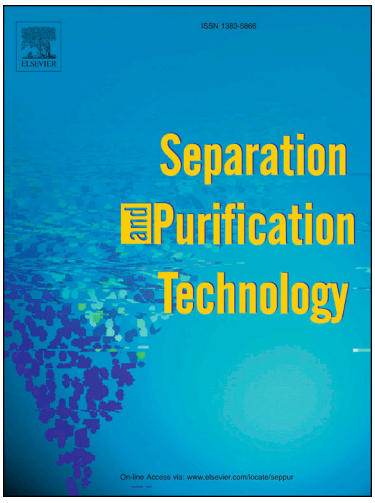

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Insights on the laccase extraction and activity in ionic-liquid-based aqueous biphasic systems

Emanuel V. Capela[1], Ana I. Valente[1], João C. F. Nunes[1], Flávia F. Magalhães[1], Oscar Rodriguez[2], Ana Soto[2], Mara G. Freire[1], Ana P. M. Tavares[1,2*]

[1]CICECO – Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal

[2]Department of Chemical Engineering, Universidade de Santiago de Compostela, Rua Lope Gómez de Marzoa, s/n. E15782 Santiago de Compostela, Spain

*Corresponding author:

Phone: +351 234370200;

E-mail: aptavares@ua.pt

ABSTRACT

Due to their catalytic properties, selectivity, and efficiency, enzymes are excellent biocatalysts. In particular, laccases are versatile multi-copper oxidases with great interest for a wide plethora of biotechnological and environmental applications. Even though several laccase-catalysed processes have been reported at an industrial level, the high costs of their downstream processing required to provide biocatalysts with high purity levels, stability and activity remains one of the main drawbacks when economically evaluating the overall processes. Aqueous biphasic systems based on ionic liquids (ILs) can be foreseen as a promising alternative approach for the extraction and activity maintenance/improvement of enzymes, essentially due to the designer solvents ability of ionic liquids. However, to take advantage of this feature and to use the full potential of IL-based aqueous biphasic systems, it is necessary to understand the effect of ILs as phase-forming constituents and how they affect the enzymes extraction and activity. In order to overcome the lack of information on this topic in the literature, in this work, IL-based aqueous biphasic systems were investigated to extract and enhance the laccase activity, in order to gather evidences that could be used to improve the enzymes downstream processing. To this end, a wide screening of imidazolium-, pyridinium-, pyrrolidinium-, piperidinium-, tetraalkylphosphonium-, and tetraalkylammonium-based ILs as phase-forming components of ABS was carried out. Furthermore, these ILs were used to create ABS combined with salts, polymers and used as adjuvants in polymer-based ABS. Most ABS comprising ILs revealed to be highly efficient extraction platforms, allowing the complete extraction of laccase for all the conditions tested, and with an enzyme activity enhancement by more than 50%. Overall, the obtained results demonstrate that laccase preferentially partitions to the most hydrophilic phase in ABS comprising ILs, both used as adjuvants or as phase-forming components, corresponding to the phase in which the IL is enriched. Furthermore, the IL chemical structure of the IL plays a significant role in the enzyme activity, where ILs with a higher number of hydroxyl groups seem to be relevant to improve the laccase activity.

Keywords: Laccase; Oxidative Enzymes; Extraction; Activity; Aqueous Biphasic Systems; Ionic liquids.

1 Introduction

Enzyme biocatalysis undergone significant developments in the last decades in the production of high-value products in a variety of industries [1]. Due to their excellent catalytic properties, selectivity, efficiency, low toxicity and biodegradability, enzymes are excellent biocatalysts to be used in mild reaction conditions. Oxidative enzymes have been applied in a wide variety of processes [2], such as delignification and biobleaching [3, 4], dye degradation [5-7], bioremediation [8], ethanol production [9], biosensors [10] and in the production of pharmaceutical drugs [11, 12]. In fact, within their relevant applications, the pharmaceutical sector can be highlighted, in which the first drug synthesized by an oxidative enzyme was actinocin, an effective compound to fight cancer by blocking the transcription of the tumour cell DNA [13]. An additional example includes the coupling of katarantine and vindoline to produce vinblastine, an anti-cancer drug [14].

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are versatile multi-copper oxidases [15], capable of oxidizing a large number of phenolic and non-phenolic molecules due to their low substrate specificity, using oxygen as electron acceptor and generating water as a by-product [16]. Due to their wide range of biotechnological and environmental applications [17], laccase is one of the most used oxidative enzyme. In particular, laccase presents a great potential in different processes related with pulp and paper [18], food [19] and textile industries [20], as well as in novel fields such as bioremediation [21], biosensing [22] and lignocellulosic biorefineries [23].

Even though several laccase-catalysed processes have been reported at an industrial level over the last decades [24, 25], due to its high relevance and wide plethora of applications, the high costs of the downstream processing of enzymes required to provide biocatalysts with high purity levels, stability and activity remains one of the main drawbacks when economically evaluating the overall processes [26]. On an attempt to overcome these limitations, the use of ionic liquids (ILs) has been investigated in the development of more efficient downstream processes for enzymes, as well as to provide more sustainable enzymatic reactions, particularly when the substrates have low water solubility [27]. The interest in ILs as a novel class of solvents for protein purification and biocatalytic transformations is mainly due to their designer solvents ability and excellent performance to act as extraction and/or reaction media [28-33].

Depending on the enzymes purity requirement, multiple bioprocessing steps may be required, including chromatography and other polishing steps such as ultrafiltration. Among these

processes, aqueous biphasic systems (ABS) have been recognized as an economic and efficient downstream processing tool to be used in the separation and purification of biomolecules from complex biological matrices [34]. Traditional ABS consists of two immiscible aqueous-rich phases based on polymer/polymer, polymer/salt or salt/salt combinations. However, typical polymer/polymer ABS display two hydrophobic phases, while polymer/salt ABS show a highly hydrophobic polymer-rich phase and a hydrophilic salt-rich phase. Therefore, there is always a restricted polarity difference between the two phases, which may limit their performance in the selective extraction and purification of proteins. The introduction of ILs in ABS was first reported by Rogers and co-workers [35], and since then the potential of ILs as phase-forming components of ABS has been recognized in the presence of several salts, amino acids, carbohydrates or polymers [31, 36-38]. Essentially due to their capability to act as designer solvents, tailored polarities of the ABS coexisting phases can be obtained, thus leading to tailored affinities and improved extractions [39].

ABS based on ILs and salts or polymers have been proposed as effective separation and purification strategies for several biomolecules, such as proteins and enzymes [28, 30, 40-44]. IL-based ABS have been also reported as effective in the extraction and in maintaining the activity of enzymes [42]. Moreover, the stability of oxidative enzymes in presence of ILs has been proved [45, 46]. Although traditional polymer/polymer and polymer/salt ABS have been investigated for the extraction of oxidative enzymes, including laccase [47], to the best of our knowledge, the application of IL-based ABS for the extraction or purification of laccase, was not previously addressed.

Since ILs are designer solvents and in order to take advantage of that characteristic to reach the full potential of IL-based ABS, in this work, a wide range of imidazolium-, pyridinium-, pyrrolidinium-, piperidinium-, tetraalkylphosphonium-, and tetraalkylammonium-based ILs were evaluated in ternary and quaternary ABS, as phase-forming compounds or adjuvants, respectively, for the extraction of laccase and their effect upon the enzyme activity. Ternary ABS composed of ILs and potassium citrate buffer ($C_6H_5K_3O_7/C_6H_8O_7$) at pH 8, potassium citrate ($C_6H_5K_3O_7$) or polypropylene glycol 400 (PPG 400) were studied; quaternary ABS composed of polymer/salt (PPG 400 + K_2HPO_4) and polymer/polymer (PPG 400 + poly(ethylene)glycol 400/PEG 400) with ILs as adjuvants at three different concentrations (1, 3 and 5 wt%) were also investigated. The phase-forming abilities of the different ILs were determined through the characterization of the respective liquid-liquid phase diagrams, followed by studies on the extraction of laccase in these systems and enzyme activity. Several operational conditions, namely the type of system, the IL,

salt, polymer and enzyme concentrations were investigated, allowing to address some of the main molecular mechanisms and driving forces ruling the laccase partition in IL-based ABS.

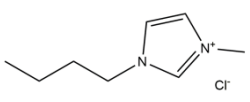
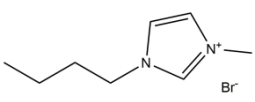
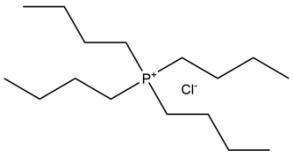
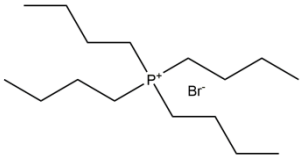
2 Experimental Section

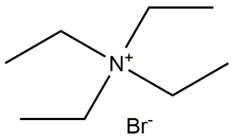
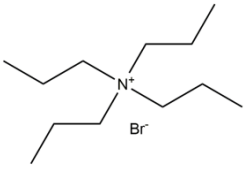
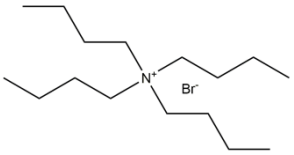
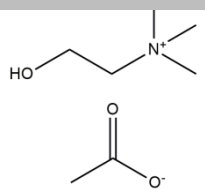
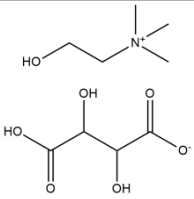
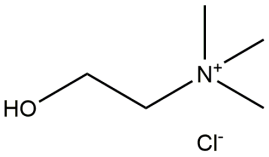
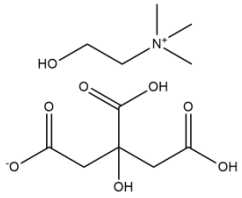
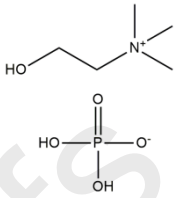
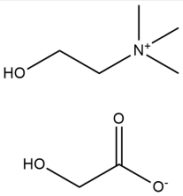
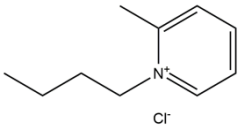
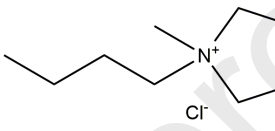
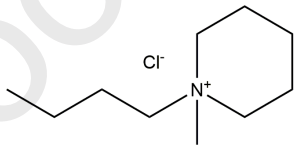
2.1 Materials

2.1.1 Chemicals

The ILs investigated in this work were: 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl; purity > 99%), 1-butyl-3-methyl-imidazolium bromide ([C_4mim]Br; purity > 99%), tetrabutylphosphonium chloride ([P_{4444}]Cl; purity > 95%), tetrabutylphosphonium bromide ([P_{4444}]Br; purity > 95%), tetraethylammonium bromide ([N_{2222}]Br; purity > 98%), tetrapropylammonium bromide ([N_{3333}]Br; purity > 98%), tetrabutylammonium bromide ([N_{4444}]Br; purity > 98%), ([Ch][Ac]; purity > 98%), cholinium bitartrate ([Ch][Bit]; purity > 98%), cholinium chloride ([Ch]Cl; purity > 99%), cholinium dihydrogen citrate ([Ch][DHCit]; purity > 98%), cholinium dihydrogen phosphate ([Ch][DHP]; purity > 99%), cholinium glycolate ([Ch][Gly]; purity > 97%), 1-butyl-2-methylpyridinium chloride ([C_4mpy]Cl; purity > 99%), 1-butyl-1-methylpyrrolidinium chloride ([C_4mpyrr]Cl; purity > 99%), 1-butyl-1-methylpiperidinium chloride ([C_4mpip]Cl; purity > 99%). Phosphonium- and ammonium-based ILs were purchased from Cytec (USA) and Sigma–Aldrich (Spain), respectively. Imidazolium-, cholinium- and pyridinium-based ILs were supplied by Iolitec (Germany), except [Ch][Bit], [Ch]Cl, [Ch][DHCit] that were acquired from Sigma-Aldrich (Spain) and [Ch][Gly] that was synthesized by us according to well-established protocols [48]. The chemical structure of all the ILs investigated on this work are presented in Table 1.

Table 1 – Chemical structures of the investigated ILs.

			
[C_4mim]Cl	[C_4mim]Br	[P_{4444}]Cl	[P_{4444}]Br

			
[N_{2222}]Br	[N_{3333}]Br	[N_{4444}]Br	[Ch][Ac]
			
[Ch][Bit]	[Ch]Cl	[Ch][DHCit]	[Ch][DHP]
			
[Ch][Gly]	[C_4mpy]Cl	[C_4mpyrr]Cl	[C_4mpip]Cl

Potassium citrate ($C_6H_5K_3O_7$), citric acid ($C_6H_8O_7$), polypropylene glycol with an average molecular weight of 400 g·mol^{-1} (PPG 400), polyethylene glycol with an average molecular weight of 400 g·mol^{-1} (PEG 400) and 2,2'-azino-bis(3-ethylbenzathiazoline-6-sulfonic) acid (ABTS) were supplied by Sigma-Aldrich (Spain). The water employed was treated with a Milli-Q® Integral water purification apparatus from Merck Millipore. A summary of all the chemicals employed on this work is provided on Table 2.

Table 2 – Summary of the chemical reagents used on this work.

Chemicals	Purity (%)	Supplier	CAS number
[C_4mim]Cl	> 99	Iolitec	79917-90-1
[C_4mim]Br	> 99	Iolitec	85100-77-2
[P_{4444}]Cl	> 95	Cytec	2304-30-5
[P_{4444}]Br	> 95	Cytec	3115-68-2
[N_{2222}]Br	> 98	Sigma-Aldrich	71-91-0
[N_{3333}]Br	> 98	Sigma-Aldrich	1941-30-6

$[N_{4444}]Br$	> 98	Sigma-Aldrich	1643-19-2
[Ch][Ac]	> 98	Iolitec	14586-35-7
[Ch][Bit]	> 98	Sigma-Aldrich	87-67-2
[Ch]Cl	> 99	Sigma-Aldrich	67-48-1
[Ch][DHCit]	> 98	Sigma-Aldrich	77-91-8
[Ch][DHP]	> 99	Iolitec	83846-92-8
[Ch][Gly]	> 97	Synthesized *in-home*	
$[C_4mpy]Cl$	> 99	Iolitec	112400-85-8
$[C_4mpyrr]Cl$	> 99	Iolitec	479500-35-1
$[C_4mpip]Cl$	> 99	Iolitec	845790-13-8
$C_6H_5K_3O_7$	99	Sigma-Aldrich	866-84-2
$C_6H_8O_7$	99.5	Sigma-Aldrich	77-92-9
PPG 400	-	Sigma-Aldrich	25322-69-4
PEG 400	-	Sigma-Aldrich	25322-68-3
ABTS	> 98	Sigma-Aldrich	30931-67-0

2.1.2 Biological reagents

Commercial laccase (Novozym® 51003; EC 1.10.3.2; 1000 LAMU.g^{-1}), from *Myceliophthora thermophila* was kindly supplied by Novozymes (Denmark). This enzyme was produced by submerged fermentation of genetically modified *Aspergillus oryzae*. According to product datasheet, provided by Novozymes, this laccase is stored at pH 8.2.

2.2 Experimental Procedure

Determination of binodal curves, tie-lines and tie-line lengths. The cloud point titration method was used to determine the new binodal curves at 25±1°C, according to a protocol previously described [49]. The binodal curves were then fitted using Equation 1, proposed by Merchuk *et al.* [50]:

$$Y = A \exp\left[\left(BX^{0.5}\right) - \left(CX^{3}\right)\right] \quad (1)$$

where X and Y correspond to the weight fractions percentages of each phase-forming compound; X is the salt or PPG and Y is the IL or PEG depending on the constituents of the ABS. A, B and C are fitting parameters obtained by least-squares regression from the experimental data.

Tie-lines (TLs) were gravimetrically determined at 25±1°C according to the method proposed by Merchuk *et al*. [50], and already applied to IL-based ABS [51]. Additional details on the TLs and tie-line lengths (TLLs) determination can be found in the Supplementary Information (SI).

Screening of IL-based ABS for active laccase extraction. The compositions of the ternary mixtures used in the extractions studies of laccase were chosen based on: **i)** phase diagrams determined in this work for the systems composed of [C_4mim]Cl, [C_4mim]Br, [P_{4444}]Cl, [P_{4444}]Br, [N_{2222}]Br or [N_{4444}]Br + $C_6H_5K_3O_7$/$C_6H_8O_7$ + H_2O and **ii)** phase diagrams already published, namely the ABS formed by [Ch][Ac], [Ch][Bit], [Ch]Cl, [Ch][DHCit], [Ch][DHP] or [Ch][Gly] + PPG 400 + H_2O [40], [C_4mpy]Cl, [C_4mpyr]Cl or [C_4mpip]Cl + $C_6H_5K_3O_7$ + H_2O [51], and [N_{3333}]Br + $C_6H_5K_3O_7$/$C_6H_8O_7$ + H_2O [52]. The experimental phase diagrams data (in weight fraction) for the systems not reported in the literature are presented in the SI, Table S1. Ternary mixtures within the biphasic region were prepared with $C_6H_5K_3O_7$, $C_6H_5K_3O_7$/$C_6H_8O_7$ at pH 8 or PPG 400, IL and laccase solution in distilled water (7.5 μL·mL^{-1}). A summary of the compositions of the ternary mixtures investigated are presented in Tables S2 – S3 in the SI. Each system containing laccase was vigorously stirred in a vortex, left to equilibrate for at least 2 h at 25±1°C and centrifuged at 10000 rpm for 20 min to achieve the thermodynamic equilibrium and separation between the two phases. Then, the phases were separated, their volumes were ascertained, and laccase activity was measured in both phases with the procedure described below.

Determination of the extraction efficiency and partition coefficient of active laccase in IL-based ABS. Laccase activity measured according to the protocol described below provides a measurement of the concentration of active enzyme present in each phase of each ABS. The extraction efficiency of active laccase (EE%) was considered as the percentage ratio between the laccase activity in the IL-rich phase to that in the opposite phase, according to Equation 2:

$$EE\% = \frac{[lac]_{IL} \times v_{IL}}{[lac]_{IL} \times w_{IL} + [lac]_{salt/PPG} \times w_{salt/PPG}} \quad (2)$$

where $[lac]$ is the activity of laccase (U·L^{-1}), v represents the volume of each phase, and the subscripts IL, $salt$ and PPG represent the IL-, salt-, and PPG-rich phases, respectively.

The activity partition coefficient K_{act} of laccase in each phase was obtained using Equation 3:

$$K_{act} = \frac{[lac]_{IL}}{[lac]_{salt/PPG}} \quad (3)$$

where the subscripts IL and $salt$ or PPG indicate the phase were the laccase activity is measured, respectively.

Screening of ILs as adjuvants in polymer-salt and polymer-polymer ABS for laccase partition/activity. The best ILs identified in IL-based ABS were tested as adjuvants in representative polymer-salt and polymer-polymer ABS, namely formed by PPG 400 + K_2HPO_4 and PPG 400 + PEG 400. The selected ILs used as adjuvants were [Ch][Ac], [Ch][DHCit] and [Ch][DHP]. The experimental liquid-liquid equilibrium data (weight fraction) for these systems are presented in the SI, Tables S4-S7. The ternary mixtures were prepared with PPG 400, K_2HPO_4 or PEG 400, IL and laccase aqueous solution (7.5 μL·mL^{-1}). The laccase activity measurement procedure was the same as described below, and its extraction efficiency ($EE\%$) was determined according to Equation 4:

$$EE\% = \frac{[lac]_{salt/PEG} \times v_{salt/PEG}}{[lac]_{salt/PEG} \times v_{salt/PEG} + [lac]_{PPG} \times v_{PPG}} \quad (4)$$

where $[lac]$ is the activity of laccase (U·L^{-1}), v represents the volume of each phase, and the subscripts $salt$, PEG and PPG represent the salt-, PEG- and PPG-rich phases, respectively.

For each phase, the pH values were determined using a Mettler Toledo U402-M3-S7/200 micro electrode, and the presence/quantification of the IL was determined by ^{1}H NMR spectroscopy. Further details on the IL determination/quantification procedure can be found in the SI.

Laccase activity. The laccase activity was determined according to a method previously described [53]. For that purpose 100 μL of laccase solution was mixed with 0.5 mL of ABTS 0.2 mM, and 1.4 mL of citrate/phosphate buffer 0.05/0.1M at pH 4.5. The increase in absorbance was measured in kinetic model of a UV-Vis spectrophotometer (Agilent 8453) at 420 nm. The laccase activity was estimated using Equation 5:

$$\frac{\mathrm{U}}{\mathrm{L}} = \frac{abs{\cdot}min^{-1} \times \mathrm{f_{dil}} \times 10^6}{\varepsilon} \quad (5)$$

where ε is ABTS molar extinction coefficient (36000 M^{-1}cm^{-1} at 420 nm), abs·min^{-1} is the increase in absorbance per minute, f_{dil} is the dilution factor of the sample, 10^6 is the conversion factor from M to μM. One unit (U) of laccase activity is defined as the amount of enzyme required to form 1

μmol of $ABTS^{\bullet+}$ per minute, and laccase activities are expressed in $U{\cdot}L^{-1}$. Blank control samples were always analysed to ascertain possible interferences of the phase-forming components on the activity assays.

Proteins profile assessment. The proteins profile of each phase was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using an Amersham ECLTM Gel from GE Healthcare Life Sciences. The samples were mixed with the buffer sample (1:1, v/v) and dithiothreitol and heated at 95±1°C for 5 min. Electrophoresis was run on polyacrylamide gels (stacking: 4 % and resolving: 20 %) with a running buffer consisting of 250 mM Tris HCl, 1.92 M glycine, and 1 % (w/v) SDS at 135V for 90 min. The gel was stained with Coomassie Brilliant Blue G-250 and then distained. A molecular weight full-range marker (VWR) was used as protein standards.

3 Results and Discussion

3.1 Extraction/activity of laccase in IL-based ABS

In the last years there has been an increasing interest in the use of ILs in the field of biocatalysis and enzyme purification [54, 55]. Previous works showed that ILs may have beneficial or deleterious effects on the stability of enzymes and their activity [56, 57]. To take advantage of the ILs designer solvents aptitude and to use the full potential of IL-based ABS, it is necessary to understand the effect of ILs as a phase-forming constituents and how they affect the enzymes extraction and activity. For that purpose a wide range of ILs used as ABS phase-forming constituents were screened, namely tetraalkylammonium-, tetraalkylphosphonium-, imidazolium-, pyridinium-, pyrrolidinium- and piperidinium-based IL combined with the bromide, chloride, acetate, bitartrate, dihydrogen citrate, dihydrogenphosphate and glycolate anions. The binodal curves of each system, required for the design of appropriate extraction routes, were obtained in this work for the systems composed of $[C_4mim]Cl$, $[C_4mim]Br$, $[N_{2222}]Br$, $[N_{4444}]Br$, $[P_{4444}]Br$ and $[P_{4444}]Cl + C_6H_5K_3O_7/C_6H_8O_7 + H_2O$ and are presented in the SI, Figure S1. The experimental weight fraction data are also given in the SI (Table S1). The remaining ternary phase diagrams used in this work were obtained from the literature: ABS formed by [Ch][Ac], [Ch][Bit], [Ch]Cl, [Ch][DHCit], [Ch][DHP] or [Ch][Gly] + PPG 400 + H_2O [40]; $[C_4mpy]Cl$, $[C_4mpyr]Cl$ or $[C_4mpip]Cl + C_6H_5K_3O_7 + H_2O$ [51]; and $[N_{3333}]Br + C_6H_5K_3O_7/C_6H_8O_7 + H_2O$ [52]. In all the IL + salt systems, the top phase corresponds to the IL-rich phase and the bottom phase corresponds to the salt-rich phase; for the IL + polymer systems, the top phase corresponds to the polymer-rich phase while the bottom phase corresponds to the IL-rich phase.

Initial studies were carried out with different concentrations of laccase, ranging from 3.75 to 15 $\mu L \cdot mL^{-1}$, in selected ABS, to investigate the effect of increasing protein concentration on laccase partition and activity and to infer the most appropriate concentration to be used. This laccase concentration range was selected to give similar enzyme activity to those achieved during its production by fermentation, based on a previously published paper by us [58]. The results are presented in Figure S2 in the SI, showing that it is possible to extract laccase for the IL-rich phase using concentrations up to 15 $\mu L \cdot mL^{-1}$, with no saturation of the phases and/or any precipitation of the enzyme at the interface. Therefore, all the investigated ABS in this work were loaded with an intermediate laccase concentration – 7.5 $\mu L \cdot mL^{-1}$, whose compositions of the ABS investigated are given in Table 3. All the mixture points studied were selected based on the corresponding phase diagrams in order to be close to the binodal curves, maximizing the amount of water in the systems,

which is beneficial when dealing with proteins/enzymes. Also, it is important to highlight that all the ABS composed of $C_6H_5K_3O_7$/$C_6H_8O_7$ buffer were set at pH 8, since it consists in the optimum pH value of the laccase under study, based on a preliminary study of the laccase activity as a function of the pH (see SI, Figure S3) – being in accordance with the information provided by the supplier in the product datasheet. The comparison between the laccase activity in the top and bottom phases, as well as the extraction efficiencies (*EE*%) and partition coefficient of the active laccase (K_{act}) are given in Table 3.

Table 3 – Results for active laccase partition (K_{act}), extraction efficiency (*EE*%), and laccase activity in the top and bottom phases using different IL-based ABS.

ABS	Mixture composition (wt%)	K_{act}	*EE*%	Laccase activity ($U \cdot L^{-1}$) top phase	Laccase activity ($U \cdot L^{-1}$) bottom phase
$[C_4mim]Br$ + $C_6H_5K_3O_7$/$C_6H_8O_7$ pH 8	32% IL 22% salt	1.76	64	70.2	123.6
$[C_4mim]Cl$ + $C_6H_5K_3O_7$/$C_6H_8O_7$ pH 8	35% IL 22% salt	--	--	ND	ND
$[N_{2222}]Br$ + $C_6H_5K_3O_7$/$C_6H_8O_7$ pH 8	31% IL 20% salt	1.45	59	483.6	702.0
$[N_{3333}]Br$ + $C_6H_5K_3O_7$/$C_6H_8O_7$ pH 8	30% IL 20% salt	--	--	53.4	ND
$[N_{4444}]Br$ + $C_6H_5K_3O_7$/$C_6H_8O_7$ pH 8	28% IL 12% salt	--	--	ND	ND
$[P_{4444}]Cl$ + $C_6H_5K_3O_7$/$C_6H_8O_7$ pH 8	28 % IL 15% salt	--	--	ND	ND
$[P_{4444}]Br$ + $C_6H_5K_3O_7$/$C_6H_8O_7$ pH 8	28 %IL 10% salt	--	--	ND	ND
$[C_4mpip]Cl$ + $C_6H_5K_3O_7$	30% IL 25% salt	--	--	ND	ND
$[C_4mpyr]Cl$ + $C_6H_5K_3O_7$	30% IL 25% salt	--	--	176.4	ND
$[C_4mpy]Cl$ + $C_6H_5K_3O_7$	30% IL 25% salt	--	--	ND	ND
[Ch]Cl + PPG 400	15% IL 25% PPG	2.21	69	160.2	353.4
[Ch][Ac] + PPG 400	12% IL 20% PPG	--	--	SP	464.4
[Ch][Gly] + PPG 400	10% IL 25% PPG	--	--	SP	343.2

[Ch][DHP] + PPG 400	15% IL 15% PPG	--	--	SP	469.8
[Ch][DHCit] + PPG 400	21% IL 25% PPG	2.05	67	226.8	698.4
[Ch][Bit] + PPG 400	12% IL 35% PPG	--	--	ND	5.06

ND: laccase activity not detected; SP: small phase – laccase activity not measured

The results summarized in Table 3 allow to analyse the impact of ILs on the laccase partitioning in IL-based ABS and its activity. No laccase activity was detected for the top and bottom phases in the ABS composed of $[C_4mim]Cl$, $[N_{4444}]Br$, $[P_{4444}]Cl$, $[P_{4444}]Br$, $[C_4mpip]Cl$ and $[C_4mpy]Cl$. In addition, laccase activity is very low in the ABS composed of $[C_4mim]Br$, $[N_{3333}]Br$, [Ch]Cl, [Ch][Gly], [Ch][Bit] and $[C_4mpyr]Cl$. Since ABS composed of these ILs were not able to maintain laccase activity, probably due to their interference with the copper-active site of the enzyme [59], they appear to be not-suitable for the design of purification processes for this high-value biomolecule, and thus these ABS were discarded for further studies. The inhibition of the laccase activity in presence of some imidazolium-based ILs was previously reported by us [60]. On the other hand, $[N_{2222}]Br$, [Ch][Ac], [Ch][DHCit] and [Ch][DHP] are the most effective ILs concerning both laccase extraction and activity preservation, meaning that quaternary alkyl ammoniums, with short alkyl side chains, are beneficial to deal with the laccase oxidative enzyme. These ILs were then selected for further studies of laccase extraction and activity.

3.1.1 Effect of mixture composition on laccase partition/activity in IL-based ABS

The partition and activity of an enzyme in an ABS depends on the properties, composition and volumetric ratio of both top and bottom phases. ABS mixtures of different compositions were evaluated for the systems previously selected: $[N_{2222}]Br + C_6H_5K_3O_7/C_6H_8O_7 + H_2O$; [Ch][Ac] + PPG 400 + H_2O; [Ch][DHCit] + PPG 400 + H_2O and [Ch][DHP] + PPG 400 + H_2O. Laccase partition and activity results are presented in Table 4.

Table 4 – Results for active laccase partition (K_{act}), extraction efficiency (*EE*%), and laccase activity in the top and bottom phases using different mixture compositions of each IL-based ABS.

ABS	**Mixture composition (wt%)**	**TLL**	K_{act}	***EE* (%)**	**Laccase activity (U·L^{-1}) top phase**	**Laccase activity (U·L^{-1}) bottom phase**

$[N_{2222}]Br$ + $C_6H_5K_3O_7/C_6H_8O_7$	31% IL 20% salt	55	0.69	73	484	702
	24% IL 26% salt	55	0.83	31	558	672
[Ch][Ac] + PPG 400	12% IL 20% PPG	44	--	--	SP	470
	9% IL 35% PPG	44	--	100	ND	708
	6% IL 41% PPG	44	--	100	ND	326
[Ch][DHP] + PPG 400	15% IL 15% PPG	44	--	100	ND	464
	10% IL 30% PPG	44	--	100	ND	828
	7% IL 35% PPG	44	--	100	ND	804
[Ch][DHCit] + PPG 400	21% IL 25% PPG	45	--	--	SP	698
	15% IL 40% PPG	45	--	100	ND	1044
	12% IL 48% PPG	45	--	100	ND	1098

ND: laccase activity not detected; SP: small phase – laccase activity not measured

Considering the complete partition of laccase obtained with cholinium-based ABS, the increase of the PPG 400 concentration and decrease of the IL concentration in the initial mixture composition was further evaluated, while maintaining the same tie-line length (TLL) and changing the volume ratio for all the extractions. It is important to remark that for all ABS evaluated, no degradation and precipitation of the enzyme, at the interface or in any phase, was observed. From the obtained results, it was found that the cholinium-based ILs present an excellent performance concerning the extraction of laccase towards the IL-rich phase, achieving extraction efficiencies of 100% for all studied systems in which the volume of the IL-rich phase was sufficient to perform the determination of laccase activity. This partitioning behaviour was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE); the protein profile of each

fraction can be found in the SI, Figure S4. Although some delay on the gel run was found on the wells containing IL-rich samples, due to the interference of the ILs (composed of ions) with the electric flow of the electrophoresis, it was possible to proper qualitatively identify laccase in each sample. Laccase preferentially partitions to the IL-rich bottom phase, with the exception of the $[N_{2222}]$Br-based ABS, where the extraction was not complete and the laccase partitions between the two phases. It is important to highlight that the ABS composed of $[N_{2222}]$Br is formed by an IL and $C_6H_5K_3O_7/C_6H_8O_7$ pH 8, and in these IL-salt-systems, the IL-rich phase corresponds to the most hydrophobic phase [40], which is the opposite to that occurring in the IL-PPG 400-systems in which the phase enriched in IL is the most hydrophilic phase. Thus, these results suggest that the hydrophobicity/hydrophilicity of the ABS phases plays a major role in the partition behaviour of active laccase, although other specific interactions occurring with the IL chemical structure may also play a role [61].

Although the complete extraction of laccase towards the IL-rich phase occurs in systems formed by cholinium-based ILs and PPG 400, a strong effect of the IL anion is however observed in laccase activity. The results given in Table 4 show that the laccase activity decreases in the following order of the cholinium-based anions: $[DHCit]^-$>$[DHP]^-$>$[Ac]^-$. Furthermore, the results obtained for cholinium-based ILs, shown in Table 4, indicate that the modification of the initial mixture composition (and consequently the systems' volume ratio) has a significant influence on the laccase activity in the IL-rich phase. In general, the activity of the enzyme decreases as the concentration of IL increases in the mixture composition. Thus, laccase activity differences obtained for the same IL at different mixture compositions, with a similar TLL and thus similar differences in the compositions between the two phases, are essentially related with the phases' volumes and saturation effects that may occur (related with the changes on the volume ratio).

Among the investigated cholinium-based ILs, [Ch][DHCit] is the most effective IL in the enzyme activity preservation/enhancement in the IL-rich phase, allowing to infer that also the IL chemical structure, and particularly the IL anion, plays an important role in the tailoring of the ABS polarity and subsequent partition of laccase. For these reasons, this study suggests that [Ch][DHCit]-based ABS is the best option, since it allowed the complete extraction of laccase in a single step with an enzyme activity higher than 1000 $U \cdot L^{-1}$.

3.2 Extraction/activity of laccase in ABS comprising ILs as adjuvants

The formation of ABS using ILs requires large amounts of these chemicals. A more economical alternative is the formation of conventional polymer-polymer or polymer-salt ABS

using ILs as adjuvants, at lower concentrations. This possibility was explored using the best ILs identified before. For this purpose, two conventional ABS were selected: a polymer-salt ABS (PPG 400 + K_2HPO_4) and a polymer-polymer ABS (PPG 400 + PEG 400), using ILs as adjuvants at 1, 3 and 5 wt%.

The effects of the IL and its concentration upon the binodal curve of PPG 400 + K_2HPO_4 + H_2O were evaluated and are presented in SI, Figure S5. In general, the curves show that, for all the ILs studied, the IL concentration of 5 wt% increased the biphasic region of the systems, meaning that lower amounts of the phase-forming compounds are required to form the ABS. After performing the extraction of laccase using these ABS composed of 5 wt% of IL, other percentages of adjuvant were further tested, namely 1 and 3 wt%, and for which the binodal curves were also determined (SI, Figure S5). The binodal curves using 1 wt% of IL are very close to those without any adjuvant, meaning that at such low concentrations the IL has a negligible effect in ABS formation. Higher concentrations are required in order to obtain a more pronounced effect in the phase diagrams and respective monophasic/biphasic regimes. In all these systems, the top phase corresponds to the PPG 400-rich phase and the bottom phase corresponds to the K_2HPO_4-rich phase.

The influence of ILs as adjuvants at the three previously mentioned concentrations (1, 3 and 5 wt%) in IL-salt ABS on laccase partition was investigated. For all the conditions tested, including for the systems without IL, a preferential partition of laccase to the bottom salt-rich phase was found, with its complete extraction achieved in one-step for all cases (*EE*% = 100%). The previous results regarding the ternary systems composed of IL + PPG 400 + H_2O show that laccase migrates preferentially to the IL-rich phase – the most hydrophilic phase in the system. The results for the quaternary systems composed of PPG 400 + K_2HPO_4 + H_2O + IL show the same behaviour, with the preferential partition of laccase to the salt-rich phase – the most hydrophilic phase in these systems, and in which the IL is retained (the complete partition of the IL was determined; the salt-rich phase is completely enriched in IL). A common mixture composition was chosen in the biphasic region of these systems: 27 wt% of PPG 400 + 5 wt% of K_2HPO_4 + (1, 3 or 5) wt% IL. Although complete extraction was obtained for all the conditions in this study (see SI, Table S8), significant differences were found regarding the laccase activity in the salt-rich phase, as depicted in Figure 1. The respective detailed values and pH values of the phases are given in the SI, Table S8.

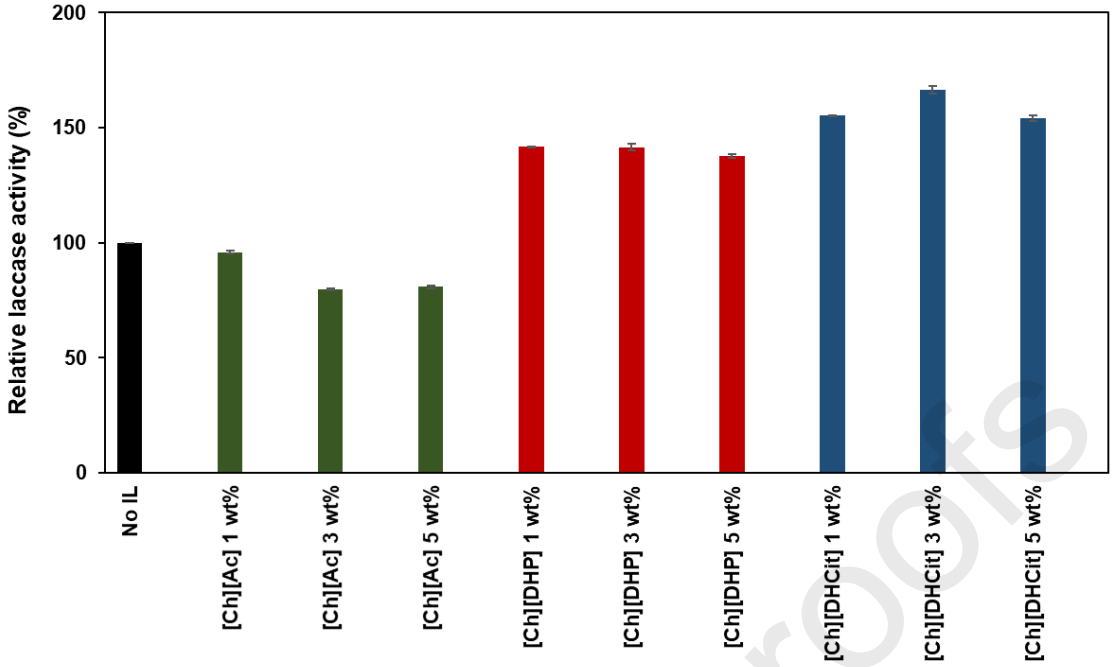


Figure 1 – Relative activities (%) of laccase in the bottom salt-rich phase of the systems composed of PPG 400 + K_2HPO_4 + H_2O + 1, 3 or 5 wt% of ILs as adjuvants.

It is clear from Figure 1 that the IL anion plays a critical role in the activity of the enzyme, in agreement with the literature [62]. Laccase is more active in systems with the anions $[DHCit]^-$ and $[DHP]^-$, when compared to the system in which no IL is added, while a decrease in activity is found for the IL containing the anion $[Ac]^-$. The systems formed by [Ch][DHCit] and [Ch][DHP] as adjuvants provide a 50 % and 40 % increase of activity, whilst for [Ch][Ac] a 20 % decrease in activity was observed when compared to the control system (without IL). Overall, the laccase activity decreases in the order: $[DHCit]^- > [DHP]^- >> [Ac]^-$. This trend is in agreement with that obtained in ABS formed by the same ILs and PPG 400, meaning that even at lower concentrations and in different type of systems the effect of the ILs chemical structure prevails. Furthermore, there are no significant differences on the enzyme activity between the three concentrations tested, which means that the IL chemical structure appears to be more relevant than the IL amount present in the systems.

In the studied polymer-salt quaternary ABS, high activity of laccase after the extraction process was attained in the best conditions tested, being even higher to those obtained with the respective ternary systems, namely for ABS composed of water, PPG 400 and [Ch][DHCit] or [Ch][DHP]. This is quite relevant since there is the possibility of achieving excellent results concerning both the extraction and activity of laccase using lower concentrations of IL.

As a last approach, ILs were also tested as adjuvants in ABS composed of two different water-soluble polymers for the partition and activity of laccase. It has been shown that polymer-polymer ABS can be easily formed depending on the type of polymers combined [36]. The polymers PEG 400 and PPG 400 were chosen since both are water soluble, nontoxic (considered as safe) [63], and have been approved for human injections and oral application, being widely applied by the chemical, food and pharmaceutical industries [64]. In order to evaluate the effect of ILs as adjuvants in this type of polymer-polymer-based ABS, the ILs previously selected – [Ch][Ac], [Ch][DHP] and [Ch][DHCit] – were studied at the same concentrations (1, 3 and 5 wt%). The experimental phase diagrams were obtained, and the schematic representation of the binodal curves are shown in the SI, Figure S6. As observed before with ABS formed by polymers and salts, it was found that by using ILs as adjuvants (1, 3 and 5 wt%) in polymer-polymer systems it is possible to lower the amounts of phase-forming compounds required to form the ABS. In all these systems, the top phase corresponds to the PPG-400-rich phase and the bottom phase corresponds to the PEG-400-rich phase.

After the characterization of the phase diagrams, the impact of the cholinium-based IL as adjuvants in each PPG 400 + PEG 400 ABS was studied in the laccase partition and activity. According to the gathered results, laccase completely partitions to the bottom PEG-400-rich phase, achieving an extraction efficiency of 100% for all the conditions tested without using IL, and also with the three ILs at the three different concentrations. For this type of systems and for all the studied concentrations of IL, the complete partition of the IL to the bottom PEG-rich phase was observed. These results allow to corroborate the idea that ILs and laccase present a higher affinity for more hydrophilic phases, being the phosphate-rich phase in the systems formed by polymers and salts and the PEG-400-rich phase in the systems formed by two polymers.

Although the complete extraction was obtained for all the conditions under study (see SI, Table S9), significant differences were found regarding the laccase activity in the various systems, as depicted in Figure 2. The detailed values and pH values of the coexisting phases are presented in the SI, Table S9.

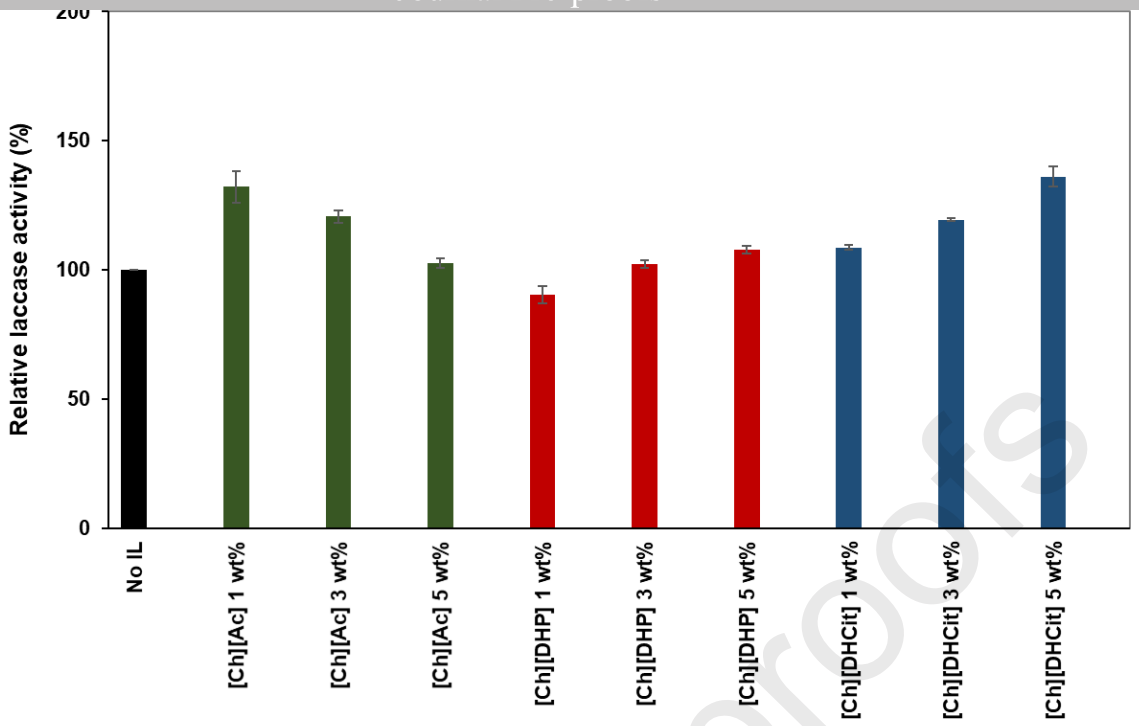


Figure 2 – Relative activities (%) of laccase in the PEG-400-rich phase of the systems composed of PPG 400 + PEG 400 + H_2O + (1, 3 or 5 wt%) ILs.

For all the conditions tested, the enzyme activity increases in the bottom PEG-rich phase when compared with the control system (without IL), except for the system composed of 1 wt% [Ch][DHP] where no signficant differences are seen. The obtained results also show that the laccase activity decreases in polymer-polymer ABS, since higher values were obtained using the system composed of PPG 400 + K_2HPO_4 + ILs as adjuvants. It is interesting to notice that the obtained laccase activity results and partitioning among the ABS phases reinforce the high hydrophilic nature of the enzyme: the enzyme partitions, once again, to the most hydrophilic phase in polymer-polymer systems.

Comparing the different adjuvants used, [Ch][DHCit] displays the best capacity to improve the laccase activity, as previously observed for polymer-salt systems. Once again it is proved that the IL chemical structure plays a significant role. This trend is in close agreement with our previous work on the finding of alternative solvents to improve the laccase activity, in which eutectic mixtures formed by [Ch][DHCit] and polyols were found as the most promising [65]. Overall, chemical structures with a higher number of hydroxyl groups are beneficial to improve the laccase activity [65], which is the case of [Ch][DHCit] amongst the several cholinium-based ILs investigated.

Overall, the obtained results allow to conclude that laccase preferentially partitions to the most hydrophilic phase in systems formed by ILs and polymers or salts, both used as adjuvants or as phase-forming components, and particularly to the phase in which the IL is enriched. Furthermore, the IL chemical structure of the IL has a significant impact in the enzyme activity, where ILs with a higher number of hydroxyl groups appear as promising to improve the laccase activity. In this sense, evidences were gathered that could be useful to improve the extraction efficiency of enzymes downstream processing, and that may take advantage of the designer solvent ability of ILs to selectively extract laccase from complex fermentation broths.

4 Conclusions

In this work, we studied different types of ABS comprising ILs to extract and improve the activity of laccase aiming at gather evidences that could allow the design of effective downstream processes. Three types of IL-based ABS have been evaluated: ternary systems composed of IL + (polymer or salt) + water and quaternary systems composed of (polymer + salt + water + ILs as adjuvants) and (polymer + polymer + water + ILs as adjuvants). From the wide range of ILs evaluated, cholinium-based ILs were identified as the most promising candidates concerning the development of an effective extraction process for laccase, providing also high activity values. Several evidences support a clear preference of laccase to the most hydrophilic phase of the ABS, independently of the type of system investigated, which is also the phase in which the IL is enriched. Both ternary and quaternary IL-based ABS showed promising results; however, ABS composed of polymers, salts and ILs as adjuvants should be highlighted as they provided the complete extraction of laccase (extraction efficiency = 100%) in a single-step, with an improvement on its activity to values higher than 150%. Furthermore, the use of ILs as adjuvants presents the advantage of requiring lower amounts of the ABS phase-forming compounds, confirmed by the shift in the respective binodal curves of the phase diagrams, as well as a small concentration of IL, thus contributing to decrease the costs associated with the process. [Ch][DHCit], the cholinium-based IL with more hydroxyl groups in its chemical structure, was identified as the most promising IL studied, corroborating the idea that the chemical structure of the IL plays a significant role in the enzymes partitioning and activity in ABS, and as such giving insights on the best strategy to develop effective downstream processes for laccase.

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References

[1] J.-M. Choi, S.-S. Han, H.-S. Kim, Industrial applications of enzyme biocatalysis: current status and future aspects, Biotechnology advances, 33 (2015) 1443-1454.

[2] M. Yadav, H. Yadav, Applications of ligninolytic enzymes to pollutants, wastewater, dyes, soil, coal, paper and polymers, Environmental chemistry letters, 13 (2015) 309-318.

[3] G. Singh, K. Kaur, S. Puri, P. Sharma, Critical factors affecting laccase-mediated biobleaching of pulp in paper industry, Applied microbiology and biotechnology, 99 (2015) 155-164.

[4] A.P.M. Tavares, J.A. Gamelas, A.R. Gaspar, D.V. Evtuguin, A.M. Xavier, A novel approach for the oxidative catalysis employing polyoxometalate–laccase system: application to the oxygen bleaching of kraft pulp, Catalysis communications, 5 (2004) 485-489.

[5] A.P.M. Tavares, R.O. Cristóvão, J.A. Gamelas, J.M. Loureiro, R.A. Boaventura, E.A. Macedo, Sequential decolourization of reactive textile dyes by laccase mediator system, Journal of chemical technology and biotechnology, 84 (2009) 442-446.

[6] A.P.M. Tavares, R.O. Cristóvão, J.M. Loureiro, R.A. Boaventura, E.A. Macedo, Optimisation of reactive textile dyes degradation by laccase–mediator system, Journal of chemical technology and biotechnology, 83 (2008) 1609-1615.

[7] R.M. Bento, M.R. Almeida, P. Bharmoria, M.G. Freire, A.P.M. Tavares, Improvements in the enzymatic degradation of textile dyes using ionic-liquid-based surfactants, Separation and purification technology, 235 (2020) 116191.

[8] D.E. Dodor, H.-M. Hwang, S.I. Ekunwe, Oxidation of anthracene and benzo[a]pyrene by immobilized laccase from *Trametes versicolor*, Enzyme and microbial technology, 35 (2004) 210-217.

[9] A.D. Moreno, D. Ibarra, J.L. Fernández, M. Ballesteros, Different laccase detoxification strategies for ethanol production from lignocellulosic biomass by the thermotolerant yeast Kluyveromyces marxianus CECT 10875, Bioresource technology, 106 (2012) 101-109.

[10] M.M. Rodríguez-Delgado, G.S. Alemán-Nava, J.M. Rodríguez-Delgado, G. Dieck-Assad, S.O. Martínez-Chapa, D. Barceló, R. Parra, Laccase-based biosensors for detection of phenolic compounds, TrAC Trends in analytical chemistry, 74 (2015) 21-45.

[11] S. Nicotra, M.R. Cramarossa, A. Mucci, U.M. Pagnoni, S. Riva, L. Forti, Biotransformation of resveratrol: synthesis of trans-dehydrodimers catalyzed by laccases from Myceliophtora thermophyla and from Trametes pubescens, Tetrahedron, 60 (2004) 595-600.

[12] M. Mogharabi, M.A. Faramarzi, Laccase and laccase-mediated systems in the synthesis of organic compounds, Advanced synthesis & catalysis, 356 (2014) 897-927.

[13] J. Osiadacz, A.J. Al-Adhami, D. Bajraszewska, P. Fischer, W. Peczyñska-Czoch, On the use of Trametes versicolor laccase for the conversion of 4-methyl-3-hydroxyanthranilic acid to actinocin chromophore, Journal of biotechnology, 72 (1999) 141-149.

[14] A. Kunamneni, S. Camarero, C. García-Burgos, F.J. Plou, A. Ballesteros, M. Alcalde, Engineering and applications of fungal laccases for organic synthesis, Microbial cell factories, 7 (2008) 32.

[15] P. Giardina, V. Faraco, C. Pezzella, A. Piscitelli, S. Vanhulle, G. Sannia, Laccases: a never-ending story, Cellular and molecular life sciences, 67 (2010) 369-385.

[16] L. Arregui, M. Ayala, X. Gómez-Gil, G. Gutiérrez-Soto, C.E. Hernández-Luna, M.H. de los Santos, L. Levin, A. Rojo-Domínguez, D. Romero-Martínez, M.C. Saparrat, Laccases: structure, function, and potential application in water bioremediation, Microbial cell factories, 18 (2019) 200.

[17] A.D. Moreno, D. Ibarra, M.E. Eugenio, E. Tomás-Pejó, Laccases as versatile enzymes: from industrial uses to novel applications, Journal of chemical technology & biotechnology, 95 (2020) 481-494.

[18] R. Saleem, M. Khurshid, S. Ahmed, Laccases, manganese peroxidases and xylanases used for the bio-bleaching of paper pulp: an environmental friendly approach, Protein and peptide letters, 25 (2018) 180-186.

[19] V.E. Manhivi, E.O. Amonsou, T. Kudanga, Laccase-mediated crosslinking of gluten-free amadumbe flour improves rheological properties, Food chemistry, 264 (2018) 157-163.

[20] J. Su, J. Noro, J. Fu, Q. Wang, C. Silva, A. Cavaco-Paulo, Coloured and low conductive fabrics by in situ laccase-catalysed polymerization, Process biochemistry, 77 (2019) 77-84.

[21] F. Reda, R. El-Mekkawy, N. Hassan, Detoxification and bioremediation of sulfa drugs and synthetic dyes by Streptomyces mutabilis A17 laccase produced in solid state fermentation, Journal of pure and applied microbiology, 13 (2019) 85-96.

[22] J.H. Coelho, A.P. Eisele, C.F. Valezi, G.J. Mattos, J.G. Schirmann, R.F. Dekker, A.M. Barbosa-Dekker, E.R. Sartori, Exploring the exocellular fungal biopolymer botryosphaeran for laccase-biosensor architecture and application to determine dopamine and spironolactone, Talanta, 204 (2019) 475-483.

[23] S. Giacobbe, A. Piscitelli, F. Raganati, V. Lettera, G. Sannia, A. Marzocchella, C. Pezzella, Butanol production from laccase-pretreated brewer's spent grain, Biotechnology for biofuels, 12 (2019) 47.

[24] C. Pezzella, L. Guarino, A. Piscitelli, How to enjoy laccases, Cellular and molecular life sciences, 72 (2015) 923-940.

[25] A. Kunamneni, F.J. Plou, A. Ballesteros, M. Alcalde, Laccases and their applications: a patent review, Recent patents on biotechnology, 2 (2008) 10-24.

[26] A. Antecka, M. Blatkiewicz, T. Boruta, A. Górak, S. Ledakowicz, Comparison of downstream processing methods in purification of highly active laccase, Bioprocess and biosystems engineering, 42 (2019) 1635-1645.

[27] M.K. Potdar, G.F. Kelso, L. Schwarz, C. Zhang, M.T. Hearn, Recent developments in chemical synthesis with biocatalysts in ionic liquids, Molecules, 20 (2015) 16788-16816.

[28] X. Chen, J. Liu, J. Wang, Ionic liquids in the assay of proteins, Analytical methods, 2 (2010) 1222-1226.

[29] M.G. Freire, A.F.M. Claudio, J.M. Araujo, J.A.P. Coutinho, I.M. Marrucho, J.N.C. Lopes, L.P.N. Rebelo, Aqueous biphasic systems: a boost brought about by using ionic liquids, Chemical society reviews, 41 (2012) 4966-4995.

[30] J.F.B. Pereira, S.P.M. Ventura, F.A. e Silva, S. Shahriari, M.G. Freire, J.A.P. Coutinho, Aqueous biphasic systems composed of ionic liquids and polymers: a platform for the purification of biomolecules, Separation and purification technology, 113 (2013) 83-89.

[31] E.V. Capela, M.V. Quental, P. Domingues, J.A.P. Coutinho, M.G. Freire, Effective separation of aromatic and aliphatic amino acid mixtures using ionic-liquid-based aqueous biphasic systems, Green chemistry, 19 (2017) 1850-1854.

[32] S. Galai, A. de los Rios, F. Hernández-Fernández, S.H. Kacem, F. Tomas-Alonso, Over-activity and stability of laccase using ionic liquids: screening and application in dye decolorization, RSC Advances, 5 (2015) 16173-16189.

[33] A. Domínguez, O. Rodríguez, A.P.M. Tavares, E.A. Macedo, M.A. Longo, M.Á. Sanromán, Studies of laccase from Trametes versicolor in aqueous solutions of several methylimidazolium ionic liquids, Bioresource technology, 102 (2011) 7494-7499.

[34] P.-Å. Albertsson, Partition of proteins in liquid polymer–polymer two-phase systems, Nature, 182 (1958) 709-711.

[35] K.E. Gutowski, G.A. Broker, H.D. Willauer, J.G. Huddleston, R.P. Swatloski, J.D. Holbrey, R.D. Rogers, Controlling the aqueous miscibility of ionic liquids: aqueous biphasic systems of water-miscible ionic liquids and water-structuring salts for recycle, metathesis, and separations, Journal of the american chemical society, 125 (2003) 6632-6633.

[36] A.L. Grilo, M.R. Aires-Barros, A.M. Azevedo, Partitioning in aqueous two-phase systems: fundamentals, applications and trends, Separation & purification reviews, 45 (2016) 68-80.

[37] M. Iqbal, Y. Tao, S. Xie, Y. Zhu, D. Chen, X. Wang, L. Huang, D. Peng, A. Sattar, M.A.B. Shabbir, Aqueous two-phase system (ATPS): an overview and advances in its applications, Biological procedures online, 18 (2016) 18.

[38] K.S. Nascimento, P. Rosa, K. Nascimento, B. Cavada, A. Azevedo, M. Aires-Barros, Partitioning and recovery of Canavalia brasiliensis lectin by aqueous two-phase systems using design of experiments methodology, Separation and purification technology, 75 (2010) 48-54.

[39] J.F.B. Pereira, L.P.N. Rebelo, R.D. Rogers, J.A.P. Coutinho, M.G. Freire, Combining ionic liquids and polyethylene glycols to boost the hydrophobic–hydrophilic range of aqueous biphasic systems, Physical chemistry chemical physics, 15 (2013) 19580-19583.

[40] M.V. Quental, M. Caban, M.M. Pereira, P. Stepnowski, J.A.P. Coutinho, M.G. Freire, Enhanced extraction of proteins using cholinium-based ionic liquids as phase-forming components of aqueous biphasic systems, Biotechnology journal, 10 (2015) 1457-1466.

[41] Y. Pei, J. Wang, K. Wu, X. Xuan, X. Lu, Ionic liquid-based aqueous two-phase extraction of selected proteins, Separation and purification technology, 64 (2009) 288-295.

[42] F. Deive, A. Rodriguez, A. Pereiro, J. Araújo, M. Longo, M. Coelho, J.C. Lopes, J. Esperança, L. Rebelo, I. Marrucho, Ionic liquid-based aqueous biphasic system for lipase extraction, Green chemistry, 13 (2011) 390-396.

[43] M. Taha, M.V. Quental, E.V. Capela, M.G. Freire, S.P.M. Ventura, J.A.P. Coutinho, Good's buffer ionic liquids as relevant phase-forming components of self-buffered aqueous biphasic systems, Journal of chemical technology and biotechnology, 92 (2017) 2287-2299.

[44] E.V. Capela, A.E. Santiago, A.F.C.S. Rufino, A.P.M. Tavares, M.M. Pereira, A. Mohamadou, M.R. Aires-Barros, J.A.P. Coutinho, A.M. Azevedo, M.G. Freire, Sustainable strategies based on glycine-betaine analogues ionic liquids for the recovery of monoclonal antibodies from cell culture supernatants, Green chemistry, 21 (2019) 5671-5682.

[45] A.P.M. Tavares, O. Rodriguez, E.A. Macedo, Peroxidase biocatalysis in water-soluble ionic liquids: activity, kinetic and thermal stability, Biocatalysis and biotransformation, 30 (2012) 417-425.

[46] A.P.M. Tavares, O. Rodriguez, E.A. Macedo, Ionic liquids as alternative co-solvents for laccase: Study of enzyme activity and stability, Biotechnology and bioengineering, 101 (2008) 201-207.

[47] S.C. Silvério, O. Rodríguez, A.P.M. Tavares, J.A. Teixeira, E. Macedo, Laccase recovery with aqueous two-phase systems: enzyme partitioning and stability, Journal of molecular catalysis B: enzymatic, 87 (2013) 37-43.

[48] N. Muhammad, M.I. Hossain, Z. Man, M. El-Harbawi, M.A. Bustam, Y.A. Noaman, N.B. Mohamed Alitheen, M.K. Ng, G. Hefter, C.-Y. Yin, Synthesis and physical properties of choline carboxylate ionic liquids, Journal of chemical & engineering data, 57 (2012) 2191-2196.

[49] C.M.S.S. Neves, S.P.M. Ventura, M.G. Freire, I.M. Marrucho, J.A.P. Coutinho, Evaluation of cation influence on the formation and extraction capability of ionic-liquid-based aqueous biphasic systems, The journal of physical chemistry B, 113 (2009) 5194-5199.

[50] J.C. Merchuk, B.A. Andrews, J.A. Asenjo, Aqueous two-phase systems for protein separation: studies on phase inversion, Journal of chromatography B: biomedical sciences and applications, 711 (1998) 285-293.

[51] H. Passos, A.R. Ferreira, A.F.M. Cláudio, J.A.P. Coutinho, M.G. Freire, Characterization of aqueous biphasic systems composed of ionic liquids and a citrate-based biodegradable salt, Biochemical engineering journal, 67 (2012) 68-76.

[52] T.E. Sintra, R. Cruz, S.P.M. Ventura, J.A.P. Coutinho, Phase diagrams of ionic liquids-based aqueous biphasic systems as a platform for extraction processes, The journal of chemical thermodynamics, 77 (2014) 206-213.

[53] P. Ander, K. Messner, Oxidation of 1-hydroxybenzotriazole by laccase and lignin peroxidase, Biotechnology techniques, 12 (1998) 191-195.

[54] S.P.M. Ventura, F.A. e Silva, M.V. Quental, D. Mondal, M.G. Freire, J.A.P. Coutinho, Ionic-liquid-mediated extraction and separation processes for bioactive compounds: past, present, and future trends, Chemical reviews, 117 (2017) 6984-7052.

[55] U. Kragl, M. Eckstein, N. Kaftzik, Enzyme catalysis in ionic liquids, Current opinion in biotechnology, 13 (2002) 565-571.

[56] S.-F. Wang, T. Chen, Z.-L. Zhang, D.-W. Pang, Activity and stability of horseradish peroxidase in hydrophilic room temperature ionic liquid and its application in non-aqueous biosensing, Electrochemistry communications, 9 (2007) 1337-1342.

[57] H. Zhao, Methods for stabilizing and activating enzymes in ionic liquids — a review, Journal of chemical technology and biotechnology, 85 (2010) 891-907.

[58] A.P.M. Tavares, M. Coelho, J.A.P. Coutinho, A. Xavier, Laccase improvement in submerged cultivation: induced production and kinetic modelling, Journal of chemical technology & biotechnology: international research in process, Environmental & clean technology, 80 (2005) 669-676.

[59] H. Liu, L. Zhu, M. Bocola, N. Chen, A.C. Spiess, U. Schwaneberg, Directed laccase evolution for improved ionic liquid resistance, Green chemistry, 15 (2013) 1348-1355.

[60] O. Rodríguez, R.O. Cristóvão, A.P.M. Tavares, E.A. Macedo, Study of the alkyl chain length on laccase stability and enzymatic kinetic with imidazolium ionic liquids, Applied biochemistry and biotechnology, 164 (2011) 524-533.

[61] A. Benedetto, P. Ballone, Room temperature ionic liquids meet biomolecules: a microscopic view of structure and dynamics, ACS sustainable chemistry & engineering, 4 (2015) 392-412.

[62] J.L. Kaar, A.M. Jesionowski, J.A. Berberich, R. Moulton, A.J. Russell, Impact of ionic liquid physical properties on lipase activity and stability, Journal of the american chemical society, 125 (2003) 4125-4131.

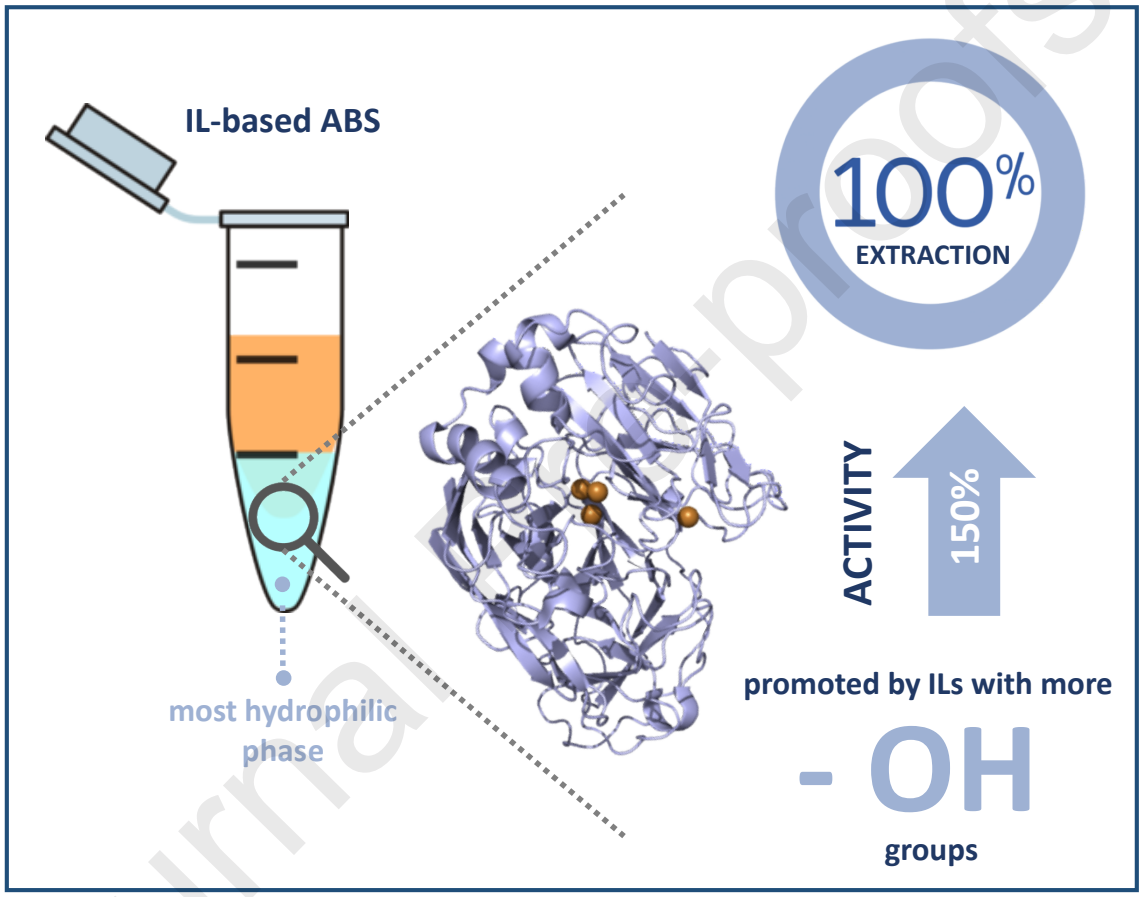
[63] R. Sadeghi, M. Maali, Toward an understanding of aqueous biphasic formation in polymer–polymer aqueous systems, Polymer, 83 (2016) 1-11.

[64] H.J. Moon, M.H. Park, M.K. Joo, B. Jeong, Temperature-responsive compounds as in situ gelling biomedical materials, Chemical society reviews, 41 (2012) 4860-4883.

[65] M.L. Toledo, M.M. Pereira, M.G. Freire, J.P. Silva, J.A.P. Coutinho, A.P.M. Tavares, Laccase activation in deep eutectic solvents, ACS sustainable chemistry & engineering, 7 (2019) 11806-11814.

Highlights:

- Several ionic liquids were investigated in aqueous biphasic systems to extract laccase and improve its activity.
- Aqueous biphasic systems containing [Ch][DHCit] leads to the best results.
- The complete extraction of laccase was achieved in a single step.
- The activity of the biocatalyst was enhanced by 50%.
- The number of hydroxyl groups in the IL plays an important role in the activity improvement.



Declaration of Interests

× The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Author statement

Capela, Emanuel V.	Investigation; Formal analysis; Writing Original Draft and Review & Editing
Valente, Ana I.	Investigation; Validation
Nunes, João C.F.	Investigation
Magalhães, Flávia F.	Investigation
Rodriguez, Oscar	Funding acquisition; Resources; Methodology; Writing – review & editing
Soto, Ana	Funding acquisition; Resources; Methodology; Writing – review & editing
Freire, Mara G.	Funding acquisition; Resources; Methodology; Writing – review & editing
Tavares, Ana P. M.	Conceptualization; Methodology; Project administration; Supervision; Writing – review & editing