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# Non-isothermal bioremediation of waters polluted by phenol and some of its derivatives by laccase covalently immobilized on polypropylene membranes

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ABSTRACT

In view of the heath problems induced by the presence into the environment of endocrine disruptors, laccase from *Trametes versicolor* was covalently immobilized on a chemically modified polypropylene membrane in order to remove phenol and its derivatives from polluted waters. Using phenol as substrate model the optimal immobilization conditions were determined. The immobilized laccase exhibited maximal enzyme activity at pH 5.5 and optimal temperature at 55 °C. These operative parameters have been compared with those obtained with the soluble laccase in order to ascertain the immobilization effect.

When employed in a bioreactor operating under isothermal conditions the immobilized laccase was able to oxidize a wide range of phenolic substrates. In particular it was found that some phenol derivatives (2-CP, 3-CP, 4-CP, NP and chlorophene) were oxidized at a similar rate than phenol, other derivatives (paracetamol, 3-MP and chloroxyphenol) at a smaller rate, while others (2,4-DCP and BPA) at higher rate.

When the catalytic membrane was employed in a non-isothermal reactor the reaction rate increased with the increase of the applied temperature difference. Practically the increase of the laccase oxidative power under the non-isothermal conditions followed the same sequence observed under isothermal conditions. Interesting enough, the percentage increase of enzyme reaction rate under non-isothermal conditions resulted higher in the cases in which the isothermal reaction rate was smaller.

When the reduction of the production times by the presence of a temperature gradient is considered, the measured values strongly candidate the technology of non-isothermal bioreactors as a useful tool in processes of detoxification of waste waters polluted by endocrine disruptors of phenolic origin.



## 1. Introduction

There is considerable concern that some chemical compounds may affect the reproductive systems of wildlife and humans by mimicking or interfering with the action of endogenous gonadal steroid hormones [1]. These compounds have been named endocrine-disrupting chemicals (EDCs). Some pesticides, phthalates, phytoestrogenes, pharmaceuticals and several phenolic compounds are recognized as endocrine disruptors.

Bisphenol A (2,2-bis(4-hydroxyphenyl)propane; BPA) and Nonylphenol (NP), major components of various consumer products, including plastic packing materials and detergents, are known EDCs. They are released on a large scale and accumulated in the environment [2].

Phenol and some of its substituted, such as 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 3-methoxyphenol are also toxic and, under some conditions, persistent pollutants in aqueous systems, soils, and waste materials. These compounds are used on a large scale as wood preservatives, pesticides, and precursors of herbicides [3].

In addition many pharmaceuticals, some with endocrine-disrupting functions, enter sewage systems and through the sewage treatment system are discharged into the environment if not fully removed [4,5]. These compounds, biologically active, have the potential to affect exposed populations. This

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class of chemicals includes paracetamol (4-acetamidophenol), chlorophene (2-benzyl-4-chlorophenol) and chloroxylenol (4-chloro-3,5-dimethylphenol).

The continuous lowering of their tolerable values in effluents imposed by government regulations, leading to more sophisticated and expensive treatments, makes current methods no more satisfactory. Phenols removal has been commonly realized by physical (i.e. adsorption on active carbon) or chemical (i.e. oxidation) methods.

In early 1980s, Klibanov et al. [6] proposed an enzymatic method based on the phenol oxidation by polyphenoloxidases (laccase or tyrosinase). Laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) is a particularly promising enzyme for the above-mentioned purposes. This enzyme catalyses the oxidation of *ortho*- and *para*-diphenols, aminophenols, chlorophenols, polyphenols, polyamines, lignins and aryl diamines as well as some inorganic ions through the reduction of molecular dioxygen to water [7,8]. The laccase catalytic cycle and the proposed mechanisms for the reduction and reoxidation of the copper sites can be found in Ref. [9].

As for the majority of enzymes, laccase can be employed in the insoluble form. The advantages of immobilized enzymes in respect to their free counterpart are well-known. Various supports have been used for enzyme immobilization [10–14], including polymer membranes. In the case of catalysis under isothermal conditions hydrophilic membranes are preferred as matrices for enzyme immobilization because they provide a better microenvironment for enzymes. For enzymatic reactions under non-isothermal conditions, instead of hydrophilic membranes, hydrophobic catalytic membranes, such as polypropylene, teflon or nylon, must be used. Indeed, when a temperature difference is applied across the catalytic hydrophobic membrane, the enzyme reaction rate increases considerably, proportionally to the values of the applied transmembrane temperature gradients [15–21]. This has been explained on the basis of the thermodynamics of irreversible processes [22–24], according to which temperature gradients drive matter fluxes in bulk solutions (thermal diffusion) or across hydrophobic membranes (thermodialysis) separating liquid mixtures. In particular, when hydrophobic and unselective porous membranes are employed in a reactor/bioreactor to separate aqueous solutions kept at different temperatures, selective solvent and solute (substrate in the case of catalysis) fluxes occur across them. Water fluxes are directed towards the cold half-cell, while solutes are moving towards the cold or warm half-cell in dependence on their nature and that of the solvent. Both solvent and solute fluxes are proportional to the applied temperature gradient. As a consequence, when a catalytic hydrophobic membrane separates two solutions kept at different temperatures, the immobilized enzymes encounter in the unit of time more substrate molecule that under isothermal conditions and, consequently, the reaction rate increases.

The goal of the present work is to study the optimal conditions for immobilization of laccase from *Trametes versicolor* on a chemically modified polypropylene membrane and its application in the biotransformation of phenolic derivatives, including phenol, using non-isothermal bioreactors.

## 2. Apparatus, materials and methods

### 2.1. The bioreactor

The apparatus consisted of two cylindrical half-cells, filled with the working solutions and separated by a catalytic membrane [15–21]. Solutions, containing the substrates, were recirculated in each half-cell by a peristaltic pump, through hydraulic circuits starting and ending in a common cylinder. Each half-cell was thermostated at a temperature $T_i$ ($i = 1, 2$). When the apparatus worked under isothermal conditions $T_1$ was equal to $T_2$. Thermocouples, placed at 1.5 mm from each of the membrane surfaces, measured the temperatures inside each half-cell. These measurements allowed the calculation of the temperature profile across the catalytic membrane. The temperatures measured by the thermocouples are indicated as $T$, while those calculated at the membrane surfaces are indicated as $T^*$. The temperatures of the warm and cold half-cell are indicated by the subscripts w and c, respectively. Under these assumptions $\Delta T = T_w - T_c$ is the temperature difference measured at the thermocouples position; $\Delta T^* = T_w^* - T_c^*$ is the actual temperature difference across the membrane, and $T_{av} = (T_w + T_c)/2$ and $T_{av}^* = (T_w^* + T_c^*)/2$ are the average temperatures of the bioreactor and the membrane, respectively. Since our system is made up of only one membrane, it is symmetric and therefore $T_{av} \equiv T_{av}^*$. In non-isothermal experiments $T_w^* < T_w$, $T_c^* > T_c$, and $\Delta T^* < \Delta T$. Indeed $\Delta T^* = \gamma \Delta T$, where $\gamma$ is smaller than 1. In our case $\gamma = 0.11$. The $\gamma$ value has been calculated (see Refs. [21,23]) on the basis of principle of heat continuity and taking as the values of thermal conductivity of water and polypropylene membranes by [25] and Touloukian et al. [26], respectively.

### 2.2. Materials

Laccase (EC. 1.10.3.2, 20 U/mg) from *Trametes versicolor* (Fluka Chemie GmbH, CH-9471, Buchs SG, Switzerland) was used as catalyst.

As solid support polypropylene membranes [11] from Sterlitech (Kent, WA, USA) were used. These hydrophobic membranes, 150 μm in thickness, had a nominal pore size of 0.2 μm.

Phenol, 3-methoxyphenol (3-MP), nonylphenol (NP), bisphenol A (BPA), 2-chlorophenol (2-CP), 3-chlorophenol (3-CP), 4-chlorophenol (4-CP), 2,4-dichlorophenol (2,4-DCP), paracetamol (4-acetamidophenol), chlorophene (2-benzyl-4-chlorophenol), chloroxylenol (4-chloro-3,5-dimethylphenol) were the phenolic derivatives used in this research (Fig. 1). All these products were purchased by Sigma (Sigma–Aldrich GmbH, CH-9471, Buchs SG, Switzerland). Also the other chemicals, such as ethylenediamine, sodium dihydrogen phosphate dihydrate, disodium hydrogen phosphate, sodium carbonate, sodium acetate, acetic acid, copper(II) sulfate pentahydrate, sodium hydroxide, sodium chloride, potassium dichromate, acetone and Folin-Ciocalteu's phenol reagent were obtained from Sigma. Hydrochloric acid, glutaraldehyde and sulfuric acid were obtained from Fluka (Fluka GmbH, CH-9471, Buchs SG, Switzerland). All chemicals were of analytical grade and were used without further purification.

### 2.3. Methods

#### 2.3.1. Catalytic membranes preparation

The preparation of the catalytic membranes was carried out by means of two steps: (a) chemical modification and (b) enzyme immobilization.

(a) Chemical modification

Chromic acid solution for modification of PP membrane surfaces was prepared by slow mixing $K_2Cr_2O_7$ with $H_2O$ and $H_2SO_4$ in proportion 1:19:29.4 by weight. PP membranes were first immersed in acetone for 5 min and subsequently dipped into a glass vessel with the chromic acid solution. The vessel was covered with a glass plate and placed in an oven at 30 °C for 10 min. Then PP membranes were dipped into a 10% (v/v) ethylenediamine aqueous solution in a vessel covered with a glass plate and placed in an oven at 40 °C for 60 min. After this step, the membranes were washed under running distilled

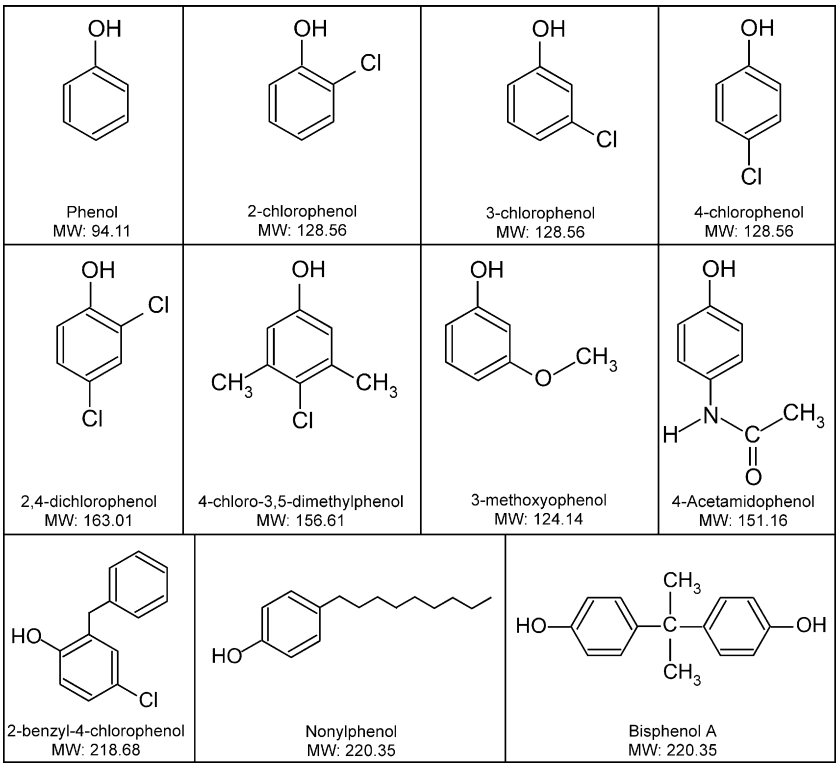


**Fig. 1.** Chemical structures of phenol and phenol derivatives.

water to remove the unreacted diamine. PP membranes, so modified, were kept overnight under distilled water.

(b) Enzyme immobilization

In order to immobilize the enzymes, the modified membranes were treated for 1 h at room temperature with a 25% (v/v) glutaraldehyde aqueous solution. After further washings with distilled water and 0.1 M Na-acetate buffer solution (pH 5.5), the membranes were immersed for 14 h at 4 °C in the same buffer solution (100 mL) containing laccase at a concentration of 0.1 mg/mL. Coupling of laccase to glutaraldehyde occurred through the amine groups of the enzyme and the free aldehyde groups generated on the membrane surface [27]. At the end of this step, the membranes were washed for 1 h with the buffer solution in order to remove the unbound enzymes.

### 2.3.2. Enzyme activity determination

The average enzyme activity was determined in experiments with soluble laccase by adding 15 μL of enzyme solution (0.1 mg/mL) to 30 mL of aqueous solution polluted by phenol or its derivatives with final concentration 50 μM in the 0.1 M Na-acetate buffer (pH 4.6) and temperature 40 °C, 10 min. The reaction was carried out under stirring. At regular time intervals, 2 mL of the enzyme treated solution were withdrawn, filtered across a Millex Filter Unit equipped with a MF-Millipore Membrane (0.45 μm) to eliminate the enzyme, and assayed according to the Folin-Ciocalteau's method for the determination of phenolic hydroxyl groups [28]. In particular the filtered samples were mixed with 0.5 mL of Folin-Ciocalteu's phenol reagent. The mixture was shaken for 5 min and 2 mL of 5% sodium carbonate solution were then added. The samples were allowed to react for 10 min. Aliquots of approximately 2 mL were used to measure the absorbance at 750 nm. Calculations of the amount of phenolic hydroxyls groups were carried out by means of different calibration curves for phenol and its derivatives. Specific activity was calculated as activity unit per milligram of immobilized enzyme (one unit is 1 $\mu mol\,min^{-1}$).

The initial enzyme reaction rate, expressed as $\mu mol\,min^{-1}$, is given by the slope of a linear plot of the substrate decrease as a function of time.

In the experiments with immobilized laccase the membrane (35 $cm^2$, 0.036 $mg\,cm^{-2}$) was allowed to react with 30 mL of aqueous solution polluted by phenol or its derivatives with final concentration 50 μM in the 0.1 M Na-acetate buffer (pH 5.5) and temperature 55 °C, 10 min. At the end of the experiment 2 mL of the enzyme treated solution were withdrawn and processed, without filtering, according to the Folin-Ciocalteau's method. The calculation of the enzyme activity was performed as for the soluble laccase.

### 2.3.3. Determination of thermal stability

To assess the thermal stability of soluble and insoluble laccase, we adopted the same procedure described in Section 2.3.2 with the only difference that before the reaction with the phenol solution (at 30 °C) either the enzyme solution or the catalytic membrane (in 0.1 M Na-acetate buffer solution, pH 5.5) were maintained, in absence of substrate, at 60 °C for the requested time. At the chosen time 15 μL of enzyme solution or the membrane were allowed to react with the phenol solution (50 μM phenol in 0.1 M Na-acetate buffer solution with pH 5.5 for immobilized enzyme and pH 4.6 for soluble enzyme) for 30 min.

### 2.3.4. Removal of phenol derivatives by immobilized laccase

Phenol and phenol derivatives were first dissolved in the appropriate amount in 2 mL 96% ethanol aqueous solution and then further diluted with 0.1 M Na-acetate buffer pH 5.5 to a final volume of 100 mL. The final concentration of ethanol was below 2% (v/v) in all the assays and final concentration of phenol derivates 50 μM.

The phenol derivatives removal was studied using the bioreactor working under isothermal and non-isothermal conditions. Four temperature differences were employed: $\Delta T = 0$, 10, 20, 30 °C. In all cases the average temperature was $T_{av} = 25$ °C. The substrate

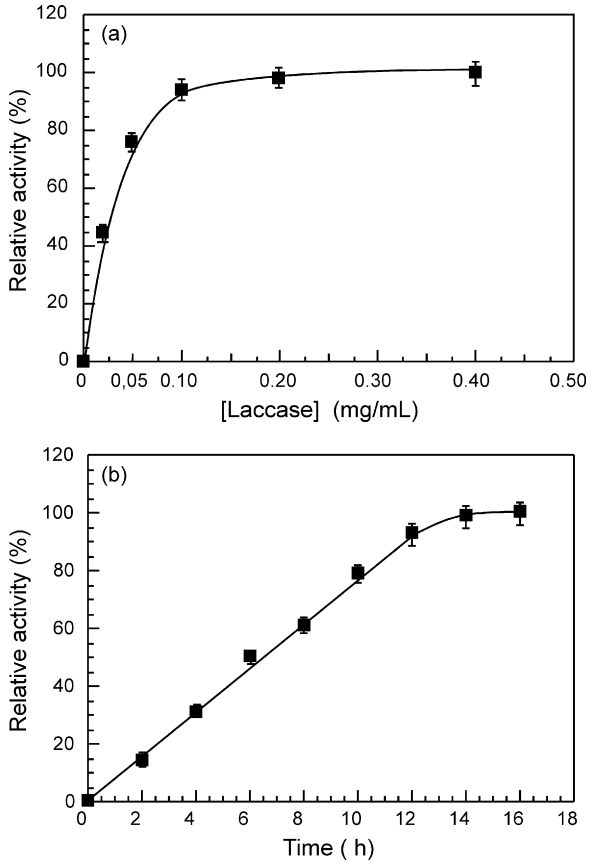


**Fig. 2.** Relative activity of immobilized laccase from *Trametes versicolor* as a function of laccase concentration (a) and immobilization time (b).

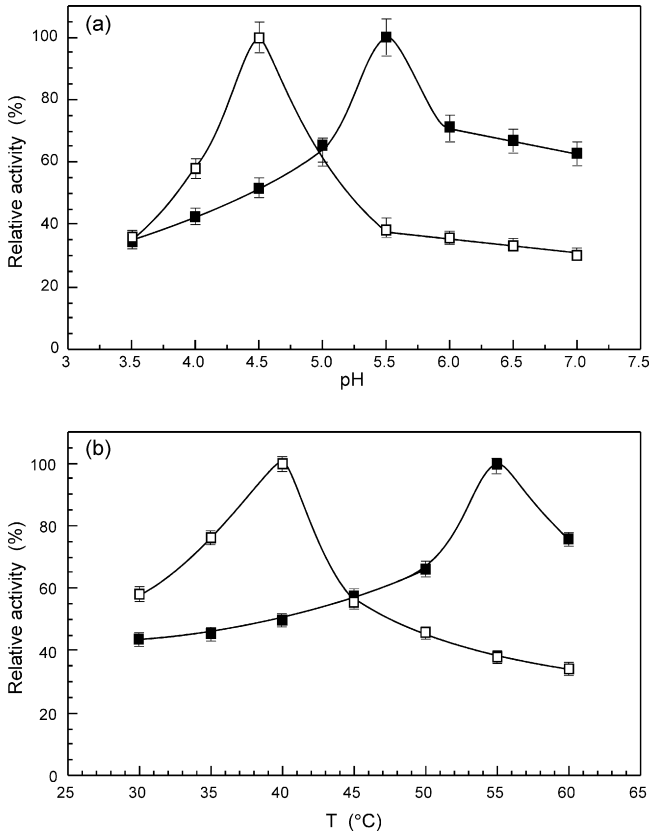


**Fig. 3.** pH (a) and temperature (b) optima of the free (□) and immobilized (■) laccase from *Trametes versicolor*.

solution feed was 3 mL/min. The catalytic membrane (35 $cm^2$) was put in contact with 30 mL of phenols solutions with concentration 50 μM in 0.1 M Na-acetate buffer, pH 5.5, circulating by means of two peristaltic pumps through the bioreactor for 30 min.

The yield of the process of substrates removal [15] was calculated by measuring every 10 min the substrate concentrations in the samples taken out from the common cylinder. Calculation was performed through the expression:

$$SR(t) = \frac{[S]_0 - [S]_t}{[S]_0} \times 100 \quad (1)$$

where $[S]_0$ and $[S]_t$ are the value of the substrate concentrations at the beginning of the run and at $t$ time, respectively.

#### 2.3.5. Experimental data treatment

Every experimental point in the figures represents the average value of five experiments performed under the same conditions. The maximal deviation from the mean value never exceeded 4.5%. The duration of each experiment, the composition of our solutions and the hydrophobic nature of the membrane excluded the occurrence of membrane fouling. In any case, to avoid membrane fouling due to membrane reuse, a cleaning 0.1 M Na-acetate buffer solution, pH 5.5, was circulated for 60 min through the bioreactor and the membrane between two subsequent experiments.

The catalytic membrane state was assessed by measuring every day the membrane activity under phenol standard conditions. When the membrane activity exhibited a value 9% lower than that of the initial value, i.e. a value twice the experimental error, the membrane was discharged.

## 3. Results and discussion

### 3.1. Determination of optimal immobilization conditions

In order to find the best conditions for obtain the highest catalytic activity of the membrane we have investigated: (i) the dependence of the membrane catalytic activity on the enzyme concentration used for the immobilization process; (ii) the dependence on the incubation time of the functionalized membrane with the enzyme solution.

First of all the optimal laccase concentration to be used for laccase immobilization was determined. To this aim the enzyme concentration in the solution used in the immobilization step was varied from 0.025 to 0.4 mg/mL. In Fig. 2a the membrane catalytic activity, expressed as relative activity, is reported as a function of laccase concentration. The substrate solution was 50 μM phenols in 0.1 M Na-acetate buffer, pH 5.5 and temperature 25 °C. From Fig. 2a clearly it emerges that an enzyme concentration of 0.1 mg/mL is practically enough to reach a relative activity of almost 100%.

Having ascertained that 0.1 mg/mL of laccase are sufficient to obtain the maximum activity of the membrane, we have determined the optimal incubation time for enzyme immobilization. In this case we varied the incubation time of the enzyme solution with the modified membrane up to 16 h. The other conditions were those listed to obtain the results in Fig. 2a. From Fig. 2b it is evident that after 8 h the relative enzyme activity is down to 60.6%, after 14 h is constantly almost 100%.

Considering all above results, we decided to use henceforth an enzyme concentration of 0.1 mg/mL and an incubation time of 14 h.

The average specific activity of immobilized enzyme at optimal conditions is 69.4 μmol $min^{-1}$ $mg^{-1}$ (50 μM phenol, 0.1 M Na-acetate buffer, pH 5.5, temperature 55 °C, 10 min) and the aver-

age amount of bound protein is 0.036 mg cm$^{-2}$. Reproducibility of membrane activity during different immobilization experiments is ±4.3 μmol min$^{-1}$ mg$^{-1}$.

### 3.2. Characterization of immobilized laccase

#### 3.2.1. Determination of pH and temperature optima

It is well know that the pH and temperature profiles of an insoluble enzyme are generally different from those of the soluble counterpart. To verify if this occurs also with our immobilized laccase we have investigated the dependence of the catalytic activity either on the pH or on the temperature.

The pH-activity profile of an immobilized enzyme depends on the support and immobilization method used. The support affects the pH value around the catalytic site, so determining differences between the catalytic behavior of the soluble and insoluble form of the catalyst. This effect is known as partitioning effect [29]. The partitioning effect, depending on the surface charge on the carrier, may cause changes in the concentrations of the charged species (e.g., hydrogen or hydroxyl ions) in the microenvironment in which the immobilized enzymes are operating.

To know how the partitioning effect affects the catalytic behavior of our enzyme derivatives, we have investigated the laccase activity, in the free and immobilized form, as a function of pH in the range from 3.5 to 7.0. The results of this investigation are reported in Fig. 3a where the relative activities of soluble and insoluble laccase are reported as a function of pH. The experimental conditions were: phenol concentration 50 μM in 0.1 M Na-acetate buffer and the temperature equal to 30 °C. Inspection of Fig. 3a shows a marked difference in the value of the optimum pH between the free and insoluble forms of laccase. The optimum pH value for soluble laccase occurs at 4.6, whereas the value for the insoluble laccase occurs at 5.5. At pH 3.5 both free and immobilized laccase show the same relative activity. Vice versa at pH 7.0, the immobilized laccase retains about 58.6% of its maximum activity, while the soluble form retains only 30%.

The isothermal characterization of the membrane activity is one of the principal parameters required to know how the immobilization procedure affects the enzyme activity. Generally, enzymatic derivatives show optima temperatures shifted toward temperatures higher than those exhibited by the soluble counterpart. In Fig. 3b the relative activity of the catalytic membrane is reported as a function of temperature. In this case the phenol concentration was kept constant at 50 μM in 0.1 M Na-acetate buffer at pH 5.5, while the solution temperature was varied. The temperature profile of the soluble laccase has been added, for comparison. Results in Fig. 3b show for the insoluble laccase a shift of the optimum activity toward higher temperatures in comparison to the position of the soluble enzyme. Soluble laccase has an optimum temperature of approximately 40 °C, whereas the optimum temperature of immobilized laccase is shifted to about 55 °C. This means that the immobilization process, strengthening the enzyme structure, gives to the macromolecule a protective effect against the heat denaturation. More interesting is the observation that at 60 °C the immobilized laccase retains 78.3% of its maximum activity, while the free laccase retains only 38.2%.

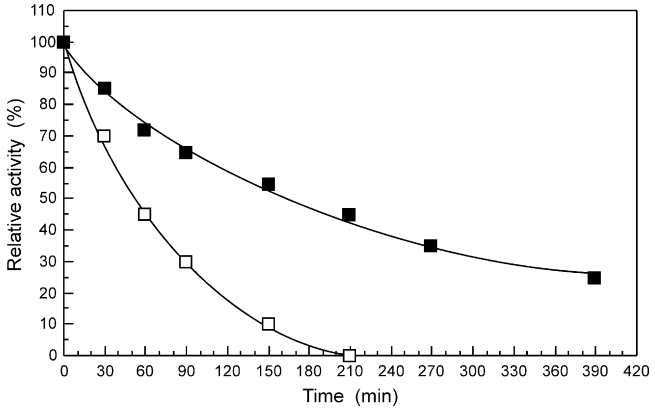


**Fig. 4.** Thermal stability at 60 °C of soluble (□) and immobilized (■) laccase.

**Table 1**
Removal efficiency of phenol and phenol derivatives under isothermal conditions at 25 °C (Δ$T$ = 0 °C, 50 μM substrate solution and treatment time 30 min).

| Substrate | Time (min) | Δ$T$ = 0 °C | | Average reaction rate (μmol min$^{-1}$) |
|---|---|---|---|---|
| | | [$S_0$] − [$S_t$] (μM) | SR (%) | |
| Phenol | 10 | 9 | 18 | 21 |
| | 20 | 15 | 30 | |
| | 30 | 20 | 40 | |
| 2-Chlorophenol | 10 | 10 | 20 | 24 |
| | 20 | 18 | 36 | |
| | 30 | 22 | 44 | |
| 3-Chlorophenol | 10 | 10 | 20 | 22 |
| | 20 | 17 | 34 | |
| | 30 | 21 | 42 | |
| 4-Chlorophenol | 10 | 7 | 14 | 21 |
| | 20 | 14 | 28 | |
| | 30 | 21 | 42 | |
| 2,4-Dichlorophenol | 10 | 16 | 32 | 34 |
| | 20 | 26 | 52 | |
| | 30 | 30 | 60 | |
| 3-Methoxyphenol | 10 | 6 | 12 | 17 |
| | 20 | 12 | 24 | |
| | 30 | 19 | 38 | |
| Bisphenol A | 10 | 8 | 16 | 26 |
| | 20 | 20 | 40 | |
| | 30 | 25 | 50 | |
| Nonylphenol | 10 | 7 | 14 | 21 |
| | 20 | 14 | 28 | |
| | 30 | 21 | 42 | |
| 4-Chloro-3,5-dimethylphenol | 10 | 4 | 8 | 11 |
| | 20 | 7 | 14 | |
| | 30 | 12 | 24 | |
| 2-Benzyl-4-chlorophenol | 10 | 6 | 12 | 20 |
| | 20 | 13 | 26 | |
| | 30 | 21 | 42 | |
| 4-Acetamidophenol | 10 | 2 | 4 | 6 |
| | 20 | 4 | 8 | |
| | 30 | 6 | 12 | |

#### 3.2.2. Thermal stability

The thermal stabilities of free and immobilized laccase in terms of the relative activities are compared in Fig. 4. Measures were carried out as described in Section 2.3.3. The activity of free enzyme decreases significantly in comparison to that of the immobilized laccase. After 90 min of incubation at 60 °C the relative activity being only the 30% of the initial value in the case of the soluble enzyme, while the insoluble form retains 65% of its initial value. At 210 min of incubation the free enzyme lost all its initial activity, while the catalytic membrane retains about 45% of its initial value. At 400 min the catalytic membrane still retains 30% of its initial value. Laccase immobilization on PP membrane led to a significant stabilizing effect towards heat deactivation.

### 3.3. Enzymatic removal of phenol derivatives

#### 3.3.1. Isothermal treatment

Removal efficiency of phenol and phenol derivatives was determined first under isothermal conditions at 25 °C ($\Delta T = 0$ °C) and with a 50 μM concentration of substrate solutions. The enzyme treatment time was 30 min. The results are listed in Table 1, were $[S_0] - [S_t]$ indicates the concentration difference of the substrate at the beginning ($S_0$) and after t minutes of enzyme treatment, and SR is the percentage removal of substrate obtained according to Eq. (1). The listed values show that our immobilized laccase is able to oxidize a wide range of phenolic substrates. Taking as reference the phenol removal, i.e. the removal percentage of 40% after 30 min of enzyme treatment under isothermal conditions, it is possible to conclude that some phenol derivatives (2-CP, 3-CP, 4-CP, NP and chlorophene) are oxidized by laccase at a similar rate, other derivatives (paracetamol, 3-MP and chloroxyphenol) at a smaller rate, and others (2,4-DCP and BPA) at higher rate.

To better quantify this observation in Fig. 5 we have reported the values of the concentration of three substrates as a function of the enzyme treatment time. Fig. 5a refers to phenol (the substrate taken as reference), Fig. 5b refers to 2,4-DCP (the phenol derivative having the smallest resistance to laccase action), Fig. 5c to paracetamol (the substrate among the phenol derivatives employed in this research, having the highest resistance to laccase oxidation). With the symbol (●) are indicated the experimental values obtained under isothermal conditions. Similar results have been obtained for the other phenol derivatives (data not shown).

The values of the average enzyme reaction rate, reported in Table 1 as μmol $min^{-1}$, confirm the previous considerations about the resistance of our substrates to oxidation by laccase. The average enzyme reaction rates have been obtained by multiplying for the volume (30 mL) the slopes of the lines best fitting in Fig. 5 the substrate decrease as function of the time.

A last consideration. The substituted phenol derivatives with chlorine atoms in *ortho*- and *para*-positions resulted more susceptible to the laccase and showed increased reactivity as the number of the chlorine atoms increased. In contrast, the chlorine atoms at *meta*-position showed decreased reactivity [30,31].

In conclusion, among the studied substituted phenol derivatives, the isothermal bioremediation power of laccase resulted to increase in the following order: paracetamol < chloroxyphenol < 3-MP < chlorophene < NP ≈ Phenol ≈ 4-CP < 3-CP < 2-CP < BPA < 2,4-DCP.

#### 3.3.2. Non-isothermal enzyme treatment

In order to establish the influence of a temperature gradient on the activity of the immobilized laccase, the concentration of the residual phenolic compounds during the enzyme treatment must be determined under non-isothermal conditions. The difference between the initial and residual concentration of phenolic derivatives gives the concentrations of oxidized phenolic compounds turned in product (Table 2). The investigation has been conducted at the following experimental conditions: 50 μM initial concentration of all phenolic derivatives in 0.1 M Na-acetate buffer, pH 5.5, $T_{av}$ = 25 °C, $\Delta T$ = 10, 20 and 30 °C. $\Delta T$ is the temperature difference measured at the position of the thermocouples. The substrate solutions, as in the case of isothermal experiments, were feed at 3 mL/min. The run lasted 30 min. Concentration measures were carried out at $t$ = 10, 20 and 30 min. As it can be seen, at each time an increase on the temperature difference results in an increase of the percentage removal efficiency.

The comparison between the results obtained under non-isothermal conditions with those obtained under isothermal conditions is done in Fig. 5, where now the symbols (◊), (□) and (○) refer to $\Delta T$ = 10, 20 and 30 °C, respectively. Results in Fig. 5 clearly demonstrate that the rate of substrate removal increases with the increase of the temperature difference applied across the catalytic membrane. A similar behavior has been obtained also with the other phenol derivatives employed in this research.

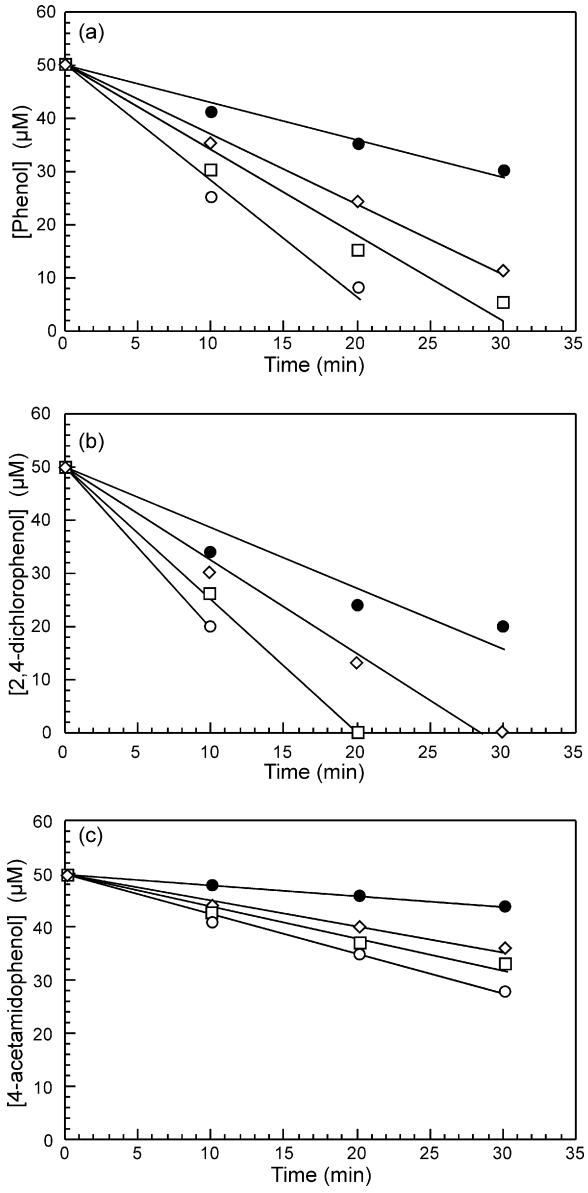


**Fig. 5.** Substrate concentrations as a function of the enzyme treatment time at $\Delta T$ = 0 °C (●); $\Delta T$ = 10 °C (◊); $\Delta T$ = 20 °C (□); $\Delta T$ = 30 °C (○): (a) phenol; (b) 2,4-dichlorophenol; (c) 4-acetamidophenol (paracetamol).

The effect of the temperature gradients is better evidenced in Fig. 6 where the changes in substrate concentrations are reported as a function of the applied temperature difference. As previously done, Fig. 6a–c refers to phenol, to 2,4-DCP and to paracetamol, respectively. The curve parameter is the enzyme treatment time: (♦), (□) and (○) refer to $\Delta t$ = 10, 20 and 30 min, respectively. The points in Table 2 giving zero concentration have not been considered since we do not know the precise time in which the zero concentration was reached. Data in Fig. 6 clearly show the effect of the temperature gradient at each considered time. The slope of each curve divided by the respective time give the parameter $\chi$ which indicate the percentage change in concentration for minute and for degree centigrade of temperature difference. We have done this cal-

**Table 2**
Removal efficiency of phenol and phenol derivatives under non-isothermal conditions at $\Delta T$ = 10, 20, 30 °C; 50 μM substrate solution and treatment time 30 min.

| Substrate | Time (min) | $\Delta T$ = 10 °C | | $\Delta T$ = 20 °C | | $\Delta T$ = 30 °C | |
|---|---|---|---|---|---|---|---|
| | | $[S_0]-[S_t]$ (μM) | SR (%) | $[S_0]-[S_t]$ (μM) | SR (%) | $[S_0]-[S_t]$ (μM) | SR (%) |
| Phenol | 10 | 15 | 30 | 20 | 40 | 25 | 50 |
| | 20 | 26 | 52 | 35 | 70 | 42 | 84 |
| | 30 | 39 | 78 | 45 | 90 | 50 | 100 |
| 2-Chlorophenol | 10 | 18 | 36 | 23 | 46 | 27 | 54 |
| | 20 | 30 | 60 | 37 | 74 | 50 | 100 |
| | 30 | 45 | 90 | 50 | 100 | 50 | 100 |
| 3-Chlorophenol | 10 | 18 | 36 | 13 | 26 | 17 | 34 |
| | 20 | 30 | 60 | 37 | 74 | 50 | 100 |
| | 30 | 45 | 90 | 50 | 100 | 50 | 100 |
| 4-Chlorophenol | 10 | 12 | 24 | 16 | 32 | 19 | 38 |
| | 20 | 25 | 50 | 32 | 64 | 38 | 74 |
| | 30 | 35 | 70 | 42 | 84 | 50 | 100 |
| 2,4-Dichlorophenol | 10 | 16 | 32 | 20 | 40 | 24 | 48 |
| | 20 | 26 | 52 | 37 | 74 | 50 | 100 |
| | 30 | 30 | 60 | 50 | 100 | 50 | 100 |
| 3-Methoxyphenol | 10 | 10 | 20 | 12 | 24 | 15 | 30 |
| | 20 | 20 | 40 | 23 | 46 | 27 | 54 |
| | 30 | 30 | 60 | 34 | 68 | 39 | 78 |
| Bisphenol A | 10 | 16 | 32 | 22 | 44 | 25 | 50 |
| | 20 | 31 | 62 | 42 | 84 | 50 | 100 |
| | 30 | 46 | 92 | 50 | 100 | 50 | 100 |
| Nonylphenol | 10 | 12 | 24 | 15 | 30 | 18 | 36 |
| | 20 | 24 | 48 | 18 | 36 | 36 | 72 |
| | 30 | 35 | 70 | 33 | 66 | 50 | 100 |
| 4-Chloro-3,5-dimethylphenol | 10 | 7 | 14 | 10 | 20 | 11 | 22 |
| | 20 | 14 | 28 | 18 | 36 | 21 | 42 |
| | 30 | 21 | 42 | 27 | 54 | 31 | 62 |
| 2-Benzyl-4-chlorophenol | 10 | 9 | 18 | 12 | 24 | 15 | 30 |
| | 20 | 21 | 42 | 25 | 50 | 30 | 60 |
| | 30 | 32 | 64 | 38 | 76 | 45 | 90 |
| 4-Acetamidophenol | 10 | 6 | 12 | 7 | 14 | 8 | 16 |
| | 20 | 10 | 20 | 13 | 23 | 15 | 30 |
| | 30 | 14 | 28 | 17 | 34 | 22 | 44 |

culation on the data relative to $\Delta t$ = 20 min, since they appear the more useful for this calculation. The values for all the substrates are reported in Table 3.

Supposing that the time dependence of the best fitting lines of results reported in Fig. 6 can be extrapolated to zero substrate concentration (a plausible hypothesis considering the very small initial concentration) it is possible obtain the values $\tau_{0,\Delta T=0}$, $\tau_{0,\Delta T=10}$, $\tau_{0,\Delta T=20}$ and $\tau_{0,\Delta T=30}$ indicating the time for the complete substrate removal under $\Delta T$ = 0, 10, 20 and 30 °C, respectively. These values are reported in Table 4 for all our phenolic derivatives. In order to establish if these times are correlated by a specific low we have plotted the $\tau_{0,\Delta T}$ values as a function of the applied $\Delta T$. Just to give an example of this plot in Fig. 7 we have reported the data relative to phenol (◯), 2,4-DCP (●) and paracetamol (□), respectively. Interesting enough, all curves (and also those of the other phenol derivatives – data not shown) are well interpolated ($R$ = 0.99 for all substrates) by an equation of the type

$$\tau_{0,\Delta T\neq 0} = \tau_{0,\Delta T=0} - \tau_x[1-\exp(-k\Delta T)] \quad (2)$$

where $\tau_x$ is the value such as $(\tau_{0,\Delta T=0} - \tau_x)$ gives the asymptotic value of the time for the complete substrate removal and $k$ (°C$^{-1}$) is the rate by which $\tau_{0,\Delta T\neq 0}$ decrease as a function of the applied $\Delta T$. $k$ is related to the rate by which a temperature gradient increase the enzyme reaction rate in comparison to the rate found under isothermal conditions. In Table 3 the $k$ values for all our phenol derivative are reported. At first glance no dependence on chemical structure appears evident. Taking the $k$ value of phenol as reference, it is possible to observe that some substances have $k$ values similar to that of phenol, others smaller values, other higher values. When the $k$ values are compared with $\tau_{0,\Delta T=0}$, i.e. the times for complete pollutant removal under isothermal conditions, a cautious observation can be done: the $k$ values appear in some way proportional to the $\tau_{0,\Delta T=0}$. This means that as smaller is the enzyme reaction rate under isothermal conditions as more effective is the temperature gradient in accelerating the reaction rate. An analytical confirm of this observation comes from Fig. 8 where the $\tau_{0,\Delta T=0}$ values for each substrate have been reported as a function of their $k$. Even if the points are characterized by a large scattering they are fitted by a polynomial curve of the second order characterized by the

**Table 3**
Values of $k$ (°C$^{-1}$) and $\chi$ (%) coefficients.

| Substrate | $k$ (°C$^{-1}$) | $\chi$ (% min$^{-1}$ °C$^{-1}$) |
|---|---|---|
| Phenol | 0.097 | 4.5 |
| 2-Chlorophenol | 0.108 | 4.7 |
| 3-Chlorophenol | 0.118 | 5.0 |
| 4-Chlorophenol | 0.096 | 4.0 |
| 2.4-Dichlorophenol | 0.072 | 5.5 |
| 3-Methoxyphenol | 0.117 | 2.4 |
| Bisphenol A | 0.099 | 5.5 |
| Nonylphenol | 0.097 | 2.5 |
| 4-Chloro-3,5-dimethylphenol | 0.117 | 2.4 |
| 2-Benzyl-4-chlorophenol | 0.086 | 2.3 |
| 4-Acetamidophenol | 0.176 | 1.8 |

**Table 4**
Time values for the complete substrate removal under $\Delta T = 0$, 10, 20 and 30 °C.

| Substrate | $\tau_{0,\Delta T=0\,^\circ C}$ (min) | $\tau_{0,\Delta T=10\,^\circ C}$ (min) | $\tau_{0,\Delta T=20\,^\circ C}$ (min) | $\tau_{0,\Delta T=30\,^\circ C}$ (min) |
|---|---|---|---|---|
| Phenol | 70.7 | 38.9 | 31.1 | 22.9 |
| 2-Chlorophenol | 62.5 | 32.9 | 25.7 | 19.7 |
| 3-Chlorophenol | 65.8 | 32.9 | 25.8 | 19.7 |
| 4-Chlorophenol | 71.4 | 41.9 | 34.0 | 26.3 |
| 2,4-Dichlorophenol | 44.3 | 28.7 | 20.5 | 16.7 |
| 3-Methoxyphenol | 80.5 | 50.0 | 43.7 | 37.6 |
| Bisphenol A | 56.9 | 32.4 | 23.6 | 20.0 |
| Nonylphenol | 71.4 | 42.4 | 34.9 | 27.7 |
| 4-Chloro-3,5-dimethylphenol | 129.5 | 71.4 | 55.1 | 47.9 |
| 2-Benzyl-4-chlorophenol | 73.7 | 47.6 | 39.8 | 33.3 |
| 4-Acetamidophenol | 250.0 | 102.8 | 83.3 | 71.4 |

expression

$$\tau_{0,\Delta T=0} = ak^2 + bk \quad (3)$$

with $a = 7396$ and $b = 30.2$. The trend in Fig. 8 appears significant notwithstanding the $R$ value is small ($R = 0.88$).

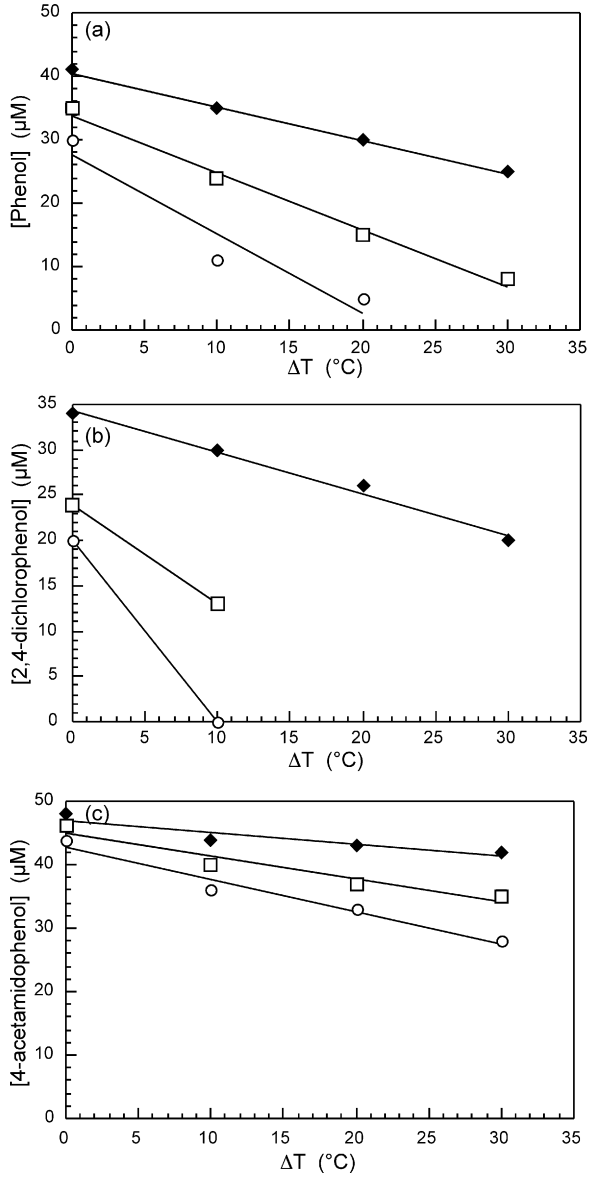


**Fig. 6.** Substrate concentrations as a function of the applied temperature difference at the enzyme treatment time $\Delta t = 10$ min (♦); $\Delta t = 20$ min (□); $\Delta t = 30$ min (○): (a) phenol; (b) 2,4-dichlorophenol; (c) 4-acetamidophenol.

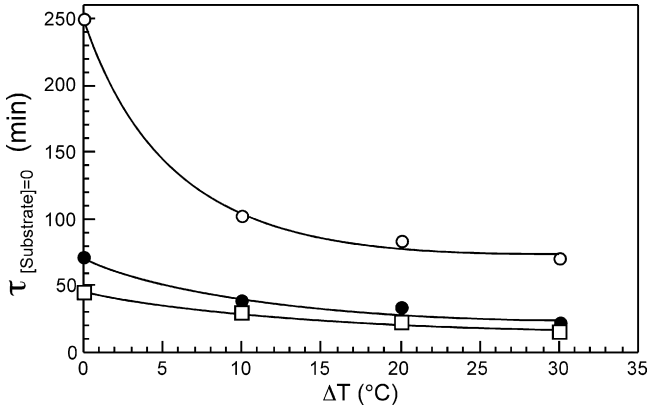


**Fig. 7.** Time values ($\tau_{0,\Delta T\neq 0}$) for complete substrate removal as a function of the applied temperature difference: phenol (●), 4-acetamidophenol (○) and 2,4-DCP (□).

Before concluding, let now calculate a parameter very significant for practical applications of the non-isothermal bioreactors technology. This parameter is the process reduction time, $\tau_r$ (%), occurring under non-isothermal conditions and it is defined as:

$$\tau_{r,\Delta T}(\%) = \left[\frac{\tau_{0,\Delta T=0} - \tau_{0,\Delta T\neq 0}}{\tau_{0,\Delta T=0}}\right] \times 100 \quad (4)$$

where $\tau_{\Delta T\neq 0}$ and $\tau_{\Delta T=0}$ are the time required to obtain the same percentage of biodegradation of our substrates under non-isothermal and isothermal conditions, respectively.

In Fig. 9 the $\tau_r$ values for each of our phenol derivatives, calculated for $\Delta T = 30$ °C, are reported. Taking, as usual, the phenol as reference it is possible to see that some phenol derivatives have $\tau_r$ values higher than the phenol value, while others have smaller

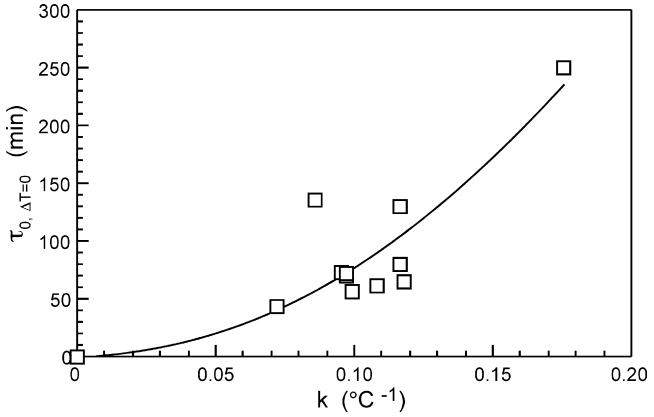


**Fig. 8.** $\tau_{0,\Delta T=0}$ values for each substrate as a function of their rate $k$ by which the $\tau_{0,\Delta T\neq 0}$ decrease as a function of the applied $\Delta T$.

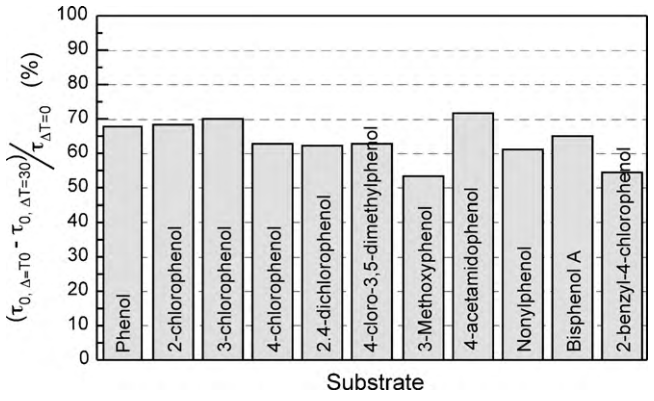


**Fig. 9.** Reduction time ($\tau_r$, %) values for each phenol derivatives calculated for $\Delta T = 30\,^{\circ}C$.

values. The $\tau_r$ values range from 55% for chlorophene to 72% for paracetamol. So high absolute values for $\tau_r$ remark the usefulness of employing non-isothermal bioreactors in processes of pollution removal from contaminated water systems.

## 4. Conclusions

All the results obtained indicate that the immobilization process of laccase from *T. versicolor* on chemically modified polypropylene membrane strongly affects the catalytic behaviors of the enzyme. The immobilized laccase has been found able to oxidize a wide range of phenol derivatives. In particular it was found that some phenol derivatives (2-CP, 3-CP, 4-CP, NP and chlorophene) are oxidized by laccase at a similar rate than phenol, other derivatives (paracetamol, 3-MP and chloroxyphenol) at a smaller rate, while others (2,4-DCP and BPA) at higher rate. Practically the same sequence was observed for the increase of the laccase oxidative power when the catalytic membrane was operating under non-isothermal conditions. Interesting enough, the percentage increase of enzyme reaction rate under non-isothermal conditions resulted higher in the cases in which the isothermal reaction rate was smaller.

When the reduction of the production times by the presence of a temperature gradient is considered, the measured values strongly candidate the technology on non-isothermal bioreactors as a useful tool in processes of detoxification of waste waters polluted by endocrine disruptors.

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