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Isothermal and non-isothermal bioreactors in the detoxification of waste waters polluted by aromatic compounds by means of immobilised laccase from *Rhus vernicifera*

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Abstract

Laccase from *Rhus vernicifera* was immobilised on a nylon membrane chemically grafted with glycidyl methacrylate (GMA). Hexamethylenediamine (HMDA) and glutaraldehyde (GLU) were used as spacer and bifunctional coupling agent, respectively. Quinol was used as substrate.

To know how the immobilisation procedures affected the enzyme reaction rate the catalytic behaviour 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 from the experimental data: (i) the narrower pH-activity profile of the insoluble enzyme in comparison to that of the soluble counterpart; (ii) the increase of the affinity of the immobilised enzyme for its substrate.

The behaviour of the catalytic membrane 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 increases with the increase of the temperature difference. These results have been discussed in the frame of reference of the process of thermodialysis driving thermodiffusive transmembrane substrate fluxes, which add to the diffusive ones.

The advantages of the catalytic process carried out under non-isothermal conditions have been thrown in relief through the evaluation of the reduction of the production times and of the percentage increases of the enzyme activity.

Keywords: Immobilised enzymes; Laccases; *Rhus vernicifera*; Phenols; Waste water detoxification; Catalytic membranes; Isothermal and non-isothermal bioreactors

1. Introduction

Aromatic amines and phenols are found as industrial waste from chemical and pharmaceutical industries, such as those operating in resin and plastic synthesis, textile and photographic operation, drugs production, etc. The majority of these compounds are pollutant, highly toxic, carcinogenic or mutagenic, thus imposing serious hazards to mankind.

Conventional physical–chemical processes, such as activated carbon adsorption or solvent extraction, are not selective with reference to the pollutant removal during treatment. For this reason, the enzymatic approach appears more interesting and effective. Enzymes such as peroxidase (in the presence of hydrogen peroxide) and laccases (in the presence of oxygen) catalyse the oxidation of a wide variety of phenols, biphenols, anilines, benzidines, and other aromatic compound. Various peroxidases and laccases have been used to treat waste waters. Laccase, in particular, has been used in the oxidation of phenolic dyes [1], phenols and chlorophenols [2–5], lignin-related diphenylmethanes [6,7], and organophosphorus compounds [8]. An extensive review

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of potential application of laccases has been recently published by Duran and Esposito [9].

Because the use of soluble enzymes is expensive, insoluble enzymes have been employed in several industrial processes [10,11]. This was due to the circumstance that immobilised enzymes are generally endowed with great advantages such as continuous operation, adaptability to reactor type, improved control of reaction, and enhanced stability and reusability [12]. Also laccases have been used in insoluble form, such as absorbed to porous glass [13], entrapped in alginate beads [14,15] and gelatine gel [16], or covalently attached [17–19].

In spite of the advantages obtained with the enzyme immobilisation, one of the most serious problems towards the industrial employment of the technology of the immobilised enzymes is the diffusion limitation. This problem, consequence of the immobilisation process, is mainly due to the steric hindrance put by the activated solid matrix to the free diffusion of substrate and reaction products towards or away from the catalytic site. What is observed, in general, is an increase of the value of the apparent kinetic constant K_m, i.e. an apparent decrease of the affinity of the enzyme for its substrate. Besides the diffusion limitations, the immobilisation process frequently induces twisting in the enzyme structure so that some loss in the relative activity of the catalyst is also measured.

Few years ago, it was proposed a new technology [20–32] to overcome the disadvantages of the immobilisation process, particularly the apparent affinity loss of the enzyme for its substrate. This technology was based on the use of membrane bioreactors operating under non-isothermal conditions. It was demonstrated that when a catalytic and hydrophobic membrane is interposed between two substrate solutions kept at different temperatures, an increase of the enzyme reaction rate in respect to that measured under comparable isothermal conditions is observed. The percentage activity increases (P.A.I.) resulted proportional to the transmembrane temperature difference and were dependent on the hydrophobicity degree of the membrane. These effects were explained on the basis of the thermodynamics of irreversible processes [33,34], with particular reference to the process of thermodialysis [35,36]. Provided that the membrane was hydrophobic, a thermodiffusive transmembrane substrate flux was produced across the catalytic membrane under the action of the temperature gradient. Since this flux adds to the diffusive one, the immobilised enzymes under non-isothermal conditions encounter in the unit of time a higher substrate concentration in respect to that encountered under isothermal conditions, so that the reaction rate is increased. This phenomenology has been analytically described in some our previous papers [24,25]. The enzymes studied so far were hydrolases or synthetases.

In this paper, the catalytic activity of a laccase immobilised on a hydrophobic nylon membrane, chemically grafted with a suitable acrylic monomer and working in a non-isothermal bioreactor, will be compared with that measured in the same bioreactor operating under isothermal conditions. The effect of temperature gradients will be evaluated in terms of reduction of the production times, percent increases of the catalytic activity and apparent increases of affinity of the immobilised enzyme towards its substrate. When possible, comparison between the catalytic behaviour of soluble and insoluble laccase will be done to know the effect of the immobilisation process on the reaction rate, with particular reference to the kinetic parameters and to the pH and temperature dependence. A three-dimensional (3D) model of our laccase has been elaborated to help the choice of the amminoacidic residues to be involved in the immobilisation method and to obtain the more useful exploitation of our catalytic membrane in non-isothermal bioreactors employed in the treatment of industrial effluents polluted by phenols.

2. Apparatus, materials and methods

2.1. The bioreactor

The apparatus employed, shown in Fig. 1, consists of two metallic flanges in each of which it is bored a shallow cylindrical cavity, 70 mm in diameter and 2.5 mm depth, constituting the working volume to be filled with the substrate solutions. The catalytic membrane is clamped between the two flanges so as to separate and, at the same time, to connect the solutions filling the half-cells. Solutions were recirculated in each half-cell by means of a peristaltic pump through hydraulic circuits starting and ending in a common cylinder *C*, not represented. By means of independent thermostats, the half-cells are maintained at predetermined temperatures. Thermocouples, placed 1.5 mm away from the membrane surfaces, measure the temperature of the solutions in each half-cell. This measure allows 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 subscripts W and C indicate temperatures corresponding to the warm and cold side, respectively. Under these assumptions, $\Delta T = T_W - T_C$ is the temperature difference measured at the position of the thermocouples; $\Delta T^* = T_W^* - T_C^*$ is the actual temperature difference across the membrane; $T_{av} = (T_W + T_C)/2$ and $T_{av}^* = (T_W^* + T_C^*)/2$ are the average temperatures of the bioreactor and membrane, respectively. Since our system is symmetric $T_{av} \equiv T_{av}^*$. In non-isothermal experiments $T_W^* < T_W$, $T_C^* > T_C$, and $\Delta T^* < \Delta T$.

To estimate the real effects of temperature gradients on the activity of immobilised enzymes, the actual temperatures on the surfaces of the catalytic membrane must be known. Being impossible to measure the temperatures on each membrane face, they can be calculated from those measured at the position of the thermocouples, if the solution motion in the two half-cells is laminar. In each half-cell, the solution

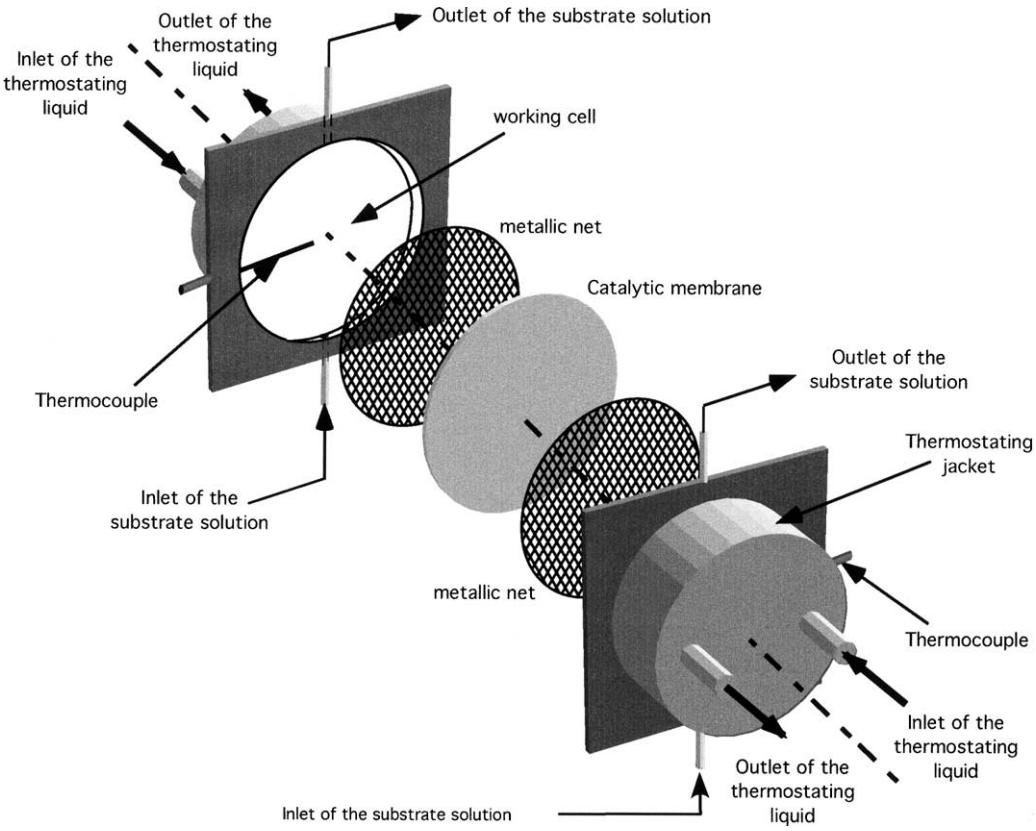

Fig. 1. A 3D picture, not to scale, of the core of the bioreactor. Hydraulic circuits (through which the substrate solutions are re-circulated) and the common cylinder C (containing the working solution volume) have been omitted.

motion is constrained by two fins with rounded tips, and it has a flow rate of 3.5 ml min^{-1}. Under these conditions, the Reynolds number *Re* had values lower than Re_{crit}, being *Re* lower than 10 [21–31]. Accordingly, the fluid motion is laminar. Thus, heat propagation in the bioreactor occurs by conduction between isothermal liquid planes perpendicular to the direction of the heat flow. In this case, by knowing the thermal conductivities and thicknesses of both filling solutions and membrane, it is possible to calculate the temperatures on the membrane surfaces by means of the heat flux continuity principle:

$$J_q = -K_i \left(\frac{\Delta T}{\Delta x}\right)_i = \text{constant} \tag{1}$$

where K_i is the thermal conductivity of the *i*th medium crossed by the heat flux, and $(\Delta T/\Delta x)_i$ is the temperature gradient existing in the same medium, Δx_i thick. Using computer simulation, the values of the temperatures at each point of the bioreactor and at the two surfaces of the catalytic membrane can be calculated. In this way, the temperature profile in the bioreactor is known. In this calculation, as values of the thermal conductivity of our solutions we have assumed those of pure water [37], whereas the value of thermal conductivity of the membrane was taken from Touloukian et al. [38]. It was found that the correlation between the temperatures read at the thermocouple positions and the ones on the surfaces of the catalytic membranes is given by:

$$\begin{cases} T_W^* = T_W - a\,\Delta T \\ T_C^* = T_C - a\,\Delta T \\ \Delta T^* = \Delta T(1 - 2a) \end{cases} \tag{2}$$

where *a* is a numerical constant. In our case, we have found $a = 0.445$.

In Fig. 2, the actual temperature profile in the bioreactor is reported, when $T_W = 35$ and $T_C = 15\,^\circ$C, i.e. under the conditions $\Delta T = 20$ and $T_{av} = 25\,^\circ$C.

2.2. Materials

As solid support to be grafted, nylon Hydrolon membranes from Pall Italia, Milano, Italy) were used. These hydrophobic membranes, 150 μm thick, had a nominal pore size of 0.2 μm. Glycidyl methacrylate (GMA) was used as monomer to be grafted. Hexamethylenediamine (HMDA) and glutaraldehyde (GLU) were used as spacer and coupling agent, respectively. The presence of the spacer was required to minimise the effect of the negative electric charges of the Nylon support on the macromolecule structure and on the micro environment in which the enzyme operates.

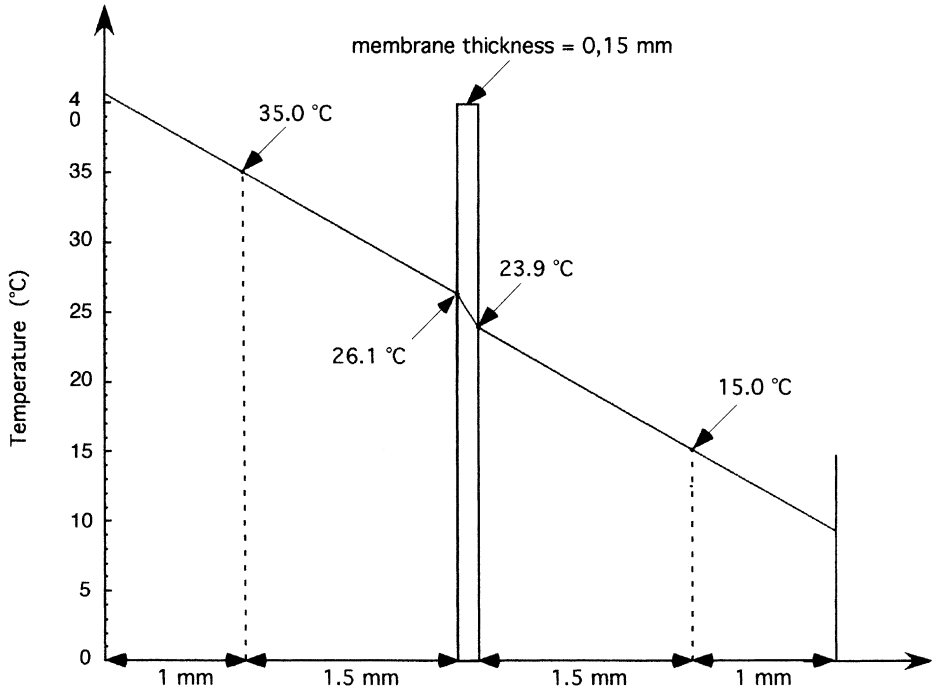

Fig. 2. Temperature profile into the bioreactor and across the catalytic membrane. Experimental conditions $\Delta T = 20$ and $T_{av} = 25\,^{\circ}C$.

Laccase (EC. 1.10.3.2) from *Rhus vernicifera* was used as catalyst. Laccases are cuproproteins belonging to the group of blue oxidase enzymes [39]. Laccase is a polyphenol oxidase catalysing the reaction of several inorganic substances (such as phenols) and aromatic compounds with concomitant reduction of oxygen to water [40]. The reduction of oxygen to water is accompanied by the oxidation of a phenolic substrate. Laccases exhibit four neighbour copper atoms, distributed among different binding sites and classified in three types. Copper type 1 is involved in electron capture and transfer, while the coppers of types 2 and 3 are involved in binding with oxygen. In particular, copper type 2 activates molecular oxygen and copper type 3, a copper dimer, is responsible for the oxygen uptake. Substrate oxidation by laccase is a one-electron reaction generating a free radical [41]. A typical laccase reaction is that used by us in this experimentation in which a quinol undergoes a one-electron oxidation to form an oxygen centred free radical. This species can be converted to quinone in a second enzyme-catalysed step or by spontaneous disproportionation. Eqs. (3) and (4) represent, according to Yaropolov et al. [42], the simplified expression for the molecular mechanism:

$$2Cu^{2+} + \text{Quinol} \rightarrow 2Cu^{+} + \text{Quinone} + 2H^{+} \quad (3)$$

$$2Cu^{+} + \frac{1}{2}O_2 + 2H^{+} \rightarrow 2Cu^{2+} + H_2O \quad (4)$$

Generally, the oxidation of a substrate by laccases leads to polymerisation of the products through C–O and C–C oxidative coupling reactions [19–43]. This process leads to the detoxification of water polluted by phenolic contaminants [44,45].

All chemical products were purchased from Sigma (Sigma Italia, Milano, Italy) and used without further purification.

2.3. Methods

2.3.1. Preparation of the catalytic membranes

The preparation of the catalytic membranes was carried out by means of two steps: (a) grafting copolymerization; and (b) enzyme immobilisation.

2.3.1.1. Grafting copolymerization. Grafting copolymerization was carried out by dissolving as initiating system $K_2S_2O_8$ and $Na_2S_2O_3$ in a 1/1 (v/v) water/ethanol mixture. To obtain the nylon-poly(GMA) membranes, the untreated nylon membranes were immersed, for 60 min at 40 °C, in a reaction vessel filled with the water/ethanol solution containing 0.6 M GMA, 0.008 M $K_2S_2O_8$, 0.008 M $Na_2S_2O_3$ and 0.03 mM $CuCl_2$. At the end of the grafting process, the membranes were treated with dimethyl ketone to remove the produced homopolymer, and dried until a constant weight was reached. Grafting percentage (G, %) was determined by the difference between the membrane masses before (M_b) and after (M_a) the grafting process through the expression:

$$G(\%) = \frac{M_a - M_b}{M_b} \times 100 \quad (5)$$

All membranes used in the experiments had a grafting percentage value of (13.5 ± 2.1%).

2.3.1.2. Enzyme immobilisation. Enzyme immobilisation was carried out by using three successive treatments. With the first one, the spacer HMDA was attached to the grafted support; with the second one, the membrane was activated by the interaction with the glutaraldehyde; with the third one, the enzyme was immobilised on the activated support through covalent attachment to the glutaraldehyde.

Nylon-poly(GMA)-HMDA membranes were obtained by immersing the grafted membranes in a 2% (v/v) hexamethylenediamine aqueous solution for 30 min at room temperature. After this step, the membranes were washed with water to remove the unreacted amines, then treated for 1 h at room temperature with a 2.5% (v/v) glutaraldehyde aqueous solution. After further washings with double-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 8 mg/ml. Coupling of laccase to glutaraldehyde is performed 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 immobilised enzyme was calculated by subtracting the amount of the laccase recovered in the solution at the end of immobilisation process and into the washing solutions from the amount of laccase initially used for the immobilisation. Protein concentration was carried out by the method of Lowry et al. [46].

2.3.2. Determination of enzyme activity

Enzyme reaction rates were calculated by separately measuring, at regular time intervals, the quinone concentrations in samples taken out from the reaction vessel (in experiments carried out with soluble laccase) or from the common cylinder C (in the case of insoluble laccase). In experiments with soluble laccase 20 μl of the extracted samples were added to 20 μl of EDTA 200 mM (to stop the enzyme reaction) and to 2 ml of 0.1 M phosphate buffer, pH 7.5. In experiments with immobilised laccase 20 μl of the extracted samples were added to 1 ml of 0.1 M phosphate buffer, pH 7.5. Quinone concentrations were spectrophotometrically measured through a calibration curve performed at 25 °C and at 245 nm. We have verified, according to Malmström et al. [47], that at this wavelength the absorbance of quinol is zero while quinone has a well-defined peak. A molar extinction coefficient $\varepsilon_{245} = 22.000\,\mathrm{mM^{-1}\,cm^{-1}}$ was found. As value of the reaction rate, expressed in μmoles it was taken the initial rate of the product formation $(\mathrm{d}p/\mathrm{d}t)_{in}$.

2.3.3. Determination of membrane stability

Time stability of the biocatalytic membranes was assessed by measuring their activity every day, under the same experimental conditions, i.e. 40 mM quinol 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 stabilised membranes were used for our study. We think that the activity loss during the first days is related to the description of some amount of enzyme absorbed during the immobilisation phase. We exclude a process of inactivation of some enzymes molecules during the first days since some inactivation occurs just during the immobilisation phase and ends with this phase. When not used, the membranes were stored at 4 °C in 0.1 M phosphate buffer, pH 7.5.

2.3.4. Experimental data treatment

Every experimental point reported in the figures represents the average value of five experiments performed under the same conditions. Each experiment lasted 30 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. Effects due to concentration polarisation, even if present, have not been taken in account for considering the hydrophobic nature of the membrane. The experimental errors never exceeded 4.5%.

2.3.5. Modelling procedure

The three dimensional structure of the laccase from *R. vernicifera* was computed using a homology building strategy. The sequence (Q94IDO in the non-redundant database) was aligned with the template structure of *Trametes versicolor* (1 gyc in the PDB database) using BLAST [48] and clustalw (http://www.ebi.ac.uk/clustalw/). The target to template sequence identity is about 26% [49]. 3D modelling was then performed using MODELLER [50]. The quality of the 3D structure of the enzyme was checked with PROCHECK [51] (biotech embl-ebi.ac.uk: 8400). Prediction of the secondary structure was performed with the PHD program [52]. Model structures were visualised using RASMOL [53].

2.3.6. Docking procedure

Docking simulation of quinol into the putative binding site of the protein was performed with the AutoDock 3 program, which allows a flexible docking by means of a Lamarckian Genetic algorithm [54]. Fifty independent runs were performed, each one processing a population of 100 conformations for 27,000 generations with rates of mutation and crossover set to 0.02 and 0.8, respectively. The elitism parameter was set to 2. The docked conformation with lowest value of estimated free binding energy was retained for further analysis. The LPC on-line software (http://bioinfo.weizmann.ac.il: 8500/oca-bin/lpccsu) was used to analyse the contacts between the protein and the substrate.

3. Results and discussion

3.1. Soluble laccase structure

Before discussing the experimental results obtained with the catalytic membrane, it is necessary to know the 3D model of our laccase and its docking with the substrate. The 3D structure of the soluble laccase and its docking with the quinol allowed us to localise the amminoacidic residues to be involved in the immobilisation process without affecting the active site and, consequently, the choice of the more useful immobilisation method to be used. At the same time, through the 3D structure it is possible to hypotise the possible chain of amino acids involved in electron transfer. In any case, the 3D structure of laccase from *R. vernicifera* is "per se" an interesting result, being unknown until now.

In Fig. 3, the alignment of the sequences of *T. versicolor* (upper line) and of *R. vernicifera* (lower line) is reported. Symbols (−) between the amino acidic sequences are "gap" artificially introduced to obtain maximum correspondence between the residues of the aligned sequences. As reported in Section 2.3 the sequence identity is about 26%. Residues neighbouring Cu1 (i.e. H438, H501, and C496) are picked out by *; while those neighbouring Cu2 and Cu3 (i.e. H497, H104, H61, H106, H495, H438, H59 and H436) are evidenced by a rectangular open box.

Taking in account all these observations, we have constructed the putative model of laccase from *R. vernicifera*, which is shown in Fig. 4a. The template used for homology building was a laccase from the fungus *T. versicolor* at 0.190 nm resolution containing a full complement of copper(II) ions (lgyc, PDB code) [55]. It is evident that the protein model has a globular structure, consisting mainly of anti-parallel beta strands. Similarly to the template, a laccase of the family of the blue copper oxidases [55], there are three cuprodoxin-like domains: the T1 site (Cu1) belongs to domain 3 (dark blue on Fig. 4a) and the T2/T3 site (Cu2, Cu3a and Cu3b) is the interface between the two other

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          10         20         30         40         50         60
AIGPAASLVVANAPVSPDGFLRDAIVVNGVFPSPLITGKKGDRFQLNVVDTLTNHTMLKS
VDVHNYTFVLQEKNFTKWCSTKSMLVVNGSFPGPTITARKGDTIFVNVINQG-----KYG
          10         20         30         40         50         60

          70         80         90         100        110        120
TSIHWHGFFQAGTNWADGPAFVNQCPIASGHSFLYDFHVPDQAGTFWYHSHLSTQYCDGL
LTIHWHGVKQPRNPWSDGPEYITQCPIKPGTNFIYEVILSTEEGTLWWHAH-SDWTRATV
    60         70         80         90         100        110

          130        140        150                160        170
RGPFVVYDPKDPHASRYDVDNESTVITLTDWYH-------TAARLGPRFPLGADATLING
HGALVIL-PANGTTYPFPPPYQEQTIVLASWFKGDVMEVITSSEETGVFPAAADGFTING
     120        130        140        150        160        170

        180        190        200        210        220        230
-LGRSASTPTAALAVINVQHGKRYRFRLVSISC-DPNYTFSIDGHNLTVIEVDGINSQPL
ELGDLYNCSKETTYRLSVQPNKTYLLRIVNAVLNEEKFFG-IAKHTLTVVAQDASYIKPI
       180        190        200        210        220        230

       240        250        260        270        280
LVDSIQIFAAQRYSFVLNANQTVGNYWIRANPNFGTVG-FAGGINSAILRYQGA------
NTSYIMITPGQTMDVLFTTDQTPSHYYMVASPFHDALDTFANFSTNAIIQYNGSYKAPKS
       240        250        260        270        280        290

   290         300        310         320                   330
PVAEPTTTQTT-SVIPLIETNLHPLARMPVP-GSPTPG----------GVDKALNLAFNF
PFVKPLPVYNDIKAADKFTGKLRSLANEKFPVNVPKVNVRRIFMAVSLNIVKCANKSCNN
       300        310        320        330        340        350

         340        350        360                        370
N---GTNFFINNASFTPPTVPVLLQILSG---------------AQTAQDLLPAGSVYPL
NIGHSTSASLNNISFALPQTDVLQAYYRNISGVFGRDFPTVQKKANFSLNTAQGTQVLMI
       360        370        380        390        400        410

   380         390        400        410        420        430
PAHSTIEITLPATALAPGAPHPFHLHGHAFAVVRSAGSTTYNYNDPIFRDVVSTGTP---
EYGEAVEIVYQGTNLGAATSHPMHLHGFNFYLVGTGAGTFNNVTDPPKYNLVDPPELNTI
       420        430     * 440        450        460        470

         440        450        460        470        480
--AAGDNVTIRFQTDNPGPWFLHCHIDFHLEA-GFAIVFAEDVADVKAANPVPKAWSDLC
NLPRIGWAAIRFVADNPGVWFLHCHFERHTTEGMATVVIVKDGGTTNTSMLPSPAYMPPC
       480        490     *  5*0        510        520        530

490
PIYDGLSEANQ  500
S----------  533
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Fig. 3. Sequence alignment of the laccase from *Trametes versicolor* (upper line) and laccase from *Rhus vernicifera*.

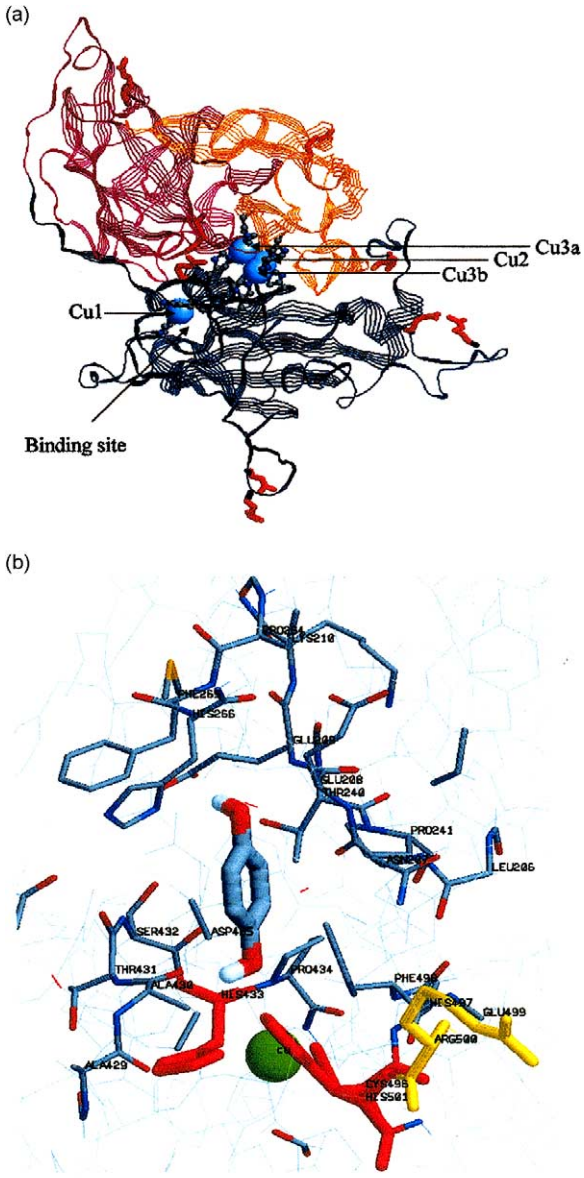

Fig. 4. (a) Three dimensional structure of laccase from *Rhus vernicifera*. The template used for homology building is a laccase from the fungus *Trametes versicolor* at 0.190 nm resolution containing a full complement of copper(II) ions. Surface exposed arginine residues, likely to be covalently bound by glutaraldheyde, are also shown in red in the computed model. (b) Docking between the quinol and the putative binding site of the *Rhus vernicifera* laccase. Copper 1 ion is highlighted in green while its co-ordinating residues (His 433, His 501, Cys 496) are highlighted in red. The quinol ligand is shown along with the protein residues within 0.85 nm. One arginine residue is found in its proximity (Arg 500).

domains (orange and magenta in the upper part of Fig. 4a). The trinuclear T2/T3 copper site is co-ordinated to eight histidines, whereas in the T1 copper site Cu1 is co-ordinated to histidines 438, 501 and cysteine 496. The model allows us to recognise all the surface exposed arginine residues, putative binding sites for glutaraldheyde. Out of 13 arginine residues in the protein, 9 are surface exposed as shown in Fig. 4a.

In a lower resolution crystal of the laccase of *T. versicolor*, a ligand (2,5-xylidine) was described in a cavity in the proximity of Cu1, suggesting that this can be the putative binding site of reduced substrates of the enzyme. Prompted by this finding, we docked the quinol substrate to the same putative binding site. In Fig. 4b, the cavity where the quinol is not tightly buried is shown. The cavity involves within a radius of 0.85 nm from the bound quinol the following amino acids: Val 205, Leu 206, Asn 207, Glu 208, Glu 209, Thr 240, Pro 241, Phe 265, His 266, Ala 429, Ala 430, Thr 431, Ser 432, His 433, Pro 434, Cys 496, His 497, Phe 498, His 50l. Cu1 is shielded by His501, possibly involved in mediating electron transfer from the quinone to Cu1 [56] and not directly exposed into the cavity. The cavity is rather polar and allows binding of quinol as indicated by our docking experiments. Interestingly, one arginine (Arg 500) is in the proximity of the binding site, indicating that in the presence of glutaraldehyde, one net charge is neutralised. Considering that also another arginine is located at 10 nm from the quinol binding site (Arg 111, not shown), we can speculate that also this circumstance may promote changes in the local pH of the cavity, inducing a higher affinity of the substrate for the binding site.

3.2. Isothemal experiments

3.2.1. Effect of pH

The pH-activity profile of an immobilised enzyme is characteristic of the enzyme, support and immobilisation method used. The support, indeed, can change the pH value around the catalytic site, thus determining appreciable differences in the catalytic behaviour of the soluble and insoluble form of the catalyst. This effect, known as partitioning effect [57], is directly related to the nature of support (and grafted monomer) which induces electrostatic or hydrophobic interactions between the matrix and the low molecular weight species present in bulk solution. Partitioning effect, for example, may cause changes in the concentrations of the charged species (e.g. hydrogen and hydroxyl ions) present in the micro environment in which the immobilised enzymes are operating.

To know how the partitioning effect affects the catalytic behaviour of our enzyme derivatives we have investigated the laccase activity, in the free and immobilised form, as a function of pH in the range between 6.0 and 8.0. The results of this investigation are reported in Fig. 5a, where the relative activities of the soluble and insoluble laccase are reported as a function of pH. The experimental conditions were: 40 mM quinol in 0.1 M phosphate buffer, pH 7.5 and $T = 25\,^{\circ}C$. Inspection of Fig. 5a shows no differences in the position of the optimum pH between the free and insoluble forms of laccase. Different authors also found optima pH values coincident for free and immobilised laccases from different sources. For example, laccase from *Pleurotus oryzae* immobilised on a polyethersulphone membrane [58] exhibited the same optimum pH of the free form (at 6.5) when syringaldazyne was

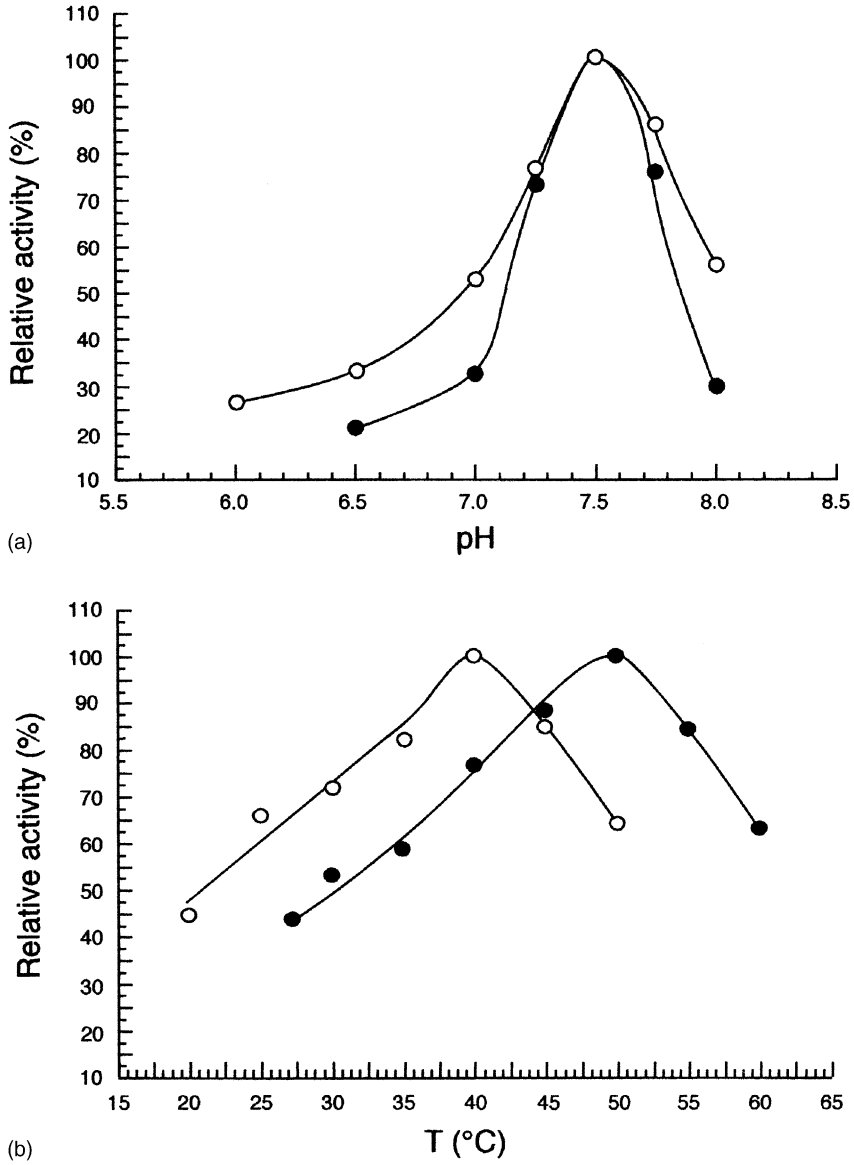

Fig. 5. (a) Relative activity of laccase as a function of pH. Symbols: (○) soluble enzyme; (●) insoluble enzyme. (b) Relative activity of laccase as a function of temperature. Symbols: (○) soluble enzyme; (●) insoluble enzyme.

used as substrate. Similarly laccase from *Lentinula edodes* was found to have an optimum pH value of 4.0 either for the free than for the form immobilised on chitosan [59]. In our case, the coincident value of soluble and insoluble laccase indicates that the chain GMA–HMDA–GLU is long enough to minimise the effect of the negative electric charge present on the nylon support. In a recent paper [60], we have demonstrated that increasing the spacer length the optimum pH value of the immobilised enzyme approached the value of the soluble one. These experiments were carried out with β-galactosidase immobilised on a nylon-poly(GMA) in which spacers of the type NH_2–$(CH)_n$–NH_2, with $n = 0$ or 4 or 6, were attached. When $n = 6$, the spacer was HMDA and the membrane was similar to that used in this research. The conclusion of this study was that a free enzyme can be considered as an enzyme immobilised on a solid support through a spacer of infinite length, in a position in which the partitioning effects induced by the carrier are zero. On the contrary, the more short was the spacer, the more effective was the partitioning effect. In other words, the hexamethylenediamine, i.e. the NH_2–$(CH)_6$–NH_2 spacer, being enough long and keeping the enzyme enough far from the electric field of the nylon membrane, creates the conditions by which the micro environment around the catalytic site of the immobilised enzyme is quite similar to that around the free form. Coming back to Fig. 5a, the narrower shape of the pH-activity curve of the insoluble laccase in respect to that of the native enzyme appears unusual for an immobilised enzymes. At our knowledge only one case has been reported [61]. The present behaviour can indicate that the immobilisation procedure reduces the conformational transitions (or the flexibility) of the immobilised enzyme thus delimiting

the range of resistance to pH changes of the immobilised laccase and, consequently, the optimum pH range. The "optimum pH range" is the range in which the relative enzyme activity is higher than 90%. From Fig. 5, it is possible to see that this range occurs between 7.4 and 7.7 for the free enzyme; between 7.4 and 7.5 for the immobilised enzyme.

3.2.2. Temperature dependence

Isothermal characterisation of membrane activity is one of the principal parameters required to know how the immobilisation procedure affects the enzyme activity. Generally, enzymatic derivatives show optimum temperatures shifted towards higher temperatures than those corresponding to the soluble counterpart.

In Fig. 5b, the relative activity of the catalytic membrane is reported as a function of temperature. The temperature dependence of the soluble laccase has be added, for comparison. Results in Fig. 5b show for the insoluble laccase a shift of the optimum activity towards higher temperatures in comparison to the position of the soluble enzyme, evidencing in this way that the immobilisation procedure strengthens the enzyme structure. The optimum temperature for the free enzyme occurs at about 40 °C, while for the catalytic membrane at about 50 °C. This means that the immobilisation process, strengthening the enzyme structure, gives to the macromolecule a protective effect against the heat denaturation. While similar behaviour was found for the soluble (50 °C) and insoluble (60 °C) laccase from *L. edodes* [59], opposite behaviour was found for the soluble (40 °C) and insoluble (35 °C) laccase from *P. oryzae* [58]. Calling "optimum temperature range" the range in which the relative enzyme activity is higher than 90%, it is possible to see that in our case this range is between 37.3 and 43.3 °C for the free enzyme; between 46 and 53.3 °C for the immobilised enzyme. The simultaneous existence of a large optimum temperature range and of the shift of the optimum temperature position towards higher temperatures suggests the use of our membranes in biotechnological processes requiring high running temperatures.

When the experimental points of Fig. 5b are reported in form of Arrhenius plots in the temperature range from 15 °C to the optimum temperature, one obtains the values of the activation energy of the enzymatic process for the soluble and the insoluble laccase. The values of the activation energies are 6.74 Kcal mol^{-1} for the free laccase, 7.50 Kcal mol^{-1} for the enzyme derivative. These values indicate that at least under our experimental conditions, i.e. 40 mM in 0.1 M phosphate buffer, pH 7.5, the enzyme reaction rates are diffusion controlled for both the catalyst form. This result is important for the employment of the catalytic membrane in non-isothermal bioreactor.

3.2.3. Concentration dependence

When a biocatalyst is immobilised, the kinetic parameters K_m and V_{max} undergo variations in comparison with those of the soluble enzyme. To indicate that the kinetic parameters are changed they are indicated as $K_{m,app.}$ and $V_{max,app.}$ These variations are attributed to several factors such as: (a) the changes in the protein conformation induced by the interactions between the support and the enzyme; (b) the immobilisation methods which, in the case of covalent attachment, can involve different amino acidic residues; (c) the steric hindrances and the diffusional effects introduced by the grafted monomers and by the spacer to the substrate movement towards the catalytic site; (d) the electrostatic interactions between the activated carrier and the substrate. These factors may operate simultaneously or separately. Consequently the $K_{m,app.}$ value may increase [62,63] or decrease [64,65] in comparison with that of the soluble enzyme. A decrease of the $K_{m,app.}$ value leads to a faster reaction rate, whereas an increase of the $K_{m,app.}$ implies the use of a higher substrate concentration in order to get the same reaction rate observed for the free enzyme. The $K_{m,app.}$ increases if, for example, the electric charges on support and substrate are of the same sign. Also the V_{max} values are affected by the immobilisation process. In general, similar values of V_{max} have been found for the free and the immobilised form of the enzyme, even if increases or decreases have also been reported. Nevertheless, it is difficult to compare the values of the $V_{max,app.}$ with the ones of the soluble enzymes, as the reaction rates are proportional to the amount of active enzyme molecules. In the case of immobilisation, indeed, even if we know the amount of immobilised enzymes, we do not know the percentage of active enzymes.

To determine the kinetic parameters for the immobilised laccase, the activity of the catalytic membrane was studied as a function of substrate concentration. The pH and temperature of the solutions were 7.5 and 25 °C, respectively. Results reported in Fig. 6a show that the catalytic membrane exhibits a Michaelis–Menten behaviour. In Fig. 6b, the kinetic behaviour of the soluble 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. 6a. When the experimental points of Fig. 6a and b are reported in form of Hanes plots, one obtains the values of K_m and V_{max} listed in Table 1. The $K_{m,app.}$ value shows that the affinity of the immobilised laccase towards the quinol is apparently higher than that found for the soluble one. Also this behaviour is unusual. By using the same support, i.e. a nylon-poly(GMA)–HMDA–GLU, but immobilising a β-galactosidase we have observed values of the apparent K_m higher than that of the soluble enzyme, according to the diffusion limitations encountered by the substrate in its approach to the catalytic site. Results similar to the latter ones were observed by us with urease and penicillin G acylase. The laccase behaviour in the present case is an exception, maybe correlated to the electronic transfer involvement in the enzyme reaction. The transformation of quinol to quinone is a two steps reactions, with an intermediate formation of a free cation radical which undergoes a further laccase-mediated oxidation reaction leading to the formation of quinones. Under these conditions, the electrostatic inter-

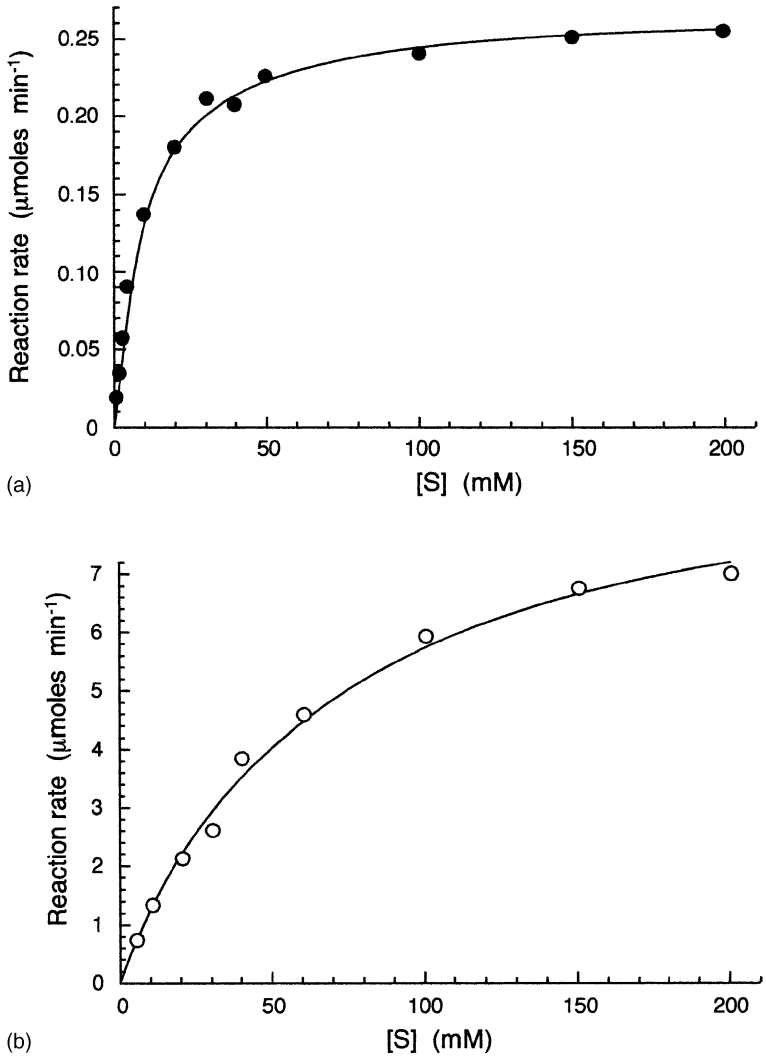

Fig. 6. (a) Reaction rate of insoluble laccase as a function of quinol concentration. (b) Reaction rate of soluble laccase as a function of quinol concentration.

actions between the cation radical and the negative charges on the nylon membranes increase the concentration of the second substrate in the case of the immobilised laccase. This mechanism offers a tentative explanation of the apparent higher affinity of the immobilised laccase for its substrate, even if our argumentation is not very solid. The existence of a cation radical at the end of the first step of the oxidation reaction has been amply discussed in references [66,67].

3.3. Non-isothermaI experiments

To understand in detail the effect of the temperature gradients on the activity of the immobilised laccase in Fig. 7a the values of the quinone production under isothermal and non-isothermal conditions are reported as a function of time. The experimental conditions were: 3 mM quinol in 0.1 M phosphate buffer, pH 7.5 and $T_{av} = 25\,^{\circ}C$. The curve

Table 1
Kinetic parameters to relative to the soluble and insoluble laccase from *Rhus vernicifera*

Enzyme status	Experimental conditions	Km (mM)	v_{max} (μmoles min^{-1})
Free enzyme	$T_{av} = 25\,^{\circ}C$; $\Delta T = 0\,^{\circ}C$	69.00	9.58
Immobilised enzyme	$T_{av} = 25\,^{\circ}C$; $\Delta T = 0\,^{\circ}C$	11.30	0.27
Immobilised enzyme	$T_{av} = 25\,^{\circ}C$; $\Delta T = 10\,^{\circ}C$	5.85	0.28
Immobilised enzyme	$T_{av} = 25\,^{\circ}C$; $\Delta T = 15\,^{\circ}C$	5.85	0.30
Immobilised enzyme	$T_{av} = 25\,^{\circ}C$; $\Delta T = 20\,^{\circ}C$	5.85	0.32

The values for Km and v_{max} for the soluble laccase has been taken from reference [34].

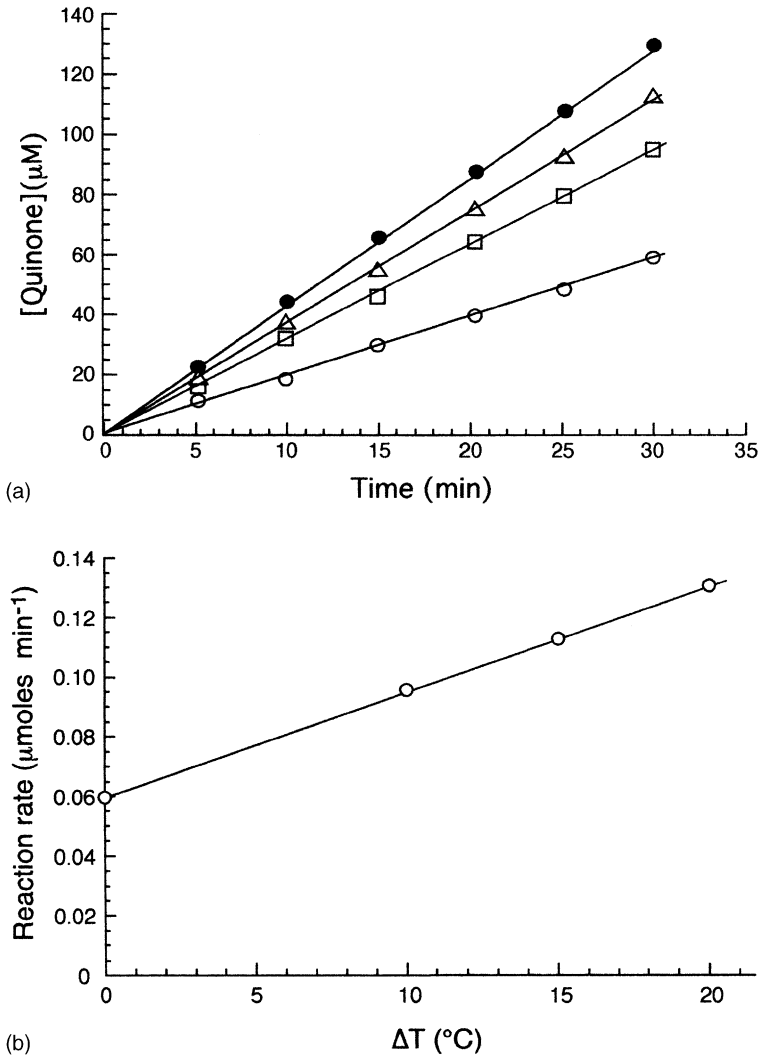

Fig. 7. (a) Isothermal and non-isothermal quinone production as a function of time. Symbols: (○) $\Delta T = 0$; (□) $\Delta T = 10$; (△) $\Delta T = 15$; (●) $\Delta T = 20\,^{\circ}C$. In all cases $T_{av} = 25\,^{\circ}C$. (b) Enzyme reaction rates as a function of the macroscopic temperature difference ΔT read at the thermocouple positions. $T_{av} = 25\,^{\circ}C$.

parameter is the temperature difference ΔT read at the position of the thermocouples. The angular coefficient of each straight line, multiplied by the solution volume (30 ml), gives directly the value of the membrane activity, expressed as $\mu moles\,min^{-1}$. In Fig. 7b, the catalytic activity of the membrane is reported as a function of the applied ΔT. Data in Fig. 7b show that membrane activity increases linearly with the magnitude of the applied ΔT. The straight line fitting the experimental points is expressed by an equation of the type $y = y_0 + a_0\ \Delta T$, where, at least in the range of the temperature differences employed in this investigation, y represents the value of the non-isothermal activity of the membrane under a determined value of ΔT, y_0 is the value of the isothermal activity, and $(y - y_0)$ represents the activity difference when $1\,^{\circ}C$ of temperature difference is measured at the thermocouple positions.

Results in Fig. 7a and b show that the catalytic activity of the membrane under non-isothermal conditions is higher than that measured under isothermal conditions and increases with the increase of the temperature difference ΔT. These results are indicative of the usefulness of the employment of non-isothermal bioreactors in biotechnological processes of industrial interest. Indeed, when the advantages of using non-isothermal bioreactors are considered, a significative parameter to be taken in consideration is the reduction of the production times, τ_r, defined as:

$$\tau_r = \frac{\tau_{iso} - \tau_{non\text{-}iso}}{\tau_{iso}} \tag{6}$$

where $\tau_{non\text{-}iso}$ and τ_{iso} are the times required to obtain the same amounts of reaction products under non-isothermal and isothermal conditions, respectively. On the basis of this definition and considering the results in Fig 7a, one obtains the reductions of the production times reported in Fig. 8a as a function of the macroscopic temperature difference ΔT.

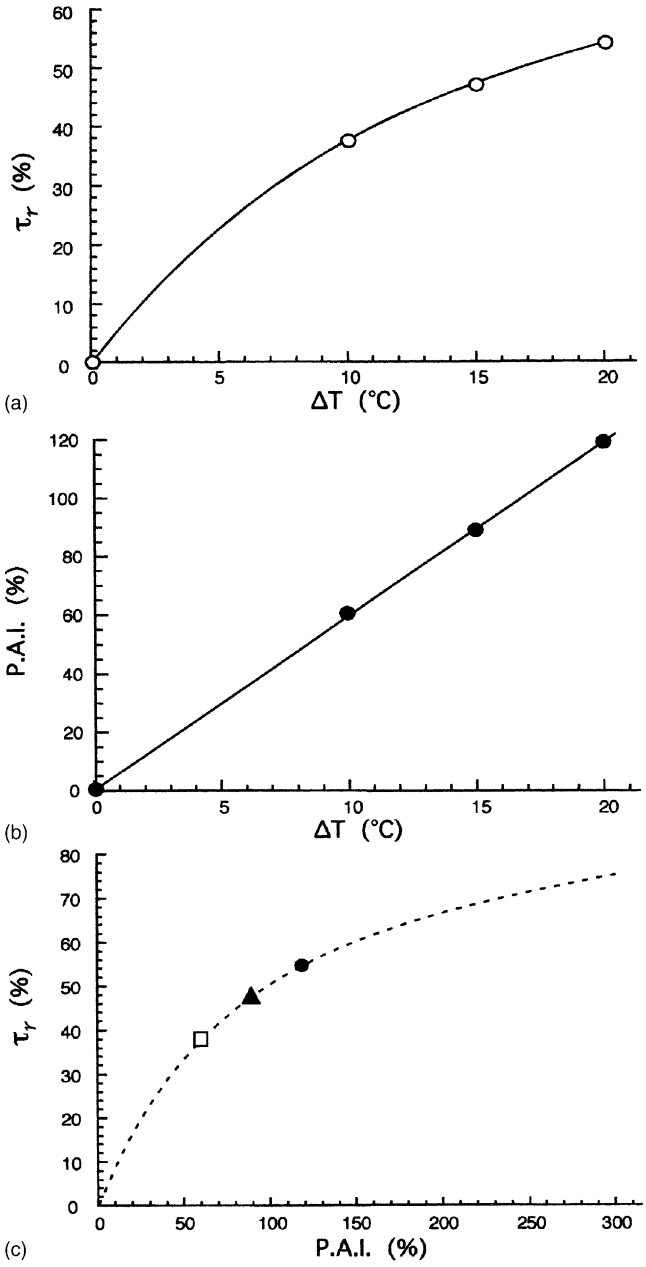

Fig. 8. (a) Reduction of the production times, τ_r, as a function of the macroscopic temperature difference ΔT at the thermocouple positions. (b) Percentage activity increases (P.A.I.) as a function of the transmembrane temperature difference ΔT read at the thermocouples positions. $T_{av} = 25\,^{\circ}C$. (c) Reduction of the production times, τ_r, as a function of P.A.I. Symbols: (---) theoretical curve; (□) $\Delta T = 10$; (△) $\Delta T = 15$; (●) $\Delta T = 20\,^{\circ}C$.

Results in Fig. 7b can be utilised to obtain a more interesting parameter correlating the efficiency increase of the catalytic process with the applied ΔT. This parameter is the percentage activity increase, defined as:

$$\text{P.A.I.} = \frac{(y - y_0)}{y_0} = \frac{a_0}{y_0}\Delta T = \alpha \Delta T \tag{7}$$

where α (%, °C) represents the percentage activity increase when a temperature difference of 1 °C is measured at the thermocouple positions. In Fig. 8b, the P.A.I. values relative to the experiments of Fig. 7 are reported as a function of ΔT. The angular coefficient of the straight line crossing the experimental points gives the value of the α coefficient which, in the present case, is 5.8 (%, °C). This means that 1 °C of temperature difference read at the positions of the thermocouples produces an increase of membrane activity equal to 5.8%.

It is interesting, at this point, to correlate the P.A.I. values with the ones of τ_r. By indicating with R the activity of a catalytic membrane under isothermal conditions, results in Fig. 7a have shown that in the presence of temperature gradients the catalytic activity of the membrane can be expressed as $R\,(1 + \text{P.A.I.})$. Therefore, the concentration of the reaction product $[P]$ will increase with the time according to the following expressions:

$$[P]_{iso} = Rt \tag{8}$$

$$[P]_{non\text{-}iso} = R(1 + \text{P.A.I.})t \tag{9}$$

From Eqs. (6)–(9) one obtains:

$$\tau_r = \frac{t_{iso} - t_{non\text{-}iso}}{t_{iso}} = \frac{\text{P.A.I.}}{\text{P.A.I.} + 1} \tag{10}$$

A theoretical plot of Eq. (10) is reported in Fig. 8c, where the experimental points refer to results reported in Fig. 7a and b.

Having ascertained that at 3 mM quinol concentration the non-isothermal conditions affect the catalytic activity of the enzyme membrane, we have extended these studies to the whole concentration range from 0 to 200 mM. The results of this investigation are reported in Fig. 9, where the catalytic activity of the catalytic membrane is reported as a function of quinol concentration. The curve parameter is the temperature difference ΔT measured by the thermocouples, the average temperature being 25 °C. Results in Fig. 9 show that: (i) all the curves interpolating the experimental points exhibit a Michaelis–Menten behaviour either in the presence or in the absence of temperature differences; and (ii) at each quinol concentration the activity increases are proportional to the size of the applied ΔT. When the experimental points in Fig. 9 are reported in forms of Hanes plot one obtains the values of the apparent K_m and V_{max}, listed in Table 1. The data in Table 1 show that: (i) the immobilised enzymes, either under isothermal or non-isothermal conditions, have apparent K_m values lower than that of the free counterpart; (ii) the apparent values of K_m under non-isothermal conditions are lower than the corresponding values under isothermal conditions; (iii) the K_m values under non-isothermal conditions are independent from the macroscopic temperature difference measured by the thermocouples.

A possible explanation for the behaviour described under (i) can be given by considering the electronic transfer involvement in the enzyme reaction. The transformation of quinol to quinone is a two steps reactions, with an intermediate formation of a free cation radical [66,67] which undergoes a further laccase-mediated oxidation reaction leading

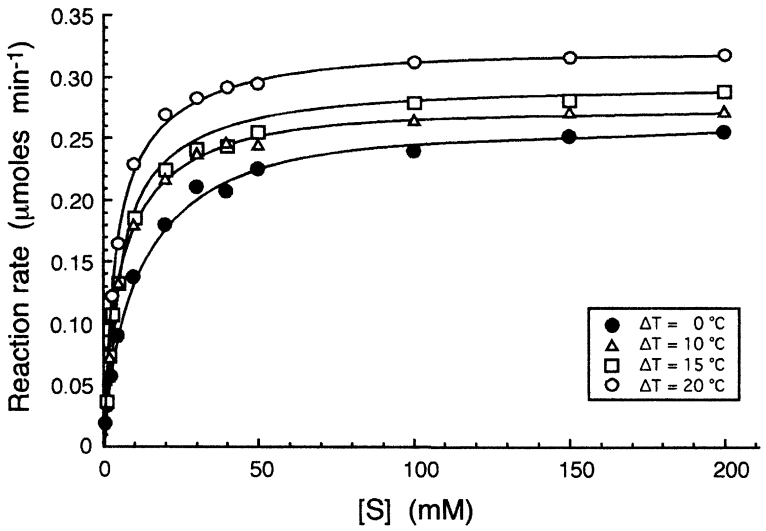

Fig. 9. Reaction rate under isothermal and non-isothermal conditions as a function of quinol concentration. Symbols: (●) $\Delta T = 0$; (△) $\Delta T = 10$; (□) $\Delta T = 15$; (○) $\Delta T = 20\,^{\circ}C$. In all cases $T_{av.} = 25\,^{\circ}C$.

to the formation of quinone. Under these conditions, the electrostatic interactions between the cation radical and the negative charges on the nylon membranes increase the concentration of the second substrate in the micro environment around the catalytic site of immobilised laccase. The result of the increase of free cation radical concentration nearby the catalytic site appears as an apparent higher affinity of the immobilised laccase for its substrate.

The behaviour described under (ii) finds explanation in the circumstance that the temperature gradient increases the substrate and product fluxes across the catalytic membrane reducing, in this way, the diffusion limitations for these substances during their movement towards or away from the catalytic site. Moreover, the increases of the enzyme reaction rates with the applied ΔT are analogous to the increases of non-isothermal transmembrane mass fluxes induced by the process of thermodialysis [35,36]. A detailed analysis of these fluxes and of the substrate concentration profiles into a catalytic membrane under isothermal (diffusion) and non-isothermal (thermal diffusion/thermodialysis) conditions, in the presence or in the absence of catalysis, has been recently published [24,25].

The independence of the K_m values on the macroscopic temperature difference across the catalytic membrane can be explained on the basis of the changes in the protein structure and dynamics induced on the immobilised enzyme by the flux of thermal energy associated to the presence of the temperature gradient. The temperature gradients should play on the enzyme structure the same role than positive effectors, which affect the K_m value of an enzyme reaction independently on their concentration. In this analogy, the size of the temperature gradient results equivalent to the size of the effector concentration.

Coming back to the results of Fig. 9, it is evident that at all the quinol concentrations studied it is possible to apply the approach used for the experiments reported in Fig. 7a and b. In this way, it is possible to calculate, at all the concentrations, the P.A.I. values, and consequently the α values, which correspond to the P.A.I. values when $\Delta T = 1\,^{\circ}C$. In Fig. 10a, the α-values relative to the results of Fig. 9 are reported as a function of quinol concentration. From Fig. 10a, it clearly emerges that the P.A.I. values decrease with the increase of the substrate concentration. Similar dependence of the α values on the substrate concentration has been found with different enzymatic derivatives [26–30]. This behaviour has been phenomenologically explained by considering that when the immobilised enzymes work at substrate concentrations near to those saturating, the addition of further substrate fluxes driven by the temperature gradient is less effective in increasing the enzyme activity. The reverse, naturally, occurs at low substrate concentration. A detailed analytical explanation of this effect has been done elsewhere [24,25], where the substrate profiles into the catalytic membrane have been deduced by using mass balance equations accounting for diffusive, thermodiffusive substrate fluxes together with the substrate consumption by the enzyme reaction.

A more interesting parameter for the industrial application of the technology of the non-isothermal bioreactors is the coefficient α', which is the percentage activity increase when an actual temperature difference $\Delta T^* = 1\,^{\circ}C$ is applied across the catalytic membrane. This new coefficient is defined as:

$$\alpha' = \alpha\left(\frac{\Delta T}{\Delta T^*}\right) = \frac{\text{P.A.I.}}{\Delta T^*} \tag{12}$$

In Fig. 10b, we have reported the α' values relative to the experimental results reported in Fig. 9.

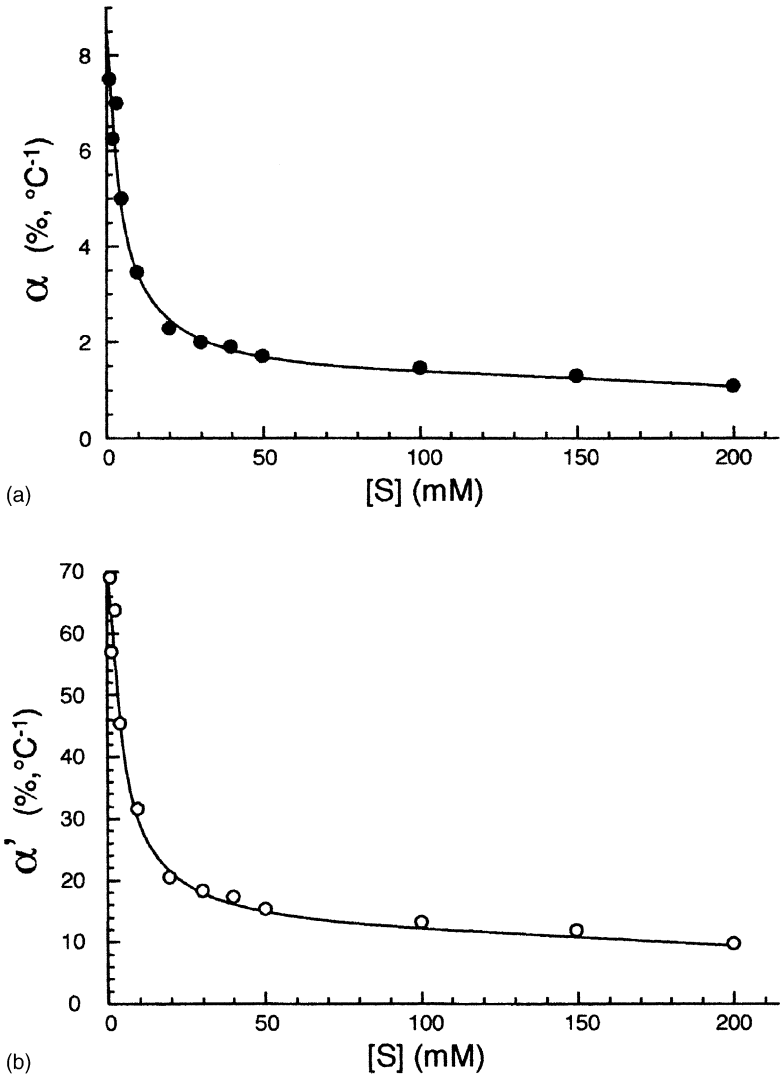

Fig. 10. (a) α coefficients as a function of quinol concentration. (b) α' coefficients as a function of quinol concentration.

4. Conclusions

All the results obtained under isothermal conditions indicate that the immobilisation process of laccase from *R. vernicifera* on a nylon-poly(GMA)–HMDA–GLU membrane strongly affects the catalytic behaviour of the enzyme. In particular differences in the amplitude of the pH-activity profile and in the position of the optimum temperature have been found between the free and insoluble forms of laccase. At the same time smaller value of the apparent K_m of the immobilised laccase in comparison to that of the soluble counterpart candidate the nylon-poly(GMA)–HMDA–GLU membrane as an useful support for a biosensors endowed with high sensitivity towards phenolic compounds. In addition, since some phenols are known to be intermediate products of pesticide degradation, the proposed catalytic membrane could be used successfully also for reducing the harmful effects of pesticides in the environment.

With respect to the results obtained under non-isothermal conditions, it clearly emerges that also hydrophobic membranes binding laccase show an increase of the catalytic activity in the presence of a temperature gradient. These results are particularly interesting considering that this is the first example of a study of the behaviour under non-isothermal conditions of a catalytic membrane loaded with an enzyme involving electron transfer in its reaction.

Interesting enough it appears the finding that the α' values obtained with the insoluble laccase are equal to those measured with the same nylon-poly(GMA)–HMDA–GLU membrane loaded with β-galactosidase [32]. The indication is that, at least under our experimental conditions, the presence of a temperature gradient increases the reaction rate of an oxidative enzymatic reaction more than in enzymatic processes of hydrolysis or synthesis.

When the reduction of the production times by a temperature gradient is considered, the values measured with the actual membranes strongly candidate the technology on

non-isothermal bioreactors as an useful tool in the process of detoxification of waste waters, polluted by phenolic compounds.

Acknowledgements

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