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PAPER

Over-activity and stability of laccase using ionic liquids: Screening and application in dyes decolorization

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The use of a wide range of water miscible and immiscible ionic liquids (ILs) as reaction media for ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) oxidation by *Trametes versicolor* laccase was studied. Thirteen ILs were shown to be suitable media for the laccase oxidation reaction, increasing the activity with respect to conventional media. Among them, the water-miscible IL choline dihydrogen phosphate [Chol][H_2PO_4] allowed over-laccase activity with an enhancement rate of 451% at 25°C and pH 7.0. This ionic liquid improved the stability of the enzyme in the face of high temperature and high pH, while storage at room temperature in aqueous medium was increased up to 4.5 times. Moreover, it was found that its use in the reaction medium for decolourizing dyes (antraquinonic and azoic) using laccase increased the decolourization rate by up to 216% and 137% for the azoic dyes Acid Black 1 and Remazol Brillant Blue R, respectively. A high decolorization rate was also obtained for a mix of dyes (80% within 8 h).

To understand the effect of [Chol][H_2PO_4] on the secondary protein structure of the laccase, several spectroscopic techniques were used such as Circular Dichroism (CD), Fourier transform infrared (FT-IR) and Fluorescence, all of which demonstrated that the β sheets structure was affected. A shift to α-helix structure [Chol][H_2PO_4] could be responsible of the enhancement of the enzyme activity observed at 300 mM

Introduction

The importance of enzymes has been demonstrated in many scientific fields; enzymes were discovered in nature and are exploited in industry for their inherent catalytic properties in complex chemical processes under mild experimental and environmental conditions [1, 2]. Laccases (EC 1.10.3.2) are versatile enzymes used in a wide spectrum of catalyses: such as the biodegradation of phenols [3], decolourization of dyes [4], lignin-cellulose treatments [5] or, more recently, for the enzymatic cross-linking of alkyd resins in a new generation of paints [6]. The desired industrial purpose is often difficult to achieve using the native form of the enzyme, and recent developments in enzyme engineering have revolutionized the development of commercially available enzymes for a better use as industrial catalysts [2, 7].

During the last decade, the use of ionic liquids in biocatalysis has received growing attention due to the advantages they offer, such as the possibility of carrying out processes involving hydrolytic enzymes that were thermodynamically unfavourable in water, replacing organic solvents (e.g. trans-esterification reactions), their ability to increase the solubility of organic substrates and to improve process performance increasing the enzyme activity, stability and selectivity, by ensuring a comfortable medium for the enzyme [8, 9, 10]. However, IL research has focused not only on replacing organic solvents in biocatalysis but also on identifying new possibilities in water-enzyme applications or for improving the catalytic activity or/and stability inon the aqueous media [7, 11].

ILs are organic salts that remain as liquids under room temperature. They normally consist of an organic cation, the most commonly used being dialkylimidazolium and tetraalkylammomiun salts, and a polyatomic inorganic anion (e.g. tetrafluoroborate, hexafluorophosphate) (see Table 1). The main advantage of these media are their near-zero vapour pressure and their good chemical and thermal stabilities [12]; they are also considered environmentally benign solvents compared with volatile organic solvents. Furthermore, all the physico-chemical properties of ILs, including their hydrophobicity, density, viscosity, melting point, polarity and solvent miscibility, can be finely tuned by selecting appropriate combinations of cations and anions, while an optimal IL can be designed for each specific enzymatic reaction system. Interest in these compounds, often heralded as the green, high-tech media of the future, is still increasing rapidly, not only in biocatalysis but also in chemical catalysis, separation technology and analytical applications [13, 14]. Recently, the use of ILs with laccases has also been described as an efficient non-conventional catalyst system in applications such as wood treatment and phenol degradation [3, 5].

On the other hand,Textile manufacturing involves the discharge of highly colored synthetic dye effluents which are aesthetically displeasing and can damage the receiving water body by impeding the penetration of light [15]. The strict environmental legislation of European countries does not permit their release since they can have very severe consequences on river courses, including reducing photosynthetic activity and dissolved oxygen concentrations [16]. The great importance of effluent treatment has been underlined in many research-works [17]. In the textile industry, a wide variety of dyes colors have been extensively used for textile industry, anthraquinonic and azoic dyes could be

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the widest used for this task [17]. The decolourization process usually involves the use of laccase as principal or co-catalyst for dye removal.

In this work, a green decolorization process based on the use of laccase and ionic liquids was developed. Firstly, the enzymatic activity of laccase from *Trametes versicolor* was tested in fifty-six ionic liquids using the oxidation of ABTS as reaction model. The best ionic liquid was then applied for the decolorization of different synthetic dyes and of a mixture of dyes. Finally, the effect of this ionic liquid on the secondary protein structure of the enzyme was analyzed by spectroscopic techniques: Circular Dichroism (CD), Fourier transform infrared (FT-IR) and Fluorescence.

Results and discussion

Effect of ionic liquids on laccase's activity

Enzyme-catalyzed reactions are greatly influenced by the reaction medium used. To study the influence of the cationic and anionic composition of ILs on the activity of laccase, a systematic study was carried out using 56 different ILs (see Table 1), 26 of which were water-immiscible and 30 water-miscible (see Table 1). The oxidation of the ABTS by laccase at pH 7.0 and 25ºC was chosen as reaction model. The 26 water immiscible ILs chosen for this study were the following: (i)[OMIM][NTf_2]; (ii)[$P_{14,14,14,6}$][dca]; (iii)[BMPi][NTf_2]; (iv)[BMIM][PF_6]; (v)[Epy][NTf_2]; (vi)[$S_{2,2,2}$][NTf_2]; (vii)[OMIM][PF_6]; (viii) [$P_{6,6,6,14}$][NTf_2]; (ix)[BMIM][NTf_2]; (x)[EMIM][NTf_2]; (xi) [$N_{8,8,8,1}$][NTf_2]; (xii)[Chol][NTf_2]; (xiii) [$N_{8,8,8,1}$][Cl]; (xiv) [OMIM][BF_4]; (xv) [$P_{6,6,6,14}$][TMPPhos]; (xvi)[BMPyr][NTf_2]; (xvii)[$P_{8,8,8,8}$][Br]; (xviii) [BMPyr][PF_6]; (xix) [MOPy][dca]; (xx)[EMPyr][NTf_2]; (xxi)[$N_{8,8,8,1}$][TfO]; (xxii)[$P_{14,14,14,6}$][BF_4]; (xxiii) [$P_{14,4,4,4}$][$C_{12}BzO_3$]; (xxiv) [$P_{14,14,14,6}$][Br] and (xxv) [THP][Cl]; (xxvi) [MOMMIM][PF_6]. The 30 water miscible ILs chosen were: (i)[Chol][H_2PO_4]; (ii) [Me_3IM][$MeSO_4$]; (iii) [$P_{4,4,4,1}$][$MeSO_4$]; (iv) [BMIM][acetate]; (v) [BMIM][Cl]; (vi) [BMIM][dca]; (vii) [BMPy][dca]; (viii) [BMPyr][Cl]; (ix) [MOEMIM][BF_4]; (x) [$P_{4,4,4,4}$][Bu_2Phos]; (xi) [EMIM][BF_4]; (xii) [MOEMIM][dca]; (xiii) [BMIM][TFES]; (xiv) [Epy][$EtSO_4$]; (xv) TEGO IL P9; (xvi) [BMIM][SCN]; (xvii) [BMIM][BF_4]; (xviii) [HMIM][Cl]; (xix) [MOEMIM][BF_4]; (xx) [$P_{6,6,6,14}$][C_9COO]; (xxi) [MOMMIM][dca]; (xxii) [OMIM][dca]; (xxiii) [BMIM][MeCOO]; (xxiv) [BMIM][HSO_4]; (xxv) [Moxa][$MeSO_4$]; (xxvi) [MMPy][$MeSO_4$]; (xxvii) TEGO IL T16ES; (xxviii) TEGO IL K5MS; (xxix) [$P_{14,14,14,1}$][TOS] and (xxx) [BMPy][BF_4]. The enzyme reaction was also carried out in the absence of ionic liquid under the same conditions to compare the efficiency of ILs for use as reaction media.

Screening of the ILs identified 13 ILs that enhanced the laccase activity and 3 that did not affect the enzymatic activity. The rest of the ILs decreased the laccase activity significantly. These results provided evidence that ILs can strongly affect enzyme structure. The effect can be positive, e.g., modification of the native structure, or negative, such as the denaturation or/and deactivation of the laccase.

[Insert Rev. Table 1 about here]

Of the 13 ILs that enhanced laccase activity, 10 were water-immiscible ILs. As seen in Table 1, the presence of the ionic liquid [Chol][H_2PO_4] in the reaction medium greatly enhances the catalytic activity of the enzyme (residual relative activity of 451%). Other ionic liquids in which the laccase enzyme showed greater activity than the control (residual relative activity between 6 and 30%) were: [OMIM][NTf_2], [$P_{14,14,14,6}$][dca], [BMPi][$NTfF_2$], [BMIM][PF_6], [PEy][NTf_2], [$S_{2,2,2}$] [NTf_2], [OMIM][PF_6], [Me_3IM][$MeSO_4$], [$P_{6,6,6,14}$][NTf_2], [BMIM][NTf_2], [EMIM][NTf_2] and [$P_{4,4,4,1}$][$MeSO_4$]. In the ionic liquid [$N_{8,8,8,1}$][NTf_2], [Chol][NTf_2] and [BMIM][acetate] the enzyme behaved in a similar way as it does in the buffered medium. Moreover, some of the ionic liquids used originated strong enzyme deactivation: [BMPy][BF_4], [$P_{14,14,14,1}$][TOS], [MOMMIM][PF_6], TEGO IL K5MS, TEGO IL T16ES, [Moxa][$MeSO_4$], [THP][Cl], [BMIM][HSO_4], [$P_{14,14,14,6}$][Br], [BMIM][MeCOO], [MMPy][$MeSO_4$] and [$P_{14,4,4,4}$][$C_{12}BzO_3$], among others.

Regarding the cation or anion composition of the ionic liquid, as can be seen from Table 1, the activity of laccase was mostly dependent on the anion composition, the highest laccase activity being obtained with ionic liquids based on the dihydrogen phosphate (H_2PO_4) and bis [(trifluoromethyl)sulfonyl]imide (NTf_2) anions, neither of which is very nucleophilic in character due to the delocalization of the negative charge on its resonance forms. In contrast, anions with a strongly localized charge, and therefore more nucleophilic in character, such as tetrafluoroborate (BF_4), are able to interact more strongly with the enzyme, affecting its active conformation by interacting with the positively charged sites in the enzyme structure, and thus adversely affecting its catalytic activity. A similar behaviour has been described for the enzyme Candida antarctica lipase B in ionic liquid media [8]. In this context, it was found that eight [NTf_2]-based ionic liquids improved the laccase activity: [OMIM][NTf_2], [EPy][NTf_2], [$S_{2,2,2}$][NTf_2], [BMP_i][NTf_2], [$P_{6,6,6,14}$][NTf_2], [BMIM][NTf_2] and [EMIM][NTf_2] - by 32%, 25%, 13%, 23%, 16%, 15% and 6%, respectively. These results are in agreement with those reported by Rehmann et al [18], who also found that [NTf_2] $^-$-based ionic liquids in combination with the cations pyridinium, [EPy][NTf_2], quaterny-ammonium, [$N_{8,8,8,1}$][NTf_2], and tetraalkylphosphonium, [$P_{6,6,6,14}$][NTf_2], are biocompatible with T. versicolor laccase. However, we identified one water-miscible IL, [Chol][H_2PO_4], that greatly enhances the enzymatic activity of lacasse., Xue et al [19] also found that [Chol][acetate] ionic liquid enhanced the catalytic performance of lipase before confirming the ability of choline derivative-based ionic liquids to enhance several enzymatic activities. On the other hand, Yu et al. [20] evaluated the effect of three trifluoromethanesulfonate ionic liquids on the activity of laccase (1-butyl-3-methylimidazolium trifluoromethanesulfonate [BMIM][TfO], 1-butyl-1- methylpyrrolidinium trifluoromethanesulfonate [BMPyr][TfO], tetramethylammonium trifluoromethanesulfonate [TMA][TfO]) and found that these ILs are not good activating agents, as is confirmed in the present work. Recently, Moniruzzaman and Ono [5] tested [EMIM][acetate] as reaction medium in the enzymatic delignification of wood using laccase and they found that the activity of laccase decreased slightly. In contrast, in this study we found that a quite similar ionic liquid [BMIM][acetate] does not

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affect the enzyme activity. Recently, another approach showed that [C_6MIM][AOT] protects the laccase against redox mediator deactivation and helps retain up to 50% of the residual activity [11].

As mentioned above, the presence of the ionic liquid [Chol][H_2PO_4] in the reaction medium greatly enhanced the catalytic activity of laccase. In aqueous solution, the effect of ions on enzyme activity has been found to correlate with kosmotropicity according to the Hofmeister series [21]. In fact, in previous works it was found that kosmotropic anion and chaotropic cation stabilize the enzyme [21, 22], while the inverse causes a destabilising effect [18]. For example, Hinckley et al [23] described, as we do in this work, that [MBPy][BF_4] greatly decreases the laccase activity and then confirmed that, in case of a chaotropic anion such as BF_4^-, the effect on enzymatic protein can be highly destructive [24]. In the case of the IL [Chol][H_2PO_4], the laccase showed a significantly high activity, which could be attributed to the kosmotropicity of the [H_2PO_4] anion and to the chaotropicity of the [Chol] cation [25]. In fact, the [H_2PO_4] anion stabilizes the water environment of the enzyme due to its small size and its strong charge density and the [Chol] cation contributes to decreasing the hydrophobicity in the hydrophobic region of the enzyme and then acts on the secondary structure of the enzyme [25]. Furthermore, the protic anion [H_2PO_4] may be self-buffered and have hydrogen bond-accepting and -donating properties which could favor enzymatic stability [26, 27, 28]. ILs based on ammonium cations have also been found to be an excellent choice for green application due to their high biodegradability and low toxicity [29]. Due to the great ability of the IL [Chol][H_2PO_4] to enhance laccase activity, this ionic liquid was chosen for the subsequent studies.

Effect of [Chol][H_2PO_4] concentration on laccase activity and related kinetic parameters

The effect of [Chol][H_2PO_4] concentration on laccase activity was evaluated using IL solutions with concentrations ranging from 0 to 1000 mM in sodium acetate buffer (50 mM; pH 7.0). As can be seen from Fig. 1-A, the activity of laccase dramatically increased up to an ionic liquid concentration of 300 mM, above which the activity fell slightly. Therefore, the enzyme does not seem to be negatively affected by high ionic liquid concentrations. This latter is in contrast with the results reported by other authors [5, 20] who found that the increase of IL concentration significantly affected the laccase activity and, in some cases, it caused the denaturation of the enzyme. ***[Insert Table 2 about here]***

In order to understand the effect of IL concentration on the enzyme kinetic, a Lineweaver-Burk plot was made (see Fig. 1-B) using three ionic liquid concentrations: 10, 300 and 1000 mM. As shown in Fig. 1-B, the kinetic plot is similar to that for competitive-uncompetitive substrate compounds in Michaelis Menten theory. Table 2, which summarizes the kinetic parameters, shows that Vm increased with increasing IL concentration, demonstrating the improvement in the catalytic action of the enzyme that can be obtained with increasing concentrations of IL. The apparent K_m increased from 36 µM (in absence of IL) to 52.6 µM (at 1M [Chol][H_2PO_4]), which suggests that the affinity of the enzyme towards ABTS decreases when the [Chol][H_2PO_4] concentration increases, reflecting a substrate-competitive effect. However, the catalytic efficiency ($K_{cat}/K_{m\text{-}app}$) increased about 5 times and the number of turn-over, K_{cat}, increased about 8 times (see Tab. 2) when the [Chol][H_2PO_4] concentration was raised to 1000 mM, demonstrating in this case an uncompetitive effect. It seems that the IL [Chol][H_2PO_4] affects the enzyme conformation which in turn affects both the affinity of the enzyme and its activity. This hypothesis was confirmed by CD and FT-IR analysis (see section 3.5).

[Insert Rev. Figure 1 about here]

Improving the stability of laccase vs. temperature and pH by using [Chol][H_2PO_4]

Many authors have focused on improving enzyme stability by using water-immiscible ILs [5, 20]. However, very few works have reported on the stability of enzymes in water-miscible ILs. In this work, the water-miscible IL [Chol][H_2PO_4] was evaluates for its effect on the laccase stability in the face of changes in temperature and pH. As seen in Fig. 2-A , the use of [Chol][H_2PO_4] allowed a significant increase in the laccase activity at 50°C, 60°C and 70°C (10.5%, 46.5% and 19.5 % of residual activity, respectively), which confirms the thermal stabilizing effect of [Chol][H_2PO_4]. Similarly, Kurniawati and Nicell [30] reported that the rate of inactivation of *T. versicolor* laccase increases as the temperature is raised from 10 to 60°C, which, they suggested, points to the sensitivity of this enzyme to temperature and the importance of enhancing its activity at high temperature such as 70°C.

As regards pH stability, Fig. 2-B provides evidence that [Chol][H_2PO_4] was a very efficient IL for increasing the stability of the enzyme against basic pHs from 9 to 12. This IL was able to recover almost 100% of the residual laccase activity at pH 12 in contrast to the control medium, in which the enzyme was almost completely deactivated. This stability effect could be related to the buffer effect recorded by the anion [H_2PO_4]. Normally, laccase activity and stability decrease as the pH of the medium increases [31], especially fungal laccase which is more stable and active at an acidic pH than at basic values. In fact, Kurniawati and Nicell [30] reported that the maximal stability of *T. versicolor* laccase is between pH 6 and 7. However, recently a new alkali-stable laccase (at pH 7 to 9) isolated from *Trametes* sp. has been described as an efficient laccase for olive oil mill treatment[32]. Enhancing the laccase stability at a highly basic pH by means of [Chol][H_2PO_4] would be very interesting for applying this enzyme in textile or olive oil mill wastewater treatment since the pH of the conventional medium is usually high.

[Insert Rev. Figure 2 about here]

Use of [Chol][H_2PO_4] as preservative agent for storing laccase at room temperature

The storage stability at room temperature was measured by incubating the enzyme in solutions containing 50% (*v/v*) [Chol][H_2PO_4], [OMIM][NTf_2] or glycerol, respectively. The activity of the enzyme was followed for 6 months by means of the standard laccase activity experiment (see section 2.2) and the results were compared with those obtained with the control medium, 100% H_2O. As shown in Table 3, the half-life time of laccase (*T. versicolor*) in the medium containing 50% (*v/v*) [Chol][H_2PO_4]/H_2O was almost 80 days, which is 3.5 times

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higher than that obtained with 100% H_2O. Almost the same improvement was obtained for two another bacterial laccases: 3.6 fold improvement in the case of SmLac from *S. maltophilia* and 2.6 fold in the case of PPO from *M. mediterrannea* MMB1 (see Tab. 3). The medium containing the water-immiscible IL [OMIM][NTf_2] allowed a slight improvement in the half life time with respect to the control medium (see Figure 3). This might be because traces of this ionic liquid dissolve in the aqueous solution and slightly improve the stability, since the stabilizing effect of this ionic liquid has been demonstrated for other enzymes [33]. However, glycerol solution, which is a conventional medium for enzyme preservation had an inhibiting effect since it decreased the half life time of laccase by about 28% with respect to the control medium. Therefore, the glycerol solution is not recommended for preserving laccase activity. However, it is very difficult to maintain laccase stability at room temperature as described in this work (almost 25°C), although many authors have reported that the laccase stability can be maintained for several months at 4°C in buffer solutions such as sodium acetate buffer [34] or at -20°C in phosphate buffer [35] (*Cerrena unicolor* and *Agaricus bisporus* laccase, respectively). Smith et al. recovered *T. sanguinea* laccase activity using the organic additive acetonitrile (1%) at 40°C [36] but denaturation occurred rapidly and the activity lost after 24h. For the same purpose, by a classical way, *T. sanguinea* laccase has been lyophilized but the powder needed to be conserved at 4°C [36] and re-solubilisation of the protein can affect residual activity. In all cases, therefore, authors mention the importance of storing laccase in cool or freezing conditions, which underlines the importance of using [Chol][H_2PO_4] as preserving agent since this IL allowed the stability of three different types of laccase (without purification and concentration) to be maintain during storage at room temperature.

[Insert Rev. Table 3 about here]

[Insert Figure 3 about here]

Study of the effect of [Chol][H_2PO_4] on the molecular structure of laccase

1 FT-IR

FT-IR has been used to detect the vibration of bands in the chemical analysis of many chemical compounds since the 1950s [37]. It has also been used to determine the secondary structure of proteins based on the vibration of the amide bond linking two consecutive amino-acids. Amide I and amide II bands are two major bands of the protein infrared spectrum, while Amide band III has been poorly described since the absorbance is very weak in the infrared region [38]. Amide type I band is mainly associated with the C=O stretching vibration and is directly related with the backbone conformation, and Amide type II band results from the N-H bending vibration and from the C-N stretching vibration [39, 40]. These bands are sensitive to second conformation changes. Depending on whether the amide band radical is engaged or not into with hydrogen bond of α-helix, β-sheets structure or random coil, the IR spectrum will change, making it possible to deduce the secondary structure [41].

Fig. 4-A shows that the absorbance of Amide II changes with the concentration of [Chol][H_2PO_4]: the absorbance decreases as the [Chol][H_2PO_4] concentration increases from 0 to 10 mM, but the peaks decrease when the [Chol][H_2PO_4] concentration increases from 10 mM to 1M. Depending on whether NH Amide II binds to water molecules or not, the enzymatic protein will have a different affinity for the ionic liquid. The balance between IL and H_2O changes with the IL concentration, which affects the interaction between the enzyme and the water. In fact, at 10 mM [Chol][H_2PO_4], the IL/H_2O balance affects protein affinity since the competition between water and IL to bind to the NH Amide II bond is very high. In contrast, at 1M, the IL/H_2O balance does not significantly affect the protein affinity since the IL bind to almost all the NH Amide II band (See Figure 5). At 300 mM, increasing the balance IL/H_2O could contribute to lower competition to link the protein and consequently increase the absorbance of the enzyme at this concentration. However, at 1M, the conformation of the enzyme is recovered since the IL will have fully occupied the micro-environment of the protein, and therefore the competition between water and IL is lower and the enzymatic protein will have returned to a conformation similar to the control, meaning that the absorbance has been recovered.

The second derivative (Fig. 4-B) pointed to the dominance of β-sheets conformers at 1618 cm^{-1} , 1645 cm^{-1} and β-turn and/or anti-parallel β sheets structure (1675 $cm^{-1}$and 1681 cm^{-1}). The large peak detected at 1645 cm^{-1} confirms that the laccase from *T. versicolor* is a β-sheet protein. Moreoever, two large peaks assigned to Amide II and Amide I, respectively, were recorded at 1583 cm^{-1} and 1648 cm^{-1}.

[Insert Figure 4 about here]

Then the second derivative was necessary to learn more about the secondary transformations due to [Chol][H_2PO_4] addition. Fig. 4-B shows that the increasing of [Chol][H_2PO_4] concentration gradient have a decreasing effect on β-sheet structure peaks absorption at 1645 cm^{-1}. In another hand, the α-helix band detected at 1658 cm^{-1} increased almost nine fold by the increasing of IL concentration to 10 mM, but decreased at 300mM and 1M (Fig. 4-B). The peaks modifications recorded at 1645 and 1658 cm^{-1}, previously described, indicated an influence on both α and β structures in the enzyme protein by IL addition. The shift of the Amide II band from 1583 cm^{-1} to 1593 cm^{-1}, when increasing the IL concentration to 1M, indicates that the pending signal of NH band have changed and confirms the results obtained in Fig. 4-A concerning the competitiveness between water and IL in the hydrogen NH band. In fact, the Amide II band was recorded at 1583 cm^{-1} for 0, 10 and 300 mM of ionic liquid, which indicates that the water dominate the NH band in these cases. However, at 1M, the peak was recorded at 1593 cm^{-1}, which indicates that ($H_2PO_4^-$) dominates the NH band in this concentration. Hydrogen bounding could also be the key to understanding the interaction of proteins and ionic liquids. Figure 5 showed that the anion ($H_2PO_4^-$) can easily have a stable hydrogen bond with the polypeptide backbone.

[Insert Rev. Figure 5 about here]

2 Circular Dichroism (CD) and Fluorescence

Circular dichroism is the most widely used technique to determine the secondary structure of proteins. The development of mathematical methods has enabled scientists to recognize not only protein structures like β sheets and α helix [20, 42], but also changes in tertiary global structure. In fact, analysis in the far-UV domain (180 – 250 nm) could be useful for the calculating the secondary structure, while analysis in the near-UV domain (250 –

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350 nm) could provide information about the tertiary structure. Figure 5 shows the profile (5-A) deduced from the original CD machine spectrum and the profile estimated (5-B) by the calculation method used in DICHROWEB website, K2D [43, 44], one of the few neural network programs available. The neural network operates via an input layer with neurons interconnecting with the output layer. The output layer (secondary structure) is calculated as a function of the input layer (CD data) by assigning weightings to each neuron. In K2D, the weights file is fixed and so there is no choice of reference dataset. The results for beta sheet and mixed proteins tend to be far less accurate than those for helical proteins, although, compared with other methods [45], these results are an improvement.

[Insert Rev. Figure 6 about here]

The far-UV spectrum (Fig. 5-A) showed that, no significant alterations,were found in the secondary structure of laccase at the concentrations of 10 mM and 1 M, compared with the control system without the IL [Chol][H_2PO_4]. In fact, the K2D calculation pointed to 2% α-helix, 51% β-sheets and 47 % random coil in three cases (See Table 4). A slight difference in the secondary structure was observed at 300 mM [Chol][H_2PO_4], which corresponds to the optimal concentration determined for laccase activity (see Figure 1). In this case, a conversion of 1% β-sheets to α-helix was observed, which indicates that modification of the secondary structure was accompanied by an overall change in the enzyme protein structure. The specific interaction between a peptide chain and a dihydrogen phosphate ion (presented in Fig. 5) could also explain the shift of 1% β-sheet to α-helix form.

[Insert Table 4 about here]

The near-UV spectrum (Fig. 6) showed a change in the band at 294 nm, corresponding to Tryptophan residue dissymmetry, and a change in the band at 275 nm ,corresponding to Tyrosine residue dissymmetry. In this context, it was observed that the CD signal at 294 nm decreased from 0.227 to -0.234 at 10 mM [Chol][H_2PO_4] and to -0.071 at 300 mM [Chol][H_2PO_4] (Fig. 6-A, 6-B). However, the same signal increased to 0.338 at 1M [Chol][H_2PO_4] (Fig. 6-C). Furthermore, at 275 nm, the CD signal decreased steadily from 0.947 to 0.007,-0.643 and -1.08792, respectively with the increase in [Chol][H_2PO_4] concentration from 10 mM to 1M. In fact, the laccase of *Trametes versicolor* used in this work contains 6 Tryptophan residues in positions 87, 97, 129, 173, 470 and 505, and 14 Tyrosine residues, many of which were concentrated in the middle of the aminoacid sequence, between 138 and 279. As reported in the literature [7], the active site of *T. versicolor* laccase involves four coppers fixed by 9 His residues and one Cys. It is important to consider that some Tryptophan residues are close to the aminoacids participating in the constitution of the three copper centers I, II and III. In fact, Try_{87} is engaged between His_{86} and His_{88}, and is responsible for fixing the copper between types II and III, respectively; meanwhile, Try_{129} is very close to another pair of His (His_{131} and His_{133}) which are engaged in the copper center type III. Therefeore, any change in the CD signal of the Try residues could indicate a change in the conformation of the active site. The decrease in the CD signal when adding 10 or 300 mM [Chol][H_2PO_4] led to a change in the conformational structure of the protein, meaning a less exposed active site. However, at 1M of [Chol][H_2PO_4], the active site could be more exposed. This results confirmed those found by CD by far-UV, which showed that the least square distances decreased at 300 mM but increased at 1M of [Chol][H_2PO_4].

[Insert Rev. Figure 7 about here]

CD analysis by far and near UV confirmed that the protein underwent profound conformational changes, which positively affected the activity and stability of the enzyme.

Fluorescence showed that the absorbance peak is almost 6 times higher in water than in 300 mM [Chol][H_2PO_4] (data not shown). The *T. versicolor* laccase contains 1.2% Trp residues, 2.7% Tyr residues and 6.2% Phe residues. The decrease in fluorescence in the case of the enzyme incubated with [Chol][H_2PO_4] confirmed the modification in the secondary structure of the laccase. Moreover, it showed that the modification affects the whole 3D structure of the enzymatic protein. However, the most important residues are the four Trp localized near the laccase active site. The decreasing fluorescence signal provides information about the shift of the active site location in the overall structure of the enzyme.

Using [Chol][H_2PO_4] to enhance green decolorization bioprocesses

As previously described, the ionic liquid [Chol][H_2PO_4] is an excellent enhancer of laccase activity not only towards ABTS, but also towards other substrates. Some dyes have been seen to be removed by the laccase catalysis. In this respect, depending on the class of dye and enzyme structure, the catalytic pathway differs, and, in some cases, depends on redox mediators such as ABTS, acetosyringone, syringaldehyde or hydroxybenzotriazole [4, 46]. Recently, Rehmann et al [11] reported the protective role of hydrophobic ionic liquid of the laccase against the redox mediator deactivation. However, no report has described a similar effect for a soluble IL in improving laccase decolorization. The present study used two of the most widely used dye classes: azoic (Amido Black: Acid Black 1: CI 20470) and anthraquinonic (Remazol Brillant Blue R: Reactive Blue 19: CI 61200). Some authors have described that the laccase is efficient at decolorizing this type of dye but usually involve slow rate kinetics or electrochemical potential incompatibilities, which make the reaction impossible to run [46, 47, 48]. The use of redox mediators would seem to be a possible solution but involves certain problems such as the toxicity of some compounds in environmental processes and the deactivation effect on the enzyme itself [11, 42]. In the present study, the rate of dye decolorization by *T. Versicolor* laccase was improved by the addition of [Chol][H_2PO_4], which circumvents the use of redox mediators. In Fig. 7 the kinetic spectrum points to a substantial enhancement of the decolorization activity reached by the laccase. In fact, enhancements of 216 % and 137 % were observed for Amido Black (AB) (see Fig. 7-A, 7-B) and Remazol Brillant Blue R (RBBR) (see Fig. 7-C, 7-D), respectively. RBBR is an anthraquinonic dye which was described frequently in the decolorization by some fungal laccasessuch as that used by Soares *et al.,* [49] whoreported the essential need of redox mediators to decolorize RBBR. Recently, the use of laccase from *Trametes trogii* for decolorization has been confirmed to be strictly dependant on the use of redox mediators (natural or synthetic) to obtain a high rate of colour removal from pure dyes or textile effluent [47, 50]. If we consider the disadvantages of using

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redox mediators as described above, [Chol][H_2PO_4] would seem a good choice for this purpose.

[Insert Rev. Figure 8 about here]

Furthermore, an assay with a mix of dyes gave a very good decolorization rate of 80% by shaking for 8 h (as shown in Fig. 7-E). An important point to consider in this assay is that all the reactions were made at pH 7.0 with possibility to make the reaction in water (data not shown). The enhancement of laccase stability provided by [Chol][H_2PO_4] at basic pH values is quite encouraging for its use in the decolorization of textile effluents which exhibit have a high pH of up to 12, for example, in the case of the effluent used by Khelifi *et al.* [47]. The green character of the ionic liquid [Chol][H_2PO_4], and the wide range of applications of laccase , make [Chol][H_2PO_4] a promising choice for further applications in bio-catalysis and bio-processes.

Experimental

Materials

The enzyme used for this study, a fungal laccase (EC 1.10.3.2) produced by *Trametes versicolor,* was purchased from Sigma-Aldrich (Madrid, Spain). The substrates, ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), Amido Black (AB) (Acid Black 1: CI 20470) and Remazol Brillant Blue R (RBBR) (Reactive Blue 19: CI 61200) were purchased from Sigma-Aldrich and Fluka Chemicals Co. (Madrid, Spain) with the highest purity available. The Ionic Liquids (ILs) were obtained from different sources: The [BMIM][PF6], [OMIM][PF6], [EMIM][BF4], [BMIM][BF4] and [BMIM][HSO4] were purchased from Solvent Innovation. The ILs [EMIM][NTf2], [BMIM][NTf2], [BMIM][acetate], [BMIM][TFES], [BMIM][SCN], [HMIM][Cl] and [BMIM][MeCOO] were purchased from Sigma-Aldrich-Fluka Chemical Co. The ILs [$P_{6,6,6,14}$][NTf_2], [$P_{4,4,4,1}$][$MeSO_4$], [$P_{6,6,6,14}$][TMPPhos], [$P_{14,14,14,6}$][BF_4], [$P_{6,6,6,14}$][C_9COO], [$P_{14,4,4,4}$][$C_{12}BzO_3$], [$P_{14,14,14,6}$][Br], [THP][Cl] and [$P_{I4,I4,I4,1}$][TOS] were purchased from Strem Chemical Inc. The ILs [Epy][NTf_2], [BMPy][dca] and [MOPy][dca] were purchased from Lonza Chemical Inc. And [OMIM][NTf2] was from Merck KgaA. All the other ILs were purchased from IoLiTec Chemical Co (Germany).The purity of the ILs was high, generally 98 to 99%.

Laccase activity assay

Based on previous works [4, 46, 51], the laccase activity was measured by following the oxidation of ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), monitoring the kinetic absorbance at 420 nm (ε_{420}=36.000 mol l^{-1} cm^{-1}) using a UV-1650 PC Schimadzu Spectrophotometer. The reaction mixture contained 0.5 mM ABTS and 20 µg ml^{-1} laccase in 50 mM sodium acetate buffer pH 7.0. One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzed the appearance of 1 µmol of product per minute at 25°C.

Laccase activity assay using ionic liquids

To study the influence of ILs on laccase activity, a systematic study was carried out using 56 different ionic liquids, 26 of them water-immiscible and 30 water-miscible ILs (see Table 1). For this, 20 µg ml^{-1} of the enzyme was incubated in a 10 mM solution of each IL in sodium acetate buffer (50 mM; pH 7.0), shaking continuously for 30 min. After this, the reaction was started by adding 0.5 mM ABTS in the standard laccase assay conditions described above (pH 7.0 and 25ºC). Similar experiments were also carried out to evaluate the effect of IL concentration on laccase activity, using IL solutions ranging from 0 to 1000 mM in sodium acetate buffer (50 mM; pH 7.0). All experiments were carried out in duplicate and the mean values are reported. The efficiency of the catalytic action was measured by the residual relative activity defined with respect to the control experiment (in 50 mM sodium acetate buffer pH 7.0) as follows:

*Residual Relative Activity (%) = [(Activity in IL – Control Activity)/Control Activity]*100*

Laccase stability assay against temperature and pH

Enzyme stability at various pH values was determined by incubating the enzyme overnight in 50 mM buffer ranging from pH values 3.0 to 13.0. The residual relative activity was measured as described in Section 2.2 with respect to the control experiment at the standard pH 7.0.

Thermal stability was determined by incubating the enzyme at temperatures ranging from 25°C to 90°C for 60 min, after which a sample of enzyme was returned to ambient temperature and the residual enzyme activity was measured as described in Section 2.2. The IL effect was studied by incubating the enzyme at the corresponding pH or temperature, as detailed above, in the presence of 10 mM of [Chol][H_2PO_4]. The stability of the enzyme during storage at room temperature was also analysed in the presence of ionic liquids. For this purpose, the enzyme was incubated for 6 months in 50% (v/v) [Chol][H_2PO_4] / 50 mM sodium acetate buffer pH 7.0, 50% (v/v) [OMIM][NTf_2] / 50 mM sodium acetate buffer pH 7.0 and 50% glycerol / 50 mM sodium acetate buffer pH 7.0, respectively, and the activity was followed daily by using the standard laccase assay, as described above. All experiments were carried out in duplicate and the mean values are reported. The half-life times were obtained from the curves showing the relative laccase activity against time. The points obtained were fitted by using Excel software, from which a specific equation for each curve, generally following the model $Y = a*x^2 + b*x + c$, was obtained.

Decolorization assay

1.5 µl (the required volume to obtain 1.0 AU at 615 nm) Amido Black (AB) or 11 µl (the required volume to obtain 1.0 AU at 595 nm) Remazol Brillant Blue R (RBBR) was dissolved in 50 mM sodium acetate buffer pH 7.0. The reaction was started by adding 50 µg ml^{-1} of enzyme and run for 8 h or more at 25ºC. For the experiments in the presence of IL, the enzyme was incubated for 5 min in a 10 mM solution of [Chol][H_2PO_4] and 50 mM sodium acetate buffer pH 7.0 before starting the decolorization reaction. Dye degradation was monitored by the decrease in absorbance at 615 or 595 nm for AB and RBBR, respectively. The decolorization rate was calculated as the percentage of remaining absorbance per min. The kinetic evolution of absorbance spectrum of the bio-decolorization reaction was followed between 380 nm and 800 nm using a Shimadzu UV-1650PC

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spectrophotometer.

Spectroscopic analysis of the laccase

Fourier Transform Infra-Red spectroscopy (FT-IR)

The laccase (10 mg ml^{-1}) was incubated overnight in aqueous solutions of 10 mM, 300 mM and 1M of the ionic liquid [Chol][H_2PO_4] to evaluate the effect of the IL concentration on its enzymatic structure. FT-IR spectra of these samples were recorded using a Thermo Nicolet 5700FT-IR at wavelengths by 400 to 4000 cm^{-1}. In a second step, the results were refined in the wavelength range 1300 - 1800 cm^{-1}using the software Omnic. The spectra were corrected by subtraction of the background spectrum (water blank). Second derivative spectra were obtained with the Savistsky-Golay algorithm for a polynomial order 3 and 33 data point window with Omnic software.

Circular Dichroism (CD)

CD analysis was carried out using a Π*180 spectrophotometer (Applied Biophysics) equipped with N_2 purge and a Peltier system for temperature control. The enzyme (0.25 mg ml^{-1}) was incubated overnight in a water solution prepared at three different [Chol][H_2PO_4] concentrations, 10 mM, 300 mM and 1M at 4ºC. The structure of the incubated enzyme was analyzed at two different wavelength ranges "near UV, 250 – 350 nm" and "far UV,180 – 250 nm" at 25ºC. Spectra were recorded at a 10 nm/min scan speed with a response time of 1 s, and 1 nm bandwidth. For the far-UV (180 – 250 nm) spectra, 0.1 cm cells were used, while in the case of the near-UV (250-300 nm) spectra, measurements were made with 1.0 cm cells. The spectra were made in triplicate and averaged to eliminate signal noise. For each IL concentration, it was necessary to subtract a blank medium without enzyme to discard its influence on the enzyme CD spectrum. The webpage "DicroWeb" (http://www.cryst.bbk.ac.uk/cdweb/html/) provides the K2D curve-analysis algorithm which was used for estimating protein secondary structure of the enzyme in the presence of three different concentrations of [Chol][H_2PO_4] (10 mM, 300 mM and 1M).

Fluorescence Spectroscopy

The laccase (10 mg ml^{-1}) was incubated overnight in a water solution containing 300 mM [Chol][H_2PO_4] at 4ºC. Two controls were prepared to follow the fluorescence spectroscopy: 10 mg ml^{-1} laccase in water solution and a 300 mM water solution of [Chol][H_2PO_4]. The sample was analyzed in an HPLC with fluorescence detection using H_2O as mobile phase at a flow rate of 0.5 ml min^{-1} at 25ºC. The excited wavelength was fixed at 295 nm and the emission was registered from 305 to 450 nm using a 5 nm bandwidth in both excitation and emission path.

Inserting Graphics

The graphs should be inserted where they are first mentioned (unless they are equations, which appear in the flow of the text). They can be single column or double column as appropriate.

Conclusions

The ionic liquid [Chol][H_2PO_4] was seen to be very effective at enhancing and stabilizing the laccase activity, due to the modifications in the secondary structure of the enzymatic protein that it produced, modifications that correlated well with the activity measured. This transformation was related to the kosmotropic nature of the anion $H_2PO_4^-$ acting in accordance with the Hofmeister series in aqueous medium. The chaotropic nature of the cation $Chol^+$ contributed equally with the anion to the stabilization and activation of the laccase as described above. CD demonstrated that a shift of 1% β-sheet structure to α-helix at 300 mM correlated with the optimal activity recorded by changing the IL concentration. FTIR confirmed that a modification in the balance between α/β structures could be recorded as a change in the absorbance of the band Amide I and Amide II bonds. An increase in IL concentration might affect the hydrogen-donating and -accepting properties with water and affect the interaction of the NH band with the micro-environment of the enzyme. As demonstrated in this work, the anion ($H_2PO_4^-$) forms a stable hydrogen bond with the polypeptide backbone, which affects the enzyme conformation. In fact, fluorescence studies show that modification of the active site location could contribute to enhancing the laccase activity. Its environmental friendly character make the ionic liquid [Chol][H_2PO_4] an excellent added compound to the wide range of laccase applications such as the decolorization processes, since an 80 % removal rate was obtained with a mixture of different types of industrial dyes.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

‡ Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

1 S. Park, R. J. Kazlauskas, *Curr. Opin. Biotech.*, 2003, **14**, 432–437.
2 R. K. Singh, M. K. Tiwari, R. Singh and J. K. Lee, *Int. J. Mol. Sci.*, 2013, **14**, 1232–1277.

RSC Advances Accepted Manuscript

3 A. P. M. Tavares, B. Pinho, O. Rodriguez and E. A. Macedo, *Procedia Engineering*, 2012, **42**, 226–230.
4 S. Galai, H. Korri-Youssoufi and M. N. Marzouki, *J. Chem. Technol. Biot.*, 2014, **89(11)**, 1741–1750.
5 M. Moniruzzaman, T. Ono, *Biores. Technol.*, 2013, **127**, 132–137.
6 K. J. Greimel, V. Perz, K. Koren, R. Feola, A. Temel, C. Sohar, E. H. Acero, I. Klimant and G. M. Guebitz, *Green Chem.*, 2013, **15**, 381–388.
7 H. Liu, L. Zhu, M. Bocola, N. Chen, A. C. Spiessb and U. Schwaneberg, *Green Chem.*, 2013, **15**, 1348–1355.
8 A. Ruiz, A. P. de los Ríos, F. J. Hernández, M. H. A. Janssen, R. Schoevaart, F. van Rantwijk and R. A. Sheldon, *Enzyme Microbiol. Technol.*, 2007, **40**, 1095–1099.
9 A. P. de los Ríos, F. J. Hernández-Fernández, D. Gómez, M. Rubio and G. Víllora, *Appl Catal B: Environ.*, 2006, **67**, 121–126.
10 F. J. Hernández-Fernández, A. P. de los Ríos, L. J. Lozano-Blanco and C. Godínez, *J. Chem. Technol. Biot.*, 2010, **85**, 1423–1435.
11 L. Rehmann, E. Ivanova, H. Q. N. Gunaratne, K. R. Seddon and G. Stephens, *Green Chem.*, 2014, **16**, 1462–1469.
12 R. A. Sheldon, *Chem. Commun.*, 2001, **23**, 2399–2407.
13 L. J. Lozano, C. Godinez, A. P. de los Rios, F. J. Hernandez-Fernandez, S. Sanchez-Segado, F. J. Alguacil, *J. Membrane Sci.*, 2011, **376**, 1–14.
14 F. Tomás-Alonso, A. M. Rubio, R. Álvarez and J. A. Ortuño. *Int. J. Electrochem. Sci.*, 2013, **8**, 4955–4969.
15 M. S. Khehra, H. S. Saini, D. K. Sharma, B. S. Chadha and S. S. Chimni, *Dyes Pigments*, 2004, **70**, 1–7.
16 M. Bhaskar, A. Gnanamani, R. J. Ganeshjeevan, R. Chandrasekar, S. Sadulla and G**.** Radhakrishnan, *J. Chromatogr. A*, 2003, **1018**, 117–123.
17 E. Forgacs, T. Cserhàti, G. Oros, *Environ. Int.*, 2004, **30**, 953 – 971.
18 L. Rehmann, E. Ivanova, J. L. Ferguson, H. Q. N. Gunaratne, K. R. Seddon, G. M. Stephens, *Green Chem.*, 2012, **14**, 725–733.
19 L. Xue, Y. Zhao, L. Yu, Y. Sun, K. Yan, Y. Li, X. Huang and Y. Qu, *Colloid Surface B-Biointerfaces*, 2013, **105**, 81–86.
20 X. Yu, F. Zou, Y. Li, L. Lu, X. Huang and Y. Qu, *Int. J. Biol. Macromol.*, 2013, **56**, 62–68.
21 F. Hofmeister, *Arch. Exp. Pathol. Pharmakol.*, 1888, **24**, 247–260.
22 D. Constantinescu, H. Weingrtner and C. Herrmann, *Angew. Chem.,* Int. Ed., 2007, **46**, 8887–8889.
23 G. Hinckley, V. V. Mozhaev, C. Budde and Y. L. Khmelnitsky, *Biotechnol. Lett.*, 2002, **24**, 2083–2087.
24 H. Zhao, O. Olubajo, Z. Song, A. L. Sims, T. E. Person, R. A. Lawal and L. D. A. Holley, *Bioorg. Chem.*, 2006, **34**, 15–25.
25 A. Kumar and P. Venkatesu, *Int. J. Biol. Macromol.*, 2014, **63**, 244–253.
26 A. P. de los Ríos, F. van Rantwijk and R. A. Sheldon, *Green Chem.*, 2012, **14**, 1584–1588.
27 R. M. Lau, M. J. Sorgedrager, G. Carrea, F. van Rantwijk, F. Secundo and R. A. Sheldon. *Green Chem.* 2004, **6**, 483–487.
28 F. van Rantwijk, F. Secundo and R. A. Sheldon. *Green Chem.*, 2006, **8**, 282–286.
29 X. D. Hou, Q. P. Liu, T. J. Smith, N. Li and M. H. Zong, *PLOS ONE* (www.plosone.org), 2013, **8-3**, e59145-e.
30 S. Kurniawati, J. A. Nicell. *Bioresour Technol.,* 2008 , **99(16)**, 7825-7834.
31 U. N. Dwivedi, P. Singh, V. P. Pandey and A. Kumar. *J. Mol. Catal. B-Enzym.*, 2011, **68**, 117–128.
32 D. Daâssi, H. Zouari-Mechichi, A. Prieto, M. J. Martínez, M. Nasri, T. Mechichi. *World J Microbiol Biotechnol.*, 2013, **29(11)**, 2145-2155.
33 A. P. de Los Rios, F. J. Hernandez Fernandez, F. A. Martinez, M. Rubio and G. Villora. *Biocatal. Biotransfor.*, 2007, **25(2-4)**, 151–156.
34 A. Michniewicz, R.Ullrich, S. Ledakowicz, M. Hofrichter. *Appl. Microbiol. Biotechnol.*, 2006, **69**, 682–688.
35 D. A. Wood. *J. Gen. Microbiol.,* 1980, **117**, 327–338.
36 U. Munusamy, V. Sabaratnam, S. Muniandy, N. Abdullah, A. Pandey, E. Jones. *J. Biol. Sci.,* 2008, **8**, 866–873.
37 Kendrew et al. *Nature*, 1958, **18**, 662–666.
38 I. H. Parvez and F. Severcan, *J. Mol. Catal. B-Enzym.*, 1999, **7**, 207–221.
39 D. M. Byler and H. Susi, *Biopolymers*, 1986, **25**, 469–487.
40 W. K. Surewicz and H. H. Mantsch, *Biochem. Biophys. Acta*, 1988, **952**, 115–130.
41 J. K. Kauppinen, D. J. Moffatt, H. H. Mantsch and D. G. Cameron, *Appl. Spectr.*, 1981, **35**, 271–276.
42 A. Lobely, L. Whitmore and B. A. Wallace, *Bioinformatics*, 2002, **18**, 211–212.
43 L. Whitmore and B. A. Wallace. *Nucleic Acids Research*, 2004, **32**, Web Server issue (W668–W673) DOI: 10.1093/nar/gkh371.
44 L. Whitmore and B. A. Wallace, *Biopolymers*, 2008, **89-5**, 392–400 (2007 Wiley Periodicals, Inc).
45 N. J. Greenfield, *Anal Biochem.*, 1996, **235(1)**, 1–10.
46 S. Galai, P. Lucas-Elio, M. N. Marzouki and A. Sanchez-Amat, *J. Appl. Microbiol.*, 2011, **111**, 1394–1405.
47 R. Khlifi, L. Belbahri, S. Woodward, M. Ellouz, A. Dhouib, S. Sayadi and T. Mechichi, *J. Hazard. Mater.*, 2010, **175**, 802–808.
48 L. Pereira, A. V. Coelho, C. A. Viegas, M. D. S. Correia Margarida, M. P. Robalo and L. O. Martins, *J. Biotechnol.*, 2009, **139**, 68–77.
49 M. B. Soares Graça, M. T. Pessoa de Amorim, M. Costa-Ferreira, *J. Biotechnol.*, 2001, **89**, 123–129.
50 E. Grassi, P. Scodeller, N. Filiel, R. Carballo and L. Levin, *Int. Biodeter. Biodegr.*, 2011, **65**, 635–643.
51 M.L. Niku-Paavola, E. Karhunen, P. Salola and V. Raunio, *Biochem. J.* 1988, **254**, 877–884.

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Over-activity and stability of laccase using ionic liquids: Screening and application in dyes decolorization

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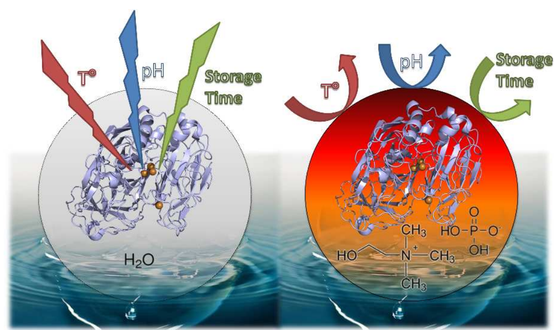

Protective effect of the ionic liquid [Chol][H_2PO_4] against temperature, pH, and storage time on*Trametes Versicolor* Laccase.

Abstract

The use of a wide range of water miscible and immiscible ionic liquids (ILs) as reaction media for ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) oxidation by *Trametes versicolor* laccase was studied. Thirteen ILs were shown to be suitable media for the laccase oxidation reaction, increasing the activity with respect to conventional media. Among them, the water-miscible IL choline dihydrogen phosphate [Chol][H_2PO_4] allowed over-laccase activity with an enhancement rate of 451% at 25°C and pH 7.0. This ionic liquid improved the stability of the enzyme in the face of high temperature and high pH, while storage at room temperature in aqueous medium was increased up to 4.5 times. Moreover, it was found that its use in the reaction medium for decolourizing dyes (antraquinonic and azoic) using laccase increased the decolourization rate by up to 216% and 137% for the azoic dyes Acid Black 1 and Remazol Brillant Blue R, respectively. A high decolorization rate was also obtained for a mix of dyes (80% within 8 h). To understand the effect of [Chol][H_2PO_4] on the secondary protein structure of the laccase, several spectroscopic techniques were used such as Circular Dichroism (CD), Fourier transform infrared (FT-IR) and Fluorescence, all of which demonstrated that the β sheets structure was affected. A shift to α-helix structure [Chol][H_2PO_4] could be responsible of the enhancement of the enzyme activity observed at 300 mM.

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Table 1. Ionic liquids nomenclature, aqueous solubility and chemical structures are described vs. the residual relative laccase activities. The reaction mixture contained 10 mM from each IL prepared in sodium acetate buffer (50 mM; pH 7.0) supplemented by 0.5 mM ABTS and 20 µg mL^{-1} of laccase and the reaction was carried out at pH 7.0 and 25ºC.

Ionic Liquid	Solubility*	Chemical structure	Residual Relative Activity (%)
[Chol][H_2PO_4]	S	Choline dihydrogen phosphate	+451 ±9.0
[OMIM][NTf_2]	I	1-methyl-3-octylimidazolium {bis(trifluoromethyl)sulfonyl}imide	+30,5 ±2.0
[$P_{14,14,14,6}$][dca]	I	Tetradecyl(trihexyl)phosphonium dicyanamide	+29,5 ±2.5
[BMPi][NTf_2]	I	1-butyl-1-methylpiperidinium bis[(trifluoromethyl)sulfonyl]amide 99% Iolitec	+29 ±6.5
[BMIM][PF_6]	I	1-butyl-3-methylimidazolium hexafluorophosphate	+27 ±5.2
[Epy][NTf_2]	I	1-ethylpyridinium bis[(trifluoromethyl)sulfonyl]imide	+25 ±2.5
[$S_{2,2,2}$][NTf_2]	I	Triethylsulfonium bis[(trifluoromethyl)sulfonyl]imide	+18 ±3.0
[OMIM][PF_6]	I	1-methyl-3-octylimidazolium hexafluorophosphate	+17 ±5.0
[Me_3IM][$MeSO_4$]	S	1,2,3-trimethylimidazolium methylsulfate	+17 ±1.0
[$P_{6,6,6,14}$][NTf_2]	I	Trihexyl(tetradecyl)phosphonium bis[(trifluoromethyl)sulfonyl]amide	+16 ±1.1

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[BMIM][NTf2]	I	1-butyl-3-methylimidazolium {bis(trifluoromethyl)sulfonyl} imide	+14 ±2.0
[EMIM][NTf2]	I	1-ethyl-3-methylimidazolium bis((trifluoromethyl) sulfonyl)imide	+11 ±2.5
$[P_{4,4,4,1}][MeSO_4]$	S	Tributylmethylphosphonium methylsulphate	+6 ±0.1
$[N_{8,8,8,1}][NTf_2]$	I	Methyltrioctylamonium bis[(trifluoromethyl)sulfonyl]imide	0 ±1.0
$[Chol][NTf_2]$	I	Choline bis(trifluoromethylsulfonyl) imide	0 ±0.1
[BMIM][acetate]	S	1-butyl-3-methylimidazolium acetate	0 ±4.0
$[N_{8,8,8,1}][Cl]$	I	Methyltrioctyl ammonium chloride	-7 ±1.2
[BMIM][Cl]	S	1-butyl-3-methylimidazolium chloride	-8 ±4.5
$[OMIM][BF_4]$	I	1-methyl-3-octylimidazolium tetrafluoroborate	-15 ±5.0
[BMIM][dca]	S	1-butyl-3-methylimidazolium dicyanamide	-15 ±0.9
[BMPy][dca]	S	1-butyl-3-methylpyridinium dicyanamide	-15 ±3.2
[BMPyr][Cl]	S	1-butyl-1-methyl pyrrolidinium chloride	-16 ±0.5
$[BMPyr][NTf_2]$	I	1-butyl-1-methyl pyrrolidinium bis[(trifluoromethyl)sulfonyl]imide	-18 ±1.3
$[P_{8,8,8,8}][Br]$	I	Tetraoctylphosphonium bromide	-20,5 ±2.5

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$[MOEMIM][BF_4]$	S	1-methoxymethyl-3-methylimidazolium tetrafluoroborate	-25 ±2.5
$[P_{4,4,4,4}][Bu_2Phos]$	S	Tetrabutylphosphonium dibutyl phosphate	-32 ±0.5
$[EMIM][BF_4]$	S	1-ethyl-3-methylimidazolium tetrafluoroborate	-34 ±1.3
[MOEMIM][dca]	S	1-methoxyethyl-3-methylimidazolium dicyanamide	-34 ±3.5
[BMPyr][PF6]	I	1-Butyl-1-methylpyrrolidinium hexafluorophosphate	-37 ±0.8
[BMIM][TFES]	S	1-butyl-3-methylimidazolium 1,1,2,2-tetrafluoroethanesulfonate	-38 ±1.8
$[Epy][EtSO_4]$	S	1-ethylpyridinium ethyl sulfate	-38 ±3.5
TEGO IL P9	S	n= 5-15	-40 ±2.0
[BMIM][SCN]	S	1-butyl-3-methylimidazolium thiocyanate	-40 ±2.0
$[BMIM][BF_4]$	S	1-butyl-3-methylimidazolium tetrafluoroborate	-46 ±1.0
[HMIM][Cl]	S	1-hexyl-3-methylimidazolium chloride	-50 ±1.8
[MOPy][dca]	I	1-methyl-3-octylpyridinium dicyanamide	-50 ±2.0
$[P_{6,6,6,14}]$ [TMPPhos]	I	Trihexyl(tetradecyl)phosphonium bis 2,4,4-(trimethylpentyl)phosphinate	-57 ±1.2

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[EMPyr][NTf_2]	I	(CF3SO2)2N 1-ethyl-1-methyl pyrrolidinium bis[(trifluoromethyl)sulfonyl]imide	-66 ±0.5
[$N_{8,8,8,1}$][TfO]	I	CF_3SO_3 Methyltrioctylammonium triflate (Methyltrioctylammonium trifluoromethanesulfonate)	-69 ±8.5
[$P_{14,14,14,6}$][BF_4]	I	C_6H_{13} P C_6H_{13} C_6H_{13} $C_{14}H_{29}$ F B F F F Tetradecyl(trihexyl)phosphonium tetrafluoroborate	-73 ±2.0
[MOEMIM][BF_4]	S	1-methoxyethyl-3-methylimidazolium tetrafluoroborate	-79 ±1.1
[$P_{6,6,6,14}$][C_9COO]	S	C_6H_{13} P C_6H_{13} C_6H_{13} $C_{14}H_{29}$ C9H19 Trihexyl(tetradecyl)phosphonium decanoate	-88 ±0.8
[MOMMIM][dca]	S	1-methoxymethyl-3-methylimidazolium dicyanamide	-89 ±0.2
[OMIM][dca]	S	1-methyl-3-octylimidazolium dicyanamide	-91 ±2.2
[$P_{14,4,4,4}$][$C_{12}BzO_3$]	I	$MeOSO_3$ Tributyltetradecylphosphonium dodecylbenzenesulfonate	-95 ±0.7
[MMPy][$MeSO_4$]	S	CH_3 1,4 dimethyl pyridinium methylsulfate	-96,5 ±0.5
[$P_{14,14,14,6}$][Br]	I	C_6H_{13} P C_6H_{13} C_6H_{13} $C_{14}H_{29}$ Br Tetradecyl(trihexyl)phosphonium bromide	-98 ±0.5
[BMIM][MeCOO]	S	OCO2 1-butyl-3-methylimidazolium methylcarbonate	-98,7 ±0.1
[BMIM][HSO_4]	S	HSO4 1-butyl-3-methylimidazolium hydrogen sulfate	-99 ±0.5
[Moxa][$MeSO_4$]	S	Methyloxazolinium methylsulfate	-99 ±1.0

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[THP][Cl] CyPhos	I	Tetradecyl(trihexyl)phosphonium chloride	-99 ±0.1
TEGO IL T16ES	S	$EtOSO_3^-$ Tallow; m,n = 6-20	-100 ±0.0
TEGO IL K5MS	S	$MeSO_4^-$ Cocos; m,n = 5-10	-100 ±0.0
[MOMMIM][PF_6]	I	1-methoxymethyl-3-methylimidazolium hexafluorophosphate	-100 ±0.0
[$P_{I4,I4,I4,1}$][TOS]	S	Triisobutyl(methyl)phosphonium tosylate	-100 ±0.0
[BMPy][BF_4]	S	1-butyl-3-methylpyridinium tetrafluoroborate	-100 ±0.0

(*) **Solubility**: (I) means Insoluble ionic liquid and (S) means Soluble ionic liquid

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Table 2. Lineweaver-Burk study on the kinetic parameters of the laccase in the absence and the presence of different concentrations of [Chol][H_2PO_4].

[Chol][H_2PO_4] concentration(mM)	Km_{app} (μmol/L)	Vm ($\cdot 10^3$) (μmol/min)	Kcat (s^{-1})	Kcat/Km (M^{-1} s^{-1})
0	35.59	5.78	0.2312	6.488
10	40.57	12.8	0.511	12.603
300	51.18	42.7	1.708	33.371
1000	52.58	45.3	1.813	34.481

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Table 3. Half-life times (days) of different laccases exhibited by incubation in different media at room temperature (25°C).

Enzyme/*Origin*	**Control H_2O**	**50% (*v/v*) Glycerol**	**50% (*v/v*) [Chol][H_2PO_4]**	**50% (*v/v*) [OMIM][NTf_2]**
Laccase/ *Trametes Versicolor*	23.3±0.1	16.8±0.1	80±0.1	25.7±0.1
SmLac/ *Stenotrophomonas maltophilia*	4.1±1.2	-	14.9±2.7	-
PPO/ *Marinomonas mediterrannea* MMB1	5.3±0.5	-	12.2±1.0	-

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Table 4. CD secondary structure analysis by K2D method (DICHROWEB)

[Chol][H_2PO_4] concentration(mM)	**Helix α (%)**	**Sheet β (%)**	**R. Coil (%)**	**Least squares distances**
0	2	51	47	1.108
10	2	51	47	1.129
300	3	50	47	955
1000	2	51	47	1.150

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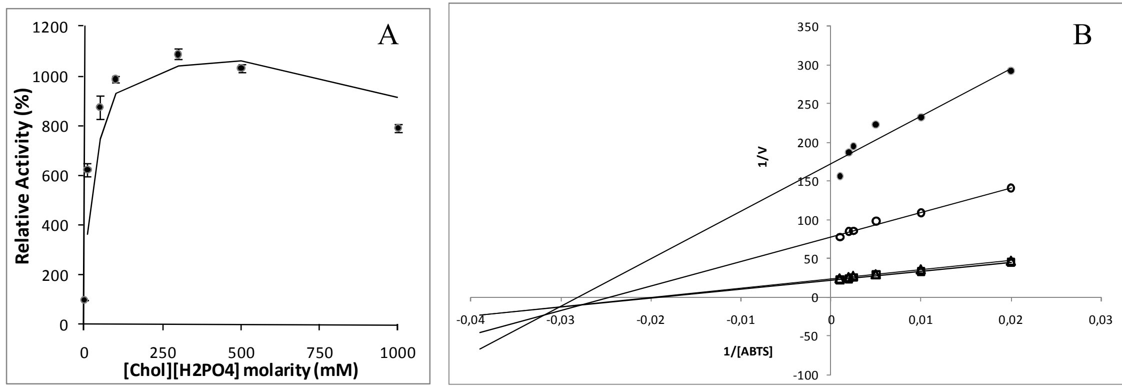

Figure 1. (A) Effect of [Chol][H_2PO_4] concentration in the enhancement of laccase activity has achieved by ABTS assay in pH7.0 and at 25°C in the absence or the presence of 10 to 1000 mM [Chol][H_2PO_4] **(B)** Lineweaver-Burk study on the kinetic parameter of the laccase has been carried out by ABTS as substrate in the absence (●) and the presence of (○) 10 mM [Chol][H_2PO_4], (△) 300 mM [Chol][H_2PO_4], (□) 1M [Chol][H_2PO_4] in pH7.0 at 25°C.

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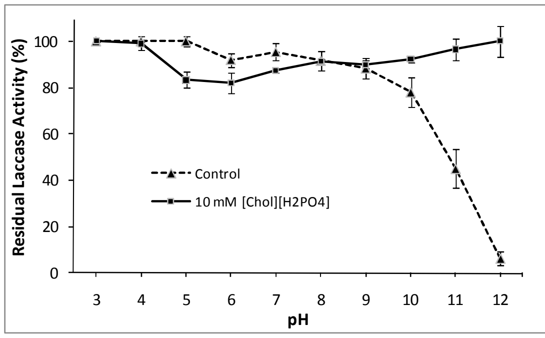

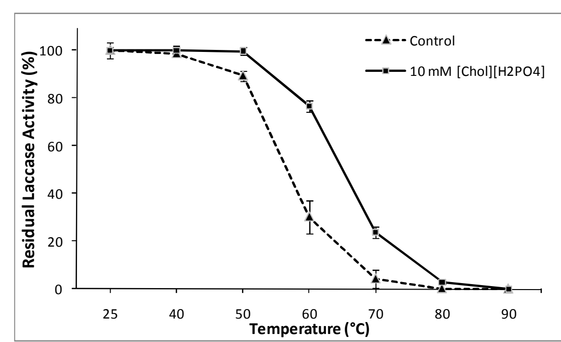

Figure 2. Effect of pH and temperature on the stability of laccase in the absence and the presence of 10 mM [Chol][H_2PO_4]. **(A)** Effect of pH on laccase activity was achieved by pre-incubation of the enzyme overnight in different pH varying from 3.0 to 12.0. **(B)** Effect of temperature on laccase activity was carried out by pre-incubation of the enzyme 1h in pH7.0 at different temperature varying between 25°C to 90°C. In the two experiments, standard ABTS assay was set up in pH 7.0 and at 25°C as described at section 2.4.

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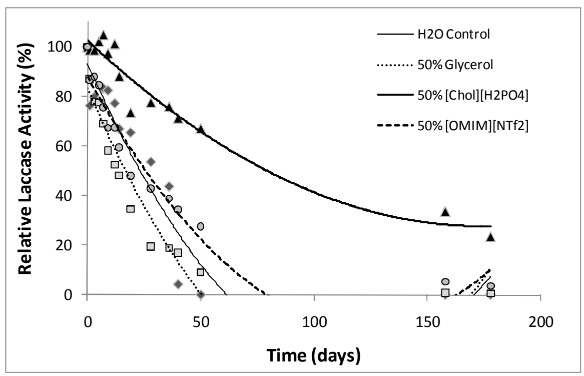

Figure 3. Storage stability at room temperature (25°C) of laccase in 100% H_2O (◆), 50% Glycerol (□), 50% [Chol][H_2PO_4] (▲), 50% [OMIM][NTf2] (●). The stability has been followed during six months according to the ABTS assay described in the material and methods section 2.2.

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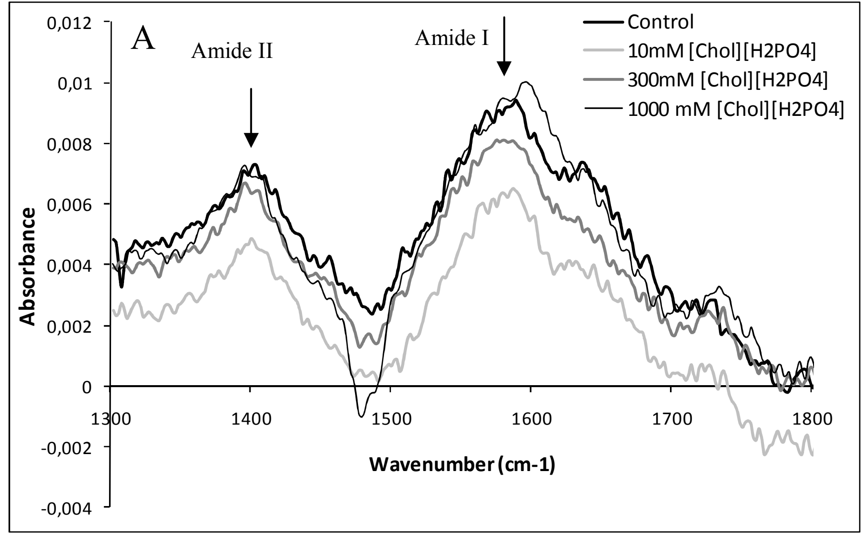

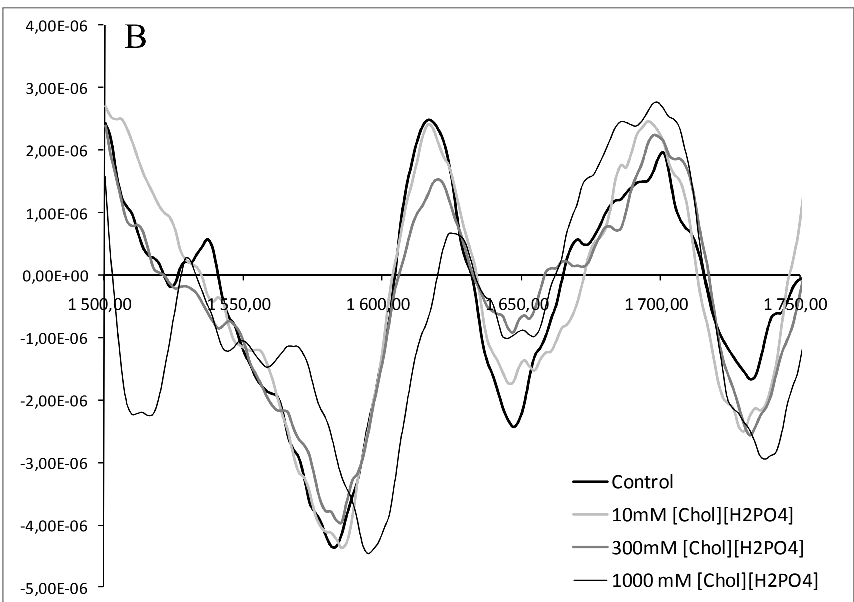

Figure 4. (A) FT-IR absorbance spectrum showing the [Chol][H_2PO_4] concentration effect on the amide structure of the enzymatic protein. **(B)** Second derivative FT-IR results showing the Amide I band and the influence of [Chol][H_2PO_4] on the secondary structure of laccase protein.

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Figure 5. (a) Schematic representation of hydrogen bonding in a beta-sheet structure; **(b)** Putative hydrogen bonds between a peptide chain and the dihydrogen phosphate ion.

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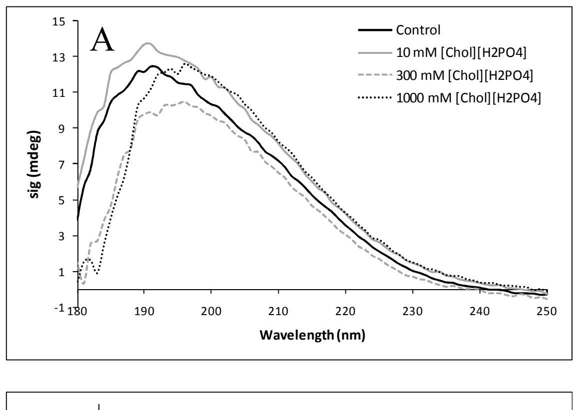

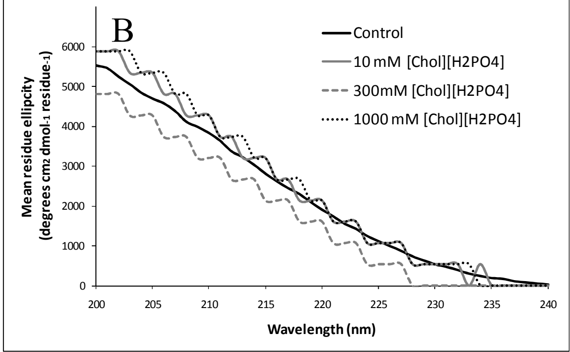

Figure 6. Far-UV CD spectra of laccase in water (Control) and in aqueous solutions containing 10 mM, 300 mM and 1M [Chol][H_2PO_4] after incubation overnight at 4ºC deduced directly from the Π*180 spectrophotometer_Applied Biophysics **(A)** and using K2D method (DICHROWEB) for secondary laccase structure calculation **(B)**.

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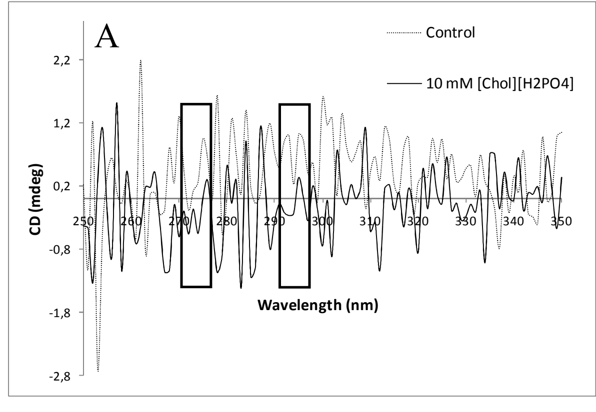

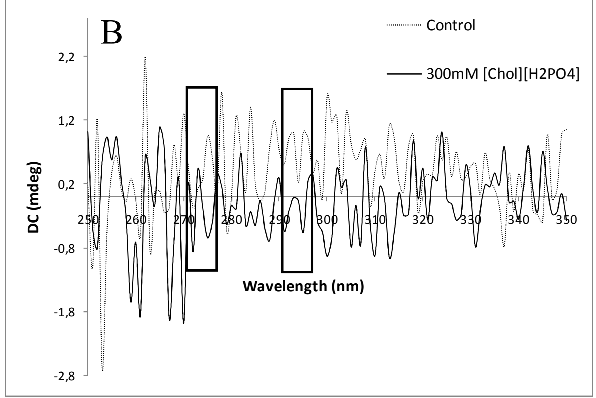

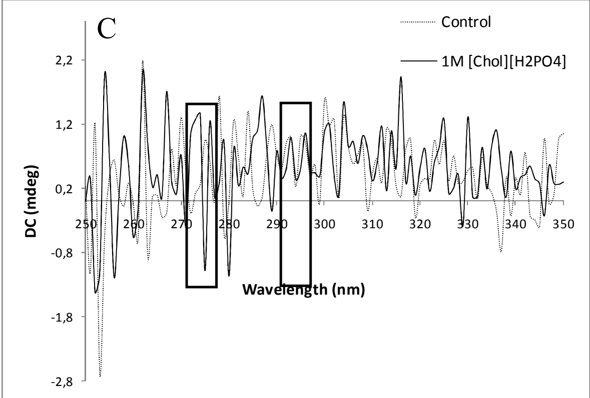

Figure 7. CD spectrums in the near UV (250-350 nm) of laccase in aqueous solution in the absence and the presence of 10 mM [Chol][H_2PO_4] and H_2O **(A)**, 300 mM [Chol][H_2PO_4] and H_2O **(B)** and 1M [Chol][H_2PO_4] and H_2O **(C)**. For each figure, the *continuous lines* represented the CD signals of laccase in the presence of [Chol][H_2PO_4] and H_2O at 25°C. *Interrupted lines* presented the CD spectrums of laccase in H_2O in the three cases.

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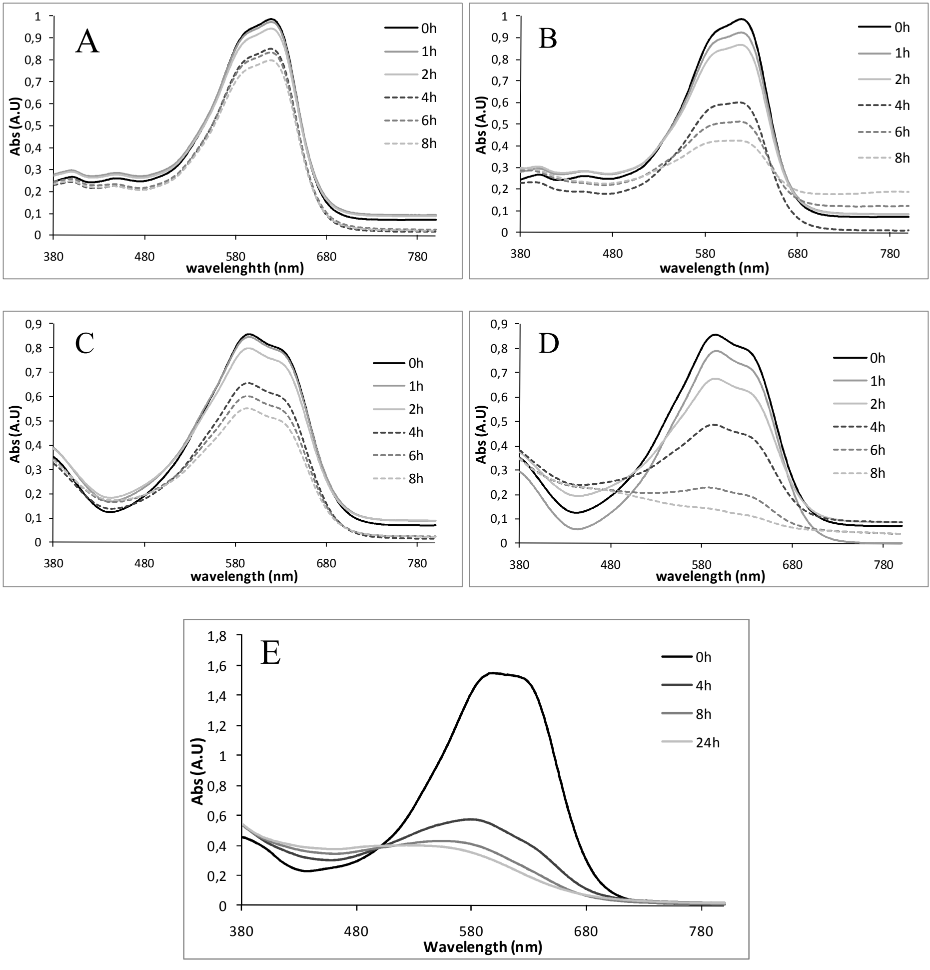

Figure 8. Decolorization achievement with laccase in the presence and the absence of 10 mM [Chol][H_2PO_4] at pH7.0 and 25°C. The addition of dyes to the laccase medium reaction has been 11µl for Remazol Brillant Blue R (RBBR) and 1.5 µl for Amido Black (AB). In the case of the mixture (RBBR+AB), the same amount has been added to the laccase reaction mixture in the presence of 10 mM [Chol][H_2PO_4]. The decolorization spectrum has followed for AB in the absence **(A)** and in the presence of 10 mM [Chol][H_2PO_4] **(B)** during 8h at 25°C and pH. The decolorization has followed for RBBR in the absence **(C)** and in the presence of 10 mM [Chol][H_2PO_4] **(D)** during 8h at 25°C. For the dyes mixture, the spectrum has followed between 380 nm and 800 nm in the presence of 10 mM [Chol][H_2PO_4] **(D)** during 24h at 25°C.

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