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A transient design model of a continuous tank reactor for removing phenol with immobilized soybean peroxidase and hydrogen peroxide

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ABSTRACT

Hydrogen peroxide and an immobilized derivative of soybean peroxidase, covalently bound to a glass support, were used in a continuous tank reactor to remove phenol from aqueous solutions. An efficiency of about 80% was attained in phenol removal in the best of the operational conditions assayed.

With the aim of scaling up the process to pilot-plant scale, the influence of the main plant operational variables, enzyme and substrate concentrations and flow rate, on the removal efficiency was studied. The continuous reactor was operated under isothermal conditions at the optimal temperature established in the literature for this system. As a first approximation to the industrial application of the process, no buffer solutions were used.

Additionally, and to check the validity of a generalized Ping Pong bisubstrate kinetics for the enzymatic reaction, obtained from an expanded version of the Dunford mechanism previously published, and assuming an ideal mixing flow for the continuous tank reactor, a transient design model was developed. By using the intrinsic kinetic parameters obtained in a previous work, where the kinetic model was developed, the reactor model was solved and the theoretical conversion values were calculated.

The nearness of the experimental and calculated conversion values confirmed that the proposed kinetic equation and the design model, as well as the intrinsic kinetic parameters previously determined, can be used to predict the behaviour of the reactor under conditions of slow enzyme deactivation, although, for further applications, an improved version of the model deactivation equation is necessary.

1. Introduction

Phenolic compounds are toxic and are often found in industrial effluents such as those generated by high-temperature coal conversion, petroleum refining and the manufacture of plastics, resins, textile, iron, steel and paper [1–4].

The removal of phenolic compounds from wastewater is an important issue, as can be seen from the number of papers that have been published [5–36]. Physical, chemical and biological methods, including incineration, adsorption on activated carbon, chemical or enzymatic oxidation, solvent extraction, microbial degradation, etc., have been proposed for removing or degrading phenols from waste waters [5–7]. However, no method stands out as being better than the others.

One of the alternatives, which has been widely studied, is based on the application of oxireductive enzymes to catalyze the removal of these compounds from wastewater. The research has focused on the use of peroxidases, mainly horseradish peroxidase (HRP), and more recently on the use of the soybean peroxidase (SBP) [8–15].

Although the use of peroxidases offers several advantages for removing phenolic compounds, the main disadvantage is enzyme deactivation during the reaction due to precipitation with the reaction products (oligomers and polymers) and, additionally, to the effect of hydrogen peroxide, if an excess of this substrate is present [16,17]. Consequently, most studies have focused in the use of protective additives to minimize the deactivation effects, with polyethylenglycol (PEG) being the most frequently used [18–21].

The use of immobilized enzymes has several advantages. For instance, they can be easy separated from the reaction products and reused. They also work better in continuous processes [30–33].

Additionally, by using free enzyme in buffered media with or without additives, studies on the kinetic of the process and design models for the reactors used have been published. In these studies, the kinetic mechanism proposed by Dunford and Stillman [36] is commonly accepted and the bisubstrate Ping Pong kinetic equation is frequently used to explain the reaction rate of the removal process, with the batch reactor being the most frequently used reactor configuration [22–29]. Only a few papers describe the use of continuous reactors with free enzyme [31,33], and this reactor

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Nomenclature

[A]	concentration of species A in the reactor (mM)
$[A]_0$	concentration of species A in the feed flow (mM)
D_p	permeability coefficient of dimer at the reactor outlet
E_{active}	active enzyme
F	volumetric feed flow (ml min^{-1})
H_2O_2	hydrogen peroxide
k_{cat1}	enzyme catalytic constant in the phenol oxidizing reaction, (mmol of substrate (g enzyme $min)^{-1}$)
k_{cat2}	enzyme catalytic constant in the dimer oxidizing reaction, (mmol of substrate (g enzyme $min)^{-1}$)
k_d	enzyme deactivation constant (g enzyme (l $min)^{-1}$)
k_{D_p}	constant for D_p calculation (min $mmol^{-1}$)
$k_{H_2O_2}$	proportionality constant for total peroxide consumption
K_{Mi}	generic Michaelis constants in the kinetic equation (i = 1, 2, 3, and 4), mM for K_{M1}, K_{M2} and K_{M3}, dimensionless for K_{M4}
$r_{\Phi H_2}$	phenol consumption rate (mM min^{-1})
$r_{H\Phi-\Phi H}$	dimer consumption rate (mM min^{-1})
$r_{H_2O_2}$	hydrogen peroxide consumption rate (mM min^{-1})
r_{dimer}	overall reaction rate of dimer (mM min^{-1})
t	time (min)
V_R	reactor volume (ml)
X	phenol conversion

Greek symbols

Δt	time increment (min)
ΦH_2	phenol
$H\Phi-\Phi H$	dimer of phenol

configuration is most frequently reserved for when immobilized derivatives of the enzyme or immobilized microorganisms are used [32–35].

The influence of temperature and pH on the phenol removal efficiency was previously analyzed, using the same commercial soybean peroxidase as used in the present work [21]. From these studies, 30 °C and pH 7 were established as the optimal temperature and pH values, respectively. In another study on phenol removal, we compared the use of an immobilized derivative of soybean peroxidase, covalently bound to a glass support, and the free enzyme. When the influence of the molar ratio of the substrates was analyzed, the range 1.3:1–1.5:1 peroxide/phenol molar ratio obtained the highest phenol removal percentages.

In a previous paper [37], we proposed an expanded version of the Dunford mechanism, which takes into account the influences of the dimers and oligomers on the reaction rate, and a generalized bisubstrate Ping Pong kinetic equation was developed. This kinetic equation also included a covered particle deactivation model for the enzyme deactivation, which was interpreted as a consequence of the precipitation of the reaction products (oligomers and polymers) formed, over the surface of the catalyst particles (enzyme sequestration). From experimental data of the hydrogen peroxide/immobilized soybean peroxidase/phenol system obtained in a batch reactor, the kinetic parameters of the model were obtained and the validity of the proposed kinetic law was confirmed [37].

For further industrial applications of the removal process, continuous tank reactors and non-buffered media should be used. For this, it is necessary to know if the kinetic of the process and the values of the kinetic parameters, previously established in [37], can be applied in the continuous reactors.

In this work, an immobilized derivative of soybean peroxidase, covalently bound to a glass support, was used in a continuous stirred tank reactor, in order to study its viability for use in the phenol removal process. The continuous reactor was operated at 30 °C, which is the optimal temperature previously established for this enzymatic system [21], and no buffered solutions were used, the pH in these conditions very close to 7, which is, also, the optimal value established in [21] for this variable. Under these fixed conditions, the influence of the main operational variables of the continuous reactor (enzyme and both substrate concentrations as well as flow rate) on the conversion of phenol was analyzed. A transient design model for the continuous tank reactor was also developed and, by using the values of the intrinsic kinetic parameters previously obtained [37], the experimental data of the phenol conversion at the reactor outlet were fitted to the model and the additional parameters of the model were also calculated. Then, the theoretical conversion values were calculated for all the experimental conditions assayed. The good approximation between the experimental and calculated values for all the experimental conditions assayed confirmed that the proposed design model and the kinetic law used, as well as the values of the intrinsic kinetic parameters, are useful for predicting the behaviour of the continuous tank reactor under conditions of slow enzyme deactivation, although, for further applications, an improved version of the deactivation equation of the model is necessary.

2. Experimental

2.1. Materials

2.1.1. Enzyme and substrates

Soybean peroxidase enzyme (SBP) (EC 1.11.1.7, 108 IU/mg), catalase (EC 1.11.1.6, 2200 IU/mg) from bovine liver, hydrogen peroxide (35%, w/v), phenol (molecular mass 94.11, minimum purity 99%), were purchased from Sigma–Aldrich Fine Chemical.

2.1.2. Colorimetric reagents

4-Aminoantipyrine (AAP) and potassium ferricyanide, were purchased from Sigma–Aldrich Fine Chemicals.

2.1.3. Immobilization reagents

Nitric acid (HNO_3) of 65% purity (Merck), γ-APTES ((3-aminopropyl) trietoxysilane) ($C_9H_{23}NO_3Si$), hydrochloric acid (HCl) of 36.5% purity (Probus S.A.) and glutaraldehyde ($OCH(CH_2)_3CHO$) of 25% purity.

2.1.4. Supports

PG 75–400, controlled pore glass 75–400, with a 200–400 mesh particle size, 77 Å average pore diameter and 182 m^2/g surface area.

2.2. Immobilization

Immobilized derivative was prepared by covalent coupling between the amine groups of the protein and the aldehyde groups of the porous glass treated with (3-aminopropyl) trietoxysilane and glutaraldehyde, as described in a previous work [30]. The immobilization procedure, protein determination and activity measurements are described below.

2.2.1. Immobilization procedure

Using 1 g of support, the immobilization process was carried out according to the following steps:

- *Preparation of the carrier*: glass beads were washed in 5% HNO_3 at 80–90 °C for 60 min and rinsed with distilled water and dried in an oven for 24 h at 110 °C.
- *Support activation*: 18 ml of distilled water along with 2 ml of γ-APTES (10%, v/v) was added to 1 g of clean PG, 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.
- *Immobilization on PG-glutaraldehyde*: 1 g of silanized glass was made to react in a jacketed column reactor (3 cm 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 plate placed 4.5 cm from the bottom. The solution was recycled for 60 min with peristaltic pump and the PG-glutaraldehyde washed with 25 ml of the same buffer. Enzyme solution (40 ml of SBP 2 mg/ml solution) was then added to the reactor and the enzyme solution recycled overnight at 4 °C. The derivative 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.

2.2.2. Protein determination

The amount of protein initially offered and in the wash-liquid after immobilization was determined by Lowry's procedure modified by Hartree [38], 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.

2.2.3. Measurement of free and immobilized enzyme activity

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 (phenol and hydrogen peroxide) were kept constant at 2 mM, while enzyme concentration was varied between 0.01 and 0.05 mg/ml. Samples were taken from the reactor every 2.5 min and phenol 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 the samples from the reactor were poured into 1 ml of catalase solution (2200 IU ml^{-1}) to stop the reaction by breaking down the hydrogen peroxide. For this, 0.2 ml of a coagulant, $AlK(SO_4)_2$ (40 g/l), were added to 1 ml of the former mixture, before centrifuging for 30 min at 10,000 × *g*. From the activity data of the free and immobilized enzyme, the activity yield was calculated.

After immobilization, an immobilized derivative, which retains 24.7% of the total protein offered and conserves 74% of the free enzyme activity, was obtained. The enzyme concentration in the derivative storage suspension was 21.3 mg enzyme/g support and 0.4 mg enzyme/ml suspension, respectively.

2.3. 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 ($[\text{phenol}] = 0.0977 \times \text{Abs}_{505}$, $r = 0.999$).

2.4. Experimental system and operational procedure

The experimental system used in the continuous assays consisted of a jacketed tank reactor, 50 ml maximum capacity, connected to a thermostatic bath, three Watson Marlow Digital peristaltic pumps (model 505 Du-RL) to feed the substrates into the reactor and to pump the effluent from the reactor to the collector tank, and two reservoir tanks for the substrate solutions. A schematic diagram is shown in Fig. 1.

For each assay, two substrate solutions were prepared in distilled water, one for phenol and another for the hydrogen peroxide, and poured into the reservoir tanks.

Operational procedure: For each assay, a sufficient volume of immobilized derivate to obtain the required initial concentration was introduced into the reactor, and the total volume (50 ml) was completed by distilled water. Next, at time zero, the substrate solutions were continuously pumped into the reactor with two of the peristaltic pumps. Simultaneously, a third pump was used to pump the reactor effluent to the collector tank. The reaction course was monitored by taking samples at the reactor exit at regular time intervals. The samples were passed through a nylon membrane (10 μm) to retain the solid biocatalyst and phenolic polymer particles in the reactor.

The temperature was kept constant at the optimal value of 30 °C by means of the thermostatic bath and the reaction mixture was continuously stirred using magnetic stirrers and Teflon-coated stir bars.

2.5. Experimental planning

To establish the experimental planning, the results obtained in previous studies were taken into account [21,30]. According to the optimal values established for this system [21], the following experimental conditions were maintained unaltered in throughout the experimental series: temperature 30 °C and pH ≈ 7 (pH without adding buffer solutions). As regards, the peroxide/phenol molar ratio, although the range 1.3:1–1.5:1 was established in a previous work [30] as the optimal ratio to attain the highest phenol conversion values, a 1:1 molar ratio for both substrates was maintained for all the experimental series described here.

There are two reasons for this decision: on the one hand, the increase in phenol conversion when the 1.3:1–1.5:1 molar ratio is used is not very great compared with the conversion obtained whit the 1:1 molar ratio, as can be seen in [37]; and, on the other hand, due to the continuous operational mode of the reactor used, equal concentration values for both substrates in the feed flow are recommended, as the best way to reach the steady state after a certain operation time. If the concentrations of both substrates are not equal in the feed flow, of the more concentrated substrate may accumulate in the reactor as time elapses it will be more difficult to reach the steady state. Additionally, and as a consequence of non-equal substrate concentrations in the feed flow, if the excess of hydrogen peroxide is great, additional deactivation effects by this substrate may occur, as indicated in the literature [16,17].

For the reactor effluent, the flow rate was kept constant as the sum of the flow rates of the two substrates, ensuring a constant reactor volume during the assay. A total reaction time of 120 min was established for all the assays.

Three series of experiments were carried out in the continuous tank reactor as follows.

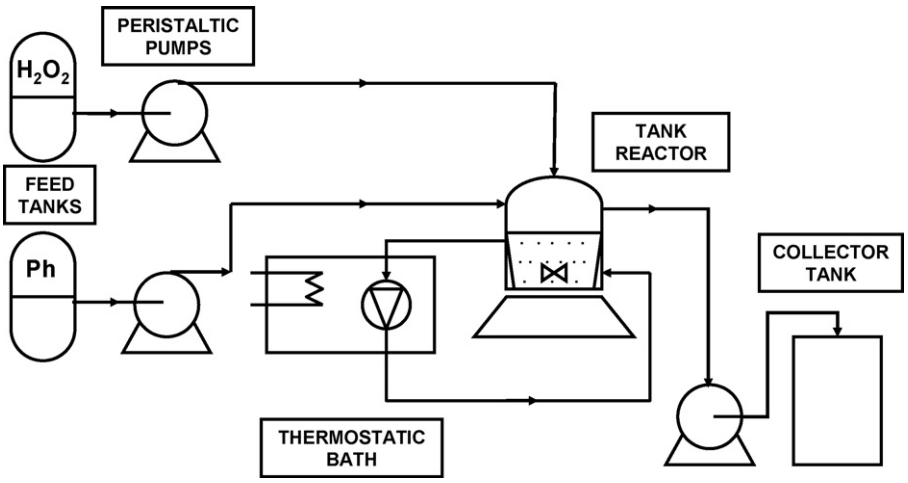

Fig. 1. Flow diagram of experimental equipment.

2.5.1. Enzyme concentration variation

Three enzyme concentrations (0.17, 0.26 and 0.34 mg/ml) were assessed. In this series, the hydrogen peroxide and phenol concentration was kept constant at 1 mM in the reactor, and the total flow rate was 5 ml/min.

2.5.2. Substrate concentration variation

The influence of substrate concentrations (hydrogen peroxide and phenol) was studied in the continuous tank reactor. Two substrate concentrations (1.0 and 2.0 mM hydrogen peroxide and phenol, molar ratio 1:1) were assessed, using the same flow rate of 5 ml/min and enzyme concentration of 0.17 mg/ml. With this enzyme concentration, and for an operational time of 120 min, the mg enzyme/total volume of treated solution ratio was similar to that used in a batch reactor in a previous work [30].

2.5.3. Feed flow variation

Several experiments were carried out with the immobilized derivative PG 75–400, using three different flow rates (5, 7.5 and 10 ml/min). In all the assays, substrate concentrations (hydrogen peroxide and phenol) were fixed at 1 mM and the enzyme concentration at 0.26 mg/ml. This is higher than in the previous series, but was intended to compensate the expected decrease in phenol conversion which takes place as a consequence of the diminishing the spatial time, V_R/F, of substrates in the reactor when the flow rate increases from 5 ml/min to 7.5 and 10 ml/min, respectively.

3. Results and discussion

To monitor the time course of the reaction, the experimentally measured variable was the phenol concentration, $[\Phi H_2]$ in the effluent, which was normalized with respect to the phenol concentration in the total feed flow, $[\Phi H_2]_0$, by defining a conversion, $X = ([\Phi H_2]_0 - [\Phi H_2])/[\Phi H_2]_0$ (Eq. (6) of design model). This permitted a scale varying from 0 to 1 to be used in the plotting and also simplified the comparison between the different experimental series.

Since the reactor volume was initially filled with distilled water, the initial value of $[\Phi H_2]$ was zero in all the assays and the initial conversion was always unity. After connecting the feed pumps, the $[\Phi H_2]$ value increased continuously and the conversion decreased until the constant value corresponding to the steady state was reached. This can be observed from Figs. 2–4, where the time course of the conversion is represented for the different experimental series.

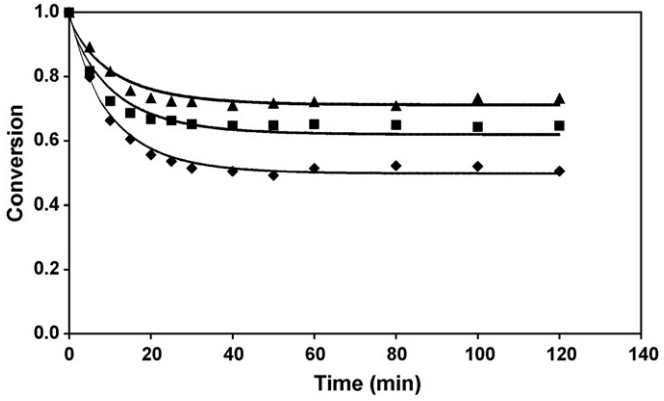

Fig. 2. Influence of the enzyme concentration. $[Phenol]_0 = [H_2O_2]_0 = 1$ mM, flow rate = 5 ml/min, $[SPB]_0$ = (♦) 0.17 mg/ml; (■) 0.26 mg/ml; (▲) 0.34 mg/ml.

3.1. Influence of the enzyme concentration

Fig. 2 shows the time course of phenol conversion in Series 1. From the initial value of unity, conversion decreased continuously until the steady state was reached.

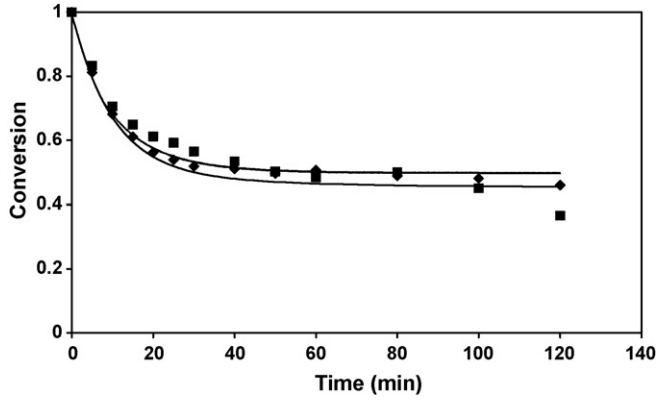

Fig. 3. Influence of substrate concentrations. $[SPB]_0 = 0.17$ mg/ml, flow rate = 5 ml/min, $[Phenol]_0 = [H_2O_2]_0$ = (♦) 1 mM; (■) 2 mM.

In the figure, it can be observed that the steady state was reached in all the situations assessed and is represented by a final horizontal straight line for each one of the conversion progress curves. The curves obtained show that the steady state conversion increased with increasing enzyme concentration, as was to be expected. According to Fig. 2, the conversion of phenol was high, 0.7, when the enzyme concentration was 0.34 mg/ml.

3.2. Influence of initial substrate concentration

The results obtained for this series are plotted in Fig. 3, where the influence of the substrate concentration (hydrogen peroxide and phenol in molar ratio 1:1) on the conversion is shown. It can be observed that similar conversion percentages were obtained when substrate concentration was varied, which leads us to affirm that substrate variation has little influence on the steady state conversion.

It can also be seen that the steady state conversion remained stable, except after 120 min of operational time in the case of 2 mM substrate concentrations, when the enzyme was deactivated. For this substrate concentration, the total amount of reaction products in the reactor after 120 min (higher than for the 1 mM substrate concentration) determined the higher recovery level of the catalytic particle surfaces and, according to the "covered particle deactivation model" proposed in [37], enzyme deactivation effects began to be significant. As a consequence, substrate concentrations of 2 mM or more are not recommendable at long operation times without significance deactivation effect.

3.3. Influence of the flow rate

In Fig. 4, variations in the conversion are plotted against time at three flow rates: 5.0, 7.5 and 10 ml/min. An increase in the flow rate, F, resulted in a decrease in spatial time, V_R/F, and a decrease in the value of the phenol conversion, again as expected.

As in the above series, the steady state was reached after several minutes. It can be seen from Fig. 4 that the maximum conversion reached was 0.65 for a flow rate of 5 ml/min. Similar to the previous Series 2, after a reaction time of 120 min enzyme deactivation occurred for a flow rate of 10.0 ml/min, although this deactivation effect was less pronounced than the one observed in the substrate concentrations series for substrate concentrations of 2 mM.

Here, this slow deactivation effect may be a consequence of both flow rate and substrate concentrations, which would determine the highest value of the substrate inlet rate for a flow rate of 10 ml/min and, as a consequence, the highest concentration of reaction products in the reactor. After an operation time of 120 min, this concentration would begin to be important and result in particle covering phenomena.

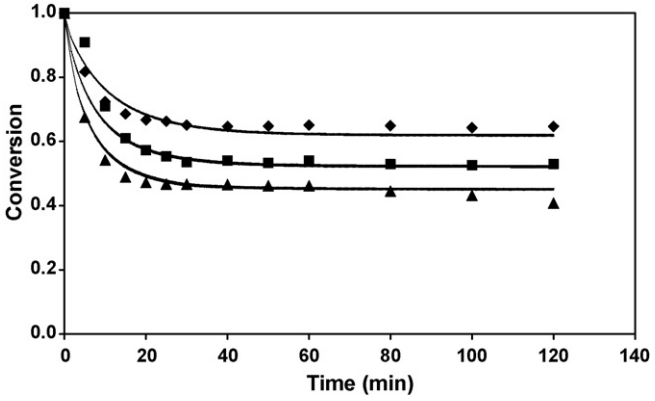

Fig. 4. Influence of flow rate. $[Phenol]_0 = [H_2O_2]_0 = 1$ mM, $[SPB]_0 = 0.26$ mg/ml, flow rate = (♦) 5 ml/min; (■) 7.5 ml/min; (▲) 10 ml/min.

4. Design model

4.1. Hypothesis

A transient design model of the reactor is proposed, based in the following hypothesis:

- The reaction of phenol with hydrogen peroxide follows the known mechanism of the free radicals of Dunford and Stillman [36], in its expanded version proposed in a previous work [37]. The reaction products (dimers, oligomers and polymers) also react, which leads to the additional consumption of hydrogen peroxide, which must be taken into account. The kinetic equations developed in [37] can also be applied to the continuous tank reactor. Those equations are shown in Appendix A.
- The values of the kinetic parameters of the above rate equations, obtained in a previous work [37] from assays in a batch reactor, are valid for the continuous tank reactor, with the exception of the deactivation constant, k_d, which differs probably because the operational conditions in the continuous reactor are different from those of the batch reactor.
- The fraction of substrates that is not consumed in the tank leaves, quantitatively, with the outlet flow. However, dimers, oligomers and polymers do not leave quantitatively, because these reaction products partially precipitate over the catalytic particles and over the nylon grid placed at the outlet of the reactor, which diminishes the effective area of the outlet orifice. This must be taken into account in the mass balance of these compounds and, a specially, for the dimer, because its concentration appears in the rate equations. To quantify these phenomena, a permeability coefficient for the dimer, D_p, was defined, with a value in the interval $0 \le D_p \le 1$. The permeability coefficient depends on the flow rate and on the substrate concentrations and it must be evaluated during the fitting of the model. The following equation for D_p was proposed:

$$D_p = e^{-k_{D_p} F[H\Phi-\Phi H]} \quad (1)$$

- The relationship between the hydrogen peroxide and phenol consumption rates, equal to 1.23 in the batch reactor, must be higher in the continuous tank due to the product accumulation, which determines the higher consumption of hydrogen peroxide. The new value of this parameter must also be evaluated during the fitting of the model.

4.2. Mass balance equations

4.2.1. Mass balance for phenol, dimer and hydrogen peroxide

For a constant flow rate, F, reactor volume, V_R, and isothermal operation, the following mass balance equations can be formulated:

$$\frac{d[\Phi H_2]}{dt} = \frac{F}{V_R}([\Phi H_2]_0 - [\Phi H_2]) - r_{\Phi H_2} \quad (2)$$

$$\frac{d[H\Phi-\Phi H]}{dt} = -\frac{F}{V_R} D_p [H\Phi-\Phi H] + r_{dimer} \quad (3)$$

$$\frac{d[H_2O_2]}{dt} = \frac{F}{V_R}([H_2O_2]_0 - [H_2O_2]) - r_{H_2O_2} \quad (4)$$

the initial conditions being:

$$t = 0; \ [\Phi H_2] = 0; \ [H\Phi-\Phi H] = 0; \ [H_2O_2] = 0 \quad (5)$$

5. Solving and fitting the model

The above mass balance equations represent an initial values non-linear ordinary differential equations problem, and they must by solved by numerical methods. Additionally, a minimization routine must be used to determine the model parameters.

As a numerical procedure, the fourth order Runge-Kutta method is commonly used because it offers good precision levels for a not very small step size. Also the Euler method can be applied, but a smaller step size must be used and, as soon as possible, double precision variables. With the new generation of computers, this requirement is not a problem, because of their large memories and very fast processors. This fact, together with the ease of obtaining a set of simple recursive expressions, led us to use the Euler method to convert the differential equations into discrete expressions.

To fit the experimental data to the model, the Nelder and Mead version of the Simplex method was used to minimize the error [39]. Other methods, too, can be used, such as the Levenberg-Marquardt algorithm, which is used in some scientific software, including Sigma Plot, and other commercially available minimization routines. The Nelder and Mead method is an enhanced version of the Simplex method, and is recommended in the book of Himmelblau [40]. Also, this method has been used successfully in the Encora 1.2 computer program, software developed by J.J Straathof to analyze enzyme kinetics by fitting the progress curve obtained in batch reactors [41], with very good results. Taking this computer program as reference, our research group developed a customized fitting software to obtain the intrinsic kinetic parameters of the immobilized SBP/phenol/hydrogen peroxide by fitting, simultaneously, a set of fifteen progress curves obtained in a batch reactor under several experimental conditions [37]. The capacity of our software 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 commercially available software. It also permits the influence of many kinetic variables, such as enzyme concentration, substrate concentration, product concentration, and others, to be included in the fitting procedure.

The objective function to be minimized was the minimum square sum of the differences between the experimental and calculated values of the phenol conversion, X, which is defined as:

$$X = \frac{[\Phi H_2]_0 - [\Phi H_2]}{[\Phi H_2]_0} \quad (6)$$

In the fitting process, the values of k_{cat1}, k_{cat2}, K_{M1}, K_{M2}, K_{M3} and K_{M4}, determined previously [37], were kept constant. Only the new parameter k_{D_p} (for the calculation of D_p) and the new values of k_d (for enzyme deactivation) and $k_{H_2O_2}$ (to evaluate $r_{H_2O_2}$), must be determined in the fitting process.

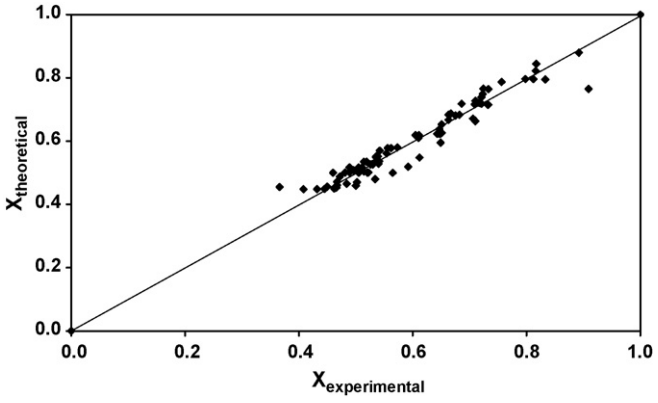

Fig. 5. Experimental (♦) and theoretical (–) conversions for all the assays in the continuous tank reactor.

Table 1
Values of the model parameters

Parameter	Value	Units	Font
k_{cat1}	36.6 ± 6.7	mmol/(g E min)	Reference [37]
k_{cat1}	$(4.6 \pm 0.8) \times 10^{-4}$	mmol/(U min)	Reference [37]
k_{cat2}	7.5 ± 1.4	mmol/(g E min)	Reference [37]
k_{cat2}	$(9.4 \pm 1.7) \times 10^{-5}$	mmol/(U min)	Reference [37]
K_{M1}	$(3.3 \pm 0.3) \times 10^{-1}$	mM	Reference [37]
K_{M2}	1.8 ± 0.3	mM	Reference [37]
K_{M3}	$(3.0 \pm 0.3) \times 10^{-1}$	mM	Reference [37]
K_{M4}	46.5 ± 6.3	Dimensionless	Reference [37]
$k_{H_2O_2}$	1.9 ± 0.2	Dimensionless	This work
k_d	$(2.0 \pm 0.3) \times 10^{-4}$	g E/(l min)	This work
k_{D_p}	236.0 ± 11.2	min/mmol	This work

The obtained typical deviation between the experimental and calculated values of the phenol conversion was 2.9% and the values of the parameters were:

$$k_{D_p} = 236.0 \pm 11.2 \ (\text{min/mmol})$$
$$k_d = (2.0 \pm 0.3) \times 10^{-4} \ (\text{g E/(l min)})$$
$$k_{H_2O_2} = 1.9 \pm 0.2$$

Figs. 2–4 show the experimental values of the phenol conversion (dots) and the calculated values (continuous lines), for all the series assayed. Finally, Fig. 5 is a comparison between the experimental and calculated values of the phenol conversion for the whole experimental series.

As regards the calculated parameters shown in Table 1, it can be observed that the deactivation constant, $k_d = 2.0 \times 10^{-4}$ g E/l min, is much lower than that obtained previously in the batch reactor, $k_d = 4.2 \times 10^{-4}$ g E/l min [37]. This is because in the batch reactor the reaction product remains in the reactor, which increases the deactivation of the enzyme, while in the continuous tank, the reaction products partially leave with the effluent and the deactivation effect diminishes.

The value of 2.9% obtained for the typical deviation, together with the value of the determination coefficient, $R^2 = 0.9696$, for Fig. 5, show good agreement between the experimental and calculated values, which indicates that the proposed model is suitable for predicting the behaviour of the reactor and, also, for potential technical purposes.

6. Conclusions

The continuous operation of a tank reactor, with no buffered media, provided a good degree of phenol elimination in all the experimental conditions assayed and, as a consequence, the present study has established a basis for further developing an industrial process for removing phenol using immobilized derivatives of soybean peroxidase and hydrogen peroxide.

Most of the continuous assays provided good recovery yields and, as a consequence, the continuous operation of the reactor makes it possible to treat large volumes of wastewater with no need for complex equipment.

The kinetic model of the process and the intrinsic parameters of the rate equations obtained in a previous work [37] were used in the design model of the continuous tank reactor. From the design equations obtained, the model was solved by numerical calculations and the experimental data were fitted to the model with a good degree of agreement between experimental and calculated values of the phenol conversion. This confirmed the validity of the design reactor model as well as the kinetic rate equations and intrinsic parameters used, despite the fact that they were obtained in assays

carried out in a batch reactor and in the presence of buffered solutions.

Finally, it can be affirmed that the results obtained in this work are a solid basis for designing a continuous industrial process for removing phenol from effluents containing this phenolic compound.

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Appendix A

Kinetics equations, taken from [37], which have been used in this work.

$$r_{\Phi H_2} = \frac{k_{cat1}[E_{active}][\Phi H_2][H_2O_2]}{K_{M1}[\Phi H_2] + K_{M2}[H_2O_2] + [\Phi H_2][H_2O_2] + K_{M3}[H\Phi{-}\Phi H] + K_{M4}[H\Phi{-}\Phi H][H_2O_2]} \quad (A1)$$

$$r_{H\Phi-\Phi H} = \frac{k_{cat2}[E_{active}][H\Phi{-}\Phi H][H_2O_2]}{K_{M1}[\Phi H_2] + K_{M2}[H_2O_2] + [\Phi H_2][H_2O_2] + K_{M3}[H\Phi{-}\Phi H] + K_{M4}[H\Phi{-}\Phi H][H_2O_2]} \quad (A2)$$

$$r_{dimer} = \frac{1}{2} r_{\Phi H_2} - r_{H\Phi-\Phi H} \quad (A3)$$

$$r_{H_2O_2} = k_{H_2O_2}(r_{\Phi H_2} + r_{H\Phi-\Phi H}) \quad (A4)$$

$$[E_{active}] = [E]_0 \exp\left(-\frac{k_d}{[E]_0} t\right) \quad (A5)$$

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