) \tag{36}$$

where $r_{H_2O_2}$ is the rate of peroxide disappearance and k_n is the proportionality constant relating total peroxide consumption in the n cycles making up the process and the consumption corresponding to the phenol and dimer cycles.

Taking Eqs. (36) and (32) into account, the above peroxide consumption rate can be expressed as:

$$r_{H_2O_2} = \frac{k_n}{2}(r_{\Phi H_2} + r_{H\Phi\text{-}\Phi H}) \tag{37}$$

or:

$$r_{H_2O_2} = k_{H_2O_2}(r_{\Phi H_2} + r_{H\Phi\text{-}\Phi H}) \tag{38}$$

whereby the new constant can been defined:

$$k_{H_2O_2} = \frac{k_n}{2}. \tag{39}$$

2.8. Deactivation of the enzyme: the covered particle deactivation model

Taking into account some of the previous deactivation models proposed by others authors [41,42], which are based on the attack of radicals on the active site of the enzyme and also on the enzyme dragging down with the precipitated end-product, oligomers/polymers ("enzyme sequestration"), a new enzyme deactivation model is presented, based fundamentally on the successive action of the radicals, according to the following hypothesis:

- In a first step, the different radicals originated in the process, besides binding among themselves to form polymers, can bind, in an irreversible process, to any of the different forms in which the enzyme is present. Afterwards, and by successive coupling of pairs of radicals, they can form end-products (oligomers/polymers) attached to the enzyme in a process that leads to the gradual covering of the surface of the catalytic particles on which the enzyme is immobilised ("*the covered particle deactivation model*").
- When the first radicals are deposited on any of the active forms of the enzyme, they produce stable enzyme-radical complexes that deactivates the enzyme through blocking its active centres. Further, other radicals can also bind to these enzyme-radical complexes to form the successive end-products (oligomers/polymers), which is equivalent to an "in situ" precipitation of the end-products on the surface of catalytic particles. This does not involve an additional deactivation of the enzyme since these centres are already blocked by the first radicals bound to them, but does involve the additional consumption of radicals and contributes to increasing the thickness of the film covering the catalytic particles.

In agreement with this hypothesis, if N_0 is the total number of catalytic centres of the enzyme, both active and inactive, N_{active} the total number of active centres at a given moment, and N_{R*} the total number of individual molecules of all the radical species present in the reaction medium at that moment, the density of the radicals with reference to the total number of catalytic centres can be defined by the equation:

$$\rho_{R*} = \frac{N_{R*}}{N_0} \tag{40}$$

At a given moment, the rate of interaction of the radicals with the free active centres must be proportional to the total number of free active centres at this moment and the density defined in Eq. (40). Denoting the proportionality constant k_R, a balance of free active centres between the instants, t and $t+\Delta t$, leads to the following equation:

$$(N_{active})_{t+\Delta t} = (N_{active})_t - k_R(\rho_{R*})(N_{active})_t \Delta t \tag{41}$$

which, for $\Delta t \to 0$, becomes:

$$\frac{dN_{active}}{dt} = -k_R(\rho_{R*})(N_{active}) \tag{42}$$

If the volume of the reaction medium is constant and equal to V_R, Eq. (42) can be expressed as:

$$\frac{d(V_R[E_{active}])}{dt} = -k_R\frac{V_R[R^*]}{V_R[E]_0}V_R[E_{active}] \tag{43}$$

and, simplifying:

$$\frac{d[E_{active}]}{dt} = -k_R\frac{[R^*]}{[E]_0}[E_{active}] \tag{44}$$

Finally, if we accept the approximation to the steady state for the total concentration of radicals in the medium, given its high reactivity, we can obtain:

$$\frac{d[R^*]}{dt} = 0 \tag{45}$$

which permits us to consider the concentration of radicals as almost constant:

$$[R^*] \approx k_{CR} \tag{46}$$

Defining one deactivation constant by the relationship:

$$k_d = k_R k_{CR} \tag{47}$$

we arrive at the following law for the variation of active species concentration of the enzyme in the reaction medium:

$$-\frac{d[E_{active}]}{dt} = k_d \frac{[E_{active}]}{[E]_0} \quad (48)$$

and, by integration:

$$[E_{active}] = [E]_0 \exp\left(-\frac{k_d}{[E]_0} t\right) \quad (49)$$

This equation, together with Eqs. (28), (35) and (38), completes the kinetic model of the process being studied. It should be emphasised that Eq. (49) does not represent a simple exponential deactivation, since the exponential term depends, not only on the actual reaction time, but also on the initial concentration of enzyme.

3. Materials and methods

3.1. Chemicals

Soybean peroxidase, SBP (EC 1.11.1.7, lyophilized powder, 108 U/mg, Mw ≈ 44 kDa), hydrogen peroxide (35%, w/v) and phenol (99%) were purchased from Sigma. Analytical chemicals 4-aminoantipyrine (AAP) and potassium ferricyanide were also from Sigma. Other chemicals were of analytical grade and were used without further purification.

3.2. Immobilization procedure

SBP was covalently immobilized on aminopropyl-glass beads activated with glutaraldehyde. The immobilization was carried out according to the procedure previously described by the authors [43]. 45.2% of the offered protein was immobilized and the protein content of the immobilized derivative was 34.9 mg SBP/g dry support. In the immobilization procedure 2 g of dry support were used. The immobilized derivative was suspended in 100 ml of 0.1 M phosphate buffer, pH 7, and stored at 4 °C until use. In the storage suspension, the enzyme concentration was 0.70 mg SBP/ml. The activity of the immobilized SBP was 74% of the corresponding free enzyme (80 U/mg immobilized enzyme).

3.3. Experimental system

Experiments were conducted in a jacketed batch reactor (30 °C) of 50 cm^3 total volume, magnetically stirred. The substrates, phenol and hydrogen peroxide, were aqueous solutions and the immobilized enzyme derivative was suspended in phosphate buffer 0.1 M pH 7. The same buffer was used to complete the reactor volume, giving a final buffer concentration of 70 mM. Phenol, hydrogen peroxide and enzyme concentrations were varied as indicated below. First, phenol and the buffer solutions were placed in the reactor. When an adequate temperature of 30 °C was reached the enzyme suspension was added followed by the hydrogen peroxide solution. The reaction course was followed by taking 1 ml samples through a nylon membrane (10 μm), and analyzing its phenol concentration up to reaction time of 150 min.

3.4. Analytical method

Phenol concentrations were measured by a colorimetric method 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 sample (phenol 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 colour 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 phenol concentrations in the sample by using a calibration curve ([phenol] = 0.0952 × Abs505, $r = 0.9997$).

3.5. Experimental planning

Six series of experiments were carried out.

3.5.1. Series 1: variation of enzyme concentration

Five enzyme concentrations were used: 0.014, 0.021, 0.028, 0.042 and 0.056 mg/cm^3 (1.120, 1.680, 2.240, 3.360 and 4.480 U/cm^3, respectively). Substrates concentration was kept constant at 2 mM.

3.5.2. Series 2: variation of phenol and hydrogen peroxide concentrations

For a molar substrates ratio of 1:1, five phenol and hydrogen peroxide concentrations were assayed: 1.00, 1.50, 2.00, 3.00 and 4.00 mM. Enzyme concentration was maintained at 0.028 mg/cm^3.

3.5.3. Series 3: variation of phenol concentration

In these five experiments, hydrogen peroxide concentration was maintained at 2.00 mM and enzyme concentration was 0.028 mg/cm^3. Phenol concentrations were: 1.00, 1.50, 2.00, 2.50 and 3.00 mM.

3.5.4. Series 4–6: variation of hydrogen peroxide concentration

In this series of experiments three phenol concentrations were used: 1.00, 1.50 and 2.00 mM (series 4–6, respectively). In series 4 and 5 the hydrogen peroxide concentrations assayed were: 0.50, 0.75, 1.00, 1.50 and 2.00 mM. In series 6, hydrogen peroxide concentrations were: 0.50, 1.00, 1.50, 2.00 and 2.50 mM. In all these experiments, enzyme concentration was kept constant at 0.028 mg/cm^3.

3.6. Stock solutions

In the preparation of the samples for the above mentioned experimental series, three stock solutions were used.

3.6.1. Immobilized enzyme stock suspension

This was not strictly speaking a solution, but the above mentioned storage suspension of the immobilized derivative. As indicated, the enzyme concentration was 0.70 mg SBP/ml. From this suspension, which was thoroughly stirred to homogenize it, the exact volume to attain the specified enzyme concentrations of the assay was added to the reactor.

3.6.2. Phenol stock solution

A 20 mM solution of phenol, in 0.1 M phosphate buffer, pH 7, was prepared and its concentration checked by using the above described colorimetric method. The solution was stored at 4 °C until use. By measuring an exact volume from this solution and by adding it to the reactor, the required initial phenol concentration for the assay can be obtained. Periodically, the concentration of the stock solution was checked with the colorimetric method.

3.6.3. Hydrogen peroxide stock solution

A 20 mM solution of hydrogen peroxide, in distilled water, was prepared and stored at 4 °C until use. The exact concentration of the solution was determined by volumetric analysis with a 0.1N solution of potassium permanganate. By measuring an exact volume from this solution and by adding it to the reactor, the required initial hydrogen peroxide concentration for the assay can be obtained. Periodically, the concentration of the stock solution was checked with the same permanganate solution.

4. Results and discussion

4.1. Fitting the model: parameters determination

To fit the model and determine the values of its parameters, we used the experimental conversion results for series 1–3, leaving those of series 4, 5 and 6 for use to check the first values.

The model contains a total of eight parameters: k_{cat1}, k_{cat2}, K_{M1}, K_{M2}, K_{M3}, K_{M4}, $k_{H_2O_2}$ and k_d. Of all these parameters, Eq. (30) representing the initial rate and identical to a ping-pong mechanism, only has 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. (28), (35), (38) and (49) must be used, along with a numerical calculation method for their integration, such as an error minimisation algorithm, to optimise the values. The way in which this was done is described below.

4.1.1. Initial rates: ping-pong equation and intrinsic parameter

Because deactivation by radicals 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. (30), which provides a family of parallel straight lines, from whose ordinates and slope the desired values of the constants can be obtained, we used the parameter determination method of Gómez et al. [44].

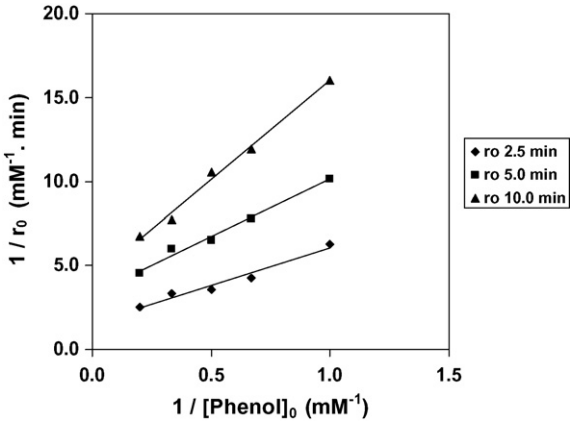


Fig. 1. Fitting the series 2 to Eq. (50).

Basically, this method makes use of three linear relationships obtained from Eq. (30) in the conditions specified below and the mean values of the reaction rate during the first moments, in this case 2.5, 5.0 and 10.0 min. With 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. (30). The equations used for the method and the corresponding conditions are detailed below.

4.1.1.1. Variable but equal substrate concentrations. When the initial concentrations of both substrates are equal, Eq. (30) adopts the form of a simple Michaelis–Menten kinetic, so that the de Lineweaver-Burk linearization is valid:

$$\frac{1}{r_0} = \frac{1}{V_{max}} + \frac{K_{M1} + K_{M2}}{V_{max}} \frac{1}{[\Phi H_2]_0} \tag{50}$$

$$\text{where :} \quad V_{max} = k_{cat1}[E]_0 \tag{51}$$

Since the intercept of Eq. (50) only contains the parameter V_{max}, this equation can be used to determine it. Therefore, with the value of the slope of Eq. (50) and the calculated value of V_{max}, the value of $(K_{M1} + K_{M2})$ can be obtained but not of the constants individually.

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 et al. [44], the mean values of the reaction rate in the five experiments of series 2 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. (50), and, by fitting the straight lines obtained, three sets of apparent values were obtained for the parameters figuring 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 with time and extrapolating to time zero, the intrinsic values of the parameters have been calculated. The obtained values were:

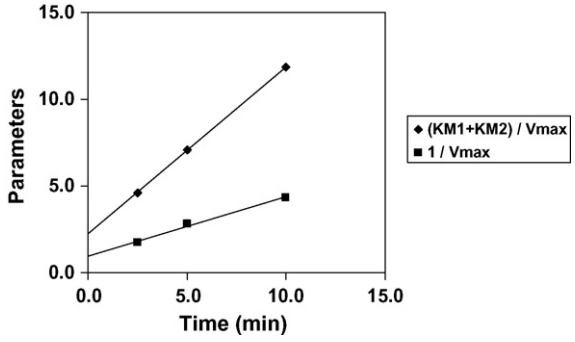


Fig. 2. Extrapolation of the apparent parameters to zero time.

$$V_{max} = 1.03 \pm 0.20 \ \text{mM min}^{-1};$$

$$K_{M1} + K_{M2} = 2.22 \pm 0.05 \ \text{mM}$$

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:

$$k_{cat1} = 36.62 \pm 6.71 \ \text{(mmoles of phenol)(g enzyme)}^{-1} \ \text{min}^{-1}$$

or, in enzyme units:

$$k_{cat1} = (4.58 \pm 0.84) \times 10^{-4} \ \text{(mmoles of phenol)U}^{-1} \ \text{min}^{-1}$$

4.1.1.2. Varying the hydrogen peroxide concentration. For a fixed phenol and variable peroxide concentration, the following form of linearization is used for Eq. (30):

$$\frac{[\Phi H_2]_0[H_2O_2]_0}{r_0} = \frac{K_{M1}[\Phi H_2]_0}{V_{max}} + \left(\frac{K_{M2} + [\Phi H_2]_0}{V_{max}}\right)[H_2O_2]_0 \quad (52)$$

which presents the advantages mentioned in the description of the method compared with other representations of Eq. (30).

In agreement with Eq. (52), for a series of experiments involving a fixed phenol and variable peroxide concentration, both the slope and the ordinate on the origin of this equation vary linearly with the concentration of phenol established for each series.

For this reason, with the data obtained for series 4–6, and taking the mean of the reaction rate during the first 2.5 min as an approximation of the initial rate, Fig. 3 has been constructed. It can be seen that the linear dependence expressed in Eq. (52) is accomplished with high values of the corresponding coefficients of determination ($R^2 = 0.9901$, 0.9881 and 0.9878, respectively). Subsequently, the slope and the intercept of the straight lines of Fig. 3 were represented versus the initial phenol concentration (Fig. 4). As can be seen, the linear dependence of both parameters with the initial phenol concentration is fulfilled to an excellent degree, in agreement with Eq. (52). From Fig. 4, together with the value of V_{max} at 2.5 min obtained in the previous section, we obtain the values:

$$(K_{M1})_{2.5min} = 0.45 \pm 0.04 \ \text{mM};$$

$$(K_{M2})_{2.5\,min} = 2.44 \pm 0.35 \ \text{mM} \quad (53)$$

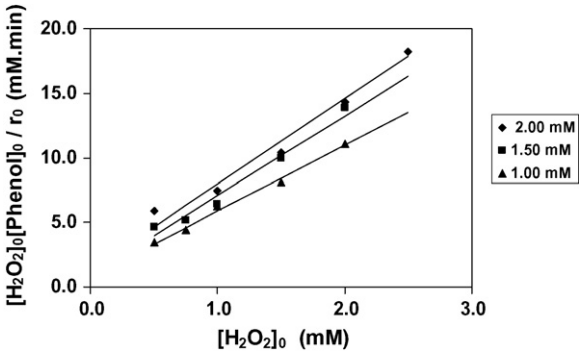


Fig. 3. Fitting the series 4–6 to Eq. (52).

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

$$(K_{M1})_0 = \frac{(K_{M1} + K_{M2})_0}{(K_{M1} + K_{M2})_{2.5}}(K_{M1})_{2.5} \quad (54)$$

$$(K_{M2})_0 = \frac{(K_{M1} + K_{M2})_0}{(K_{M1} + K_{M2})_{2.5}}(K_{M2})_{2.5} \quad (55)$$

finally giving the following values:

$$(K_{M1})_0 = 0.33 \pm 0.03 \ \text{mM};$$

$$(K_{M2})_0 = 1.81 \pm 0.26 \ \text{mM} \quad (56)$$

Data for these parameter were not found in the literature because this is, probably, one of the first works in which immobilized soybean peroxidase has been used for phenol removal. Nevertheless, for the above constants and for free HRP and phenol, Gilabert et al. [36] found the values 0.032 and 2.19 mM, respectively. Despite the fact that the peroxidase was not the same in this work and that it was used in an immobilized way, the values for the phenol Michaelis constant, were very similar.

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

To determine the five remaining parameters, k_{cat2}, K_{M3}, K_{M4}, $k_{H_2O_2}$ and k_d, the complete kinetic model needs to be integrated and an error minimisation procedure followed.

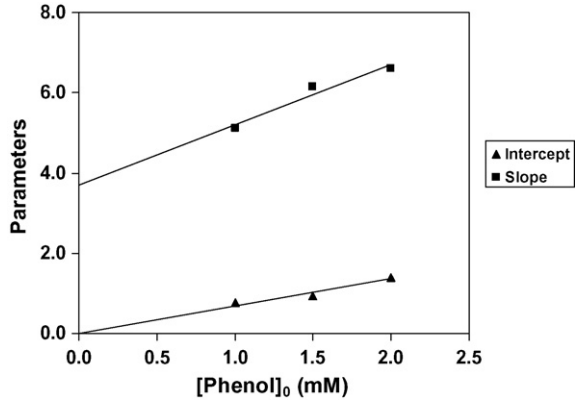


Fig. 4. Parameters dependence with initial phenol concentration in Eq. (52). (▲) Intercept and (■) slope.

Table 1
Values obtained for the eight parameters of model

k_{cat1}	36.62 ± 6.71 mmol/(g E min)	k_{cat1}	$(4.58 \pm 0.84) \times 10^{-4}$ mmol/(U min)
k_{cat2}	7.54 ± 1.36 mmol/(g E min)	k_{cat2}	$(9.43 \pm 1.70) \times 10^{-5}$ mmol/(U min)
K_{M1}	0.33 ± 0.03 mM	K_{M4}	46.47 ± 6.32 (dimensionless)
K_{M2}	1.81 ± 0.26 mM	$k_{H_2O_2}$	1.23 ± 0.14 (dimensionless)
K_{M3}	0.30 ± 0.03 mM	k_d	$(4.23 \pm 0.58) \times 10^{-4}$ g E/(l min)

In this work, the model equations were integrated using Euler's method, with a pass width of 0.05 min for the time, which acts as 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 minimisation routine based on the Simplex algorithm in the improved version proposed by Nelder and Mead [45]. 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.

In the fitting process the experimental values corresponding to series 1–3 were used, leaving those for series 4–6 for checking the goodness of the constants determined by minimisation. The values obtained for these constants, together with those determined above from the initial velocity equation, are shown in Table 1.

Fig. 5A–C show the experimental values (points) versus time for series 1–3, while the continuous lines represent the conversion values calculated by the model for these series. The typical deviation was 3.27%.

Fig. 6 is a comparison of the calculated values and experimental values for these series and demonstrates the good fit obtained with the model, as seen from the value of the coefficient of determination, $R^2 = 0.9837$, and high level of significance (98%, from ANOVA test).

4.2. Checking the model: behaviour of series 4–6

Using the values found and with the minimisation algorithm deactivated, the program for determining the constants was used to calculate the conversion values provided by the model for series 4–6, which were not used for calculating the last five parameters.

The results obtained are shown in Fig. 7A–C, corresponding to series 4–6, respectively. As in Fig. 5, the points represent the experimental conversion values and the continuous lines the values calculated with the model. The fit obtained was better than for the constants since the standard deviation was 2.72%, which is lower than the 3.27% mentioned above. The representation of the conversion values calculated versus the experimental values (Fig. 8) also provided a better coefficient of determination, $R^2 = 0.9904$, and a higher degree of significance (99%, from ANOVA test), underlining the model's validity for predicting the behaviour 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 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 through the joint action of the radicals, as already reflected, and of the peroxide, according to the literature.

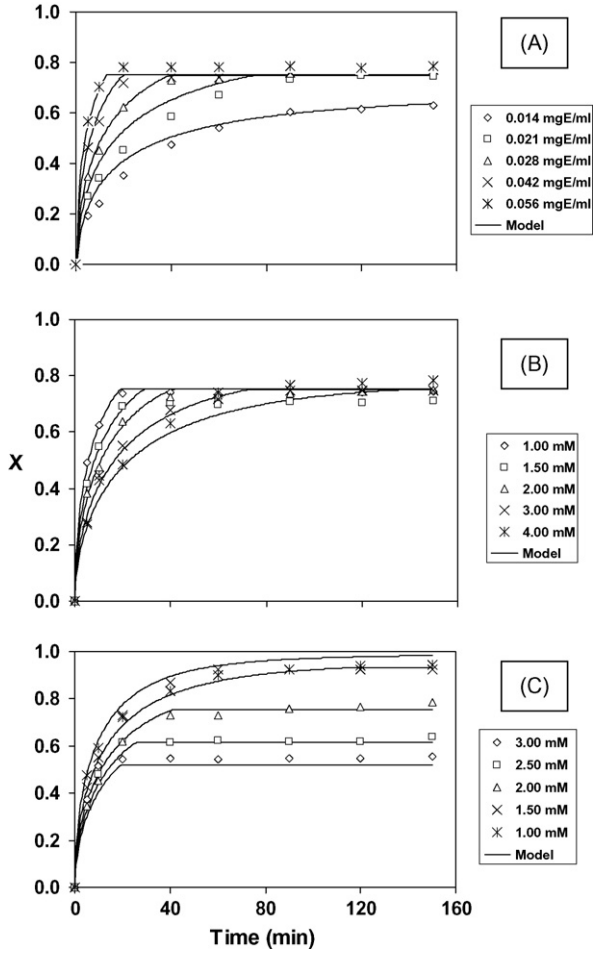


Fig. 5. Fitting the complete model: (A) Series 1, variable enzyme concentration. (B) Series 2, variables both substrate concentrations in molar ratio 1:1. (C) Series 3, variable phenol concentration.

4.3. Lifetime of the enzyme: estimation of the "turnovers"

In a previous work, carried out with free horseradish peroxidase, Nicell et al. [33] estimated a catalytic lifetime of the enzyme by calculating the "turnovers", defined as the number of molecules of phenol removed per molecule of deactivated enzyme. This is a measure of the number of times that the enzyme passes through, or turns over, its catalytic cycle. According to these authors, "turnovers", T_S, is given by the following equation:

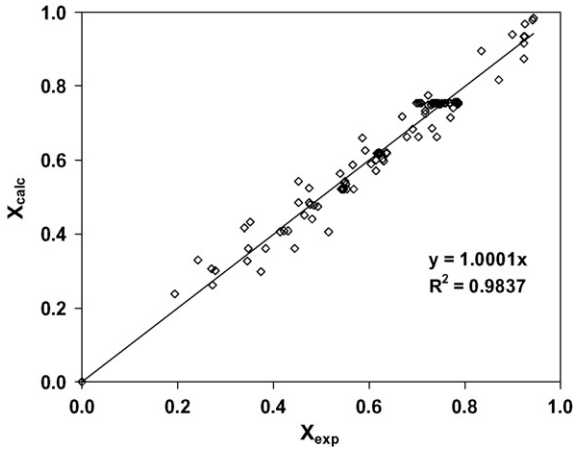


Fig. 6. Calculated and experimental conversion values. Experimental series 1–3.

$$T_S = \frac{[\Phi H_2]_0 - [\Phi H_2]}{[E]_{inactive}} \quad (57)$$

where both concentrations, for phenol and enzyme, must be given in the same units. This equation suggests that, for an individual assay, there is a linear relationship between the phenol consumed, at any time, and the concentration of the inactive enzyme at the same time:

$$[\Phi H_2]_0 - [\Phi H_2] = T_S[E]_{inactive} \quad (58)$$

the slope of this linear relationship representing the "turnovers"

In this work, no experimental data for the enzyme deactivation has been reported. But, by using the deactivation equation of the model, an estimation of the concentration of deactivated enzyme can be made as follows: at any time, from the total mass balance for the enzyme, and from the value of the concentration of active enzyme (covered particle model, Eq. (49)), the following equation can be obtained for the concentration of inactive enzyme:

$$[E_{inactive}] = [E]_0 \left(1 - \exp\left(-\frac{k_d}{[E]_0} t\right)\right) \quad (59)$$

with the value for k_d obtained in the fitting of the model and the values used for $[E]_0$ in series 1, and taking into account the molecular weight of the enzyme (≈44 kDa, from Sigma), the concentration of inactivated enzyme (mM) was estimated for this experimental series, for the same time intervals that the residual phenol concentration was measured. From values of the initial and residual phenol concentration, phenol consumption was also calculated. Fig. 9 shows the phenol consumption versus

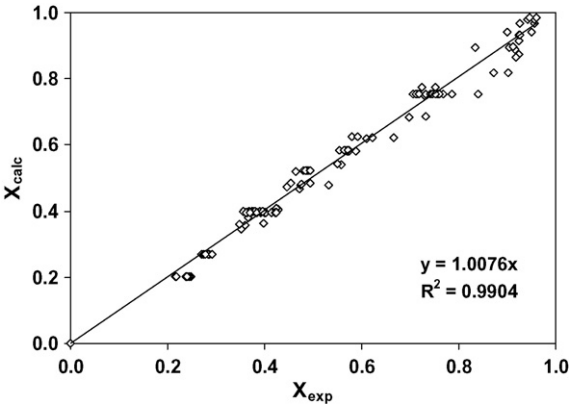


Fig. 8. Checking the model with variable peroxide concentration series. Calculated and experimental conversion values.

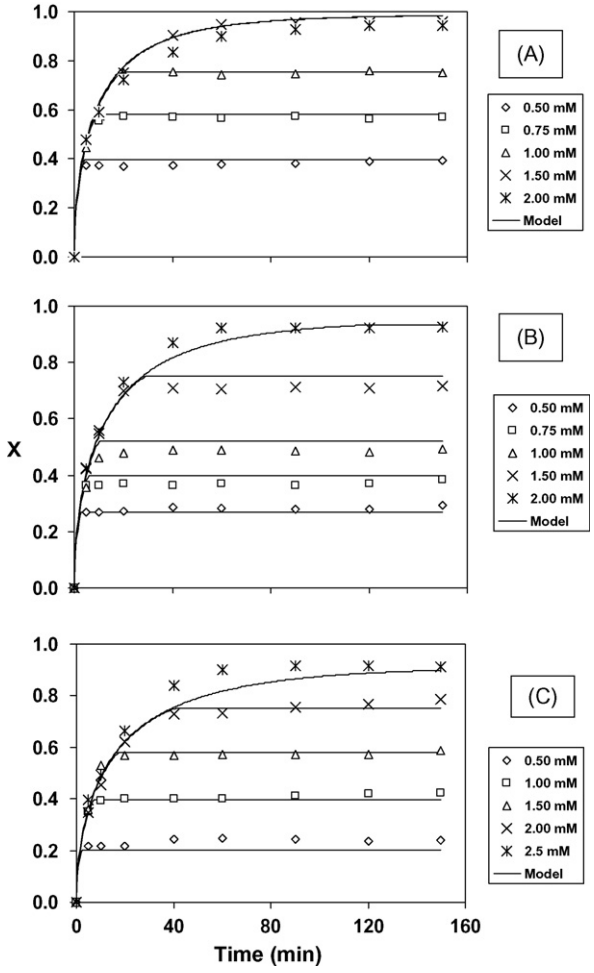


Fig. 7. Checking the model with variable peroxide concentration series: (A) series 4, fixed 1.00 mM phenol concentration, (B) series 5, fixed 1.50 mM phenol concentration, and (C) series 6, fixed 2.00 mM phenol concentration.

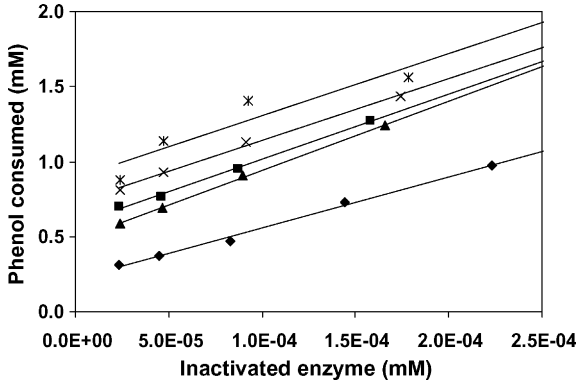


Fig. 9. "Turnovers" estimation for different initial enzyme concentrations: (♦) 0.014 mg SBP/ml, (▲) 0.021 mg SBP/ml, (■) 0.028 mg SBP/ml, (×) 0.042 mg SBP/ml, (✳) 0.056 mg SBP/ml.

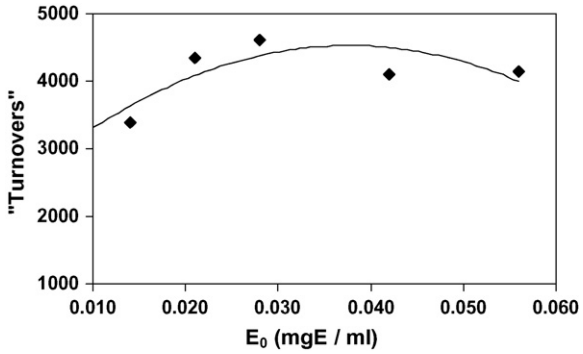


Fig. 10. "Turnovers" variation with the initial enzyme concentration.

inactivated enzyme concentration. As can be seen, there was good linear dependence ($R^2 > 0.97$) for the first time intervals (from 2.5 to 20 min) of the reaction course, which are the time intervals where, approximately, the reaction attains maximum conversion. From the slope of the straight lines, "turnovers" were obtained for the different initial enzyme concentrations assayed. As can be observed, from Fig. 10, the "turnovers" varied slightly with the initial enzyme concentration, the maximum "turnover" being obtained with 0.028 mg SBP/ml, which was the value selected for all experimental series, except series 1, in this work. The estimated values of the "turnovers" obtained in the present work (3300–4600) were very similar to those reported by Nicell et al. [33] for free horseradish peroxidase without using PEG, which were in the range 3270–4280, depending on the initial phenol concentration. The value these authors obtained for a phenol concentration of 2 mM was 3570, which is in the rage of the "turnovers" obtained in the present work, pointing to the good degree of agreement, which confirms that the proposed "covered particle model" can be useful for predicting the lifetime of the enzyme. In addition, in the above paper Nicell et al. demonstrated that T_s increases up to 90,000 when PEG was used, which suggests that the use of this additive with the immobilized derivative should be investigated as a way to improve the lifetime of the enzyme.

5. Conclusions

The model presented for the kinetic study of the immobilised SBP/phenol/hydrogen peroxide system extends, for the first time, the peroxidase cycle to the reaction products, which permits us to obtain a more general kinetic equation than has previously been proposed. At time zero, the equation is reduced to a ping-pong bisubstrate kinetic, 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 radical deactivation and/or enzyme-sequestration with the precipitated oligomers/polymers, widely described in the literature, has been modelled for the study system, as being the result of the gradual covering of the catalytic particles that contain the enzyme, which determines a growing loss of activity in the same. The deactivation model is included in the kinetic model.

To confirm the validity of the model six series of experiments were carried out in a discontinuous tank reactor. Integration of the model by numerical calculation and the use of an error minimisation algorithm, applied to three of the experimental series enabled us to obtain the values of the remaining parameters, with an excellent degree of accordance. Furthermore, since the model reproduces the behaviour of the system for the three 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 estimation of the "turnovers", for series 1, and comparison with the experimental results of other authors, confirms that the covered particle model can be useful for predict the lifetime of the immobilized enzyme.

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