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Study of the Alkyl Chain Length on Laccase Stability and Enzymatic Kinetic with Imidazolium Ionic Liquids

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Abstract The activity and stability of laccase and their kinetic mechanisms in water soluble ionic liquids (ILs): 1-butyl-3-methyl imidazolium chloride [C_4mim][Cl], 1-octyl-3-methyl imidazolium chloride [C_8mim][Cl], and 1-decyl-3-methyl imidazolium chloride [C_{10}mim][Cl] were investigated. The results show that an IL concentration up to 10% is satisfactory for initial laccase activity at pH 9.0. The laccase stability was well maintained in [C_4mim][Cl] IL when compared to the control. The inactivation of laccase increases with the length of the alkyl chain in the IL: [C_{10}mim][Cl]>[C_8mim][Cl]>[C_4mim][Cl]. The kinetic studies in the presence of ABTS as substrate allowed calculating the Michaelis–Menten parameters. Among the ILs, [C_4mim][Cl] was the suitable choice attending to laccase activity and stability. Alkyl chains in the ions of ILs have a deactivating effect on laccase, which increases strongly with the length of the alkyl chain.

Keywords Ionic liquids · Laccase · Stability · Activity · Kinetic parameters

Introduction

One of the most promising areas of research in green technologies is the application of ionic liquids (ILs) as new solvents or co-solvents. Usually, ILs present large organic cations with a variety of anions and melt at temperatures below 100°C [1]. The properties of the IL will depend on the careful choice of both the cation and the anion. Among them, it is important to highlight their basic or superacidic character, miscibility (especially with water), hydrophilicity, and hydrophobicity for their relevance to enzymatic biocatalysis [2–5]. To some extent, the anion is used to control water miscibility while the cation gives control of the hydrophobicity and hydrogen-bonding ability. The background interest of biocatalysis in ILs is replacing traditional volatile organic solvents by nonvolatile ILs. There are three

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common modes of operation with ILs in biocatalytic systems: can be used as a solvent, as a co-solvent in aqueous systems or in a biphasic system [6].

Organic solvents are widely used with enzymes, although excellent functionality of enzymes is found in water, their natural medium. The reasons have been discussed in detail elsewhere [7, 8], such as organic solvents improve the solubility of hydrophobic reagents or avoid undesired side reactions. Moreover, the unconventional solvent properties of ILs have already given rise to new and highly efficient reaction methodologies. Recent developments with enzymatic catalysis in ILs have mostly investigated enzymatic reactions with lipase [9–13]. However, some works in the field of biocatalysis in ILs or (IL+water) mixtures with oxidative enzymes have been published [14, 15].

Restrict regulatory controls have strongly targeted the reduction on the volume of industrial organic pollutants. Due to this problem, considerable research has been conducted to investigate the feasibility of applying enzymes for organic waste treatment. Currently, the enzymatic treatment of insoluble organic compounds is carried out with organic solvents. As disadvantages, the molecules of solvent may directly interact with the enzymes, thus changing their structure, exchanging water molecules in the active center of proteins, and causing irreversible inactivation of the enzymes [16]. Besides, most of the organic solvents used present intrinsic toxicity and low biodegradability. In order to develop a green biotechnological solution, the use of ILs as solvent or co-solvent in oxidative enzymatic reactions such as the degradation of insoluble organic compounds is a promissory alternative to be developed [17–19]. Approaches to reduce the traditional solvents will be of great importance in cleaning up industrial effluents and reducing atmospheric emissions.

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is among the most effective enzymes capable to catalyze the degradation of phenolic compounds [20, 21]. Phenols such as hydroquinone, catechols, guaiacol, and 2,6-dimethoxyphenol are good substrates for laccase in aqueous media. However, most phenolic compounds present a low solubility in water, such as bisphenol A [22]. The use of a different solvent or the presence of a co-solvent is necessary to increase the solubility of phenolic compounds, thus raising the capacity of a bioreactor and avoiding the appearance of two phases.

The oxidation by laccase is a one-electron reaction with the reduction of oxygen to water [23]. It has been known for two decades that laccases catalyze the direct oxidation of phenols and amines in aqueous media. Efficient and environmentally benign processes for the textile [24, 25], pulp and paper industries [26, 27], bioremediation [28], and others have increased the interest in these essentially "green" catalysts, which work with air and produce water as the only by-product.

In order to evaluate the effect of IL cations on the activity and stability of laccase, three ILs, 1-butyl-3-methyl imidazolium chloride [C_4mim][Cl], 1-octyl-3-methyl imidazolium chloride [C_8mim][Cl], and 1-decyl-3-methyl imidazolium chloride [C_{10}mim][Cl], with an identical anion (Cl^-) and different alkyl chains in the imidazolium cation were selected as reaction media for this study. The optimum reaction parameters and the kinetic mechanisms of laccase were also evaluated.

Materials and Methods

Chemicals

The ILs, 1-butyl-3-methyl imidazolium chloride [C_4mim][Cl], 1-octyl-3-methyl imidazolium chloride [C_8mim][Cl], and 1-decyl-3-methyl imidazolium chloride [C_{10}mim][Cl], were

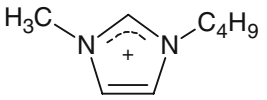
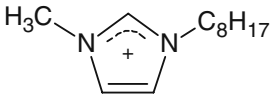
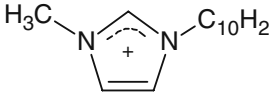
kindly provided by Prof. A. Domínguez (University of Vigo, Spain) and used without further purification. Their chemical structures are shown in Table 1. Water content of ILs was measured prior to use by Karl Fisher titration (Metrohm 756 Karl Fisher Coulometer, Switzerland) and resulted in 0.48%wt for [C_4mim][Cl], 0.52%wt for [C_8mim][Cl], and 0.25%wt for [C_{10}mim][Cl]. 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS; 98%) was obtained from Sigma (Spain). Commercial laccase (E.C. 1.10.3.2; granulated powder) from *Aspergillus* was kindly provided by Novozymes (Denmark). The enzyme is presented deposited in pellets. Prior to the experiences, a stock solution of the enzyme was prepared dissolving it into a suitable phosphate buffer, so the enzyme is always used in homogeneous solution.

Determination of Enzyme Activity and Stability

The residual laccase activity was assayed spectrophotometrically (Thermo Electron, model UV1 spectrophotometer, England). The ABTS substrate solution was prepared mixing 0.5 mL of ABTS (0.4 mM) with 1.9 mL of 0.05 mM citrate/0.1 mM phosphate buffer pH 4.5. To measure laccase activity, 0.1 mL of the incubated enzyme solution (containing ILs, buffer, or a mixture of both) was added to 1.9 mL of the ABTS solution at 40°C [29]. The change in absorbance at 420 nm (ε=36,000 M^{-1} cm^{-1}) was recorded for 30 s, and the catalytic activity was determined through the slope of the initial linear portion of the kinetic curve. One activity unit is defined as the amount of enzyme that oxidized 1 μmol of ABTS per minute, and the activity is expressed in UL^{-1}.

Laccase stability at different pH values and for different concentrations of ILs was determined by incubating the enzyme solution in a (IL+buffer) solution. The concentration of the ILs was set to 5%, 10%, 20%, and 40% (*v*/*v*). The water content of the ILs is neglected (see the "Chemicals" section above) and does not affect this assay. The effect of pH was studied using potassium phosphate buffer solutions of pH 5.0, 7.0, or 9.0. A sample

Table 1 Cations and anion of the ionic liquids used in this study

Cations		Anion
	$[C_4mim]^+$	$[Cl]^-$
	$[C_8mim]^+$	
	$[C_{10}mim]^+$	

incubated in pure phosphate buffer (50 mM) at the corresponding pH (5.0, 7.0, or 9.0) was used as reference for each case. These samples are named "control" in the tables and figures presented. The initial laccase activity was measured after addition of the enzyme solution into the incubation media, with a final enzyme activity of 2,000 UL^{-1}. Residual activities were determined at regular time intervals, up to 7 days, using the same standard procedure described above.

Enzyme Kinetics

The kinetics of ABTS substrate oxidation were evaluated experimentally. The enzyme was incubated in a 50-mM phosphate buffer solution (pH 9.0) with 20% (*v*/*v*) of IL. The initial oxidation rates for different ABTS concentrations were measured experimentally using the spectrophotometric assays explained above. The experimental data obtained were analyzed by nonlinear least-squares regression to the Michaelis–Menten equation using Sigma Plot v5.0 (SPSS Inc.).

Results and Discussion

Influence of the IL Cations on Laccase Activity and Stability

Laccase initial activities and stabilities in three alkylimidazolium chloride ILs, [C_4mim][Cl], [C_8mim][Cl], and [C_{10}mim][Cl], have been studied with ABTS as substrate. The oxidation mechanism of ABTS by laccase is presented in Fig. 1. The product oxidation of ABTS by laccase is a stable dark green cationic radical, $ABTS^+$ [30] which was monitored spectrophotometrically, with the concomitant reduction of oxygen to water [31].

The results for three different pH values (5.0, 7.0, and 9.0) and IL contents varying from 5% to 40% (*v*/*v*) are presented in Table 2. Initial laccase activities in the presence of moderate [C_4mim][Cl] and [C_8mim][Cl] concentrations (5% *v*/*v*–10% *v*/*v*) were promising. The activities in these both ILs were close or high to those of control samples depending on the condition (pH and IL content) as is explained in details below (see Table 2). However, for [C_{10}mim][Cl] a high decrease in initial activity was obtained. When the IL concentration increases to 40% *v*/*v*, a decrease in initial enzyme activity between 6 and 11 times was observed for all ILs. At this concentration of IL (40% *v*/*v*), the highest initial activity was measured for [C_4mim][Cl].

The effect of pH on initial laccase activity was a function of the nature of IL and its concentration. For [C_4mim][Cl] at pH 5.0 and 40% *v*/*v*, the initial enzyme activity (≅600 UL^{-1}) remained above the control (≅400 UL^{-1}), while for the other ILs, the activity decreased 82% approximately. However, for pH 7.0 and 9.0, the enzyme activity values in [C_4mim][Cl] were slightly below that of the control. Nevertheless, it is to note that these activities were higher than the corresponding value at pH 5.0. In the presence of [C_{10}mim]

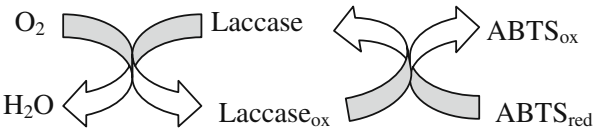


Fig. 1 Mechanism of ABTS oxidation by laccase

Table 2 Residual activity and activity loss of laccase in ionic liquid (IL) at different conditions

Co-solvent	C (% *v*/*v*)	pH	Residual activity (UL^{-1})				Activity loss (%)		
			Day (s)						
			0	2	4	6	2	4	6
Control		5	380	297	242	182	22.0	36.6	52.1
[C_4mim][Cl]	5	5	668	502	500	462	24.8	28.1	30.8
	10	5	776	600	590	580	22.6	23.4	25.3
	20	5	711	560	545	530	21.2	23.4	25.5
	40	5	616	334	300	240	39.3	51.3	61.0
[C_8mim][Cl]	5	5	515	420	342	197	18.4	33.5	61.6
	10	5	478	460	402	358	3.75	15.9	25.0
	20	5	101	102	70	65.1	8.0	30.7	35.5
	40	5	53.8	53.0	50.0	49.8	37.5	5.2	5.6
[C_{10}mim][Cl]	5	5	150	27.0	30	38	82.0	86.7	74.5
	10	5	151	35.5	nd	15.5	76.5	nd	89.8
	20	5	120	4.45	20	17.2	96.3	83.3	85.7
	40	5	81	80.6	30	82	0.5	62	0
Control		7	1,123	752	629	561	33.0	44.0	50.0
[C_4mim][Cl]	5	7	1,083	858	760	663	20.8	29.8	38.7
	10	7	965	707	451	400	26.7	53.3	58.5
	20	7	677	592	461	386	12.6	31.9	43.0
	40	7	550	101	45.2	16	81.6	91.8	97.1
[C_8mim][Cl]	5	7	803	376	236	197	53.3	70.7	75.5
	10	7	771	399	326	300	48.2	57.7	61.1
	20	7	303	34.0	15.6	26.7	88.8	94.8	91.2
	40	7	78.5	10.1	63.8	24.8	81.6	91.8	97.1
[C_{10}mim][Cl]	5	7	196	18.8	31	48.6	90.4	84.1	75.1
	10	7	94.2	22.3	20	23.7	76.6	78.8	74.8
	20	7	92.4	21.8	21.3	21.7	76.4	76.9	76.5
	40	7	66.2	18.1	33	59.9	72.7	50.2	9.5
Control		9	985	966	885	750	2.0	10.1	23.8
[C_4mim][Cl]	5	9	767	776	759	750	8.9	10.9	12.0
	10	9	856	797	762	759	6.9	10.9	11.3
	20	9	752	697	672	656	7.3	10.6	12.8
	40	9	588	135	82	21	77.0	86.1	96.4
[C_8mim][Cl]	5	9	895	406	231	137	54.6	74.1	84.7
	10	9	892	359	299	323	60.0	66.4	63.8
	20	9	304	53.5	47	53	82.4	84.5	82.6
	40	9	92.1	26.5	66	41	71.2	28.3	55.5
[C_{10}mim][Cl]	5	9	233	28.1	23	42.1	87.9	90.1	81.9
	10	9	80.7	4	4.1	4.6	95.0	94.9	94.3
	20	9	86.8	15	19.2	21.9	82.7	77.9	74.8
	40	9	84.7	48.3	33.6	56.4	42.9	60.3	33.4

C co-solvent concentration

[Cl], very low initial laccase activities were obtained for all conditions tested (concentration of IL, pH).

Figure 2 compares the effect of pH on laccase stability for reaction media using the same concentration of IL co-solvent (10% *v/v*). There is a clear reduction on laccase activity and stability for acidic pH with [C_{10}mim][Cl]. However, the initial enzyme activity and residual activity for [C_4mim][Cl] and [C_8mim][Cl] are quite higher than those corresponding to the control experiment at this pH. The same tendency was not observed for pH 7.0 and 9.0: the enzyme activity values are below those of the control for all ILs. Although the activities were below to the control for pH 7.0 and 9.0, the activities were higher when compared to pH 5.0 for all ILs. These results are in accordance with a previous study for the same

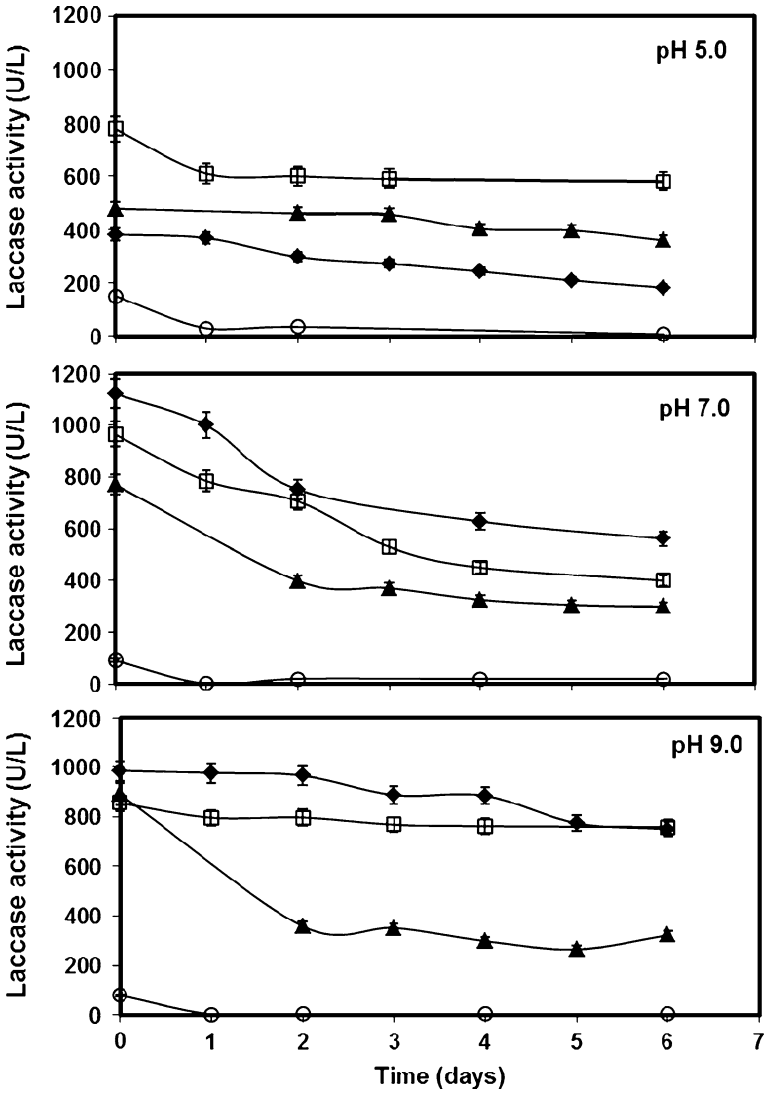


Fig. 2 Effect of ionic liquid (10% *v/v*) on laccase stability at different pH values. *Black diamond*—control, *white square*—[C_4mim][Cl], *black triangle*—[C_8mim][Cl], *white circle*—[C_{10}mim][Cl]

enzyme [32] which could be the result of rapid inactivation of the enzyme at pH 5.0. The best conditions in terms of activity and stability correspond to pH 9.0 for either [C_4mim][Cl] and [C_8mim][Cl], while for [C_{10}mim][Cl], no significant enzyme activity nor stability was observed for all conditions. The influence of the cations (namely the length of alkyl side chains in the imidazolium ring) on laccase stability at different pH values can be easily observed in Fig. 2.

Since the results above clearly proved that IL concentrations up to 10% are satisfactory for laccase stability, this content was chosen for the comparison of stabilities. The laccase stability was well maintained in [C_4mim][Cl] IL when compared to control samples. The results show that enzyme activity decreased with the length of the alkyl chain: [C_4mim][Cl]>[C_8mim][Cl]>[C_{10}mim][Cl]. The same tendency was found in a previous report on horseradish peroxidase biocatalysis [33], which proved that peroxidase activity decreased within the series [C_2mim][Cl]>[C_4mim][Cl]>[C_6mim][Cl]>[C_8mim][Cl]. The presence of the alkyl chain has important consequences on the enzyme activity and stability which should come from changes on the conformational structure of laccase. The reasons for these changes may be found on the differences produced by the ILs on the reaction media, namely in solvation properties such as polarity and polarizability, hydrophobicity, or hydrogen-bonding capacity. It has been reported that changes in these properties will have an effect on the enzyme conformation and consequently its reactivity [34]. For the ILs used in this work, the only difference is the length of an alkyl chain in the imidazolium cation. This difference produces a clear change in the physical properties of the ILs: as the alkyl chain increases, melting point temperatures, glass transition temperatures, and densities decrease, while viscosities increase [35]. The increase in viscosity with alkyl side chains was also found in alkyl-methylimidazolium chloride ILs [36]. This increase of viscosity is associated to larger van der Waals interactions between longer alkyl chains. The stronger destabilizing effect of longer alkyl chains can be understood as an increased "salting in" effect of the alkyl chain on buried hydrophobic groups of the enzyme because of increased van der Waals interactions [37]. A different line of reasoning was adopted by H. Zhao [38–40] using the chaotropic/kosmotropic character of the ions (also referred to as Hofmeister series). But the final conclusion is the same: Enzymes are stabilized by chaotropic cations and destabilized by kosmotropic cations. As the alkyl chain in the cation increases, its kosmotropicity increases and thus, enzymes are more strongly destabilized or even unfolded and deactivated.

Kinetic Study for Laccase Biocatalysis in ILs

In order to gain some insights on the effect of the presence of ILs in the kinetic mechanism of laccase reaction, some experiments with different ABTS concentrations for given conditions of pH (9.0) and IL concentration (20% *v*/*v*) were performed. Although it was found that IL concentration of 10% (*v*/*v*) was the best for laccase stability, it was too low for the kinetic experiments (no significant activity loss was obtained for the best IL, [C_4mim][Cl]). Thus, the kinetic experiences were carried out at 20% (*v*/*v*) for all ILs.

The correlation between initial oxidation rate and ABTS concentration can be adequately described by the Michaelis–Menten equation, the most studied model for laccase kinetics [41–44]:

$$V = \frac{V_m[S]}{K_m + [S]} \tag{1}$$

Table 3 Kinetic parameters of ABTS oxidation by laccase in reaction systems with different ILs and the respective correlation coefficient (R^2)

Ionic liquids	V_m (mM/min)	K_m (mM)	Catalytic efficiency	R^2
Control	0.0277	0.0066	4.178	0.967
[C_4mim][Cl]	0.0230	0.0113	2.043	0.960
[C_8mim][Cl]	0.0059	0.0082	0.722	0.779
[C10mim][Cl]	0.0059	0.0682	0.087	0.990

The catalytic efficiency was defined as the ratio of V_m to K_m

where V is the initial reaction rate (mM/min), V_m is the maximum rate (mM/min), and [S] is the ABTS concentration (mM). The Michaelis–Menten constant (K_m) and the maximum oxidation rate (V_m) of laccase were determined by nonlinear regression for all ILs and they are collected in Table 3 together with the regression coefficients obtained.

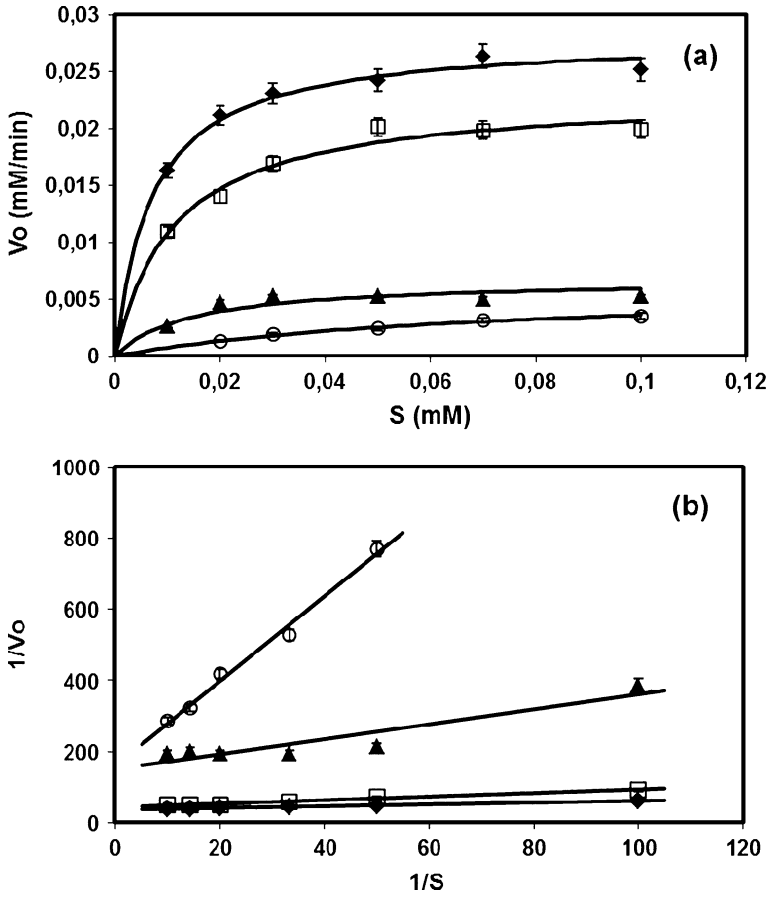


Fig. 3 Effect of ILs on ABTS oxidation rate by laccase. **a** *Points*—experimental, *lines*—Michaelis–Menten equation. **b** Lineweaver-Burk plot. *Black diamond*—control, w*hite square*—[C_4mim][Cl], *black triangle*—[C_8mim][Cl], w*hite circle*—[C_{10}mim][Cl]

The reaction kinetic profiles were determined by monitoring the increase of absorbance during the catalytic reaction of laccase with ABTS (initial rates obtained at varying ABTS concentrations while the amount of enzyme was held constant), and they are presented in Fig. 3. Additionally, the Lineweaver–Burk graphical approach, which is shown in Fig. 3b, shows a good linear relationship ($1/V_0$ vs. $1/S_0$), thus confirming that the enzymatic reactions can be represented by Michaelis–Menten kinetics. This conclusion is in agreement with other literature data for enzymatic reactions with laccase [45]. It is important to mention that kinetic parameters were calculated by nonlinear regression and the linearization approach (Lineweaver–Burk plot) was used only for graphical visualization.

K_m values can be understood as a measurement of the substrate affinity with the enzyme and K_m decreases for increasing affinities. From the results in Table 3, it follows that ABTS shows higher affinity with laccase in the presence of [C_4mim][Cl] and [C_8mim][Cl] than in [C_{10}mim][Cl], as indicated by the respective K_m values. The catalytic efficiency (V_m/K_m ratio; 46] was also used as an indication of the ability of enzyme to convert substrate under given conditions. Table 3 shows that the catalytic efficiency of laccase reaction in the presence of [C_4mim][Cl] IL was much higher. ABTS showed lowest affinity with laccase and lowest catalytic efficiency in the presence of [C_{10}mim][Cl]. The value was 23 times lower than that for [C_4mim][Cl], and a reduction of 48 times when compared to the value in buffer solution was obtained.

Conclusions

The overall findings show that maximal efficiency of laccase catalysis was demonstrated in the [C_4mim][Cl] IL, while poor results were obtained in [C_{10}mim][Cl]. The most suitable pH for laccase stability was 9.0, but pH 7.0 provided the best initial activity. The obtained results showed that the oxidation performance and the process can be satisfactorily described by the Michaelis–Menten kinetic model. The length of alkyl side chains often used in imidazolium-based ILs has proven to have negative effects on laccase activity, stability, and kinetics and thus, short chains would be preferable when ILs are used as co-solvents.

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