Sustainable liquid supports for laccase immobilization and reuse: Degradation of dyes in aqueous biphasic systems

Ana M. Ferreira¹, Ana I. Valente¹, Leonor S. Castro, João A. P. Coutinho,
Mara G. Freire* and Ana P. M. Tavares*

CICECO – Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal.

*Corresponding authors:
E-mail address: maragfreire@ua.pt and aptavares@ua.pt
¹These authors contributed equally to this work.

Abstract

Novel liquid supports for enzyme immobilization and reuse based on aqueous biphasic systems (ABS) constituted by cholinium-based ionic liquids (ILs) and polymers for the degradation of dyes are here proposed. The biocatalytic reaction for dye decolorization using laccase occurs in the biphasic medium, with the enzyme being “supported” in the IL-rich phase and the dye and degradation products being enriched in the polymer-rich phase. An initial screening of the laccase activity in aqueous solutions of ABS constituents, namely cholinium dihydrogen citrate ([Ch][DHC]), cholinium dihydrogen phosphate ([Ch][DHP]), cholinium acetate ([Ch][Acet]), polypropylene glycol 400 (PPG 400), polyethylene glycol 400 (PEG 400) and K₂HPO₄ was carried out. Compared to the buffered control, a relative laccase activity of up to 170%, 257% and 530% was observed with PEG 400, [Ch][DHP] and...
[Ch][DHC], respectively. These ABS constituents were then investigated for the in situ enzymatic biodegradation of the Remazol Brilliant Blue R (RBBR) dye. At the optimized conditions, the ABS constituted PPG 400 at 46 wt% and [Ch][DHC] at 16 wt% leads to the complete degradation of the RBBR dye, further maintaining the enzyme activity. This ABS also allows an easy immobilization, recovery and reuse of the biocatalyst for six consecutive reaction cycles, achieving a degradation yield of the dye of 96% in the last cycle. In summary, if properly designed, high enzymatic activities and reaction yields are obtained with ABS as liquid supports, while simultaneously overcoming the safety and environmental concerns of conventional organic solvents used in liquid-liquid heterogeneous reactions, thus representing more sustainable biocatalytic processes.

**KEYWORDS:** Aqueous Biphasic Systems, Dye degradation, Enzyme Immobilization and Reuse, Laccase, Liquid Support, Ionic Liquids

**1. Introduction**

In the last decades, biocatalysis became a powerful alternative to classical chemical catalysts, being applied in a wide variety of processes (Bornscheuer et al., 2012). Enzymes allow the development of sustainable catalytic processes compared to their chemical counterparts, since they are more specific to target substrates, allow a lower consumption of chemicals, and are
natural, renewable and biodegradable (Datta, Christena, & Rajaram, 2013). For these reasons, enzymes are important catalysts, exhibiting high potential in many industrial applications, such as in the chemical, textile, food, and pharmaceutical industries (Chapman, Ismail, & Dinu, 2018; Rajhans, Sen, Barik, & Raut, 2020). However, to make these enzymatic processes economically viable, it is crucial to achieve the reuse of the selected biocatalyst while maintaining its catalytic activity. One of the main strategies used to improve biocatalytic processes is based on the immobilization of enzymes onto insoluble solid supports, further allowing the enzymes reuse and recycling (Chapman et al., 2018). Various immobilization techniques have been developed, including adsorption, covalent binding, entrapment, encapsulation and cross-linking (Fernández-Fernández, Sanromán, & Moldes, 2013). The selected solid supports must be inert, stable, allow enzyme regeneration, and be able to increase, or at least maintain, enzyme activity (Chapman et al., 2018). It is however important to remark that the ideal support materials depend on the type of the enzyme and biocatalytic process to be developed (Zdarta, Meyer, Jesionowski, & Pinelo, 2018). Overall, the free homogeneous form of the enzyme changes to its heterogeneous immobilized form, usually allowing its use in severe conditions of temperature, pH and pressure (Zdarta, Meyer, Jesionowski, & Pinelo, 2018). Nevertheless, immobilized enzymes on solid supports may also display a reduced performance due to enzyme–matrix interactions that may compromise the diffusion of the substrate to the enzyme active center, low enzyme loading, mass transfer limitations (Xiu, Jiang, & Li, 2001). Other possible disadvantages may include difficult manufacturing and cleaning of the support, unspecific or weak interactions between the enzyme and the support, high cost and restricted reusability (Zdarta et al., 2018; Zdarta, Meyer, Jesionowski, & Pinelo, 2019). Moreover, as these systems are typically operated in a continuous mode, leaching of the enzyme from the solid support may occur (Misson, Zhang, & Jin, 2015). Thus, the development of new strategies to efficiently immobilize enzymes and keep their biocatalytic performance is still a key challenge.

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To overcome the described shortcomings of solid supports for enzymes immobilization, liquid-liquid processes have been used to perform enzymatic reactions. Lugaro et al. (Lugaro, Carrea, Cremonesi, Casellato, & Antonini, 1973) reported enzymatic reactions (using laccase from *Polyporus versicolor*) in a heterogeneous medium composed of one organic solvent (hexane, toluene, tetrahydrofuran, acetone, among others) comprising the substrate and products, and an aqueous phase containing the enzyme. Nicotra et al. (Nicotra, Intra, Ottolina, Riva, & Danieli, 2004) isolated and characterized four C-C and C-O dimers from the steroid hormone estradiol, using laccase from *Trametes pubescens*. The catalytic reaction occurred in a heterogeneous medium, formed by ethyl acetate and an aqueous buffered salt solution, with a yield of ca. 27% (Nicotra et al., 2004). Arca-Ramos et al. (Arca-Ramos, Eibes, Moreira, Feijoo, & Lema, 2012) demonstrated the potential of liquid-liquid bioreactors for the laccase-catalyzed degradation of anthracene, in a heterogeneous medium constituted by silicone oil and water. The surfactant Triton X-100 (above its critical micelle concentration) was used to enhance the anthracene solubility and facilitate its degradation (Arca-Ramos et al., 2012).

In the previously described works, organic solvents not miscible with water were used, which may compromise the biocatalyst performance (Doukyu & Ogino, 2010). Since most enzymes are more active in aqueous media because of their biological origin (Albertsson, 1958), aqueous biphasic systems (ABS), a particular type of liquid-liquid systems where both phases are aqueous, can be considered as promising alternatives to act as liquid supports (instead of solid supports) for enzymes, while simultaneously allowing enzyme immobilization and reuse. ABS are ternary systems, typically formed by two phase-forming components (e.g. polymers, salts and surfactants) and water that above given concentrations lead to the creation of two phases (Freire et al., 2012; Jiménez et al., 2020), allowing the combination of several processing steps (reaction, separation and reuse). Some works describing the use of ABS as a medium for biocatalytic processes have been reported (Cacace & Keating, 2013; Ferreira et al., 2018). Cacace and Keating (Cacace & Keating, 2013) used ABS composed of polyethylene glycol (PEG) and dextran to perform the mineralization of calcium carbonate.
(CaCO₃) using urease as a biocatalyst. However, the enzyme and products are enriched in the same ABS phase, not allowing their separation and enzyme reuse. Coutinho and co-workers (Ferreira et al., 2018) designed ABS composed of polymers and zwitterions, which allowed not only the quick oxidation of the model substrate 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) by laccase, but also its separation from the enzyme to the opposite phase. In this work (Ferreira et al., 2018) the reaction was carried out in homogeneous medium, requiring an external stimulus (change of temperature) to induce the formation of two phases to separate the enzyme from the products.

In addition to the well-studied polymer-based ABS, a new type of systems formed by aqueous mixtures of ionic liquids (ILs) and inorganic salts was proposed by Rogers and co-workers (Gutowski et al., 2003). Compared to polymer-based ABS, IL-rich systems show a set of important advantages, such as low viscosity, fast phase separation, and high extraction efficiency and selectivity achieved by the proper tailoring of the IL chemical structure (Ferreira, Coutinho, Fernandes, & Freire, 2014; Freire et al., 2012). Furthermore, ILs, if properly selected, lead to improved activity and stability of a wide number of enzymes and improved selectivity in biocatalysis (Bisht et al., 2017; Capela et al., 2020; dos Santos et al., 2018; Santos et al., 2018; Schindl, Hagen, Muzammal, Gunasekera, & Croft, 2019).

Although many studies have been reported on IL-based ABS, the majority of these are composed of imidazolium-based ILs and inorganic salts and applied as liquid-liquid strategies to purify biocompounds (Freire et al., 2012) and. Trying to overcome some of the toxicity and biodegradability concerns associated to imidazolium-based ILs, ABS formed by biocompatible phase-forming components, e.g. constituted by cholinium-based ILs and polymers, have been later reported (Hulsbosch, De Vos, Binnemans, & Ameloot, 2016). Cholinium-based ILs, designed from a water-soluble essential nutrient that supports several biological functions (Blusztajn, 1998), may be designed to be non-toxic and biodegradable. Polymers such as PEG or polypropylene glycol with a molecular weight of 400 g·mol⁻¹ (PPG
400) are able to form ABS with ILs and display high biodegradability, low toxicity, low cost, relatively low melting temperatures, and low volatility (Li, Liu, Pei, Wang, & He, 2012).

In this work, ABS composed of cholinium-based ILs and PPG 400 are proposed as novel liquid supports for enzymes, i.e. as alternatives to the commonly used solid supports and liquid-liquid systems using volatile organic solvents, in order to develop a sustainable and integrated catalytic-separation process. Traditional ABS composed of polymer-salt or polymer-polymer combinations were also evaluated for comparison purposes. The enzyme laccase was chosen due to its ability to degrade a wide variety of substrates, being currently considered a relevant biocatalyst in diverse industries (Alshabib & Onaizi, 2019; Fernández-Fernández et al., 2013). An initial screening of the ABS phase-forming components and associated pH on the enzyme biocatalytic performance was carried out. Subsequently, the ABS potential as liquid support for laccase was evaluated through the study of the enzyme ability to decolorize a textile dye, namely Remazol Brilliant Blue R (RBBR) (Zimbardi et al., 2016). RBBR was chosen as the substrate since about 10-15% of the synthetic dyes produced are discharged into industrial effluents (Spadaro, Gold, & Renganathan, 1992). Due to the textile dyes color, chemical structure and application, these compounds are usually resistant to degradation, causing serious environmental problems (inhibition of sunlight transmission in aquatic environments) and health concerns (gene mutations, cancers and allergies). Alternative wastewater treatments based on enzymes to degrade dyes have been reported as promising strategies (Antecka et al., 2018). Enzymatic dye decolorization further allows the reuse of decolorized aqueous effluents in industrial processes (e.g. to rinse products) (Bento, Almeida, Bharmoria, Freire, & Tavares, 2020; Mani, Chowdhary, & Bharagava, 2019; Moreira, Milagres, & Mussatto, 2014; Xu, Sun, Wang, Song, & Wang, 2018).

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2. Experimental

2.1. Materials

To prepare ABS, the following ILs were used: cholinium dihydrogen citrate, [Ch][DHC] (≥ 98 wt%), from Sigma-Aldrich; cholinium dihydrogen phosphate, [Ch][DHP] (> 98 wt%) and cholinium acetate, [Ch][Acet] (98 wt%), from IoLiTec. Polyethylene glycol 400 (PEG 400) was acquired from Cmd Chemicals, polypropylene glycol 400 (PPG 400) was from Sigma-Aldrich, and dipotassium hydrogen phosphate trihydrate (K₂HPO₄·3H₂O) (purity of 98 % wt) was purchased from Scharlau.

Commercial laccase from *Trametes versicolor* (light brown powder, ≥ 0.5 U·mg⁻¹), 2,2’-azino-bis3-ethylbenzathiazoline-6-sulfonic acid (ABTS) and phosphate buffered saline (PBS, 0.01 M, pH ≈ 7.4) were acquired from Sigma. Citric acid (C₆H₈O₇·H₂O) (> 99%) was acquired from Fisher Scientific, sodium phosphate dibasic (Na₂HPO₄) (> 99%) was acquired from Merck, and Remazol Brilliant Blue R (RBBR) was purchased from ACROS Organics.

The water employed was double distilled, passed across a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus.

2.2. Laccase activity in presence of ABS phase-forming components

In this work, three types of ABS were studied, *i.e.*, traditional ABS constituted by a polymer and a salt, ABS constituted by two polymers, and ABS constituted by a polymer and an IL. To evaluate the effect of the ABS constituents in the laccase activity, several aqueous solutions containing [Ch][Acet], [Ch][DHP], [Ch][DHC], K₂HPO₄, PEG 400 and PPG 400 at concentrations ranging from 20 wt% to 50 wt% were prepared. The laccase activity registered in PBS buffer (0.01 M, pH ≈ 7.4) was considered as the control. In all assays, laccase concentration was kept at 1 g·L⁻¹. Mixtures were incubated for 10 min at room temperature and samples were taken for laccase activity assays (Ander & Messner, 1998). To measure the enzyme activity, aqueous solutions of ABTS (substrate) with a concentration of 0.8 g·L⁻¹ were used. 50 μL of the sample (laccase solution in the presence of the different constituents) was
added to 250 µL of ABTS and 700 µL of citrate/phosphate buffer (0.1M), pH 4.5. The increase in absorbance/min was recorded using a UV-Vis spectrophotometer (Shimadzu UV-1800 Spectrometer). Laccase activity is expressed in U·L⁻¹, where one unit (U) is the amount of enzyme that oxidizes 1 µmol of ABTS·min⁻¹ at 420 nm (ε = 36,000 M⁻¹cm⁻¹). For each aqueous solution prepared, the enzymatic activity was determined at least three times. Possible interferences of the ABS compounds in the enzyme activity measurements were taken into account, and control samples were always prepared using the solutions without laccase and with the ABTS.

2.3. Evaluation of the ABS potential as liquid supports for laccase

The ternary mixture compositions for the laccase partition studies were chosen based on the phase diagrams reported in the literature (Quental et al., 2015; Sadeghi & Maali, 2016; Xie et al., 2010) (for details see the Supporting Information, experimental section and results section - Figure S1). To avoid discrepancies in the results that could arise from the different compositions of the two phases, studies were performed at a constant tie-line length (TLL). Moreover, a mixture point that led to an equal weight ratio of the phases (parameter α ≈ 0.5, ratio between the top and the total weight of the mixture) was chosen to ensure that there was enough volume to use ABS as enzymatic liquid supports. The mixture compositions chosen, which correspond to a TLL of circa 65 ± 3 and α approximated to 0.5 (cf. the Supporting Information, experimental section and results section - Table S1 and S2), are: 45 wt% of PPG 400 + 7 wt% of [Ch][DHP], 46 wt% of PPG 400 + 16 wt% of [Ch][DHC], 51 wt% of PPG 400 + 6 wt% of [Ch][Acet], 33 wt% of PPG 400 + 8 wt% of K₂HPO₄ and 47 wt% of PPG 400 + 24 wt% of PEG 400. The laccase extraction efficiencies were investigated using laccase stock solutions at circa 1 g·L⁻¹ in PBS solution (0.01 M, pH ≈ 7.4). In each system, 29 wt% of laccase stock solution was added to reach a total weight of 1 g. After the equilibrium was reached (centrifugation at 3500 rpm for 30 min), both phases were carefully separated, and laccase activity was quantified as previously described. At least three individual experiments were performed to determine the average extraction efficiency, as well as the respective

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standard deviations. Control samples were always prepared to compare the possible interferences of the compounds used in ABS. The extraction efficiency ($EE_{laccase\%}$) of the studied ABS for laccase is defined as the percentage ratio between the activity of laccase in the salt-, PEG- or IL-rich phase to that in the two aqueous phases (total of the two phases), according to Equation 1.

$$EE_{laccase\%} = \frac{L_{A_x} \times w_x}{L_{A_x} \times w_x + L_{APPG_{400}} \times w_{PPG_{400}}} \times 100\%$$  \hspace{1cm} (1)

where $L_{A_x}$ and $L_{APPG_{400}}$ are the laccase activity in the salt-, PEG- or IL-rich and in the PPG 400-rich phase, respectively. $w_x$ and $w_{PPG_{400}}$ are the weight of the salt-, PEG- or IL-rich phase and the weight of the PPG 400-rich phase, respectively.

The ABS that showed the highest enrichment of laccase in one phase while preserving or improving its activity, namely the ABS formed by PPG 400 and [Ch][DHC] and PPG 400 and PEG 400, were applied to determine the partition behavior of the studied dye - RBBR. The mixture compositions described before were tested with 0.03 wt % of RBBR. The dye's quantification in the two phases was carried by UV-spectroscopy, using a Shimadzu UV-1800 Spectrometer, at the maximum wavelength of RBBR, i.e. 595 nm, using an established calibration curve. The extraction efficiency of the studied ABS to RBBR ($EE_{dye\%}$) is defined as the percentage ratio between the amount of dye in the PPG-rich aqueous phase in relation to its amount in the two aqueous phases.

2.4. ABS as liquid supports for laccase recovery and reuse

The laccase catalytic performance to decolorize dyes was investigated in the most promising ABS, constituted by PPG 400 and [Ch][DHC] (1 g of ABS: 46.00 wt% of PPG 400 + 16.00 wt% of [Ch][DHC] + 29.00 wt % of laccase solution + 8.97 wt % of water + 0.03 wt % of dye RBBR). The performance of the enzymatic catalysis was investigated using laccase solutions at 1 g·L⁻¹ (dissolved in PBS, 0.01 M, pH ≈ 7.4. The ABS were then stirred for 60 min at 25 °C using a Programmable rotator-mixer Grant-Bio PTR-30, centrifuged at 3500 rpm for 30 min,
and both phases were carefully separated. The determination of the laccase activity and the
dye quantification were carried out as previously described. Each experiment was repeated at
least three times to determine the average extraction efficiency, the dye decolorization yield,
and their respective standard deviations. Possible interferences of the IL, polymer and salt in
the analytical methods were taken into account and control samples were prepared using PBS
solutions instead of laccase and dyes. Moreover, to ensure that the IL or polymer have no
interference in the decolorization, control reactions in the ABS using the same compounds,
apart from laccase, were carried out. The RBBR percentage dye decolorization yield ($Y_{dec\%}$)
is defined as equation 2:

$$Y_{dec\%} = \frac{C_{RBBR_f}}{C_{RBBR_i}} \times 100$$ (2)

where $C_{RBBR_f}$ is the RBBR concentration at the end of the reaction (after decolorization) and
$C_{RBBR_i}$ is the initial RBBR concentration.

The identification of degradation products of RBBR in each phase was carried out using an
HPLC-DAD (Shimadzu, model PROMINENCE). HPLC analyses were performed with an
analytical C18 reversed-phase column (250 × 4.60 mm), kinetex 5 μm C18 100 A, from
Phenomenex. The mobile phase used was a gradient system of acetonitrile (phase A) and 30
mM acetic acid/ammonium acetate buffer at pH 4.5 (phase B), previously degassed by
ultrasonication. The separation was conducted using the following gradient mode, 0 min 5%
of A, 15 min 20% of B, 18 min of 60% of B, 24 min 60% of B, and then returning to initial
conditions in 20 min to ensure the column stabilization (Osma, Toca-Herrera, & Rodríguez-
Couto, 2010). The separation was conducted at a flow rate of 0.8 mL·min$^{-1}$ with an injection
volume of 10 μL. DAD and set at 213 nm. Each sample was analyzed at least in duplicate.
The column oven and the autosampler were operated at 25°C.

To investigate the recovery and reuse of the enzyme, six consecutive cycles of the oxidative
reaction of RBBR by laccase were performed using the selected ABS constituted by PPG 400
and [Ch][DHC]. Each complete cycle (reaction + separation) was carried out in 105 min (60 min of reaction + 30 min of centrifugation + 5 min for the separation of the phases). The laccase activity and the quantification of the RBBR dye were determined as previously described. After the first cycle of reaction, the ABS were once more stirred for 60 min and centrifuged at 3500 rpm for 30 min. The PPG-rich phase (top phase), containing the majority of the dye reaction products, was then removed and a fresh dye solution was added (same volume of solution removed with a composition determined by the tie-line of the ABS), with a new cycle starting with the same [Ch][DHC] rich-phase (enriched in laccase, bottom phase). This procedure was repeated for six consecutive cycles of reaction. The laccase stability along the cycles was evaluated by measuring the relative enzyme activity, defined as equation 3:

\[
\text{Relative laccase activity (\%) } = \frac{LA_f}{LA_i} \times 100 \tag{3}
\]

where \( LA_i \) is the laccase activity in the first cycle and \( LA_f \) is the laccase activity at the end of each subsequent cycle.

3. Results and discussion

3.1. Laccase activity in presence of ABS phase-forming constituents

The main goal of the current work is to demonstrate the potential of ABS composed of ILs and polymers as liquid supports for enzymes, allowing the recovery and reuse of the biocatalyst with minimum loss of enzyme activity. ABS formed by cholinium-based ILs and PPG 400, whose ternary phase diagrams were taken from the literature, were used (Quental et al., 2015; Sadeghi & Maali, 2016; Xie et al., 2010). The ILs investigated correspond to [Ch][DHC], [Ch][DHP] and [Ch][Acet]. Classical ABS formed by PPG 400 and PEG 400, and by PPG 400 and K$_2$HPO$_4$, were included for comparison purposes. Since ABS may be composed of different constituents, the first step consisted of evaluating the effect of each ABS phase-forming compound on the laccase activity at different concentrations (20-50 wt%). These concentrations were chosen since they are commonly used for IL-based ABS formation (Freire et al., 2012). The results obtained are shown in Figure 1, and the respective
data are presented in the Supporting Information (cf. Table S3). The laccase activity in a phosphate buffer solution (PBS, 0.01 M) at pH 7.2 was considered as the control. Comparing laccase activity in the PBS solution used as control (100%), there is a high decrease in the relative enzyme activity in the presence of [Ch][Acet], K$_2$HPO$_4$ and [Ch][DHP] (50 wt%), whilst a slight decrease is observed in presence of PEG 400. Some works from the literature report that ILs constituted by the anion acetate lead to a decrease in the activity of different types of enzymes (Galai, P. de los Ríos, Hernández-Fernández, Haj Kacem, & Tomas-Alonso, 2015; Moniruzzaman & Ono, 2013; Stevens, Rodgers, Dumon, & Shi, 2020). In particular, for laccase, Stevens et al. (Stevens et al., 2020) suggested, by docking simulations, that the IL 1-ethyl-3-methylimidazolium acetate inhibits the activity of laccase since it interferes in the ABTS binding to the active site (competitive inhibition) and with the ABTS oxidation (uncompetitive inhibition). Moreover, previous works have described changes in the kinetic parameters of laccase in presence of ILs (Domínguez et al., 2011; Galai et al., 2015; Liu et al., 2013; Rehmann et al., 2012; Rodakiewicz-Nowak & Jarosz-Wilkołazka, 2007; Rodríguez, Cristóvão, Tavares, & Macedo, 2011). However, the effect of salts structures on enzyme activity highly depend on the enzyme and substrates nature (Patel, Kumari, & Khan, 2014; Zhao, 2016). Furthermore, pH has an important role in laccase activity on aqueous solutions of ILs and salts (Galai et al., 2015; Sun, Liu, Yang, Chen, & Fu, 2017), and cannot be discarded in the current work.

PEG 400 and [Ch][DHP] lead to increases in the laccase activity up to 170 and 257%, respectively. A remarkable increase in the relative laccase activity, up to 530%, is observed with [Ch][DHC]. Specifically, it seems that there were significant activity enhancements by increasing the number of hydroxyl groups, which is in agreement with literature results using deep eutectic solvents formed by the same IL, i.e. [Ch][DHC] (Toledo et al., 2019). These results suggest that the laccase activity strongly depends on the type of polymer used and nature of the IL anion, as discussed above. Furthermore, the increase of the IL and polymer concentration leads to a decrease in the laccase activity, except for [Ch][DHC]. Overall,
[Ch][DHC], [Ch][DHP] and PEG 400 are the most promising ABS phase-forming compounds investigated, being able to significantly improve the laccase activity. Despite not being studied in the current work, it should be remarked that laccase activity also depends on the IL cation. Galai et al. (Galai et al., 2015) studied the effect of the IL anion and cation on the laccase activity. The authors observed that the activity of laccase is mostly dependent on the IL anion nature. However, the cation also played an important role in the laccase activity, being cholinium the most promising IL cation investigated. This positive effect was correlated with the chaotropicity of the cholinium ion, simultaneously with its role in decreasing the hydrophobicity and positively acting on the secondary structure of the enzyme (Galai et al., 2015).

3.2. Evaluation of the ABS potential as liquid supports for laccase

Aiming to develop efficient supports, it is necessary to design ABS capable of completely concentrating the target enzyme in one phase, and the dye and the reaction products in the opposite phase. After identifying the phase-forming components able to improve the activity of laccase, the extraction efficiency of laccase ($EE_{laccase}$ $%$), i.e. the amount of active laccase in the IL-, salt- or PEG-rich phase in respect to the total active laccase present in both phases, was investigated in the PPG 400-based ABS (combined with PEG 400, $K_2HPO_4$, [Ch][DHP], [Ch][DHC] and [Ch][Acet]). In all studied ABS, the top phase corresponds to the PPG-rich phase, while the bottom phase is mainly composed of IL, PEG 400 or salt ($K_2HPO_4$). The results obtained are depicted in Figure 2 (respective data are presented in the Supporting Information, Table S4). All mixture compositions used in this set of experiments correspond to a similar TLL ($65 \pm 3$) and $\alpha$ close to 0.5 (additional data are given in the Supporting Information, experimental section and results section - Table S1 and S2). The reasons for this choice are given in the experimental section.

The partition of proteins and enzymes in ABS depends on dispersive interactions, electrostatic forces, hydrogen-bonding, molecular size and solubility, while their magnitudes further
depend on the two-phase systems’ compositions and nature of the phase-forming components (Quental et al., 2015). In this work, laccase does not partition to the PPG-rich phase and preferentially partitions to the salt-, IL- or PEG-rich phase. Like most proteins and enzymes, it preferentially migrates to the more hydrophilic phase in polymer-based systems (Mondal et al., 2016; Quental et al., 2015). Accordingly, $EE_{\text{laccase}}\%$ higher than 87% were obtained in the bottom phase (K$_2$HPO$_4$-, PEG 400-, or IL-rich) of all systems investigated (Figure 2). In particular, the complete extraction of laccase is observed in the ABS formed by ILs or PEG 400. These results demonstrate that the complete partition of active laccase can be achieved using an adequate ABS, thus avoiding enzyme losses when envisioning its recovery and reuse. However, it is important to keep in mind that the $EE_{\text{laccase}}\%$ considers the amount of active enzyme in one of the phases in relation to the total amount of active enzyme in both phases of the ABS. Thus, a high $EE_{\text{laccase}}\%$ means that the active enzyme is mostly enriched in one of the ABS phases. Although the [Ch][Act]- and K$_2$HPO$_4$-based ABS present a high $EE_{\text{laccase}}\%$ (i.e., the active enzyme migrates preferentially to the [Ch][Act]- and K$_2$HPO$_4$-rich phases), these systems present a low relative enzyme activity (Figure S2) when compared to the remaining ABS. These systems indeed negatively affect the enzyme activity, which is related to their alkaline pH (Figure S2), and as discussed below.

The results related to the relative laccase activity in the bottom phase of the PPG 400-based ABS and the respective pH are given in the Supporting Information (cf. Figure S2). The laccase activity in the bottom phase depends on the ABS phase-forming components, increasing in the following order: K$_2$HPO$_4$ $<$ [Ch][Acet] $<$ [Ch][DHP] $\sim$ PEG 400 $<$ [Ch][DHC]. These results are in agreement with the previous results shown in Figure 1, where the laccase activity was determined in the presence of each compound. The activity of laccase is maximum in phases where the pH ranges between 3.9 and 5.3; above this pH the laccase activity markedly decreases. These results show the catalytic activity of the enzyme depends on the pH, which in turn depends on the ABS phase-forming components. A maximum laccase activity occurs in the systems with a pH range between 4 and 5. On the
other hand, for systems with neutral or alkaline pH there is a decrease in the laccase activity (K$_2$HPO$_4$- and [Ch][Acet]-based systems). This behavior is in agreement with the previous studies demonstrating that, depending on the laccase source and subtract, the activity of laccase is improved in the pH range 4.0 – 5.0, at 25°C (Kumar, Kaur, Jain, & Kumar, 2016).

Based on the previous results, and since the laccase biocatalytic performance is better in [Ch][DHC] aqueous solutions, the ABS based on PPG 400 and [Ch][DHC] were chosen as representative systems of IL-polymer ABS. The ABS composed of PPG 400 and PEG 400 was additionally studied due to its high ability to enrich laccase in one phase. Both ABS were investigated concerning their ability to separate the target dye, which ideally should partition to the opposite phase to that where laccase is present. According to Figure 3 (detailed data given in the Supporting Information, Table S5), RBBR shows a different partition behavior in the two studied ABS, where an $EE_{\text{dye}}$ of 95% to the PPG-rich phase in the [Ch][DHC]-based ABS and an $EE_{\text{dye}}$ of 10% to the PPG-rich phase in the PEG 400-based ABS were obtained. In summary, in systems formed by PPG and [Ch][DHC], RBBR preferentially partitions to the PPG-rich phase, whereas in ABS constituted by PEG 400 and PPG 400 the dye preferentially partitions to the PEG-rich phase.

In order to develop a liquid support for enzymes based on ABS, which allows the separation of the enzyme from the dye and respective products as well as the catalyst recovery and reuse, it is mandatory to choose an ABS where laccase and the dye partition to opposite phases, and where the enzyme should maintain its activity. Based on the results obtained, the most promising liquid support for laccase is the ABS based on PPG 400 and [Ch][DHC].

3.3. ABS as liquid supports for laccase recovery and reuse

After determining the best ABS that could be used as a liquid support for laccase, the $in situ$ dye decolorization was carried out in two-phase systems, i.e. at heterogeneous conditions, using the ABS constituted by PPG 400 and [Ch][DHC]. It should be remarked that the ABS
phase-forming compounds have no effect on the degradation of dyes, with no decolorization observed without laccase, as experimentally appraised.

The bioreaction comprising both laccase and the dye occurred in a heterogeneous medium, with continuous stirring of the ABS during 60 min, at the mixture point containing 46 wt% of PPG 400 and 16 wt% of [Ch][DHC]. After the reaction was concluded, the ABS was centrifuged, and the phases recovered. As shown in Figure 4, the yield of the dye decolorization ($Y_{\text{dec}}$) is 100% and laccase maintains its activity at the end of the reaction. The presence of degradation products of RBBR was studied according to the method proposed by Osma et al. (Osma et al., 2010), being demonstrated that they partition to the PPG-rich phase (data shown in the Supporting Information, Figure S3). It should be noted that the degradation products of dyes by laccase are well reported. In particular, Antecka et al. (Antecka et al., 2018) and Ramsay et al. (Barbora, Daniela, & Miroslav, 2016) demonstrated that the degradation products of the RBBR by laccase have lower toxicity than the original dye. Laccase and the dye reaction products partition to opposite phases, allowing the removal of these products and enzyme reuse, supporting the idea of ABS as novel liquid supports for enzymes. The bottom-phase ([Ch][DHC]-rich phase) containing laccase was recovered and reused to form a new ABS by the addition of ABS constituents by considering the tie-line composition. The step of recovering and reusing the IL-rich phase containing laccase was repeated in six consecutive cycles of the decolorization reaction. The results presented in Figure 4 show that laccase is able to completely degrade RBBR in [Ch][DHC]-based ABS ($Y_{\text{dec}}$ ranging from 96 to 100%) without any laccase mediator as usually needed (Chmelova & Ondrejovic, 2016; Lueangjaroenkit et al., 2019; Soares, de Amorim, & Costa-Ferreira, 2001; Wang et al., 2018). Although circa 40% of laccase activity is lost along the six reaction cycles, the remaining active enzyme is enough to perform almost the complete decolorization of RBBR (>96%). This enzymatic activity loss may be due to the time required for the complete dye decolorization and to the physical forces applied, i.e. the continuous stirring to
increase the interfacial contact area between the two phases during the six cycles of reaction. Further optimization in this sense is thus advised in future works.

Some works (Arca-Ramos et al., 2012; Cacace & Keating, 2013; Ferreira et al., 2018; Lugaro et al., 1973; Nicotra et al., 2004) on the use of biphasic systems for biocatalysis have been reported in the literature. Most of these works correspond to biocatalytic reactions in heterogeneous liquid-liquid systems composed of volatile organic solvents and water (Arca-Ramos et al., 2012; Lugaro et al., 1973; Nicotra et al., 2004). However, these systems lead to a lower yield, perhaps due to the use of the volatile organic solvents that may lead to a decrease in the enzymatic activity. Furthermore, it is necessary to ensure the separation of the enzyme from the reaction products while maintaining the enzymatic activity to allow its reuse. These drawbacks are overcome with the ABS proposed in this work.

The decolorization of RBBR was already accomplished by immobilized laccase onto solid supports (Antecka et al., 2018; Osma et al., 2010). Osma et al. (HajKacem et al., 2020; Osma et al., 2010) showed that immobilized laccase on alumina leads to an RBBR decolorization yield of 44% after 42h of reaction, for an initial RBBR concentration of 133 mg·L⁻¹. Antecka et al. (Antecka et al., 2018) used a small concentration of RBBR, 5 mg·L⁻1, and obtained a decolorization yield of 91% after 24h of reaction immobilized laccase on oxide derivate materials. The immobilized biocatalyst was reused five times, and in each cycle of reaction the yield of dye decolorization decreased by around 15% (Antecka et al., 2018). HajKacem et al. (HajKacem et al., 2020) used a batch process membrane bioreactor based on a polymeric IL for the decolorization of RBBR by laccase. Three 24h consecutive cycles were applied, allowing an RBBR removal yield of ca.75% in the first cycle, 65% in the second cycle, and 60% in the third cycle (HajKacem et al., 2020). The authors explained the reduction in decolorization yield due to mass transfer problems (HajKacem et al., 2020). In these works, the immobilized laccase loses its capacity for the dye degradation along the cycles of reuse, contrarily to what obtained with the IL-based ABS proposed in the current work. Moreover,
in the three works with immobilized laccase on solid supports, a longer period of reaction time (42 and 24h) was necessary for the decolorization of RBBR, and with a smaller amount of dye added when compared to our conditions (1h and 300 mg·L⁻¹ of RBBR in each laccase reaction cycle).

The novel concept of “liquid supports” resorting to ABS allows to overcome the major drawbacks of using enzymes in homogeneous reactions and supported enzymes in heterogeneous reactions. In the first case, reaction yields are enhanced, but it is unfeasible to recycle the enzyme easily; in the second case, the enzyme costs may be increased and reaction yields may be decreased due to mass transfer limitations. The approach here proposed allows a beneficial compromise of both techniques: to carry out the reaction in homogeneous medium, with no mass transfer limitations, and to recover and reuse the enzyme as if it was supported onto a solid material.

In summary, it is here demonstrated that properly designed ABS allow enzymatic reactions to be carried out in water-rich biphasic systems, with a simultaneous separation of the products to the opposite phase. This selectivity allows the separation of the products from the enzyme and the direct enzyme reuse in a new biocatalytic reaction, showing that it is possible to immobilize and directly reuse the enzyme in a liquid support corresponding to the ABS phase. The results here obtained support the applicability of ABS as novel and efficient liquid supports for biocatalytic reactions, as schematically shown in Figure 5.

4. Conclusion

This work shows that ABS constituted by cholinium-based ILs and polymers can be used as liquid supports for enzyme immobilization and reuse. If properly selected, ABS can be used in heterogeneous bioreactions, simultaneously allowing products and enzyme separation and reuse. This possibility was here demonstrated for the decolorization of the dye RBBR, using laccase as the biocatalyst.
In the ABS formed by [Ch][DHC] and PPG 400, the reaction occurs in the biphasic (heterogeneous) medium, in which the enzyme and the dye/products are enriched in opposite phases. The recovery and reuse of both the IL-based-rich phase and enzyme were demonstrated. After six cycles of reaction, the yield of dye decolorization was > 96%.

This work shows that ABS are a viable and promising alternative to solid supports for enzyme immobilization, while overcoming many of the safety and environmental concerns of conventional organic solvents used in liquid-liquid heterogeneous reactions, thus contributing to the development of more sustainable biocatalytic processes.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

Author Contribution Statement

Ana P. M. Tavares, Mara G. Freire and João A.P. Coutinho conceived the project, designed the experiments, review & editing. Ana I. Valente and Leonor Castro performed experiments. Ana M. Ferreira analyzed the data and wrote the manuscript with inputs from all authors. All authors read and approved the final manuscript.

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**Figures**

FIGURE 1. Relative laccase activity (%) at 25°C in aqueous solutions of ABS phase-forming components at: 20 wt% (■), 30 wt% (■), 40 wt% (■) and 50 wt% (■). Control in PBS 100 % (---).

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FIGURE 2. Extraction efficiency of laccase ($EE_{_{\text{laccase}}} \%$) in ABS formed by PPG 400 with cholinium-based ILs (■), PEG 400 (■) and $K_2HPO_4$ (■), at 25°C.

FIGURE 3. Extraction efficiency of RBBR ($EE_{_{\text{dye}}} \%$) to the PPG-rich phase in ABS formed with PEG 400 or [Ch][DHC] at 25°C.

FIGURE 4. (A) Yield of decolorization of RBBR ($Y_{\text{dec}} \%$, bars) and relative laccase activity (%) in the IL-rich phase (symbols) in the 6 cycles comprising both the recovery and reuse of the enzyme and of the [Ch][DHC]-rich phase, at 25°C. (B) Schematic overview of ABS as novel liquid supports for enzymes immobilization and reuse.
FIGURE 5. Flowchart of the process developed using ABS as liquid supports for enzymes reaction, *i.e.*, immobilization and directly reuse of the enzyme (IL-rich phase).