Immobilization of laccase from Trametes versicolor on a modified PVDF microfiltration membrane: characterization of the grafted support and application in removing a phenylurea pesticide in wastewater

Claude Jolivalt, S. Brenon, Eliane Caminade, Christian Mougin, Maxime Pontié

Immobilization of laccase from *Trametes versicolor* on a modified PVDF microfiltration membrane: characterisation of the grafted support and application in removing a phenylurea pesticide in waste water

C. Jolivalt*[1], S. Brenon[1], E. Caminade[2], C. Mougin[2], M. Pontié[3]

[1]Laboratoire de Bioorganique et Biotechnologies de l'Environnement

[2]Unité de Phytopharmacie et Médiateurs Chimiques[2], Institut National de la Recherche Agronomique, Versailles, France.

[3]Laboratoire d'Electrochimie et de Chimie Analytique[**3**], Ecole Nationale Supérieure de Chimie de Paris, Paris, France.

Abstract

Laccase immobilization onto a hydrophilic PVDF microfiltration membrane and its application for removing a herbicide derivative, *N'*,*N'*-(dimethyl)-*N*-(2-hydroxyphenyl)urea (2-HF), from waste water were studied. 2-HF was transformed via an oxidation reaction catalyzed by laccase mostly into an insoluble product, which was simultaneously separated by filtration through the membrane. The microfiltration membrane used to graft laccase was treated with hydrazine to form a hydrazide group on its surface, which further reacts with the oxidized carbohydrate groups of the enzyme, leading to a covalent immobilization of laccase. Contact angle measurements by the sessile drop technique and X-Ray photoelectron spectroscopy (XPS) followed the successive modifications of the membrane. Streaming potential measurements as a function of the pH help show the presence of the laccase on the membrane by determining of the isoelectric point of the

grafted membrane at 4.1±0.5. This value is representative of the isoelectric point of the laccase mixture produced by the white rot fungus *Trametes versicolor*. Herbicide removal experiments were performed in a microfiltration module. The performance of the laccase grafted membrane was evaluated for different enzyme loading rates. It was shown that laccase grafted membrane with a 60 cm^2 effective microfiltration area transformed 200 mL of a 0.1 g/L 2-HF solution within less than five minutes. The main transformation product, an insoluble dark purple precipitate, was retained by the membrane.

Keywords

immobilization, laccase, microfiltration, PVDF, phenylurea, streaming potential, XPS, contact angle, fouling, wastewater.

Introduction

Among the different pollutants that enter the environment from various sources, most of them come from agricultural and industrial activities. In particular, the toxicity of phenols and their associate derivatives play an important role in the ecological balance of some compartments such as soils and water. The development of methods for removing and transforming such toxic compounds has increased in recent years. Biodegradation appears a promising technology, particularly the use of oxidative enzymes as biocatalysts, included in a micro-organism or used as a free enzyme. Laccase (EC 1.10.3.2), an enzyme widely distributed in higher plants and fungi [1], has received particular attention because of its ability to catalyze the oxidation of a wide spectrum of molecules containing an aromatic ring substituted with electron withdrawing groups. Its rather low specificity makes laccase a promising tool in transforming many toxic substituted phenols [2-5] or even non-phenolic compounds such as polycyclic aromatic hydrocarbons [6-7]. The reduction of a substrate typically involves the formation of a free cation radical which can further undergo laccase-catalyzed oxidation reactions, generally leading to the formation of quinones from phenols, or non-enzymatic transformation such as hydration or polymerization, forming high molecular weight insoluble components [8-10].

Klibanov and co-workers [11] have proposed the use of laccase for treating waste waters containing phenol derivatives, arguing that the formation of the resulting precipitate could be easily removed by filtration or sedimentation. Laccases were immobilized on various supports, chosen for two different applications [12]. First, many efforts have been undertaken to provide suitable models for laccase activity in soils [13-15], since these enzymes have been shown to catalyze coupling reactions between phenolic compounds and humic acids, leading to the binding of pollutants on the organic matter of soil, thus reducing their bioavailability and their toxicity.

The second application of immobilized laccases, or microorganisms producing laccases, was for phenolic effluent treatments [16-19]. Enzyme immobilization usually allows a good preservation of enzyme activity over time [14, 19]. The efficiency of the enzyme extract can even be enhanced by selective adsorption when immobilized, as reported by Tatsumi *et al*. [20] in the removal of chlorophenols from waste waters by peroxidase immobilized by physical adsorption on magnetite. In most cases, laccases are immobilized on porous beads and xenobiotics are degraded in bed-packed column reactors. However, immobilization of enzymes on a membrane and the use of filtration offers advantages. First, it allows the simultaneous downstream separation of the transformation products when they are insoluble. Secondly, flow rates can be higher than with packed beds, because all the substrate flows through the support instead of diffusing in the bead pores. Mass transfer is much faster as a result of this convective flow [21]. Recently, Edwards *et al*. [22] reported the use of a polyphenoloxidase, immobilized by adsorption on a polysulfone capillary membrane, to convert phenols concomitantly with the separation of the insoluble products resulting from the transformation of the phenolic compounds.

The immobilization of proteins on solid supports, mostly for use in affinity chromatography but also in manufacturing commodity products [23,24] or in organic synthesis [25], has become routine and many immobilization procedures have been described. Protein immobilisation on solid supports by covalent linkage involves interaction between an “activated” group on the support and a functional group on the protein, most commonly β−amino groups of basic residues. In the present work, laccase from *Trametes versicolor* was covalently immobilized on a hydrophilic PVDF microfiltration membrane using a site-directed immobilization technique. The membrane, previously activated to contain a hydrazide function, was then left to react with the oxidized oligosaccharide groups of the enzyme. The protein-grafted membrane was used in a

filtration module to transform *N',N'*-(dimethyl)-*N*-(2-hydroxyphenyl)urea (2-HF), a herbicide derivative known to be oxidized into an insoluble compound in the presence of laccase [10]. Particular attention was paid to characterizing the microfiltration membrane using physicochemical methods throughout its modification procedure. Streaming potential measurements, as previously described [26-28], were performed in order to determine the isoelectric point of the grafted membrane. This electrokinetic technique was coupled with XPS and contact angle measurements, which are techniques providing information on the surface properties of the membrane and show the presence of the covalently grafted laccase. Furthermore permeability measurements were taken to evaluate membrane fouling.

Experimental

Chemicals

Hydrazine hydrate (98% purity) and acetic acid were obtained from Prolabo, sodium acetate from Fluka and sodium periodate from Sigma. Benzoxasolone, triethylamine and dimethylamine hydrochloride were purchased from Aldrich Solvents used for HPLC analysis and for synthesis were from Carlo Erba (Val de Reuil, France). Electrolytes used to modulate the ionic strength (KCl) and pH (KOH, HCl) in stream potential measurements were *p.a.* grade (Merck).

Membrane and module

The frame plate reactor module Minitan S and Durapore hydrophilic membranes (GVWP type, 150×80 mm) were purchased from Millipore (Saint Quentin en Yvelines, France). The pore diameter of the Minitan S filter sheet membrane used is of 0.45 μm**.** The design of the membranes is planar and the effective filtration area in the Minitan is 60 cm^2. Before the experiments, all the

membranes were cleaned by means of standard procedures to remove preservatives and rinsed with deionized water until the conductivity of the permeate remained below 1 $\mu S.cm^{-1}$.

Enzyme

Laccase production

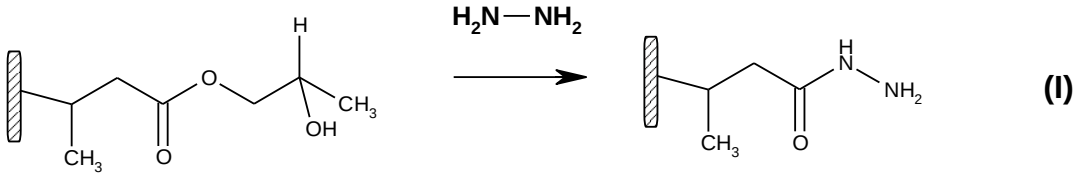
Laccase was produced as previously described in [10] from *Trametes versicolor* (culture collection of the Unité de Phytopathologie, INRA Versailles, France). At the end of the culture, the extracellular medium was filtered and stored frozen at -80 °C. The activity of the crude enzyme extract used for these experiments was 165 U/mL.

Measuring laccase activity

Laccase activity was measured as the oxidation of 2,2'-azinobis-(3-ethylphenyl-thiazoline-6-sulfonic acid) (ABTS). In case of free enzyme solution, 10 µL of enzyme solution were added to 990 µL of a 1 mmol/L ABTS solution in phosphate citrate buffer (0.1 mol/L, pH 3). The buffer solution was air saturated by bubbling prior to the experiment. The absorbency of the radical formed by oxidation of ABTS (ε=36 000 $L \times mol^{-1} \times cm^{-1}$) was measured at λ=420 nm. The experiment was performed at 30°C. Activit in units (U) is defined as the number of micromoles of ABTS oxidized in one minute and is related to 1 mL of the enzyme solution. A similar procedure was adopted to measure the activity of laccase bound on a membrane. The grafted membrane was soaked in a crystallizing dish containing an ABTS 1 mmol/L solution at pH 3. The dish was stirred on an orbital table at 30°C. The absorbency of the supernatant was measured at 420 nm as a function of time.

Immobilizing laccase on a PVDF membrane

Preparating the hydrazide membrane (scheme I): the membrane was rinsed several times in water prior to any utilisation to remove additives used by the supplier to prevent any microbial degradation during its storage. The rinsed membrane was then soaked at room temperature overnight in hydrazine hydrate (100 mL for the 150×80 mm membrane). The modified membrane was then rinsed in 3×200 mL ultrapure water. During this step, the hydroxypropyl acrylate groups on the membrane surface undergo the following reaction:



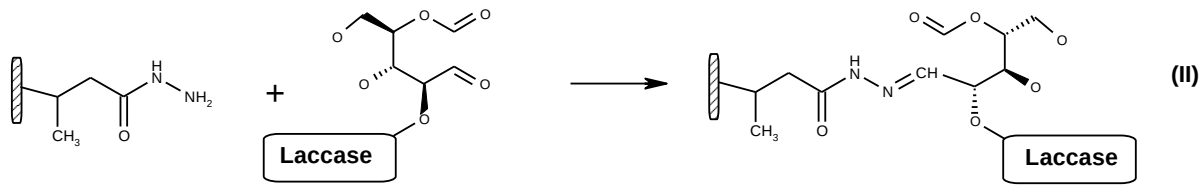
(I)

(I) membrane activation by hydrazine

Introducting aldehyde groups into laccase: we followed glycoprotein oxidation procedures described in the literature [30,31], except for the sodium periodate removal, where we used a diafiltration method instead of gel filtration. We added 230 μL of freshly prepared 38 mM sodium periodate in water (final concentration 1 mmol/L) with rapid mixing to 6 mL of a 165U/mL protein crude extract in sodium acetate buffer (15 mmol/L, pH 5.5) containing 0.15 mol/L sodium chloride. After 25 minutes in darkness at room temperature, the mixture was diafiltered over a YM10 Amicon ultrafiltration membrane to remove unreacted sodium periodate. $NaIO_4$ concentration was lowered about 2000 fold by diafiltration. The ultrafiltrate was checked throughout the experiment for laccase activity: the overall loss of laccase activity in the permeate

was less than eight activity units. Finally, 770 laccase activity units were recovered. Part of the sample was used immediately for coupling with the membrane and the remaining part was stored at -30°C in the presence of 30 %(w/w) glycerol.

Binding laccase to the hydrazide membrane (scheme II): up to 320 U of oxidized was added to 100 mL acetate buffer (50 mmol/L, pH 5.5) containing sodium chloride to a final concentration of 0.1 mol/L. The hydrazide membrane was then soaked in the mixture and agitated on an orbital shaker at 4°C. Coupling proceeded for various durations depending on the amount of laccase to be bound. When starting with an enzyme solution containing 320 U, the coupling process required about 6 hours to afford maximum activity for the immobilized laccase. A longer incubation time did not increase the activity significantly. Using this reaction scheme and 1.3 mg of the enzyme (320 U), about 0.9 mg of laccase (220 U) was immobilized on a 120 cm^2 membrane. The decrease in the laccase activity in the supernatant was measured periodically during the course of the reaction. An aliquot (2 mL) of the initial oxidized solution was used as a control: as no change in activity was detected in the control, the amount of bound laccase was calculated from the difference between the initial and the measured laccase activity in the supernatant. At the end of the reaction, the laccase solution was removed. The membrane was rinsed successively in 100 mL acetate buffer (50 mmol/L, pH 5.5), 100 mL water, 100 mL sodium chloride 0.5 mol/L and 1 mol/L successively and finally acetate buffer each for ten minutes under agitation. No laccase activity was detected in the rinsing solutions. The membrane was then stored in acetate buffer pH 5.5 at 4°C.



(II) covalent coupling of laccase forming a hydrazone

Hydroxyphenylurea substrate

Synthesizing N',N'-dimethyl-N-(2-hydroxyphenyl)urea (2-HF)

The synthesis procedure for 2-HF was adapted from Hadjieva *et al* [29]. Triethylamine diluted in ethanol was added dropwise to a stirred mixture of benzoxasolone and a large excess of dimethylamine hydrochloride in 50% aqueous ethanol. The resulting mixture was heated at 80°C under reflux for six hours. After cooling at room temperature, diluted aqueous hydrogen chloride was added until neutralization and evaporated under reduced pressure. The product was purified by flash chromatography on a silica gel column using ethyl acetate as a mobile phase.

Analyzing 2-HF and its soluble transformation products

2HF transformation was measured using Waters HPLC equipment (Controller 600, 700 Satellite WISP and software Millennium) with detection at 244 nm on a Waters 486 UV detector. A Waters Nova-Pak C_{18} column (3.9×150 mm) was used with a mobile phase described by the following gradient: 100% water during the first 3 minutes, then a linear gradient up to 100% acetonitrile at 18 minutes, followed by a 5 minute stage at 100% acetonitrile. The column was equilibrated for 10 minutes before any injection. The flow rate was 1 mL/min.

Operating the bioreactor

A schematic diagram of the continuous flow reactor system is shown in figure 1. A peristaltic pump (b1) is used to pump the substrate solution from a reservoir (a) to the planar module (f). A pair of saturated calomel electrodes (SCE) (γ_1,γ_2) is connected to a high impedance voltmeter (i) and allows to measure the potential difference across the membrane as a function of the applied pressure. The potential difference $\Delta\phi$ between the permeate (p) and the retentate (r) is defined as $\Delta\phi=\phi_r-\phi_p$. Using this convention, $\Delta\phi$ has the same polarity as the membrane surface charge. The SP is defined as the potential difference per unit of applied pressure, $\nu=\Delta\phi/\Delta P$. Each value of ν is the result of five measurements. Statistical calculations give an average error of 10%. The temperature is maintained at 22°C. The system is operated as a batch process.

Hydraulic permeability measurements were performed with acetate buffer (25 mmol/L, pH 5) at a transmembrane pressure of 50 kPa for virgin, grafted and fouled membranes, in conformity with the AFNOR guideline [32].

The filtration experiments were conducted with the module Minitan II (f), the effective filtration surface (60 cm^2) being reduced to half of total membrane surface. The substrate solution consisted of 200 mL of 2-HF (0.1 g/L in acetate buffer 25 mmol/L, pH 5). Because this work was primarily aimed at testing the feasibility of immobilized laccase to transform 2-HF, the permeate was recycled in the substrate pool, so that the system proceeded in a batch mode. The substrate solution was air-saturated by bubbling prior to its use in the filtration device, to ensure that the oxygen concentration had reached its solubility limit (1.26 mmol/L in water at 25°C). As the initial 2-HF concentration was only 0.56 mmol/L, oxygen, which is the co-substrate of the oxidation catalyzed by laccase, was not a limiting factor of the reaction course.

Contact angle measurements

The contact angle measurements were carried out by the sessile drop technique [33]: a liquid droplet is placed onto a flat homogeneous surface and the contact angle of the droplet with the surface is measured with a KRUSS G1 contact angle meter. The contact angle measurements were carried out on dried films of planar pieces (5×5 cm) of membranes. A droplet of acetate buffer solution (pH=5.5 and a volume of 1 to 2 µL) was deposited onto the surface using a micro-syringe. Reported values are the averages of the contact angles (right and left) of five droplets. During the short measurement time (less than 1 minute), no change in contact angle was observed. Contact angles do not give absolute values but make it possible to compare materials. A variation of 5 degrees in the angle is needed to differentiate each kind of material.

Results and discussion

Covalent coupling of laccase onto the membrane

The starting material used was a PVDF membrane modified by the supplier, Millipore, to provide a hydrophilic surface [34]. Acrylate groups grafted on the membrane surface behave like "activated esters" and react with hydrazine to form hydrazide groups, as described by O'Shanessy and Hoffmann [30] for Affigel○ beads, or King *et al* [35] for the coupling of proteins to low molecular weight ligands. The protocol is adapted in the present work to a membrane solid support. Then the membrane activated with the hydrazide groups reacts at pH 5.5 with the aldehyde function on the previously oxidized glycoprotein to form a hydrazone (scheme II). This type of immobilization chemistry offers the advantage of minimizing intramolecular cross-linking of polypeptide chains due to the formation of Schiff bases between aldehyde groups and free

β−amino groups of the protein [36]. Indeed, the coupling reaction takes place at pH 5.5, so that laccase β−amino groups are protonated and thus hardly reactive.

The successive membrane modifications significantly modify its initial hydrophilic feature, as confirmed by contact angle measurements. The contact angle of the membrane prior to any modification was 0°, in accordance with the hydrophilic type announced by the supplier. The substitution of the acrylate group by the hydrazide on the membrane surface markedly enhanced its hydrophobicity: after this treatment, the measured contact angle increased from 0 to 123°. A significant consequence of the hydrophobic feature of the modified surface is that protein adsorption is more likely to take place and thus to compete with the covalent linkage process, as confirmed by the following control experiment. A hydrazide-modified membrane fragment (12 cm^2) was incubated with a non-oxidized laccase solution containing 13 U activity. After six-hour long incubation, the decrease of the enzymatic activity in the supernatant was similar to that observed when conducting the experiment with oxidized enzyme at the same concentration. Contact angle measurements on both membranes, washed according to the procedure described in the experimental section, gave 0° for the covalent linkage and 60 ° in case of adsorption. In both cases, the covering of the membrane with protein led to an increase of the surface hydrophilicity. However, when protein has been only adsorbed, the hydrophilic feature was less pronounced, suggesting that successive washings sodium chloride solutions led to the partial desorption of the enzyme.

The amount of immobilized laccase remaining active was measured in batch experiments by soaking a grafted membrane fragment (1 cm×2 cm) in a 1 mmol/L ABTS solution with stirring. Comparison with the amount of grafted laccase calculated from the decline of activity in the supernatant during the grafting procedure (1.5 U activity) showed that the actual activity

determined by batch experiments was only 20% of the expected value. The measured activity increased when increasing the ABTS concentration to 3 and 10 mmol/L, whereas such concentrations are above the substrate saturation concentration determined with an equivalent amount of free laccase (K_M=0.140 mmol/L and k_{cat}=65 s^{-1} at pH 3 for ABTS. The difference in K_M between the free and immobilized laccase is likely to be due to diffusional limitations, as encountered when immobilizing enzyme on porous beads [37, 38]. Batch activity measurements thus lead to an underestimation of the total activity of the immobilized enzyme.

Stability properties

As a reference, free laccase activity was measured in different buffers (pH ranging from 3.8 to 7.8) and stored at 4°C. No significant decrease in the enzyme activity was detected over a two months period. Similar results were reported by Leonowicz et al [14] with laccase from *Trametes versicolor*. When immobilized on the membrane, laccase maintained significant activity during storage at 4°C in citrate phosphate buffer (20 mmol/L, pH 5, thimerosal 0.01 %w/w to prevent any bacterial contamination). During the first three months storage period, the activity dropped by 30 %. Thereafter, the activity remained stable during the next three months. For comparison, laccase from *Coriolus versicolor* was reported to show 38 % of denaturation in the first four days at 4°C when immobilized on activated carbon [18] whereas immobilization on porous glass beads slightly increased its storage stability at the same temperature [14].

Influence of pH on the activity of the immobilised laccase

In order to determine the optimum pH of soluble and immobilized enzymes, the activity was assayed between pH 2 and 8 using ABTS as a substrate. The effect of the pH on the activity of free and immobilized laccase is reported in figure 3. Both free and immobilized enzymes display

maximal activity near pH 3. At higher pH, the immobilized enzyme exhibits a less marked decrease in activity. However, the pH activity profiles for the free and the immobilized enzyme remain very similar.

Physico-chemical characterization of the grafted membranes

XPS measurements were performed in an attempt to follow the successive grafting steps by analysing the elementary composition of the membrane surface (instrument, spectra recording and analysing are detailed in [39]). As expected, the XPS spectrum of the hydrophilic PVDF membrane contained the representative peaks of carbon, fluorine and oxygen. After activating of the membrane with hydrazine, nitrogen was also detected on the membrane surface. From the ratio of oxygen to nitrogen peak areas, corrected for their sensitivity, it can be estimated that 60% of the initial hydroxypropyl acrylate groups were replaced by a hydrazide group.

XPS analyses on protein-grafted membrane showed an increase in the proportion of both nitrogen and oxygen detected, compared with the hydrazide-activated membrane and using fluorine as an internal standard. Such a result could be evidence of the presence of protein on the membrane surface but can not lead to a quantitative estimation of the amount of enzyme bound on the membrane surface. Indeed, coating the membrane with protein, a globular molecule with approximate dimensions 50 Å × 70 Å × 45 Å [40], forms a biological layer whose thickness prevents any accurate quantitative analysis by XPS.

In contrast with XPS, which gives information on the surface of the membrane, streaming potential determination, based on measurements made on a flow *through* the membrane, makes it possible to investigate the surface charges inside the pores of the membrane. The variations of streaming potential as a function of pH were recorded at two ionic strengths, 0.01 and 0.1 mol/L

to determine the isoelectric point of the grafted membrane (figure 2). A decrease in streaming potential values was observed for very low pH, where the ionic strength due to HCl addition cannot be negligible when compared to KCl concentration. The density of charge is dependent on the surface properties of the polymer, the type and kind of electrolyte and the adsorption behavior of the charged molecules, *i.e.* small ions or proteins [26, 27]. An increase of the ionic strength from 0.01 M to 0.1 M increases the conductivity of the solution and thus decreases the density of charge, according to Helmholtz-Schmolukowsky equation. At the isoelectric point (IEP), the surface charge and the streaming potential both vanish. The IEP of the membrane was thus deduced from figure 2, as being the pH value at $v=0$. The IEP obtained is about 4.1 ± 0.5 for both ionic strengths. The uncertainty on the IEP measurement arises from the experimental error on the streaming potential measurement. As the IEP does not depend on the ionic strength, it can be assumes that the membrane surface charges do not arise from a specific electrolyte adsorption but is an intrinsic property of the membrane, as developed elsewhere [26-27]. This value is significantly different from the IEP of the non modified PVDF material, *i.e.* 6, determined by a titration method [41]. Laccase used in this study is an extracellular enzyme from a *Trametes versicolor* culture. The protein solution is mainly a mixture of two enzyme pools exhibiting a laccase activity, which can be separated by chromatographic methods. One of the crude extract fractions exhibits an isoelectric point around 3, while IEP of the second fraction is higher, in the range of 4.6-6.8, as determined by electrofocusing experiments [42]. The overall isoelectric point of the mixture is therefore likely to lie between these two values. The IEP of the grafted membrane, when deduced from streaming potential measurements, appears to be representative of the isoelectric point of the enzyme mixture grafted on the membrane. This demonstrates the

ability of this technique to characterize the actual charges on the pore walls modified by biological matter.

Combining 2-HF transformation and microfiltration

A series of membranes with four different loading rates: 4.4, 10, 50 and 220 U activity (on the 120 cm^2 membrane) was tested in the microfiltration module for its ability to transform 2-HF. Figure 4 compares the effect of laccase loading on 2-HF concentration in the permeate. Clearly, the higher the protein loading, the more efficient the degradation is. With the membrane grafted with 220 U activity, no more 2-HF was found in the permeate at the first sampling, *i.e.* five minutes after the beginning of the experiment. Knowing the kinetic parameters obtained with 2-HF as a substrate, at pH 6 [43], it can be calculated that 110 U of laccase grafted on the useful membrane area should be able to transform a 2-HF solution with a maximum rate V_{max}=33 µmol/min. In the present work, the initial phenylurea solution contained about 110 micromoles of 2-HF. The experimental filtration results are thus consistent with the above calculated degradation rate. In the filtration device, the grafted membrane appears to be more efficient than when used in batch conditions: forced convection through the membrane overcomes the mass transfer limitation of the substrate encountered in batch experiments. Such experiment also confirmed that batch activity measurements lead to an underestimate of the total immobilized activity.

With a 50 U laccase coating, it takes more than 3 hours to remove 98% of 2-HF. Comparison between the 50 U and 10 U laccase grafted membranes seems to indicate a proportionality between the amount of laccase immobilized and the extent of degradation. However, such a comparison is not valid for the 220 U grafted membrane, whose efficiency is proportionally

higher than expected when compared to the 50 U grafted membrane. One explanation could be that the amount of laccase covalently linked onto the membrane is overestimated. Indeed, the amount of laccase immobilized was determined by the difference between that added to the system and the enzyme left in the supernatant at the end of the immobilization. This amount accounts for both adsorption and covalent coupling of the enzyme. As previously discussed, the adsorption phenomenon is significant due to the hydrophobic feature of the hydrazide activated membrane. On the other hand, the phenomenon is reversible: batch activity measurements on laccase immobilized by adsorption on a hydrazide membrane (1cm×2 cm) have shown that 40 % of the 0.3 U activity initially measured were removed by two successive washings in 0.5 M sodium chloride. Unfortunately, a direct quantification of the amount of released enzyme could not be performed because of the loss of laccase activity in concentrated salt solutions. At low protein covalent covering, the number of sites available for adsorption is high, leading to a higher overestimation of the remaining amount of active laccase on the membrane after washings , thus explaining the discrepancy in the efficiency of the degradation at low enzyme loading.

Hydraulic permeability results

The transformation of 2-HF by immobilized laccase was simultaneously accompanied by the formation of an insoluble purple colored product, which accumulated onto the membrane surface and remained confined in the retentate compartment. Optical microscopy have shown that the size of the smallest particles can be estimated around 2-3 μm, which is large enough to be retained by the microfiltration membrane, whose pore diameter is 0.45 μm. However, because of the presence of these particles, the precipitate could play a major role in the mechanism of membrane fouling [44]. Flow measurements were performed in order to quantify a possible fouling of the

membrane. Initial, grafted and fouled membranes in the presence of the solid deposit were tested with acetate buffer (25 mmol/L, pH 5) at a transmembrane pressure of 50 kPa. After grafting with laccase, the permeate flux decreased from 2.1±0.1 L/h for the initial membrane to 1.6±0.1 L/h, as the result of the steric hindrance due to the presence of the protein on the membrane surface and inside the pores. A fouling phenomenon was observed after the transformation of 2-HF into its insoluble oxidation product since the flux was reduced to 1.3±0.1 L/h, *i.e.* 38% lower than the value for the initial membrane.

A crude model can be used to evaluate the decrease in pore diameter induced by membrane fouling. Equations (1) and (2) give the fluxes as a function of the transmembrane pressure, respectively before (J_p) and after fouling (J_{p1}) in conformity with Poiseuille's law :

$$J_{p0} = K d_p^4 \Delta P \quad (1)$$

$$J_{p1} = K d_f^4 \Delta P \quad (2)$$

d_p is the pore diameter and d_f the pore diameter after fouling, K is a constant.

The flux ratio J_{p0}/J_{p1} gives the equation that allows to determine d_f knowing d_p. Knowing the pore diameter of the virgin membrane, *i.e.* 0.45 μm, the calculated pore diameter of the grafted and fouled membrane decrease to 0.42 μm and 0.40 μm, respectively. The membrane fouling due to the precipitation 2-HF transformation product has thus a limited effect on both permeability and geometrical properties of the membrane, which could be explained by the relatively large precipitate particle size.

Conclusion

The present work reports the feasibility of an integrated water treatment coupling the transformation on an herbicide derivative, *N'*,*N'*-(dimethyl)-*N*-(2-hydroxyphenyl)urea (2-HF), by enzymatic oxidation and the concomitant separation of its insoluble reaction product by crossflow filtration in a microfiltration module. Because laccase is a small size molecule, compared to the pore size of the microfiltration membrane, it is necessary to immobilize the enzyme on a solid support to avoid losses during the filtration process and allow the reuse of this expensive biological matter. A covalent linkage on the membrane was preferred to physical adsorption because it avoided any leakage of the protein. The performance of the laccase grafted membrane was evaluated for different laccase loading rates and it was shown that a membrane with a 60 cm^2 effective filtration area allows to transform 200 mL of a 0.1 g/L 2-HF solution within less than five minutes. Most of the transformation products were insoluble and were retained by the filtration membrane. However, as evidenced by the pink color of the permeate and confirmed by HPLC measurements, a small part of the oxidation products remained soluble. Therefore, a method for removing these colored products has to be found. A method adapted from the recent work of Edwards *et al.* [22] could be promising. Indeed, the authors faced a similar problem: a capillary reactor was used for the treatment of industrial effluent containing phenol with a polyphenoloxidase. In this case, no precipitation was observed, but the permeate was highly colored, suggesting the presence of o-quinone derivatives. These products were removed using a packed column containing chitosan. Chitosan is a readily available polysaccharide polymer (obtained for example from crab shell) which has nucleophilic amine groups capable of reacting

with carbonyl groups of the quinone, resulting in a covalent bond between chitosan and o-quinones. Further studies are in progress in order to assess such a method.

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Figure 1: Experimental devices used for streaming potential measurements

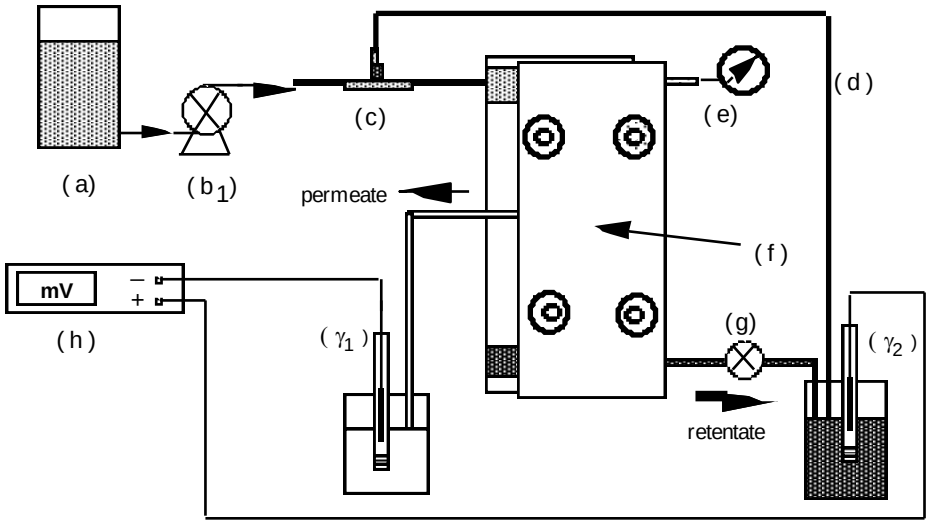

Figure 2: Effect of the pH on the activity of free and immobilized laccase *from Trametes versicolor*. (◇) free laccase ; (■) laccase covalently immobilized on a PVDF membrane [2222]ABTS 1 mM, citrate/phosphate buffer (0.1 M, pH 3), T=30°C.

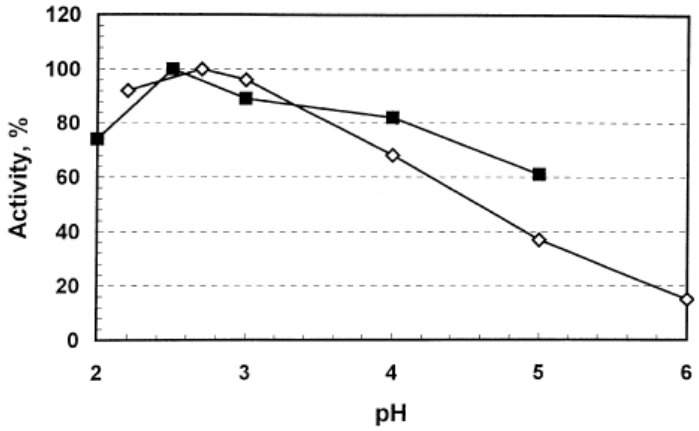

Figure 3: Streaming potential of a PVDF grafted membrane as a function of pH KCl concentration: (❍) 0.01 mol/L, (■) 0.1 mol/L.

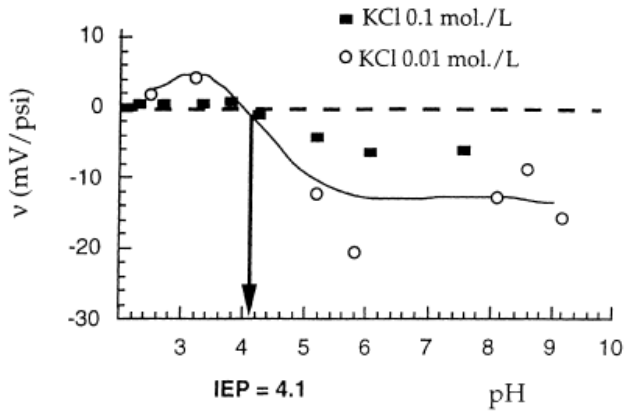

Figure 4: Decrease in 2-HF concentration in the filtrate as a function of time. 120 cm^2 membranes grafted with 4.4 (σ), 10 (◆), 50 (ν) and 220 (λ) U laccase activity. 2-HF initial concentration 0.1 g/L in acetate buffer (0.05 M, pH 5), T=22°C.