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Removal of 4-chlorophenol in a continuous membrane bioreactor using different commercial peroxidases

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ABSTRACT

Three commercial plant peroxidases, the commonly used soybean (SBP) and horseradish (HRP) and a cheap alternative, artichoke (AKPC), have been tested for the removal of 4-chlorophenol in a continuous tank reactor associated to an ultrafiltration membrane module. An important conversion fall over time was observed both in the reactor and permeate samples with AKPC. For the other peroxidases, this conversion decrease occurred in the reactor only, as a result of the enzyme retention on the membrane, and was rather insignificant in the case of SBP. Supplementary addition of enzyme in the feed stream allowed maintaining high conversions in the case of HRP. Consequently, SBP and HRP were selected as the most appropriate peroxidases. Using them, it was observed that conversion values increased when higher substrate concentrations were used and that the influence of the molar ratio H_2O_2:chlorophenol and the spatial time was negligible. In all cases, higher 4-chlorophenol conversions were attained with SBP, reaching almost 100% in the permeate. SBP was chosen to test the activity of the enzyme retained on the membrane surface, proving that the peroxidase retains its activity and reaches some kind of equilibrium between reactor and membrane module, allowing the continuous 4-chlorophenol removal for longer times.

Keywords: 4-chlorophenol; Wastewater treatment; Ultrafiltration membrane reactor; Soybean peroxidase; Horseradish peroxidase; Artichoke peroxidase

1. Introduction

Phenolic compounds and their chlorinated derivatives are used in many industrial processes releasing pollutant effluents to the environment. Most of these compounds are dangerous for both humans and animals [1,2] and they are listed as environmental pollutants [3] by the US EPA in the Clean Water Act and by the European Directive 2455/2001/EC.

During the past two decades, much effort has been devoted to the study of phenolic compounds removal from wastewater. Among the used methods physical [4–6], chemical and photochemical [7–11] and biological treatments stand out [12,13]. However, none of them has become a definitive solution to the mentioned problem [14]. All the methods have to deal with the aim of achieving economically viable and friendly environmental alternatives.

Enzymes present several advantages such as their operability within a wide range of conditions (especially under mild conditions), high specificity towards the targeted compound and minimum environmental impact [15]. Enzymes extracted from plants and microorganisms [15–17] have been used to remove phenolic compounds in the past years, being the peroxidase ezymes extracted from a vegetable source the most studied peroxidases in the recent past [18–20].

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Our research group has been studying in depth phenol and 4-chlorophenol removal with peroxidases from vegetables sources such as soybean and horseradish ones [21,22]. These studies started with the use of the soluble enzymes in order to set the optimum experimental conditions of the enzymes, but taking into account the scale up of the process the use of continuous systems is always desirable. There are two possibilities to work with continuous processes: the use of immobilized enzymes or the use of reaction/separation techniques.

4-Chlorophenol conversion values above 90% were obtained with immobilized SBP and HRP onto porous glass beads in different reactor configurations such as discontinuous and continuous reactors [23,24]. The main disadvantages of the use of enzymes are their deactivation due to the polymer formation that covers the active centre of the enzymes increasing the diffusional limitations [25] and the difficulty of the biocatalyst recovery. Besides, in most cases the immobilization procedure means a high cost that is not economically viable for an industrial process.

This work has focused on the study of a new reactor configuration consisting of an ultrafiltration membrane reactor (UFMR) for the removal of 4-chlorophenol with three vegetable peroxidases: soybean (SBP), horseradish (HRP) and artichocke peroxidase (AKPC). The strength of this reactor configuration is that the loss of the enzyme can be avoided with the association of the membrane after the bioreactor. Besides, semipermeable membranes are able to retain both the biocatalyst and the polymers formed during the reaction without the use of high cost immobilization techniques and providing a clean effluent. Despite the clear advantages of these reactor configurations not many papers have been published on the removal of phenols with this technique, and most of them were done with microorganisms [26] or immobilized enzymes on the membranes [27,28]. Our research group has previously studied the removal of 4-chlorophenol with a commercially SBP in this reactor configuration obtaining good results [29]. Since the commercially SBP is no longer manufactured different peroxidases had to be tested. In this work we have chosen three enzymes: the two most used peroxidases enzymes for phenolic compounds removal, HRP and SBP provided from a different supplier and AKPC also described by some authors [30] in order to study the removal of 4-chlorophenol in the ultrafiltration membrane reactor.

2. Materials and methods

2.1. Chemicals

Enzymes and substrates. Soybean peroxidase enzyme (SBP) (EC 1.11.1.7, 1356 units/mg) was purchased from Bio-Research Products. Artichoke peroxidase (AKPC) (EC 1.11.1.7, 3142 units/mg) was purchased from Artbiochem S.I. Horseradish peroxidase type I (HRP) (EC 1.11.1.7, 113 units/mg), 4-chlorophenol (molecular weight 128.6 g/mol and purity 99% or greater) and hydrogen peroxide (35% w/v) were supplied by Sigma-Aldrich Fine Chemical.

Analytical and auxiliary chemicals. 4-aminoantipyrine (AAP), potassium ferricyanide, aluminium potassium sulphate dodecahydrate and catalase from bovine liver (EC 1.11.1.6, 1870 units/mg) were acquired from Sigma-Aldrich Fine Chemical. Other chemicals were of analytical grade and were used without further purification.

2.2. Equipment

A Minitan-S system (Millipore Co., Bedford, MA) was used as ultrafiltration unit and polysulphone membranes (PTTK OMS, Millipore) with a surface area of 30 cm^2 and a nominal molecular weight cut-off of about 30,000 Da as reported by the manufacturer were selected. Watson Marlow Digital (Model 505 Du-RL) peristaltic pumps were used in the experiments. An eppendorf (MiniSpin) centrifuge was used to separate the precipitates in the samples and a Termo Spectronic, Helios α spectrophotometer was used for all the absorbance measurements.

2.3. Sample treatment

0.5 ml samples from the reactor were poured in 0.5 ml of catalase solution (2200 units/ml) to stop the reaction by breaking down the hydrogen peroxide. To 1 ml of the former mixture, 0.1 ml of a coagulant [$AlK(SO_4)_2$ 40 g/l] was added before centrifuging for 30 min at 10,000 g. The 4-chlorophenol present in the supernatant was analyzed by the colorimetric method described below.

2.4. Analytical method

4-Chlorophenol concentrations were measured by a standard colorimetric method [31]. 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) were prepared. Aliquots (2.4 ml) of the sample (4-chlorophenol concentration up to 0.2 mM) were placed in a spectrophotometer cuvette (3 ml) together with 0.3 ml of ferricyanide solution and 0.3 ml of AAP. 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 of ferricyanide solution and 0.3 ml of AAP solution). Absorbance values were transformed to 4-chlorophenol concentrations in the sample by using a calibration curve ([4-chlorophenol] = $0.0959 \times Abs_{505}$, $r = 0.9997$).

2.5. Experimental system

The experimental system used in the continuous assays consisted of a 100 ml jacketed tank reactor, two reservoir tanks for the substrate solutions, a collector tank, the membrane module and four Watson Marlow Digital peristaltic pumps to feed the substrates into the reactor, to pump the effluent from the reactor to the membrane module and to pump the permeate from the membrane to the collector tank. For each assay, two substrate solutions were prepared in distilled water, one for 4-chlorophenol, which eventually can be accompanied by the enzyme in the assays where additional amount of enzyme was added to the reactor with the feed flow and another for the hydrogen peroxide, before being poured into the reservoir tanks. Fig. 1 shows a scheme of the experimental system.

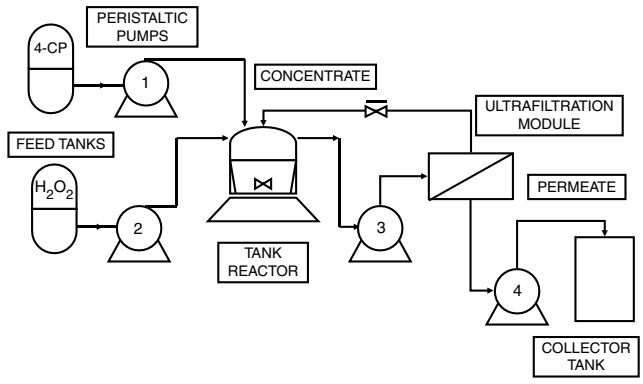

Fig. 1. Experimental system flow diagram.

2.6. Experimental planning

The following experimental conditions were maintained in all experimental series: temperature 20°C and a total reaction time of 80 min was established for all the assays as sufficient time to reach the steady state. The steady state was considered to have been reached when the difference between the 4-chlorophenol conversion values in the permeate stream in two consecutive measurements was lower than 3%. Five series of experiments were carried out in the continuous tank reactor coupled with ultrafiltration module, as shown in Table 1.

Duplicates were carried out for all the experimental series and average values are presented in all Figures. Standard deviation calculated for the whole set of experimental data was of 2.54%.

Table 1
Experimental planning

[*E*] (mg cm^{-3})	[4-chlorophenol] (mM)	[H_2O_2] (mM)	Residence time (min)	$[E]_{Feed}$ (mg cm^{-3})
Varying the enzyme concentration				
0.04	2	2	20	0
0.08				
Varying the enzyme concentration in the feed flow				
0.04	2	2	20	0.000
				0.001
				0.002
				0.004
Varying the residence time				
0.04	2	2	10	
			15	0.002
			20	
Varying the substrate concentration				
0.04	0.5	0.5		
	1	1	20	0.002
	2	2		
Varying the molar ratio H_2O_2: 4-chlorophenol				
0.04	2	2		
		2.5	20	0.002
		3		
Testing the influence of the enzyme retained on the membrane				
0.04	2	2	20	0.000

3. Results

3.1. Influence of initial enzyme concentration in the reactor

To follow the time course of the reaction, the concentration of 4-chlorophenol was measured in the permeate $[4CP]_P$ and in the reactor $[4CP]_R$. The $[4CP]$ value was normalized with respect to the 4-chlorophenol concentration in the feed flow, $[4CP]_F$, by defining the conversion in the reactor and permeate as follows:

$$Permeate\ conversion = \frac{[4CP]_F [4CP]_P}{[4CP]_F} \quad (1)$$

$$Reactor\ \ conversion = \frac{[4CP]_F - [4CP]_R}{[4CP]_F} \quad (2)$$

Two enzyme concentrations of 0.04 and 0.08 mg/ml were assessed for this study. In Fig. 2 it can be observed that conversion slightly increased with higher peroxidase concentrations. Also, higher conversion values, above 90%, were attained both in the reactor (Fig. 2A) and the permeate (Fig. 2B) when SBP was used, with the conversions remaining constant over the reaction time for the two concentrations tested, which differs from a previous work [29] where a significant conversion fall took place in the reactor for all enzyme concentrations tested.

High conversion values were also reached with HRP, even above 90% for the permeate samples (Fig. 2D) and, again, remaining constant over time for the two enzyme concentrations assayed. However, a significant conversion decrease can be observed in the reactor (Fig. 2C) from 30 min reaction time ([*E*] = 0.08 mg/ml) and 20 min reaction time ([*E*] = 0.04 mg/ml).

In the case of AKPC, an important conversion decrease was observed in the reactor (Fig. 2E) from the beginning of the reaction process, while conversion values in the permeate (Fig. 2F) are higher and, for the higher enzyme concentration (0.08 mg/ml) remained constant up to 40 min reaction time.

Differences between reactor and permeate conversion values can be explained by the fact that part of the enzyme is retained by the polymer formed along the reaction process and deposited on the membrane surface. As a result there is a continuous loss of enzyme in the reactor that causes the conversion decrease and, at the same time, the enzyme retained on the polymer keeps enhancing 4-chlorophenol conversion in the permeate stream. With the sole use of a continuous tank reactor [23] a lower SBP concentration, of 0.01 mg/ml, was enough to reach steady conversion values of over 90%, which supports the hypothesis of the enzyme loss in the membrane bioreactor due to retention on the membrane.

Another possible explanation for the observed behaviour would be the enzyme passing through the membrane. However, the three peroxidases present around 40 kD of molecular size, while the cut off of the membrane is of 30 kD, suggesting that the enzyme does not pass through the membrane. Tests were done to check for the presence of enzyme in the permeate stream and no significant amount (especially in the case of SBP) was detected, supporting the idea of the enzyme retention on the membrane.

Since no significant differences in conversion were attained when the initial enzyme concentration was increased, the lowest one, 0.04 mg/ml, was selected for the subsequent experimental series.

3.2. Influence of the supplementary addition of enzyme in the feed stream

Due to the enzyme retention on the polymer deposited on the membrane surface and the subsequent conversion fall in the reactor, a series of experiments supplying additional enzyme in the feed stream, in the range 0.000–0.004 mg/ml, was carried out to compensate the enzyme loss. For this study, the lowest enzyme concentration from the previous series (0.04 mg/ml) was used.

Results corresponding to the different enzyme concentrations in the feed stream are shown in Fig. 3. It can be observed that the higher conversions were attained, for both the reactor and permeate samples, when SBP was used (Fig. 3A and 3B).

There was a decrease in the reactor conversion values over time, which took place from the very beginning of the process in the case of AKPC (Fig. 3E) and from around 15 and 40 min for HRP and SBP, respectively (Figs. 3A and 3C). The conversion decrease was much more significant in the case of AKPC and HRP and, especially for HRP, it was partially avoided by adding more enzyme in the feed stream, achieving almost constant conversion values with the addition of 0.004 mg/ml of enzyme. In the case of SBP, conversion values in the reactor remained over 80% regardless the enzyme concentration in the feed stream, while in our previous study [30], with initial SBP concentration of 0.03 mg/ml, over 0.005 mg/ml of enzyme in the feed stream were required to attain the same conversions.

As for the conversion values in the permeate, they remained practically constant over time in the case of SBP (Fig. 3B), independently of the supplementary addition of enzyme. For the other peroxidases, the conversion fall started after 30 min and went on rather slowly for HRP (Fig. 3D) while it can be observed from the beginning of the reaction when using AKPC (Fig. 3F),

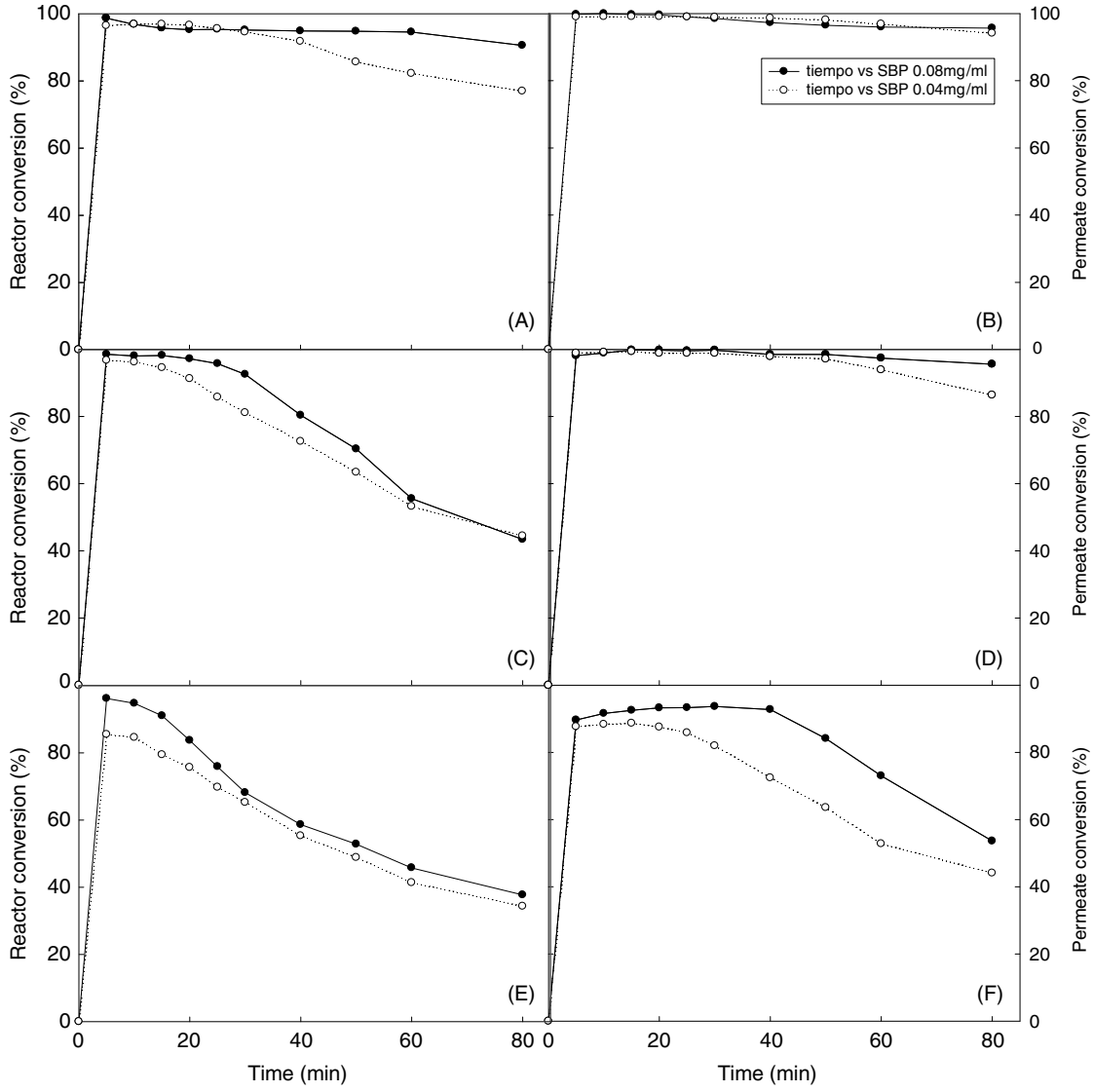

Fig. 2. Conversion in the reactor (A, C, E) and the permeate (B, D, F) over time for the different initial enzyme concentrations in the continuous tank reactor associated to a membrane module. V_R = 100 ml, Q = 5 ml/min, [4-chlorophenol]$_0$ = [H_2O_2]$_0$ = 2 mM, [E]$_0$ = (•) 0.08 mg/ml and (o) 0.04 mg/ml; (A) reactor and (B) permeate for SBP, (C) reactor and (D) permeate for HRP and (E) reactor and (F) permeate for AKPC.

being the decrease in the conversion values particularly strong even when enzyme was added in the feed stream. In the case of HRP, supplementary addition of enzyme in concentrations over 0.002 mg/ml allowed to maintain the conversion values all over the process.

As a result of this study, SBP and HRP were selected as the most appropriate peroxidases, achieving both on them conversion values close to 100% in the permeate stream, either with no need of supplementary enzyme, in the case of SBP, or for enzyme concentrations over 0.002 mg/ml in the feed stream, in the case of HRP. This result agrees with a preliminary study [32] where it was shown that SBP has the highest enzymatic activity, followed by HRP and AKPC.

3.3. Influence of the initial substrate concentration

Three different initial substrate concentrations of 0.5, 1.0 and 2.0 mM were used in this series of experiments carried out with the most efficient peroxidases, SBP and HRP. Results are depicted in Fig. 4 where it can be seen that there was a conversion increase both in the reactor (Fig. 4A, 4C and 4E) and the permeate (Figs. 4B, 4D and 4F) when the initial substrate concentrations were

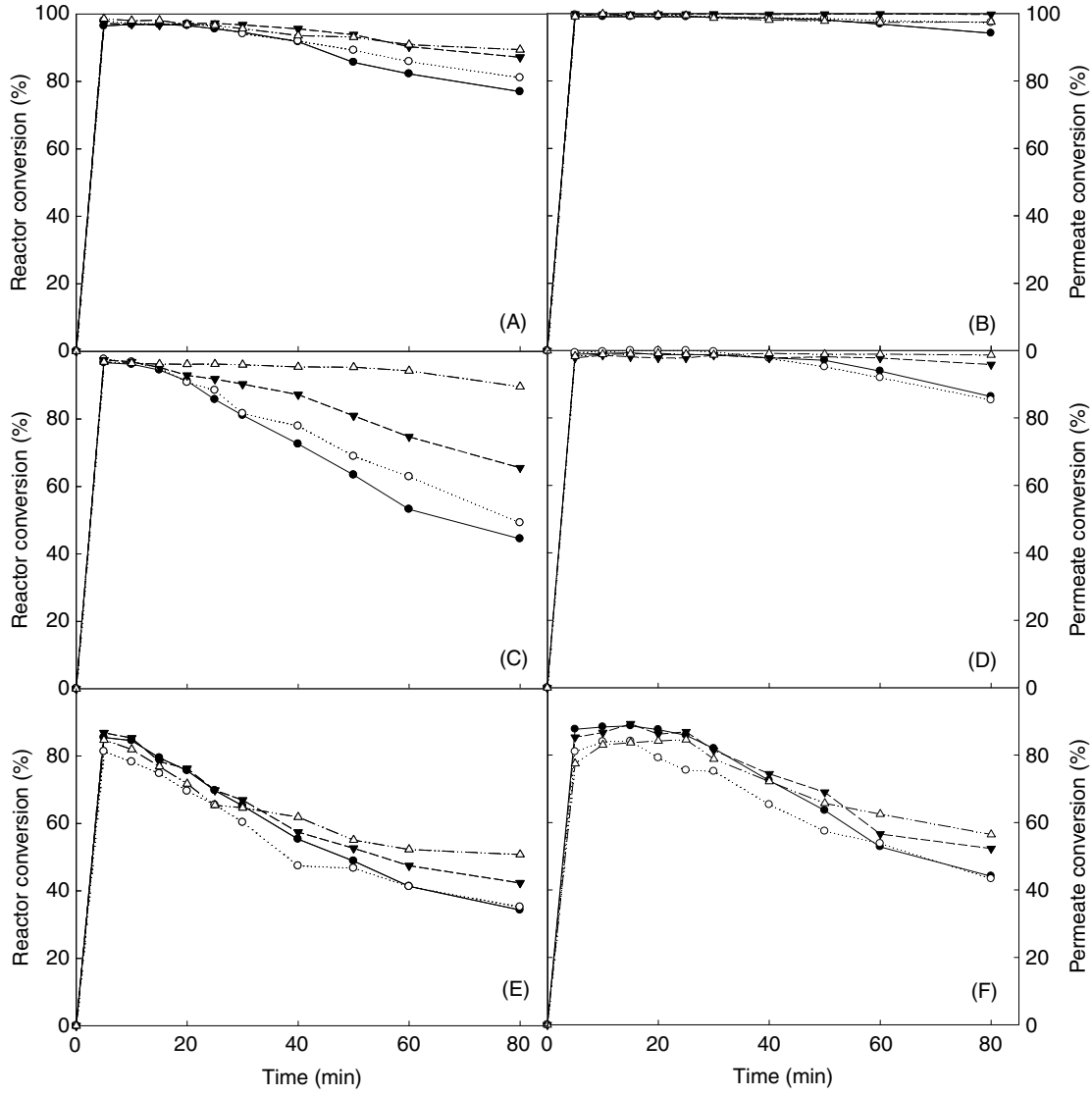

Fig. 3. Conversion in the reactor (A, C, E) and the permeate (B, D, F) over time for the different enzyme concentrations in the feed stream for the continuous tank reactor associated to a membrane module. V_R = 100 ml, Q =5 ml/min, $[4\text{-chlorophenol}]_0 = [H_2O_2]_0 = 2$ mM, $[E]_0 = 0.04$ mg/ml. $[E]_F$ = (•) 0.000, (o) 0.001, (▾) 0.002 and (Δ) 0.004 mg/ml. (A) reactor and (B) permeate for SBP. (C) Reactor and (D) permeate for HRP. (E) Reactor and (F) permeate for AKPC.

increased. This behaviour can be due to the non lineal form of the reaction rate equation, which for this process is of the Bisubstrate Ping-Pong type. The opposite tendency was observed in a CSTR [33], with lower substrate concentrations leading to higher conversions. The results obtained with the membrane bioreactor can be due to the fact that higher substrate concentrations lead to the formation of more polymeric products that once deposited on the membrane surface decrease its permeability, which makes it difficult for the substrates to pass through it, increasing the enzyme-substrates contact time and consequently the conversion values, not only in the reactor but also in the permeate which receives a stream of low chlorophenol load.

Additionally, there was a stronger enzyme deactivation and a lower enzyme to substrate ratio in the reactor when the substrate concentration increased, leading to a higher conversion fall, while in the permeate the conversion values remained constant, except for the lower concentration tested (0.5 mM).

Once again, higher conversions were obtained, for all cases, when SBP was used, ranging from almost 100% in the permeate for the higher substrate concentration to around 70% for the lowest one.

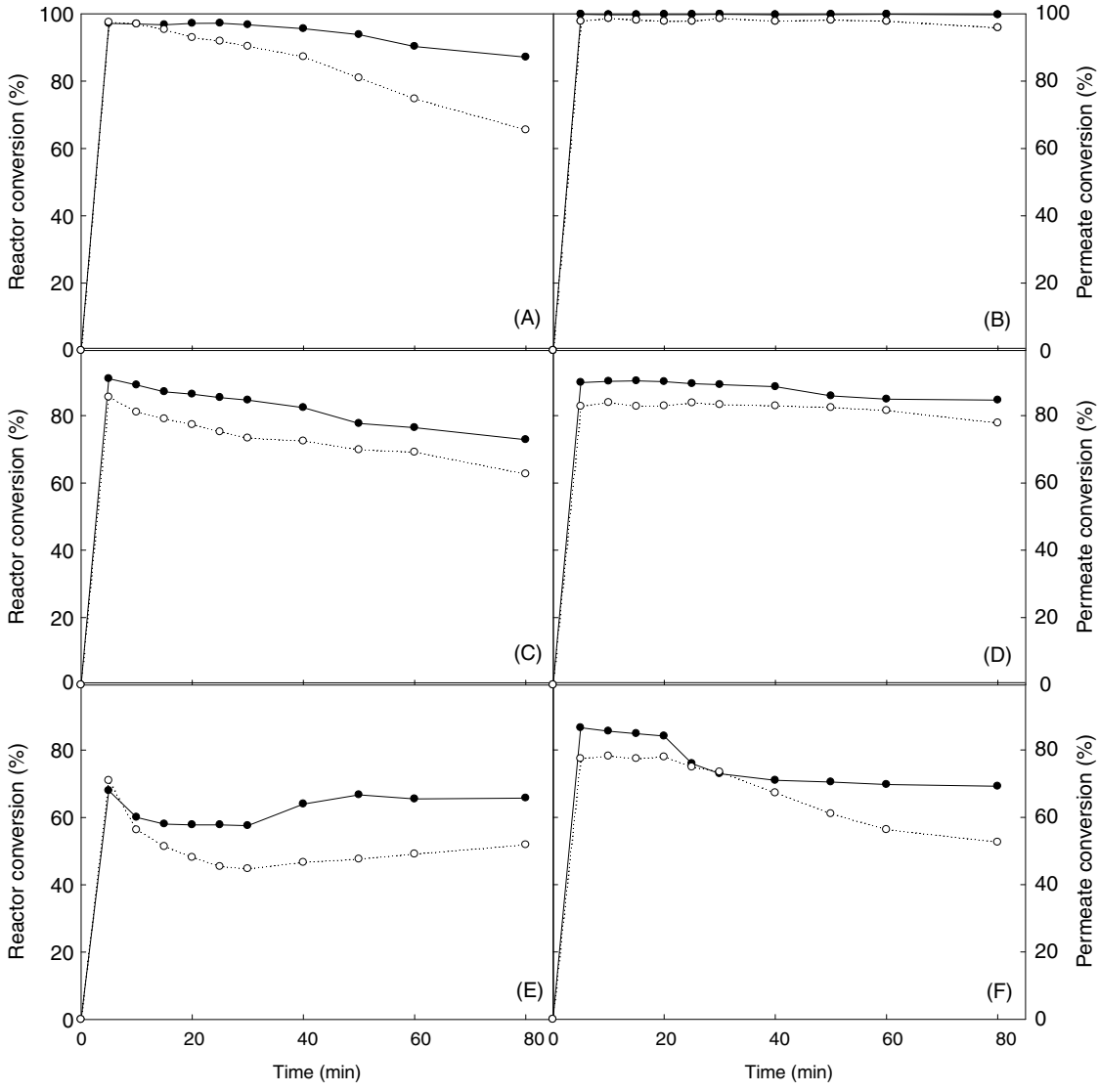

Fig. 4. Conversion in the reactor (A, C and E) and the permeate (B, D and F) over time for several substrate concentrations at fixed concentration ratio of 1:1 in the continuous tank reactor associated to a membrane module. V_R = 100 ml, Q = 5 ml/min, $[E]_0$ = 0.04 mg/ml, $[E]_F$ = 0.002 mg/ml, (•) SBP and (o) HRP, [4-chlorophenol]$_0$ = $[H_2O_2]_0$ = 2.0 mM (A and B), [4-chlorophenol]$_0$ = $[H_2O_2]_0$ = 1.0 mM (C and D) and [4-chlorophenol]$_0$ = $[H_2O_2]_0$ = 0.5 mM (E and F).

3.4. Influence of the initial hydrogen peroxide concentration

Using a fixed 4-chlorophenol concentration of 2.0 mM, three different hydrogen peroxide initial concentrations of 2.0, 2.5 and 3.0 were assessed, which leads to molar ratios H_2O_2:4-chlorophenol of 1, 1.25 and 1.5, respectively. Results shown in Fig. 5 indicate that there was no significant influence of the initial H_2O_2 concentration on the conversion values of the reactor (Fig. 5A, 5C and 5E) and permeate (Fig. 5B, 5D and 5F), with a slight conversion increase when the molar ratio 1:1 is used. This result agrees with the one previously obtained in a discontinuous tank reactor with soybean peroxidase and the same substrate [21], suggesting the deactivation effect of an excess of hydrogen peroxide on the free enzyme under the conditions tested.

For both SBP and HRP, high conversions (90% or more) were attained in the reactor at the beginning of the reaction and, later on, there was a continuous conversion fall until constant values, between 75 and 90% for SBP and around 70% for HRP, were reached. The permeate curves also showed a very similar behaviour for the three H_2O_2 concentration tested, with steady

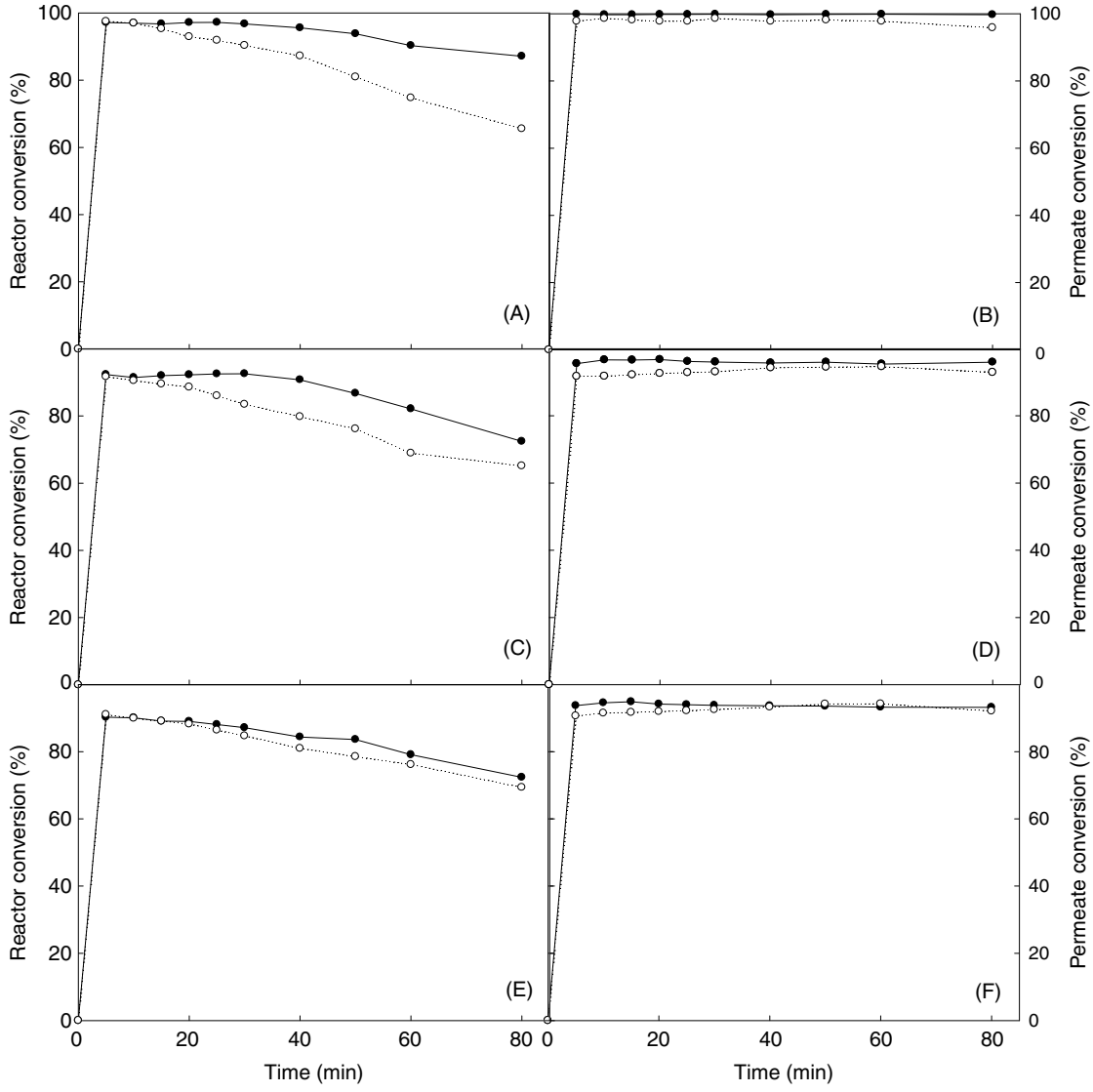

Fig. 5. Conversion in the reactor (A, C and E) and the permeate (B, D and F) over time for the different hydrogen peroxide concentrations in the continuous tank reactor associated to a membrane module. V_R = 100 ml, Q = 5 ml/min, $[E]_0$ = 0.04 mg/ml, $[E]_F$ = 0.002 mg/ml, (•) SBP and (o) HRP, [4-chlorophenol]$_0$ = 2 mM, $[H_2O_2]_0$ = 2.0 mM (A and B), 2.5 mM (C and D), 3.0 mM (E and F).

conversions from almost the beginning of the process, over 90% for both SBP and HRP.

3.5. Influence of the spatial time

In this series, three reaction volumes of 50, 75 and 100 ml were used for a fixed flow rate of 5 ml/min, which leads to spatial times of 10, 15 and 20 min, respectively.

Results corresponding to this series are depicted in Fig. 6 and, according to them, there was a conversion increase for both SBP and HRP in the reactor (Fig. 6A, 6C and 6E) when increasing the spatial time, as expected and agreeing with previous results [29,33]. However, in the permeate (Fig. 6B, 6D and 6F), the conversion remained practically constant regardless the spatial time used, so this variable has no influence under these experimental conditions where maximum conversion values had already been reached for the lower spatial times tested. Again, the best results in conversion were achieved when SBP was used, although in the case of the permeate samples differences were insignificant and 100% removal was achieved in all cases.

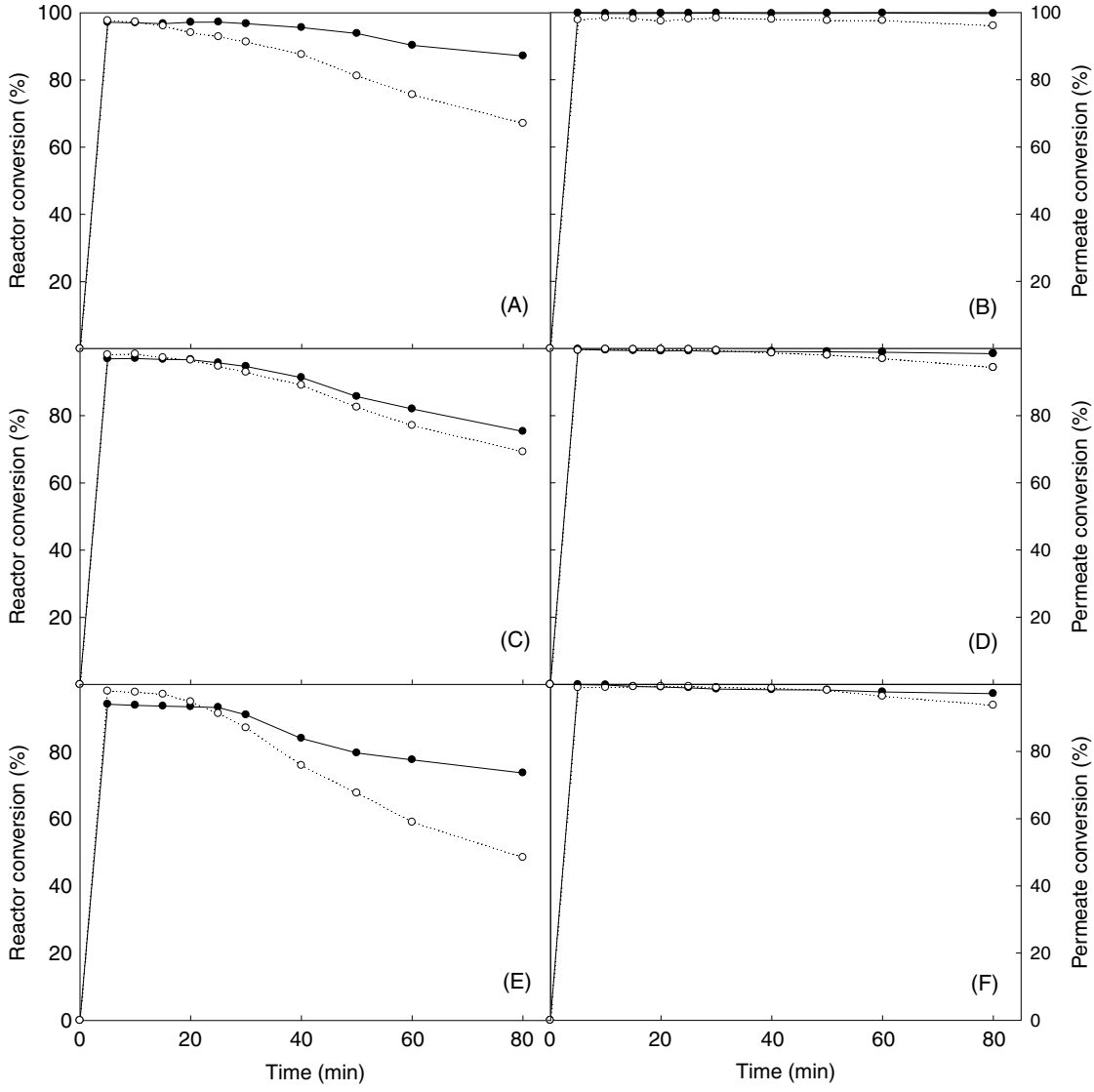

Fig. 6. Conversion in the reactor (A, C and E) and the permeate (B, D and, F) over time for the different spatial times in the continuous tank reactor associated to a membrane module. $[E]_0$ = 0.04 mg/ml, $[E]_F$ = 0.002 mg/ml, (•) SBP and (o) HRP, [4-chlorophenol]$_0$ = $[H_2O_2]_0$ = 2.0 mM, Q= 5 ml/min, τ = 20 min (A and B), τ = 15 min (C and D), τ = 10 min (E and F).

3.6. Influence of the enzyme retained on the membrane

According to the obtained results, SBP turned out to be the best one of the three peroxidases tested. So it was selected for a last assay to check if the enzyme retained on the polymer deposited on the membrane keeps acting on chlorophenol removal. This assay was carried out in two steps. In the first step, the experiment was conducted in the presence of enzyme and substrates along 80 min. Then, after stopping the reaction and cleaning, the system was reinitiated, feeding again both substrates but without adding the enzyme (neither in the reactor nor in the feed stream).

It can be observed from Fig. 7 that the 4-chlorophenol removal continued during the second step, both in the reactor and the permeate, so the enzyme does retain its activity. There was a slight conversion increase in the reactor, reaching final values of around 50%. On the contrary, conversion values showed a continuous decrease in the permeate during this second step, with initial values of around 87% and final values of around 60% after 160 min. Additionally, and as it can be observed steps 1 and 2 had different behaviours. In the case of the reactor this can be explained considering that the enzyme retained on the polymer might be partially released

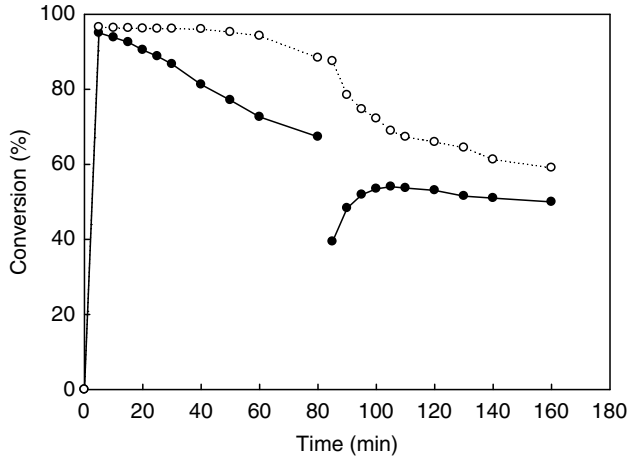

Fig. 7. Conversion over time in the continuous tank reactor associated to a membrane module. V_R = 100 ml, Q = 5 ml/min, $[4\text{-chlorophenol}]_0 = [H_2O_2]_0$ = 2 mM, $[E]_0$ = 0.04 mg/ml, $[E]_F$ = 0.000 mg/ml (•) reactor and (o) permeate.

and returns to the reaction media during the second step, leading to the conversion increase previously commented. In the case of the permeate, the release of the enzyme retained on the membrane would cause a decrease of enzymatic activity over time and, as a result, a conversion fall during the last reaction period. This result confirms the enzyme retention on the membrane surface by adsorption-precipitation with the polymers and, also, that the enzyme conserves its activity.

4. Conclusions

SBP and HRP have proven to be the most efficient peroxidases for the removal of 4-chlorophenol in the continuous tank reactor associated to a membrane module. Initial SBP concentrations in the tank reactor of 0.04 mg/ml attained high conversion values that remained constant with no need to add enzyme in the feed stream. This supplementary addition of enzyme, in concentrations of 0.002 mg/ml or higher, allowed obtaining steady conversions and high removal efficiencies for HRP, but had no effect in the case of AKPC. The removal of 4-chlorophenol improved when increasing the initial substrate concentrations, although there was a stronger conversion fall in the reactor. The increase of the molar ratio H_2O_2:4-chlorophenol and the spatial time had no influence on the permeate conversions and a rather negligible effect on the reactor conversion, so the lower values (molar ratio 1:1 and spatial time of 10 min) were selected. The higher conversion values, close to 100% in the permeate, were obtained with SBP in all cases. An additional assay with this peroxidase demonstrated that the enzyme is capable of maintaining its activity when retained on the membrane surface, being partially released along time and reaching a reactor-membrane equilibrium that facilitates the continuous 4-chlorophenol removal process.

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