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Advantages in using non-isothermal bioreactors in bioremediation of water polluted by phenol by means of immobilized laccase from *Rhus vernicifera*

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ABSTRACT

Laccase from *Rhus vernicifera* was immobilized on a polypropylene membrane chemically modified with chromic acid. Ethylenediamine and glutaraldehyde were used as spacer and bifunctional coupling agent, respectively. Phenol was used as substrate.

To know how the immobilization procedures affected the enzyme reaction rate the catalytic behavior of soluble and insoluble laccase was studied under isothermal conditions as a function of pH, temperature and substrate concentration. From these studies, two main singularities emerged: (i) the narrower pH–activity profile of the soluble enzyme in comparison to that of the insoluble counterpart and (ii) the increase in pH and thermal stability of the insoluble enzyme.

The laccase catalytic behavior was also studied in a non-isothermal bioreactor as a function of substrate concentration and size of the applied transmembrane temperature difference. It was found that, under non-isothermal conditions and keeping constant the average temperature of the bioreactor, the enzyme reaction rate linearly increased with the increase of the temperature difference.
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1. Introduction

Several types of industrial and agricultural wastes contain phenol compounds. For example, chlorophenols, largely used as wide-spectrum biocides, and nitrophenols, widely used in the chemical industry, accumulate in soils, sediments, surface waters, and animals because of their continuous usage and recalcitrant nature [1]. Moreover, a lot of phenol compounds are recognized as endocrine disruptors [2] since they possess estrogenic or anti-estrogenic activities so interfering with the endocrine system.

Phenol and phenol derivatives oxidation in the presence of the enzyme laccase has been extensively studied [3]. Laccases (EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductase) belong to the so-called blue-copper family of oxidases. Laccases catalyze the oxidation of a variety of organic compounds including methoxyphenols, phenols, *o*- and *p*-diphenols, aminophenols, polyphenols, polyamines, and lignin-related molecules.

Laccase can catalyze the oxidation of *o*-, *m*-, and *p*-benzenediols and phenol to *o*-, *m*-, *p*-quinones or radical species [4] and does not require hydrogen peroxide as co-substrate or any cofactors for the catalytic reaction. The catalytic cycle of laccase and the proposed mechanisms for the reduction and reoxidation of the copper sites can be found in Ref. [3].

As for the majority of enzymes also laccase can be employed in the insoluble form. The advantages of immobilized enzymes in respect to their free counterpart are well known. There are few publications dealing with the removal of phenol and phenol derivatives by immobilized laccase from *Rhus vernicifera*. Various supports have been used for enzyme immobilization. One of the first examples is the immobilization of laccase from *R. vernicifera* by entrapment into a polyacrylamide gel [3]. Other carries were celite and activated agar [3]. Some parameters and properties of laccase immobilized on these carries (i.e. immobilization conditions, reusability, thermal stability, K_m, pH and temperature optima) were studied and compared with the analogous of the native enzyme. Novel types of *p*-benzoquinone-activated supports (agarose, polyvinyl alcohol, chitosan) were also developed and used for laccase immobilization [5]. Laccase was also immobilized on silica treated with $TiCl_4$, $ZrCl_4$, $FeCl_3$, $CuCl_2$ or $ZnCl_2$ [3–6].

Microporous membranes have gained considerable significance not only in separation processes but also as carriers for active catalytic substances. In recent years they have been used as carriers for biocatalysts immobilization (so-called enzyme membranes) and employed in bioreactors. It is important that for such applications, immobilization is achieved without loss of activity. In comparison with native enzymes immobilized biocatalysts may demonstrate

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increased thermal stability and resistance to extreme conditions and chemical reagents. They may also be easily separated physically from the reaction products and used in continuous processes.

Polypropylene (PP) is a very attractive material as hydrophobic support owing to its capability of forming a thin film composite membrane endowed of high durability and resistance to chemicals, pH variations, and to a substantially wide range of solvents. However, a PP membrane has no active groups to be used for enzyme immobilization, and that is why a modification of the membrane is necessary. Several surface modification techniques have been developed to improve wetting, adhesion, and printing of polymer surfaces by introducing a variety of polar groups: wet chemical, UV irradiation, and ionized gas treatments [7]. Chromic acid and potassium permanganate in sulfuric acid have been used to introduce reactive groups onto PP [8,9]. The surface functionality consists primarily of carbonyl derivatives: 60% present as carboxylic acid groups and 40% as ketones or aldehydes. Alcohols do not seem to be present. Several procedures have been developed to use these carboxylic acid groups as the basis for further chemical modification of the polymer surface. There are not many publications describing the application of modified PP membranes as carriers for enzyme immobilization. Furthermore, the hydrophobic properties of a PP membrane make it suitable for the conduction of an enzyme reaction under non-isothermal conditions, where the enzyme reaction rate increases considerably [10–19].

It is well known from the thermodynamics of irreversible processes [20–22] that temperature gradients drive matter fluxes in bulk solutions (thermal diffusion) or across hydrophobic membranes separating liquid mixtures. In particular, when hydrophobic and unselective porous membranes are employed in a reactor to separate aqueous solutions kept at different temperatures, selective solvent and solute fluxes occur across it. This kind of mass transport has been called thermodialysis [23,24]. 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 dispersing phase. Both solvent and solute fluxes have been found to be proportional to the temperature gradient across the membrane. This phenomenology has been analytically described in our previous papers.

In this paper, the catalytic activity of laccase immobilized on a hydrophobic polypropylene membrane, chemically modified and working in a non-isothermal bioreactor, will be described. The behavior of immobilized laccase under isothermal and non-isothermal conditions, vice versa, will be discussed with the aim of demonstrating the useful exploitation of non-isothermal bioreactors also in the treatment of industrial effluents polluted by phenols.

2. Apparatus, materials and methods

2.1. The bioreactor

The apparatus was the same used for the experiments reported in previous works [12–14,18,19]. The bioreactor was working under non-isothermal conditions by thermostating the two half-cells at different temperatures. The temperatures read by the thermocouples will be indicated by T, while the ones calculated at the membrane surfaces by T^*. This difference is due to the fact that thermocouples are placed 1.5 mm from each surface of the catalytic membrane. The temperatures related to the warm or cold side will be indicated by the subscript “w” or “c”, respectively. Under these assumptions, it follows that $\Delta T = T_w - T_c$ and $\Delta T^* = T^*_w - T^*_c$, as well as $T_{av} = (T_w + T_c)/2$, $T^*_{av} = (T^*_w + T^*_c)/2$, $T^*_w < T_w$, $T^*_c > T_c$ and $\Delta T^* < \Delta T$. From these considerations it follows that all the effects occurring under the macroscopic temperature difference ΔT, at the catalytic membrane level must be attributed to the actual temperature difference ΔT^*. Usually, we have calculated [12–14,18,19] that $\Delta T^* = 0.1\,\Delta T$, ant this is true also in the present case.

2.2. Materials

Laccase (EC. 1.10.3.2, 120 U/mg) from *R. vernicifera* (Sigma–Aldrich Schweiz, Buchs SG, Schweiz) was used as catalyst.

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

Phenol, ethylenediamine, sodium dihydrogen phosphate dihydrate, disodium hydrogen phosphate, sodium nitrite, sodium carbonate, copper (II) sulfate pentahydrate, *p*-nitroaniline, sodium hydroxide, sodium chloride, potassium dichromate, acetone and Folin-Ciocalteu’s phenol reagent were obtained from Fluka (Buchs, Switzerland).

Hydrochloric acid, glutaraldehyde and sulfuric acid were obtained from Sigma–Aldrich Schweiz, Buchs SG, Schweiz.

All chemicals were of analytical grade and 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) chromic acid oxidation of the polypropylene membrane and (b) enzyme immobilization.

2.3.1.1. Chromic acid oxidation of the polypropylene membrane. Chromic acid solution for hydrophilization of PP membranes was prepared by slow mixing of $K_2Cr_2O_7$ with H_2O and H_2SO_4 in the proportion of 1:19:29.4 by weight. PP membranes were before immersed in acetone for 5 min and subsequently dipped into a glass vessel with chromic acid solution. The vessel was covered with a glass plate and placed in an oven at 30 °C for 10 min. PP membranes so modified were kept under water overnight.

2.3.1.2. Enzyme immobilization. Enzyme immobilization was carried out by using three successive treatments. With the first one, the spacer ethylenediamine (EDA) was attached to the support; with the second one, the membrane was activated by the interaction with the glutaraldehyde; with the third one, the enzyme was immobilized on the activated support through covalent attachment to the glutaraldehyde.

As matter of the fact, 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 [25–28]. After this step, the membranes were washed with water to remove the non-reacted diamines then treated for 1 h at room temperature with a 25% glutaraldehyde aqueous solution. After further washings with distilled water and 0.1 M phosphate buffer solution (pH 7.5), the membranes were treated for 16 h at 4 °C with the same buffer solution containing laccase at a concentration of 4 mg/mL. Coupling of laccase to glutaraldehyde occurred through the amine groups of the enzyme and the aldehyde groups of glutaraldehyde. At the end of this step, the membranes were washed with the buffer solution in order to remove the unbound enzymes.

The amount of immobilized enzyme was calculated by subtracting the amount of the laccase recovered in the solution at the end of immobilization process and into the washing solutions from the amount of laccase initially used for the immobilization. Protein concentration was determined by the method of Lowry et al. [29].

2.3.2. FT-IR measurements

FT-IR spectra of treated and untreated membranes were recorded using a Spectrum One PerkinElmer spectrometer equipped with a micro-ATR accessory. The micro-ATR uses a Germanium crystal allowing a contact area with sample of 100 μm of diameter. All spectra were collected using 16 scans in the range from 4000 to 650 nm^{-1} with a 4 nm^{-1} spectral resolution.

2.3.3. Enzyme activity determination

In experiments with soluble laccase, 1 μL of enzyme solution (concentration 4 mg/mL) was added to 2 mL phenol solutions with concentrations ranging from 5 to 200 mg/L. The enzyme and phenol were dissolved in 1 M phosphate buffer, pH 7.5.

In isothermal experiments with immobilized enzyme (batch experiments, without the bioreactor) 1 cm^2 of membrane with immobilized laccase was added to 2 mL of phenol solution at the concentration of 5 mg/L.

In isothermal and non-isothermal experiments with the bioreactor about 35 cm^2 of catalytic membrane were put in contact with 30 mL of phenol solution circulating trough the bioreactor and the common cylinder.

Enzyme reaction rates were calculated by separately measuring, at regular time intervals, the phenol concentrations in the samples taken out from the reaction vessel (in the case of soluble laccase or batch experiments) or from the common cylinder (in the case of insoluble laccase). Phenol concentrations were spectrophotometrically measured, based on the reaction of phenol with diazonium salt prepared from *p*-nitroaniline, through a calibration curve performed at 25 °C and 475 nm [30,31].

2.3.4. Time stability

Time stability of the biocatalytic membranes was assessed by measuring their activity every day, under the same experimental conditions, i.e. 5 mg/L phenol concentration in 0.1 M phosphate buffer, at pH 7.5 and 25 °C. After few days, during which the membranes lost part of their initial activity, a stable condition was reached for about 1 month. Only these stabilized membranes were used for our study. Probably the activity loss during the first days was due to desorption of some amount of enzyme absorbed during the immobilization phase. When not used, the membranes were stored at 4 °C in 0.1 M phosphate buffer, pH 7.5.

2.3.5. Thermal and pH stability

The thermal stabilities of free and immobilized laccase have been investigated under the following experimental conditions: 5 mg/L phenol in 0.1 M phosphate buffer (pH 7.5), temperature 60 °C, and at the respective pH_{opt} for free and immobilized enzyme. The incubation time in the buffer solution was 270 min.

The pH stabilities of free and immobilized laccase have been investigated under the following experimental conditions: 5 mg/L phenol in 0.1 M phosphate buffer, pH 6.0–8.5, and at the respective T_{opt} for free and immobilized enzyme. For both soluble and insoluble laccase the incubation time in the buffer solution was 60 min.

2.3.6. Determination of amino groups

The amount of amino group on the surface of modified membrane was determined by the residual potentiometric titration in heterogeneous medium. The measurement was performed by a pH meter (model-3310, Jenway Limited, England). The experimental setup is the same as that described in Refs. [32,33].

2.3.7. Experimental data treatment

Every experimental point in the figures represents the average value of five experiments performed under the same conditions.

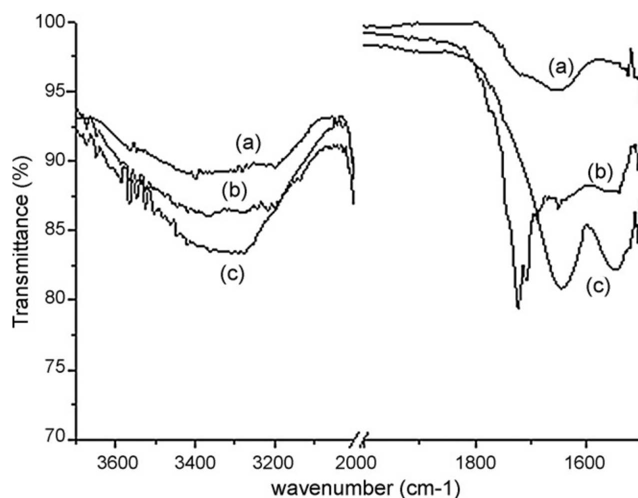


Fig. 1. FT-IR spectra of membrane before any treatment (curve a), after chromic acid treatment (curve b) and after subsequent EDA treatment (curve c).

The experimental errors never exceeded 4%. Each experiment lasted 20 min. 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 phosphate buffer solution, pH 7.5, was circulated for 60 min through the bioreactor and the membrane between two subsequent experiments.

3. Results and discussion

As reported in Section 2, in order to introduce active groups for the immobilization of the enzyme the polypropylene membrane was modified by chromic acid. The optimum chromic acid concentration at which the membrane still preserves its physical and chemical properties and shows sufficient amount of active groups was defined. The surface functionality consists primarily of carbonyl derivatives: present as carboxylic acid groups and as ketones or aldehydes. Thereafter the modified membrane was processed with ethylenediamine in order to introduce amino groups in the membrane. The ethylenediamine was bounded with carbonyl derivatives, mainly with ketones and aldehydes. The disappearance of these groups and the presence of new groups as a result of the membrane modification have been proved by FT-IR spectroscopy. The comparison between the spectrum of the untreated membrane and the spectra of the modified membranes after chromic acid oxidation and after treatment with ethylenediamine clearly demonstrates the appearance of new spectrum bands for the modified membranes. Fig. 1 shows the FT-IR spectra for a membrane before any treatment (curve a), after chromic acid treatment (curve b) and after subsequent EDA treatment (curve c) in the regions 3700–3000 cm^{-1} and 200–1600 cm^{-1}. In the former region, chromic acid treatment and subsequent EDA treatment cause an increase of the band relative to OH and NH stretching vibrations (3400–3300 cm^{-1}). In the latter wave number interval, the chromic acid treatment induces the carboxylic acid or aldehydes peak at 1724 cm^{-1} and ketones peak at 1715 cm^{-1}. The subsequent EDA treatment causes the appearance of two peaks relative to amide group at 1645 cm^{-1} and to NH bending at 1549 cm^{-1} and disappear peaks at 1724 and 1715 cm^{-1}.

The spectra clearly confirm the expected modifications. These spectra are highly reproducible for a large number on examined samples.

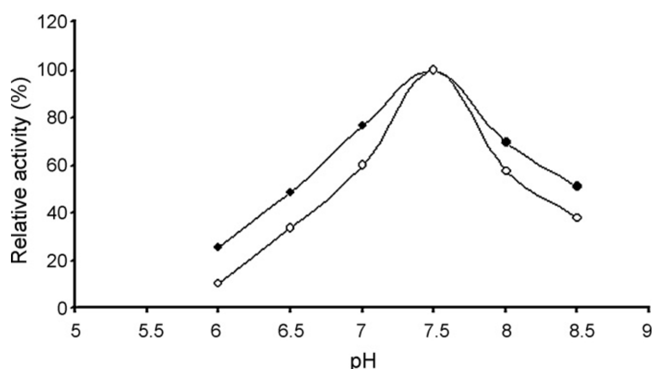


Fig. 2. pH dependence for free (○) and immobilized (●) laccase.

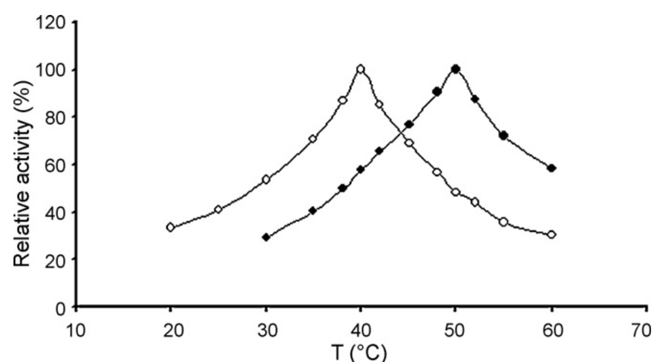


Fig. 3. Effect of temperature on activity of free (○) and immobilized (●) laccase.

The amount of amino groups on the modified membrane surfaces, reported in Section 2.3.6, resulted to be −0.8 mgequiv./g. The amount of bound protein and the relative activity of the immobilized laccase resulted to be $0.036 \pm 0.004\,\mathrm{mg/cm^2}$ and $68 \pm 5\%$, respectively.

Some authors showed that the laccase oxidized phenols to quinone and phenol polymers [34,35]. Isak Hägg and Emil Nilvebrant investigated purification of water from five phenolic compounds—vanillin, phenol, guaiacol, nonylphenol and paracetamol using enzymatic polymerization with laccase from *Trametes versicolor* [35]. All phenols were studied at a concentration of 3.3 mM. Vanillin, paracetamol and guaiacol reacted almost instantly, whereas phenol and nonylphenol appeared to be less sensitive to the laccase. Furthermore, the concentration of monomer substrate greatly influence the degree of polymerization. In our experiments we work mainly with 5 mg/L phenol concentration. This relatively low, therefore the degree of polymerization is insignificant.

3.1. Isothermal experiments

3.1.1. pH optima

Laccase activity as a function of pH was determined at 25 °C with 5 mg/L phenol solution in 0.1 M phosphate buffer over the pH range 6.0–8.5. Experiments were carried out in batch in the conditions reported in Section 2.3.3. The pH profiles of free and immobilized *R. vernicifera* laccase are shown in Fig. 2. The responses of free and immobilized laccase to pH were similar with optima activities at pH 7.5. However, immobilized laccase maintained a higher relative activity than free laccase at both lower and higher pH levels, indicating that the immobilized laccase was less sensitive to pH changes that the free laccase. At pH 6.0 immobilized laccase retains about 25% of its maximum activity, while the soluble form retains only 10%. Respectively, at pH 8.5 immobilized laccase retains about 52% of its maximum activity, while the soluble form retains only 37%.

3.1.2. Temperature optima

The temperature activity profile of free and immobilized laccase was determined in the range 30–60 °C at 5 mg/L phenol in 0.1 M phosphate buffer, pH 7.5. Experiments were carried out in batch in the conditions reported in Section 2.3.3. Fig. 3 shows the temperature dependence of the relative activity for the free and the immobilized laccase. Free laccase has an optimum temperature of approximately 40 °C, whereas the optimum temperature of immobilized laccase is shifted to 50 °C. These results indicate that the immobilization process increase the resistance of the immobilized laccase to enzyme deactivation with the increase of the temperature. More interesting is the observation that at 60 °C the immobilized laccase retains 58% of its max activity, while the free laccase retains only 30%.

3.1.3. Stability properties

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 under Section 2.3.5.

The activity of free enzyme decreases significantly after 100 min of incubation at 60 °C. One hundred and fifty minutes later, the relative activities of free and immobilized enzymes were 15.7% and 41.8% of the initial activity, respectively. Laccase immobilization on PP membrane led to a significant stabilizing effect towards heat deactivation.

The relative activities of free and immobilized laccase as a function of pH are shown in Fig. 5. For both soluble and insoluble laccase the incubation time was 90 min.

The free and immobilized laccase displayed high relative activities in a wide pH interval. For example at pH 6.5 the residual activity of the immobilized enzyme is 73.8% of the initial activity, and at pH 8 it is 80.7%. At the same pH values the activity of the free laccase decreased to 55.3% and 74.9%, respectively.

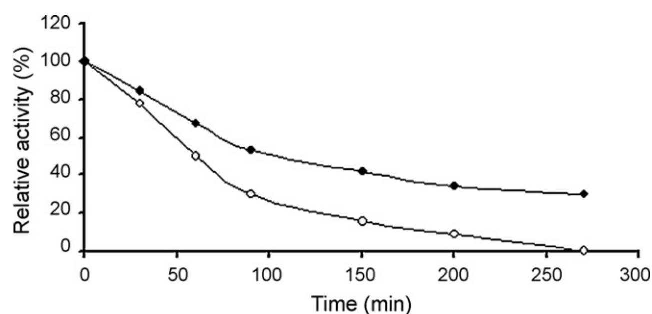


Fig. 4. Thermal stability of free (○) and immobilized (●) *Rhus vernicifera* laccase.

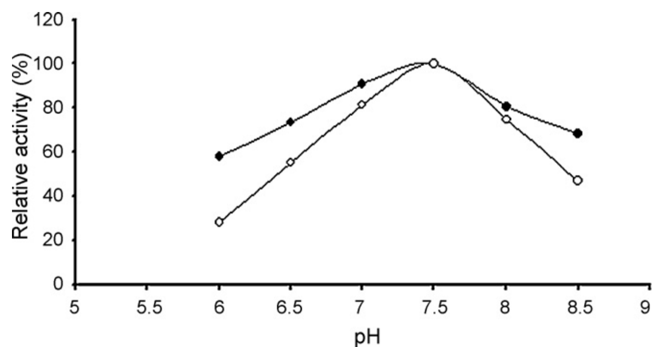


Fig. 5. pH stability of free (○) and immobilized (●) *R. vernicifera* laccase.

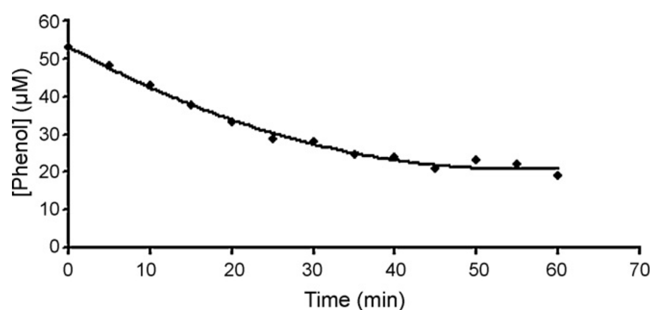
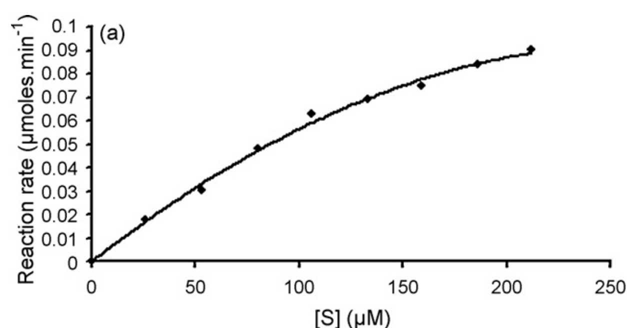


Fig. 6. Phenol degradation with immobilized laccase as a function of the time.



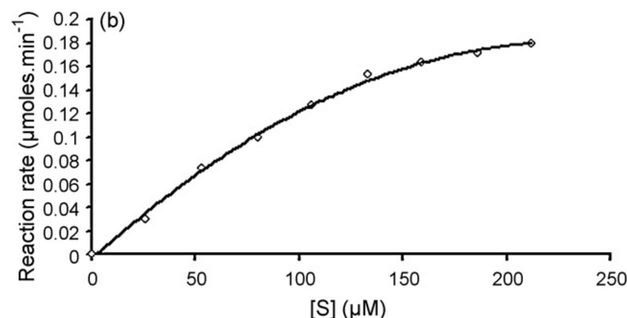


Fig. 7. (a) Reaction rate of immobilized laccase as a function of phenol concentration. (b) Reaction rate of free laccase as a function of phenol concentration.

3.1.4. Kinetic parameters

For the employment of immobilized enzymes in bioreactors for industrial purposes, it is very important to know the apparent kinetic parameters resulting from the immobilization process. In general, these parameters undergo variations indicating an affinity change for the substrate. These variations can be attributed to protein conformational changes, steric hindrances, and partitioning and diffusion effects. All these factors may occur simultaneously or separately. As a consequence, the affinity between enzyme and substrate may be modified by immobilization. This modification can result in a decrease [36,37] or increase [38,39] of the apparent K_m value. A decrease in the K_m value of an immobilized enzyme leads to a faster reaction rate than its free counterpart, whereas an increase of K_m implies the use of a higher substrate concentration to achieve the same rate of the reaction obtained with the free enzyme. The apparent K_m decreases if, for example, the charges on the support and the substrate are opposite. The conformational changes of the protein molecule and steric hindrances usually lead to an increase in the K_m values, due to a decrease in the affinity between the enzyme and the substrate. Also, the V_{max} values are affected by the immobilization procedure. In general, the values of V_{max} obtained for the immobilized enzymes, taking into account the activity loss induced by the immobilization process, are almost the same of that for the free enzyme, also if increases [40] or decreases [38] are reported.

To determine the kinetic parameters for the immobilized laccase, first the activity of the catalytic membrane was studied at isothermal condition in the bioreactor. The pH and temperature of the solution were 7.5 and 25 °C, respectively. In Fig. 6 we report, as one example, the phenol degradation (initial concentration 5 mg/L) during 60 min. The initial membrane activity, expressed as μmol min^{-1}, is given by multiplying the angular coefficient $(dS/dt)_{in}$ of the straight line interpolating the initial experimental points of the phenol degradation (μM) as a function of time for the volume of treated solution, in our case 30 mL. This means that the initial reaction rate is $(dS/dt)_{in}$ $(V)_{sol}$ and in the case under examination the value of 0.029 μmol min^{-1} is obtained.

Following this methodology, we obtained the results reported in Fig. 7a, which show that catalytic membrane activity exhibits a Michaelis–Menten behavior. In Fig. 7b, the kinetic behavior of the free laccase has been reported as a function of substrate concentration. The experimental conditions were the same that those employed for obtaining the results of Fig. 7a. The values of K_m and V_{max}, listed in Table 1, for the enzyme reaction have been derived from the Lineweaver–Burk equation (1/*S* versus 1/*V*) at isothermal condition. The increase in K_m with immobilized laccase was not negligible: it might be caused by the steric hindrances found by the substrate molecules in reaching the active site on the support.

Results reported in Fig. 7a show that catalytic membrane exhibits a Michaelis–Menten behavior. In Fig. 7b, the kinetic behavior of the free laccase has been reported as a function of substrate concentration. The experimental conditions were the same that those employed for obtaining the results of Fig. 7a. The values of K_m and V_{max}, listed in Table 1, for the enzyme reaction have been derived from the Lineweaver–Burk equation (1/*S* versus 1/*V*) at isothermal condition.

Table 1
Kinetic parameters to relative to the free and immobilized laccase from *Rhus vernicifera*

Enzyme status	Experimental conditions	K_m (mM)	V_{max} (μmol/min)
Free enzyme	T_{av} = 25 °C; ΔT = 0 °C	0.20 ± 0.04	0.37 ± 0.05
Immobilized enzyme	T_{av} = 25 °C; ΔT = 0 °C	0.36 ± 0.06	0.25 ± 0.06
Immobilized enzyme	T_{av} = 25 °C; ΔT = 10 °C	0.25 ± 0.04	0.27 ± 0.04
Immobilized enzyme	T_{av} = 25 °C; ΔT = 20 °C	0.25 ± 0.05	0.30 ± 0.05
Immobilized enzyme	T_{av} = 25 °C; ΔT = 30 °C	0.25 ± 0.04	0.33 ± 0.06

3.2. Non-isothermal experiments

In order to establish the influence of the temperature gradient over the activity of the immobilized laccase the concentration of the residual phenol has been defined at isothermal and non-isothermal conditions. The difference between the initial and residual phenol concentration gives the oxidized phenol concentration with turns in quinone (Fig. 8a). The investigation has been conducted at the following experimental conditions: 5 mg/L initial phenol concentration in 0.1 M phosphate buffer, pH 7.5, T_{av} = 25 °C, ΔT = 0, 10, 20, and 30 °C (ΔT is the temperature difference measured by the thermocouples) and phenol solution feed of 3 mL/min. The enzyme reaction rate has been calculated in μmol/min (using immobilized laccase) by multiplying the slope coefficient of each line from Fig. 8a by the volume of the respective sample solution. The data from Fig. 8b clearly demonstrate that the activity of the immobilized laccase is increasing linearly with the increase of the applied temperature difference ΔT. The straight line in Fig. 8b can be described by the equation $y = y_0 + a_0 \Delta T$, where "*y*" represents the activity of the immobilized enzyme, determined under non-isothermal condi-

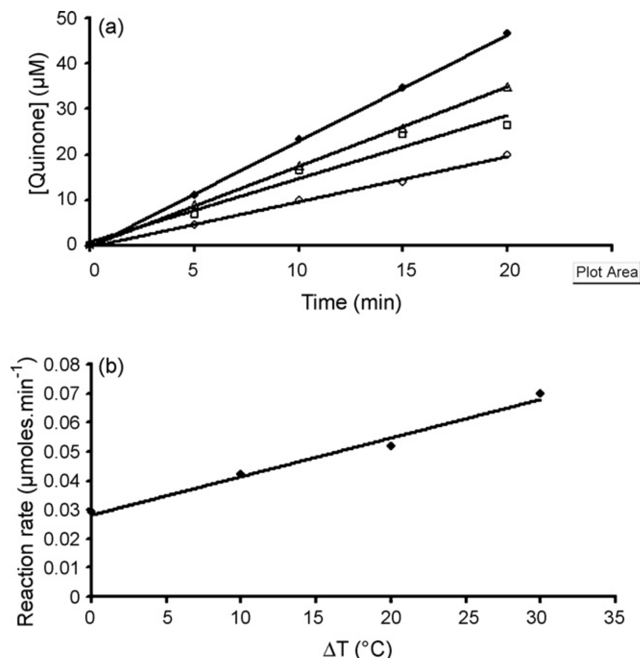


Fig. 8. (a) Isothermal and non-isothermal quinone production as a function of time. Symbols: (○) $\Delta T = 0\,^{\circ}$C; (□) $\Delta T = 10\,^{\circ}$C; (△) $\Delta T = 20\,^{\circ}$C; (●) $\Delta T = 30\,^{\circ}$C. In all cases $T_{av} = 25\,^{\circ}$C. (b) Enzyme reaction rates as a function of the macroscopic temperature difference ΔT read at the thermocouple positions. $T_{av} = 25\,^{\circ}$C.

tions and given ΔT, "y_0" represents the activity of the immobilized enzyme at isothermal conditions, and "a_0" represents the activity variation due to a temperature difference of 1 °C (measured by the thermocouples).

The experimental results, summarized in Fig. 8a and b, lead to the conclusion that the catalytic activity of the enzyme membrane under non-isothermal conditions is higher than that measured under isothermal condition. This provides a promising perspective to the application of non-isothermal bioreactors in the biotechnological industry.

In order to outline the advantages of a non-isothermal bioreactor, an important parameter can been calculated: the reduction of the production time, τ_r

$$\tau_r = \frac{\tau_{iso} - \tau_{non\text{-}iso}}{\tau_{iso}} \quad (1)$$

where τ_{iso} and $\tau_{non\text{-}iso}$ are the time intervals for the production of the same amount of products under isothermal and non-isothermal conditions, respectively. This equation has been used to elaborate the results reported in Fig. 8a for the calculation of the τ_r values. The latter are reported in Fig. 9a as a function of the macroscopic temperature difference ΔT.

The experimental results of Fig. 8b can be used for the calculation of another parameter, correlated with the effective increase of the enzyme reaction rate due to the applied ΔT. Such a parameter is the percentage activity increase, P.A.I. that can be defined as follows:

$$\text{P.A.I.} = \frac{y - y_0}{y_0} = \left(\frac{a_0}{y_0}\right)\Delta T = \alpha \Delta T \quad (2)$$

α (%, °C), now, represents the percentage increase of the enzyme activity per 1 °C of the temperature difference measured by the thermocouples. The values of P.A.I., calculated on the basis of the data from Fig. 8b, are presented in Fig. 9b as a function of the temperature difference ΔT. The angular coefficient of the resulting straight line gives the coefficient α, which, in this case, is 4.6 (%, °C). This means that 1 °C of temperature difference read at the position of the thermocouples produces an increase of membrane activity equal to 4.6%.

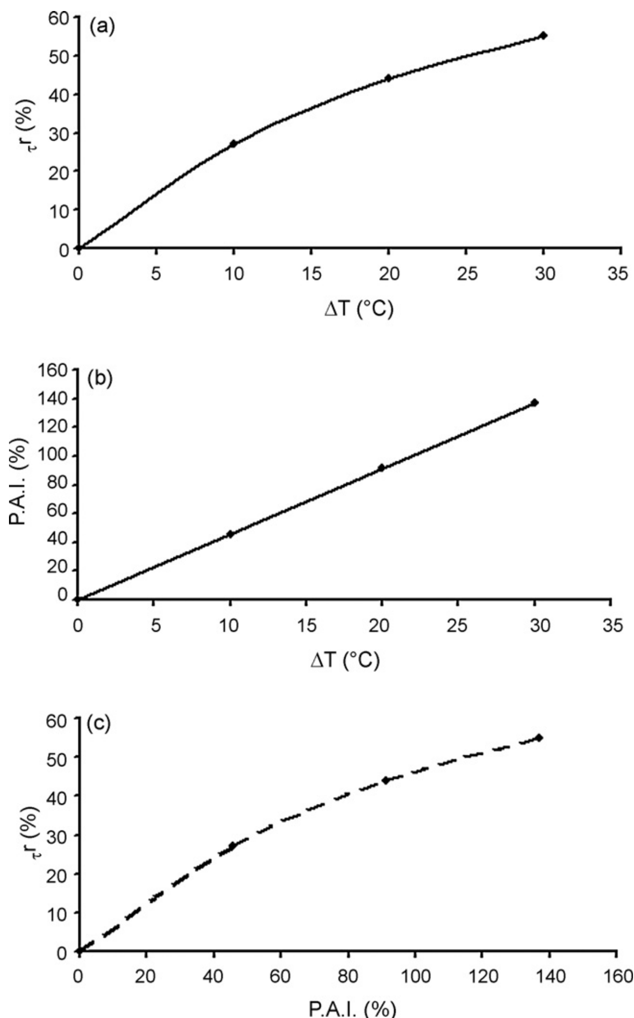


Fig. 9. (a) Reduction of the production times, τ_r, as a function of the macroscopic temperature difference ΔT at the thermocouple positions. (b) Percentage activity increases (P.A.I.) as a function of the transmembrane temperature difference ΔT read at the thermocouples positions. $T_{av} = 25\,^{\circ}$C. (c) Reduction of the production times, τ_r, as a function of P.A.I.

Another interesting aspect of our investigations concerns the correlation between the reduction of the production time τ_r and

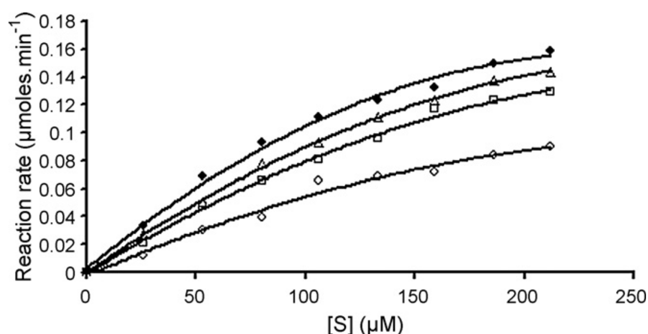


Fig. 10. Reaction rate under isothermal and non-isothermal conditions, as a function of phenol concentration. Symbols: (○) $\Delta T = 0\,^{\circ}$C; (□) $\Delta T = 10\,^{\circ}$C; (△) $\Delta T = 20\,^{\circ}$C; (●) $\Delta T = 30\,^{\circ}$C. In all cases $T_{av} = 25\,^{\circ}$C.

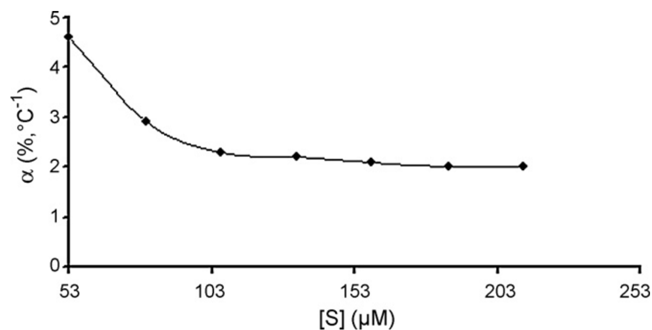


Fig. 11. α coefficients as a function of phenol concentration.

the P.A.I. This correlation can be described as follows:

$$\tau_r = \frac{\tau_{iso} - \tau_{non\text{-}iso}}{\tau_{iso}} = \frac{\text{P.A.I.}}{\text{P.A.I.} + 1} \quad (3)$$

A theoretical plot of Eq. (3) is reported in Fig. 9c, where it is possible to appreciate how the experimental points of Fig. 8a and b well fit with the theoretical curve.

The influence of the phenol concentration (5–200 mg/L) on the catalytic activity of the enzyme membrane has also been studied under non-isothermal conditions. The experimental results are summarized in Fig. 10 and their interpretation shows that:

1. All curves can be described by a Michaelis–Menten equation, either in the presence or in the absence of temperature differences.
2. The enzyme activity, i.e. the enzyme reaction rate, is proportional to the applied ΔT.

The values of K_m and V_{max} for the enzyme reaction, derived from the Lineweaver–Burk plot, are listed in Table 1. Their interpretation shows that:

1. The values of K_m under non-isothermal conditions are lower than that under isothermal conditions.
2. Under non-isothermal conditions the K_m values are independent from the macroscopic temperature difference, measured by the thermocouples.

Similar results have also been obtained with catalytic membranes on which different enzyme were immobilized [11–15].

The experimental results concerning K_m, can be explained by considering that the presence of a temperature gradient increases the substrate and the product fluxes across the catalytic membrane reducing, in this way, the diffusion limitations for these substances during their movement towards or away from catalytic site. The molecular basis for this behavior has been discussed in some previous works [12–18].

Coming back to the results in Fig. 10, it can be seen that at all the phenol concentrations studied it is possible to apply the approach used for the experiments reported in Fig. 8a and b. Thus it is possible to calculate the values of P.A.I. and the respective values of α, which are equivalent to the values of P.A.I. when the macroscopic temperature difference ΔT is equal to 1 °C. The α values are reported in Fig. 11 as a function of the phenol concentration. As it can be seen from Fig. 11 the values of α decrease with the increase of the substrate concentration. A similar dependence of α on the substrate concentration is a characteristic found by us for other different enzymes [12–19]. Such a behavior can be explained considering that at substrate concentrations close to the limit of saturation of all the active sides of immobilized enzymes, an additional substrate flux driven by the thermodialysis process would be less effective in order to increase the enzyme activity. The reverse effect is observed at low substrate concentrations.

4. Conclusions

All the results above reported show that the yield of an enzyme reaction is increased when the catalyst is immobilized on a hydrophobic porous membrane working in a non-isothermal bioreactor. The substrate fluxes, driven by the temperature gradients and which add to the isothermal diffusive ones, are responsible for this effect.

Looking at Fig. 11, it seems that the effect produced by the non-isothermal conditions is not so relevant owing to the smallness of the α values. This is not true, since we must remember that the α values reported in Fig. 11 are referred to a ΔT difference of 1 °C measured at the position of thermocouples, while the actual trans-membrane temperature difference is ΔT^* is 0.1 °C. This means that in the present case just 1 °C of the actual temperature difference across the catalytic membrane induces an increase of the reaction rate amounting to 4.6% at 53 μM phenol and 2% at 210 μM. When these indications are applied to the reduction of the production times the industrial interest of our technology appears in its right value.

To conclude the present results encourage the exploitation of the technology of non-isothermal bioreactors in industrial processes, particularly when they are applied in the degradation of toxic or estrogenic compounds.

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References

[1] L. Gianfreda, J.-M. Bollag, in: R.G. Burns, R. Dick (Eds.), Enzymes in the Environment: Activity, Ecology and Applications, Marcel Dekker, New York, 2002.
[2] B. Jimenez, Trends Anal. Chem. 16 (1997) 596.
[3] N. Duran, M.A. Rosa, A. D'Annibale, L. Gianfreda, Enzyme Microb. Technol. 31 (2002) 907.
[4] R.S. Freire, N. Duran, L.T. Kubota, Talanta 54 (2001) 681.
[5] M.A. Mateescu, G. Weltrowska, E. Agostinelli, R. Saint-Andre, M. Weltrowski, B. Mondovi, Biotechnol. Tech. 3 (1989) 415.
[6] F. Lei, B. Shi, Z. Huang, H. Wang, M. Guo, Linchan Huaxue Yu Gongye 16 (1996) 19–22;
F. Lei, B. Shi, Z. Huang, H. Wang, M. Guo, Chem. Abst. 126 (1998) 196823.
[7] J.M. Goddard, J.H. Hotchkiss, Prog. Polym. Sci. 32 (2007) 698.
[8] J.R. Rasmussen, E.R. Stedronsky, G.M. Whitesides, J. Am. Chem. Soc. 99 (1977) 4736.
[9] A.P. Korikov, P.B. Kosaraji, K.K. Sirkar, J. Membr. Sci. 279 (2006) 588.
[10] S. Stellato, M. Portaccio, S. Rossi, U. Bencivenga, G. La Sala, G. Mazza, F.S. Gaeta, D.G. Mita, J. Membr. Sci. 129 (1997) 175.
[11] F. Febbraio, M. Portaccio, S. Stellato, S. Rossi, U. Bencivenga, R. Nucci, M. Rossi, F.S. Gaeta, D.G. Mita, Biotechnol. Bioeng. 59 (1998) 108.
[12] M.S.M. Eldin, M. Santucci, S. Rossi, U. Bencivenga, P. Canciglia, F.S. Gaeta, J. Tramper, A.E.M. Janssen, C.G.P.H. Schroen, D.G. Mita, J. Mol. Catal. B: Enzym. 8 (2000) 221.
[13] N. Diano, M.M. El-Masry, M. Portaccio, M. Santucci, A. De Maio, V. Grano, U. Bencivenga, F.S. Gaeta, D.G. Mita, J. Mol. Catal. B: Enzym. 11 (2000) 97.
[14] P. Travascio, E. Zito, A. De Maio, C.G.P.H. Schroen, D. Durante, P. De Luca, U. Bencivenga, D.G. Mita, Biotechnol. Bioeng. 79 (2002) 334.
[15] H. El-Sherif, S. Di Martino, P. Travascio, A. De Maio, M. Portaccio, D. Durante, S. Rossi, P. Canciglia, D.G. Mita, J. Agr. Food Chem. 50 (2002) 2802.
[16] S. Di Martino, H. El-Sherif, N. Diano, A. De Maio, V. Grano, S. Rossi, U. Bencivenga, A. Mattei, D.G. Mita, Appl. Catal. B: Environ. 46 (2003) 613.
[17] D. Durante, R. Casadio, L. Martelli, G. Tasco, M. Portaccio, P. De Luca, U. Bencivenga, S. Rossi, S. Di Martino, V. Grano, N. Diano, D.G. Mita, J. Mol. Catal. B: Enzym. 27 (2004) 191.

[18] A. Attanasio, N. Diano, V. Grano, S. Sicuranza, S. Sergio, U. Bencivenga, L. Fraconte, S. Di Martino, P. Canciglia, D.G. Mita, Biotechnol. Prog. 21 (2005) 806.
[19] N. Diano, V. Grano, L. Fraconte, P. Caputo, A. Ricupito, A. Attanasio, M. Bianco, U. Bencivenga, S. Rossi, I. Manco, L. Mita, G. Del Pozzo, D.G. Mita, Appl. Catal. B: Environ. 69 (2007) 252.
[20] I. Prigogine, Thermodynamics of Irreversible Processes, Interscience, New York, 1954.
[21] S.R. De Groot, P. Mazur, Non-Equilibrium Thermodynamics, Noord-Hollandsche, Amsterdam, 1962.
[22] R. Haase, Thermodynamics of Irreversible Processes, Addison-Wesley, Reading, MA, 1969.
[23] D.G. Mita, F. Bellucci, M.G. Cutuli, F.S. Gaeta, J. Phys. Chem. 86 (1982) 2975.
[24] F.S. Gaeta, E. Ascolase, U. Bencivenga, J.M. Ortiz de Zarate, N. Pagliuca, G. Perna, S. Rossi, D.G. Mita, J. Phys. Chem. 96 (1992) 6342.
[25] C.C. Lin, M.C. Yang, Biomaterials 24 (2003) 1989.
[26] M.C. Yang, C.C. Lin, Biomaterials 22 (2001) 891.
[27] C.C. Lin, M.C. Yang, Biotechnol. Prog. 19 (2003) 361.
[28] C.C. Lin, M.C. Yang, Biomaterials 24 (2003) 549.
[29] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[30] J.I. Lurie, A.J. Rybikowa, Chemical Analysis of Wastewaters, Goschmizat, Moscow, 1968 (in Russian).
[31] W. Leithe, Analysis of Organic Pollutants in Water and Waste Water, Ann Arbor Science, Ann Arbor, MI, 1972.
[32] K. Saito, A. Tanioka, K. Miyasaka, Polymer 35 (1994) 5098.
[33] S. Kawaguchi, Y. Nishikawa, T. Kitano, K. Ito, A. Minakata, Macromolecules 23 (1990) 2710.
[34] J.A. Akkara, K.J. Senecal, D.L. Kaplan, J. Polym. Sci. A: Polym. Chem. 29 (1991) 1561.
[35] I. Hägg, E. Nilvebrant, Purification of water from phenolic compounds, using enzymatic polymerization, Project work, Stockholm, 2007.
[36] R. Kleine, P. Spangerberg, C. Fleming, Hoppe-Seylers Z. Physiol. Chem. 357 (1976) 629.
[37] T. Sato, T. Mori, I. Chibata, Arch. Biochem. Biophys. 147 (1971) 788.
[38] S.W. May, N.N. Li, Biochem. Biophys. Res. Commun. 47 (1972) 1178.
[39] W.E. Horndy, M.D. Lilly, E.M. Crock, Biochem. J. 107 (1968) 668.
[40] J.S.M. Cabral, J.F. Kennedy, A. Novais, Enzyme Microb. Technol. 4 (1982) 343.