

Effects of phosphonium-based ionic liquids on the lipase activity evaluated by experimental results and molecular docking

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Conflict of Interest

The authors declare that no conflict of interest exists regarding this work.

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Abstract

In this work, the effect of several phosphonium-based ionic liquids (ILs) on the activity of lipase from *Burkholderia cepacia* (BCL) was evaluated by experimental assays and molecular docking. ILs comprising different cations ($[P_{4444}]^+$, $[P_{444(14)}]^+$, $[P_{666(14)}]^+$) and anions (Cl^- , Br^- , $[Deca]^-$, $[Phosp]^-$, $[NTf_2]^-$) were investigated to appraise the individual roles of IL ions on the BCL activity. From the activity assays, it was found that an increase in the cation alkyl chain length leads to a decrease on the BCL enzymatic activity. ILs with the anions $[Phosp]^-$ and $[NTf_2]^-$ increase the BCL activity, while the remaining $[P_{666(14)}]$ -based ILs with the Cl^- , Br^- and $[Deca]^-$ anions display a negative effect on the BCL activity. The highest activity of BCL was identified with the IL $[P_{666(14)}][NTf_2]$ (increase in the enzymatic activity of BCL by 61% at $0.055 \text{ mol}\cdot\text{L}^{-1}$). According to the interactions determined by molecular docking, IL cations preferentially interact with the Leu17 residue (amino acid present in the BCL oxyanion hole). The anion $[Deca]^-$ has a higher binding affinity compared to Cl^- and Br^- , and mainly interacts by hydrogen-bonding with Ser87, an amino acid residue which constitutes the catalytic triad of BCL. The anions $[Phosp]^-$ and $[NTf_2]^-$ have high binding energies (-6.2 and $-5.6 \text{ kcal}\cdot\text{mol}^{-1}$, respectively) with BCL, and preferentially interact with the side chain amino acids of the enzyme and not with residues of the active site. Furthermore, FTIR analysis of the protein secondary structure shows that ILs that lead to a decrease on the α -helix content result in a higher BCL activity, which may be derived from an easier access of the substrate to the BCL active site.

Keywords: Phosponium-based ionic liquids, *Burkholderia cepacia* lipase, enzymatic activity, molecular docking.

Introduction

The importance of biocatalytic processes is well known, in which the relevant biocatalytic potential of enzymes is confirmed by their widespread use in research and industry (in the pharmaceutical, waste treatment, food and energy fields).¹⁻³ Enzyme-based processes display some advantages in comparison to traditional chemical catalytic processes, such as lower energy consumption and process cost effectiveness.⁴ In addition to these advantages, the most attractive factor in biocatalysis over chemical catalytic processes is their biocompatible and eco-friendly features.⁵ Lipases (glycerol ester hydrolases EC 3.1.1.3) are a sub-class of hydrolases that belong to the esterase family, which naturally hydrolyse long chain triacylglycerols.⁶ As a result, lipases have application in the food,⁷ detergents,⁸ cosmetics,⁹ textile,¹⁰ pharmaceutical,¹¹ and biofuels¹² industries.¹⁴ Amongst available lipases, *Burkholderia cepacia* lipase (BCL), also known as *Pseudomonas cepacia* lipase, displays relevant biocatalytic properties, such as high substrate specificity and selectivity, high thermal stability and high tolerance to non-aqueous solvents.¹⁵ Therefore, BCL is commonly applied in organic media, particularly when hydrophobic compounds are part of the reaction media.^{16,17} Nevertheless, most organic solvents applied display important disadvantageous, such as high vapour pressure and flammability, and high toxicity.

In order to decrease the environmental impact and non-biocompatible features of biocatalytic processes that occur in organic media, ionic liquids (ILs) have been explored as an alternative non-aqueous reaction media. In fact, after the first reports on IL-based reaction media for biocatalysis in 2000,¹⁸⁻²⁰ an enhancement in the biocatalysis research field with ILs was noticed.²¹ ILs are molten salts composed of a large organic cation and an organic or inorganic anion, which by general definition should display melting temperatures below 100 °C.²² Due to their ionic nature, most ILs display singular properties such as negligible vapour pressure, low flammability, high thermal and chemical stability, and a high solvation ability for a wide range of compounds.^{22,23} In addition to these characteristics, the adjustment of their physical properties can be achieved by tailoring the cation and anion chemical structures,

allowing the IL design for a particular propose.²⁴ ILs are thus promising solvent alternatives to volatile organic compounds in biocatalysis.²⁵

Although most studies are focused on the enzymes catalytic activity in presence of aromatic ILs, particularly imidazolium-based, non-cyclic and non-aromatic cations, such as phosphonium-based ILs, could lead to relevant improvements and contribute to the design of efficient biotechnological processes.^{26–28} Imidazolium-based ILs have been applied as solvents in biocatalytic processes involving reactions such as transesterification and hydrolysis.^{29–34} Mai et al.²⁹ studied the transesterification of sugar fatty acids catalysed by Novozym 435 in presence of imidazolium-based ILs, showing 96% of conversion of vinyl laurate with glucose in a mixture of (1:1, v/v) [C₄mim][TfO]:[C₄mim][NTf₂] (1-butyl-3-methylimidazolium triflate:1-butyl-3-methylimidazolium bistriflamide). After 10 cycles, the enzyme activity was 75% of its initial activity. In order to better understand how the *Renilla luciferase* activity is influenced by imidazolium-based ILs, Ghaedizadeh et al.³⁴ performed a combined experimental and molecular dynamics simulation study with ILs composed of fluorinated anions, namely [C₄mimM][PF₆] and [C₄mimM][BF₄] (1-butyl-3-methylimidazolium hexafluorophosphate and 1-butyl-3-methylimidazolium tetrafluoroborate). The authors showed that these ILs promote changes in the protein secondary structure leading to a decrease on the enzymatic activity. In general, mainly ILs composed of fluorinated based-anions and cations with small alkyl chain length were employed to maintain or improve the enzymes activity.^{32,35–37}

In addition to the commonly applied imidazolium-based ILs, phosphonium-based counterparts display interesting advantages. These ILs are less expensive, display higher thermal stability and are available in large quantities, being already applied in industrial processes.³⁸ Due to these advantages, some researchers already started to apply phosphonium-based ILs in order to improve the biocatalytic performance of proteases and lipases.^{27,28,39,40} Abe et al.²⁷ evaluated the transesterification catalysed by BCL in 2-methoxyethyl(tri-n-butyl)phosphonium bis(trifluoromethanesulfonyl)imide ([P₄₄₄₁][NTf₂]) as solvent. The results obtained showed high enantioselectivity in presence of the IL in comparison to traditional organic solvents (hexane, toluene and

diisopropyl ether)²⁷. Aiming to develop activating agents by BCL coating, novel phosphonium-based ILs were synthesized with a PEG-sulfate anion (3,6,9,12,15,18,21,24,27,30-decaoxahexatetracontyl sulfate).⁴⁰ The results obtained led to the conclusion that IL-coating process can improve enzyme activity, stability and reuse.⁴⁰

From the large number of studies reported on the combination of enzymes and ILs, it is well accepted that the ILs effect on the enzymes activity and stability is not similar to common salts, and depend on the enzyme and IL chemical structure and concentration. Protein stability is usually connected to the well-known Hofmeister series⁴¹ and on the salt ions salting-in and salting-out effects, which are responsible for modifications in the proteins three-dimensional architecture and changes in their biological function. However, ILs are not traditional high-charge density salts well characterized by the Hofmeister series; therefore, studies to better understand the ILs effect on proteins at the molecular level are still ongoing. Up to date, cation/anion chemical structures, hydrophobicity, polarity, viscosity, hydrogen bond basicity/acidity, among others properties, have been described to influence the enzymes activity and stability.^{42,43} Overall, these features contribute to specific interactions of the IL ions and the amino acids residues and solvation layer at the proteins surface.^{44,45} However, the multitude of IL ion-water-protein interactions cannot be generalized, and more studies are required in this field to better understand the ILs impact on enzymes. Aiming to solve this puzzle on the ILs effects on enzymes activity and stability, some studies applied computational methods.⁴⁵⁻⁴⁷ Amongst these computational tools, molecular docking arises as a promising low cost, fast and easy method to identify IL ions - enzymes interactions, while providing values on the binding affinity of macromolecules (receptor) and small molecules (ligand).⁴⁸ The AutoDock Vina program was already applied to characterize the effects of ILs ions on DNA,⁴⁹ antibodies,⁵⁰ enzymes⁵¹ and other proteins.^{52,53} Taking into account the described advantages of phosphonium-based ILs, the aim of this work is to experimentally evaluate the effect of these alternative solvents on the BCL activity and to contribute to a better understanding of the molecular-level scenario ruling the enzyme activity and stability by ILs by the application of molecular docking.

The BCL activity was experimentally determined by the hydrolysis of olive oil in presence of ILs. In addition to enzymatic activity assays, Fourier transform infrared (FTIR) spectroscopy was used to appraise the changes in the secondary structure of BCL. Several ILs composed of phosphonium-based cations of different alkyl side chain lengths and different anions were investigated.

Materials and methods

Materials

The phosphonium-based ILs studied in this work were: tetrabutylphosphonium chloride [P₄₄₄₄]Cl (> 96% pure); tributyltetradecylphosphonium chloride [P₄₄₄₍₁₄₎]Cl (> 98% pure); trihexyltetradecylphosphonium chloride [P₆₆₆₍₁₄₎]Cl (> 95% pure); trihexyltetradecylphosphonium bromide [P₆₆₆₍₁₄₎]Br (> 98% pure); trihexyltetradecylphosphonium decanoate [P₆₆₆₍₁₄₎][Deca] (> 97% pure); trihexyltetradecylphosphonium bis(2,4,4-trimethylpentyl)phosphinate [P₆₆₆₍₁₄₎][Phosp] (> 93% pure); and trihexyltetradecylphosphonium bis(trifluoromethylsulfonyl)imide [P₆₆₆₍₁₄₎][NTf₂] (> 98% pure). All ILs were kindly provided by Cytec Industries Inc. Before use, all ILs were further purified at 80°C for 48 h under constant stirring and moderate vacuum conditions (≈ 0.1 Pa). The ILs chemical structures are shown in Figure 1. BCL was purchased from Sigma Co, St. Louis, MO, USA (≥ 2.900 U g⁻¹, pH 7.0, 50 °C). Hexane (> 99% pure), acetone (> 99% pure), ethanol (> 99% pure) and gum arabic were obtained from Synth (São Paulo, Brazil), and olive oil was purchased at a local market.

Activity of BCL in the olive oil hydrolysis and spectroscopic studies

The hydrolytic activity of BCL was determined according to the method described by Soares et al.⁵⁴ The substrate was an emulsion composed of 35 mL of olive oil, 35 mL of

sodium phosphate buffer solution (0.1 M, pH 7.0) and 7% (w/v) of arabic gum (used as an oil-in-water emulsifying agent). The enzymatic reaction was carried out with 5 mL of substrate, 2 mL of the sodium phosphate buffer solution, 1 mL of milli-Q water, ILs concentrations ranging from 0.011 to 0.055 mol·L⁻¹, and 1 mL of enzyme solution (0.1 g of BCL in 1 mL of milli-Q water). The reaction temperature was maintained at 37 °C under constant agitation (250 rpm) for 5 min. The reaction was stopped by adding 2 mL of a solution constituted by acetone/ethanol/water (1:1:1, v:v:v). The fatty acids released were titrated with a solution of potassium hydroxide (0.04 mol·L⁻¹), using phenolphthalein as indicator. All reactions were performed in triplicate. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of free fatty acid per min (μmol·min⁻¹) under the assay conditions (37 °C, pH 7.0, 80 rpm). The hydrolytic activity and total activity recovery yield were determined. The hydrolytic activity (U g⁻¹) was determined by Equation 1:

$$\text{Hydrolytic activity} = \frac{(V_{TS} - V_{TB}) \times M}{R_T \times S_{MR}} \times 1000 \quad (1)$$

where V_{TS} is the volume of titrated sample (mL), V_{TB} is the volume of titrated blank (mL), M is the molarity of KOH (mol·L⁻¹), R_T is the reaction time and S_{MR} is the sample weight used in the reaction (g).

The relative activity (%) of the enzyme was calculated according to Equation 2:

$$\text{Relative activity} = \frac{\text{Hydrolytic activity}}{\text{Initial activity of the enzyme}} \times 100 \quad (2)$$

The changes in the secondary structure of BCL were analysed by FTIR spectroscopy. For FTIR analysis, the BCL infrared spectra in the presence and without IL were recorded by a Shimadzu IR, in the wavelength range from 1800 to 1200 cm⁻¹. Peak frequencies in amide I region (1700 – 1600 cm⁻¹) were identified by using the secondary derivative and deconvoluted peaks using the Origin 8.5 software.

Molecular docking analysis

The interaction sites of BCL with the IL ions were identified using the AutoDock Vina 1.1.2 software⁵⁵. The open crystal structure of BCL (PDB:3LIP) was used. AutoDockTools⁴⁸ was used to prepare the BCL by merging non-polar hydrogen atoms, adding partial charges and atom types. Ligands (IL cations and anions) 3D atomic coordinates were computed by Gaussian 03w and the rigid root was generated using AutoDockTools, setting all possible rotatable bonds defined as active by torsions. The grid center at the center of mass (x-, y-, and z-axes, respectively) to cover the whole interaction surface of BCL was $30\text{\AA} \times 40\text{\AA} \times 50\text{\AA}$.⁵⁶ The binding model chosen corresponds to the highest binding free energy and amino acids residues interactions, and was searched out from 10 different conformers for each ligand.

Results and discussion

Aiming to address the BCL catalytic activation/inactivation in presence of ILs, several phosphonium-based ILs with different cation alkyl chain lengths ($[\text{P}_{4444}]^+$, $[\text{P}_{444(14)}]^+$, $[\text{P}_{666(14)}]^+$) combined with different anions (Cl^- , Br^- , $[\text{Deca}]^-$, $[\text{Phosp}]^-$, $[\text{NTf}_2]^-$) were investigated. Furthermore, these were used in concentrations ranging from 0.011 to $0.055 \text{ mol}\cdot\text{L}^{-1}$ in the hydrolysis reaction of olive oil catalysed by BCL. FTIR spectra was recorded to appraise the influence of ILs on the protein secondary structure. Molecular docking was additionally used to better understand the interactions occurring between the IL ions and BCL responsible for the enzyme stability and activity, addressing the binding energies, types of interactions and specific amino acids residues involved.

BCL activation/inactivation

BCL has a mobile region (Lid Domain), whose mobility depends on the physico-chemical properties of the environment. The Lid Domain protects the catalytic triad responsible for the lipase catalytic activity, constituted by the three following amino acids: Ser87, His286 and Asp264 (Figure 2).^{57,58} In polar media, the Lid Domain is

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predominantly closed, and thus the active site is protected from the polar environment and inaccessible to substrates. However, in more hydrophobic (non-polar) media, the Lid Domain undergoes conformational rearrangements toward the open conformation where substrates can access the active site.⁵⁹ The conformational changes that induce the opening of the Lid Domain in presence in organic media and the water/lipid interfaces generate the interfacial activation phenomenon.⁶⁰ However, the rearrangement of the Lid Domain is not the only force ruling the lipase activity. The amphiphilic nature of lipases also exerts an important role in the enzyme activity since the hydrophilic regions - external - are directed toward the solvent media and the hydrophobic regions - internal - are directed toward to enzyme active site.⁶¹ Furthermore, two amino acids (Leu17 and Gln88) close to the active site form the BCL oxyanion hole. These amino acids are responsible for stabilizing the tetrahedral intermediate, which through donate their backbone amide protons to the substrate in the transition state during ester hydrolysis.^{62,63} A detailed analysis through molecular dynamics simulations in different media (water, octane and water/octane interface) was performed by Barbe and co-authors⁵⁹. The researchers investigate the movement of the BCL Lid Domain, showing that the Leu17 residue contributes to the oxyanion hole narrowing. It has been also shown that the oxyanion hole modification state could promote different behaviours on the BCL enantioselectivity, resultant from the substrates accessibility to the enzyme catalytic site.⁶⁴

Effect of the IL cation alkyl chain length on the BCL activity

The effect of the IL cation alkyl chain length on the BCL activity on the hydrolysis of olive oil was addressed using tetraalkylphosphonium-based ILs with a common anion, namely [P₄₄₄₄]⁺Cl⁻, [P₄₄₄₍₁₄₎]⁺Cl⁻ and [P₆₆₆₍₁₄₎]⁺Cl⁻. Figure 3 exhibits the relative activity of BCL in presence of the different ILs in concentrations ranging from 0.011 to 0.055 mol·L⁻¹. Overall, there is a significant reduction of the enzyme activity in presence of [P₄₄₄₄]⁺Cl⁻, [P₄₄₄₍₁₄₎]⁺Cl⁻ and [P₆₆₆₍₁₄₎]⁺Cl⁻ in comparison to the control (reaction without IL).

The relative activity of BCL ranges between 5.02 and 30.30 %. The inactivation of BCL is more evident and significant for higher IL contents. Moreover, ILs with longer alkyl side chains are not beneficial to the hydrolysis of olive oil. The relative activity of BCL decreases according to the ILs cation rank: $[P_{4444}]^+ > [P_{444(14)}]^+ > [P_{666(14)}]^+$. Some authors described that an increase of the cation alkyl chain length, and thus IL hydrophobicity, results in a lower access of the substrate to the enzyme catalytic site.^{65,66} Furthermore, ILs of the same family with shorter alkyl side chains and with the same cation are more viscous, rendering mass transfer effects. In summary, the obstruction of the catalytic triad of the enzyme by more hydrophobic IL cations and limitations of mass transfer may be responsible for the lower enzymatic activities observed.

In general, the ion effects on enzymatic activity are ruled by a multitude of interactions, such as hydrophobic, electrostatic, and hydrogen bonding. Molecular docking was applied to better understand the interactions established and the effect of the ILs ions on the protein structure, and thus on the enzyme activation/inactivation. The absolute value of affinity ($\text{kcal}\cdot\text{mol}^{-1}$) of each IL ion to BCL, as well as the molecular interaction diagrams, are displayed in the Supporting Information (SI) (Figures S1 to S6). The best binding pose and docking affinities, and type of interaction and geometry distance (\AA), are reported in the SI (Table S1 and S2). The enzyme activity and conformational stability of BCL imparted by ILs can be evaluated through the docking affinity energy values of each IL cation to the enzyme. The lower the affinity energy values, or the higher their absolute values, the higher is the interaction energy between the IL cation and the protein. IL cations with higher absolute values of interaction energy have a higher capacity to promote conformational changes and to decrease the enzyme activity. Accordingly, the docking affinity energy values for the IL cations in respect to the BCL structure decreases in the same order of the BCL activity: $[P_{666(14)}]^+ (-4.7 \text{ kcal}\cdot\text{mol}^{-1}) > [P_{444(14)}]^+ (-4.5 \text{ kcal}\cdot\text{mol}^{-1}) > [P_{4444}]^+ (-4.3 \text{ kcal}\cdot\text{mol}^{-1})$ (Table S1), in agreement with the experimental data. However, all IL cations studied present the same BCL bind spot, and that does not correspond to the BCL active site. According to Figure 4, the IL cation

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binding occurs preferentially to Leu17 residue, by hydrophobic interactions. Leu17 is one of the two hydrogen-bond donor amino acid residues that constitute the BCL oxyanion hole and stabilize the tetrahedral intermediate during catalysis. As described before, the two amino acids (Leu17 and Gln88) constituent's amino acids of the BCL oxyanion hole are responsible for stabilizing the tetrahedral intermediate, which donate their backbone amide protons to the substrate in the transition state during ester hydrolysis.^{62,63} It was also demonstrated that interactions with the Leu17 residue contributes to the oxyanion hole narrowing, promoting different behaviours on the BCL enantioselectivity and substrate accessibility to the enzyme catalytic site.⁶⁴ Accordingly, our results indicate that the interactions between the IL cations with Leu17 seem to be the major driven force responsible for the lower catalytic behaviour of BCL in presence of the studied ILs.

Effect of the IL anion on the BCL activity

Figure 5 shows the relative activity of BCL corresponding to the hydrolysis reaction of olive oil in presence of ILs with a common cation ($[P_{666(14)}]^+$) and different anions (Cl^- , Br^- , $[Deca]^-$, $[Phosp]^-$ and $[NTf_2]^-$), at concentrations ranging from 0.011 to 0.055 $mol \cdot L^{-1}$. Several behaviours have been reported for enzymes in presence of ILs, with most of the studies suggesting that the enzyme activity is highly dependent on the IL anion.^{67,68} This trend is also observed in this work, with relative activities of BCL ranging from 5.02 to 161.67 %. The ILs investigated promote or decrease the BCL activity, which is strongly dependent on the IL anion nature or chemical structure. ILs with the anions $[Phosp]^-$ and $[NTf_2]^-$ increase the BCL activity, with an increase of 44% and 61% at 0.055 $mol \cdot L^{-1}$, respectively, when compared to the control (no IL added). The remaining ILs display a negative effect on the BCL activity, following the anion order: $Cl^- > Br^- > [Deca]^-$. As observed before when analysing the IL cation effect, and increase in the IL concentration is not beneficial and further decrease the BCL activity. This trend is the opposite to the one observed with the ILs

[P₆₆₆₍₁₄₎][Phosp] and [P₆₆₆₍₁₄₎][NTf₂], where an increase in concentration, at least up to 0.055 mol·L⁻¹, improves the BCL activity. Overall, the interactions occurring between the IL anion and the enzyme seem to be fundamental on the opposite trends observed. Zhao⁴³ reported that the nature of the ions that constitute the IL plays an essential role in protein activity and stability, showing that Cl⁻ and Br⁻ anions, described as chaotropic anions, lead to the inactivation of many enzymes, as observed in this work with BCL.

In order to better understand the impact of the IL anions on the protein structure responsible for the obtained experimental results, the BCL docking binding sites of the IL anions were analysed. The docking affinities, interacting amino acids, type of interaction, and geometry distance (Å) of each IL anion to protein structure are presented in Table S2 in the SI. According to docking results shown in Figure 6, Cl⁻ and Br⁻ present low binding energies with the surface protein. However, these small high-charge density anions strongly bind to the solvation layer of BCL by hydrogen bonding, which may be responsible for a decrease in the protein stability and activity. The anion [Deca]⁻ has a higher binding affinity compared to Cl⁻ and Br⁻, and mainly interacts by hydrogen-bonding with Ser87, an amino acid residue which constitutes the catalytic triad of BCL, which may justify the decrease on the enzymatic activity observed with [P₆₆₆₍₁₄₎][Deca]. The anions [Phosp]⁻ and [NTf₂]⁻ have high binding energies (-6.2 and -5.6 kcal·mol⁻¹, respectively) with BCL, and preferentially interact with the side chain amino acids of the enzyme and not with residues the catalytic triad. These interactions with side chain amino acids favour the enzyme activity, but are however different in nature, being mainly hydrophobic for [P₆₆₆₍₁₄₎][Phosp] and hydrogen-bonding for [P₆₆₆₍₁₄₎][NTf₂]. It has been reported that ILs comprising the anion [NTf₂]⁻ prevent the enzyme unfolding and promote a higher activity.^{69,70} A similar conclusion can be taken from our results.

FTIR analysis of the BCL secondary structure

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FTIR is an well-known and established tool for the analysis of the secondary structure of polypeptides and proteins.⁷¹ Amide I bands (1,700 – 1,600 cm^{-1}) are the most sensitive vibrational bands of the protein backbone in what concerns the protein secondary structural components, oriented by the elements of β -turn ($\sim 1670 \text{ cm}^{-1}$), α -helix ($\sim 1660 \text{ cm}^{-1}$), random coil ($\sim 1645 \text{ cm}^{-1}$) and β -sheet ($\sim 1635 \text{ cm}^{-1}$).⁷² Changes in the BCL secondary structure in aqueous solutions of $[\text{P}_{666(14)}]\text{Cl}$ and $[\text{P}_{666(14)}][\text{NTf}_2]$ at $0.055 \text{ mol}\cdot\text{L}^{-1}$ and without IL was evaluated by FTIR spectra deconvolution in the amide I region, whose results are shown in Figure 7. According to computational analysis, BCL contains 38.13% of α -helix, 29.69% of random coil, 16.88% of β -sheet and 15.31% of β -turn. The decrease in β -sheet content and increase in random coil of BCL in presence of $[\text{P}_{666(14)}]\text{Cl}$ possibly affects the active site of the enzyme, leading to the observed experimental decrease on the BCL activity. On the other hand, in presence of $[\text{P}_{666(14)}][\text{NTf}_2]$, the α -helix content decreases and the β -sheet content slightly increases, suggesting a higher opening of the BCL active site, while promoting an improvement in the BCL activity (61% increase in presence of $[\text{P}_{666(14)}][\text{NTf}_2]$ at $0.055 \text{ mol}\cdot\text{L}^{-1}$ as observed experimentally). These results are in accordance with the studies of Pan et al.⁶⁹ and Liu et al.⁷³, who previously analysed the secondary structure of BCL and reported that a lower α -helix content in the secondary structure promotes an easier access of the substrate to the BCL active site.

Conclusions

During the past years, an extended number of enzyme-mediated reactions in ionic liquid media has been described. If properly tailored, ILs may lead to higher enzyme activities and stabilities. It has been reported that both the alkyl chain length of the cation and type of anion influence the enzyme activity. Even so, the most studied ILs up to date are imidazolium-based. In this work, we evaluated the performance of seven phosphonium-based ILs, in which the chemical structures used allowed us to conclude on the IL cation alkyl chain length and anion nature effects on the *Burkholderia cepacia* lipase activity. It was found that an increase in the alkyl chain length ($[\text{P}_{4444}]^+$, $[\text{P}_{444(14)}]^+$,

[P₆₆₆₍₁₄₎]⁺) and increase in the concentration of these ILs comprising a common anion (Cl⁻) promote negative effects on the BCL activity, with relative activity values ranging from 5.02 and 30.30 % in 0.011 mol·L⁻¹. However, when analysing the IL anion effect, both the enzyme activation and inactivation effects have been observed. The ILs [P₆₆₆₍₁₄₎]Cl, [P₆₆₆₍₁₄₎]Br and [P₆₆₆₍₁₄₎][Deca] caused the inactivation of the enzyme, observed by a strong decrease in the enzyme relative activity, whereas the ILs [P₆₆₆₍₁₄₎][Phosp] and [P₆₆₆₍₁₄₎][NTf₂] improve the enzymatic activity of BCL, by 144.06 and 161.67%, respectively, at 0.055 mol·L⁻¹. These effects were further appraised at the molecular-level by molecular docking. All IL cations studied preferentially interact with Leu17 residue (amino acid constituent of the BCL oxyanion hole) by hydrophobic interactions, an amino acid of the BCL oxyanion hole that further influences the substrate accessibility to the enzyme catalytic site. Regarding the IL anions, Cl⁻ and Br⁻ present low binding energies with the surface protein. The anion [Deca]⁻ has a higher binding affinity compared to Cl⁻ and Br⁻, and mainly interacts by hydrogen-bonding with Ser87, an amino acid residue which constitutes the catalytic triad of BCL, supporting the decrease on the enzymatic activity observed. The anions [Phosp]⁻ and [NTf₂]⁻ have high binding energies with BCL and preferentially interact with side chain amino acids of the enzyme. These interactions with side chain amino acids enhance the lipase activity. The BCL secondary structure changes in presence of ILs were also investigated by FTIR, which showed that the α -helix content decreased and the β -sheet content increased with the increase of enzyme activity. In summary, the molecular docking results show that the different IL ions studied interact with different amino acid residues and by different types of interactions, which are responsible for the different behaviours observed in the enzyme activity.

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Figures

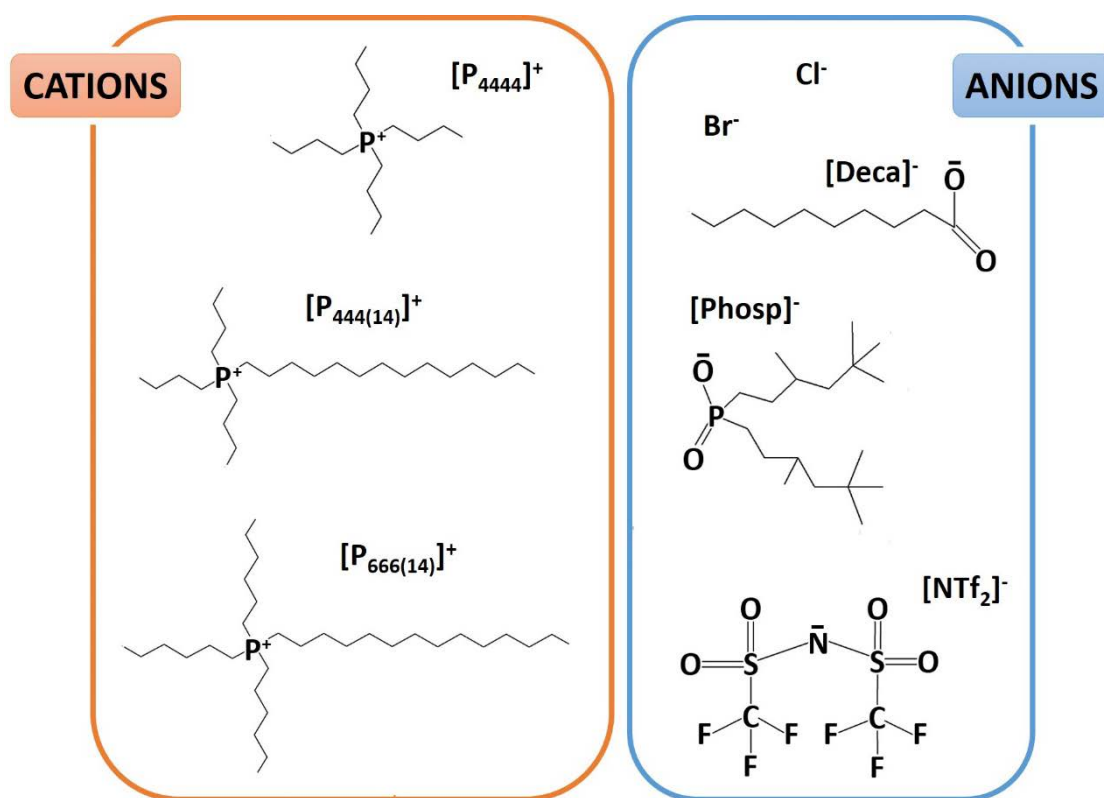


Figure 1. Chemical structures and abbreviations of the studied tetraalkylphosphonium-based ionic liquids.

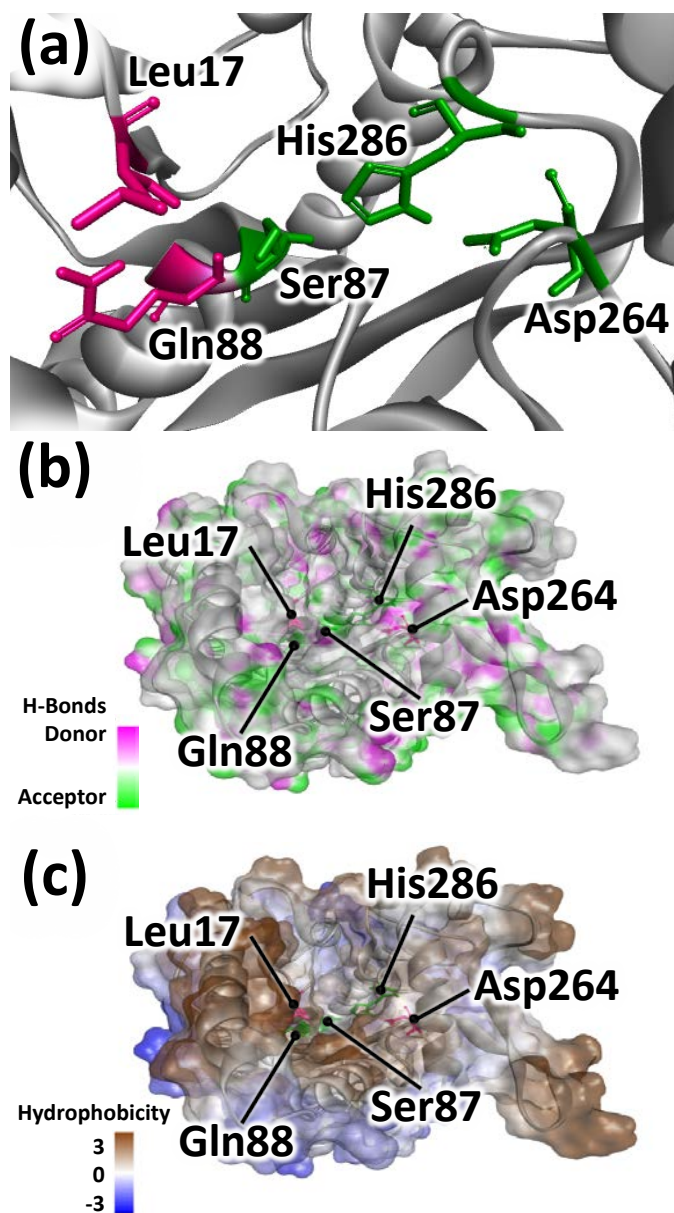


Figure 2. 3D structure of BCL (PDB:3LIP): (A) BCL catalytic triad is shown in green (Ser87, Asp264 and His286) and oxyanion hole in magenta (Leu17 and Gln88), (B) H-bond donor (pink) and acceptor ability (green) of amino acids residues, and (C) Scale of hydrophobicity of amino acids residues on BCL surface (brown – high hydrophobicity to blue – lower hydrophobicity).

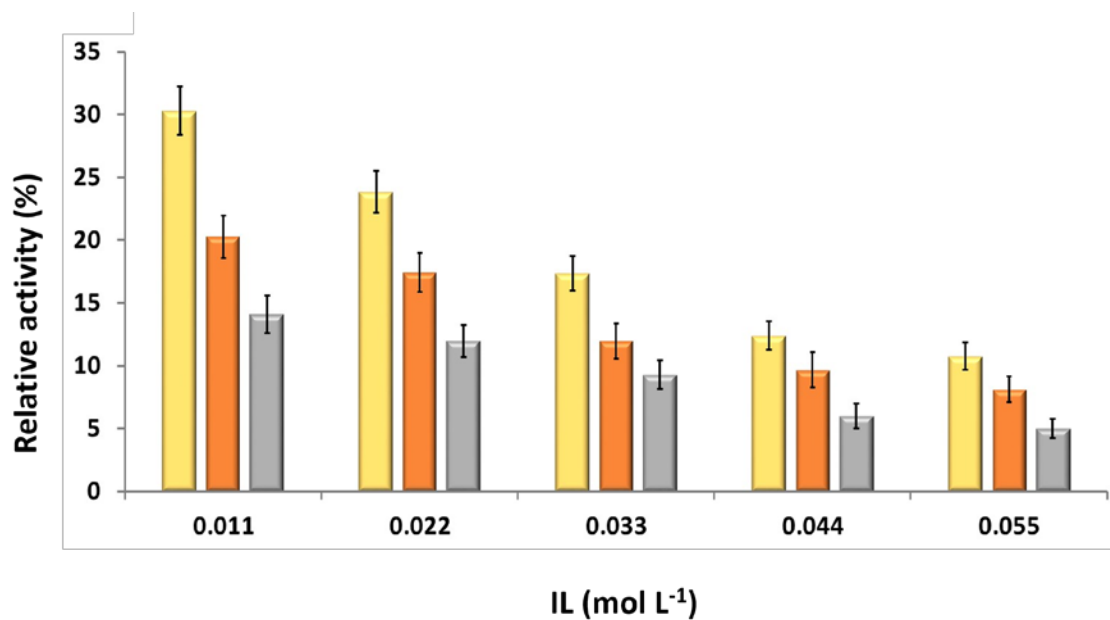


Figure 3. BCL activity in presence of tetraalkylphosphonium chloride ILs at different concentrations: [P₄₄₄]Cl (■), [P₄₄₄₍₁₄₎]Cl (■) and [P₆₆₆₍₁₄₎]Cl (■).

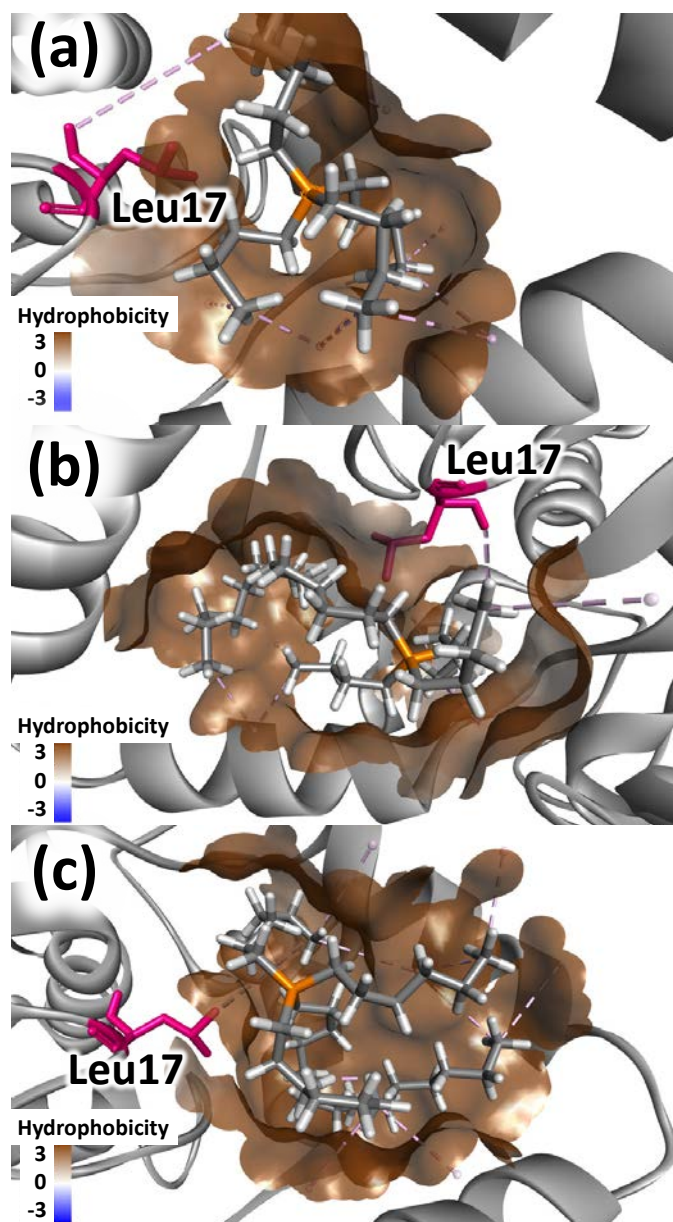


Figure 4. Molecular docking with tetraalkylphosphonium-based IL cations and BCL: (A) [P₄₄₄₄]⁺, (B) [P₄₄₄₍₁₄₎]⁺ and (C) [P₆₆₆₍₁₄₎]⁺. Leu17 is shown in magenta.

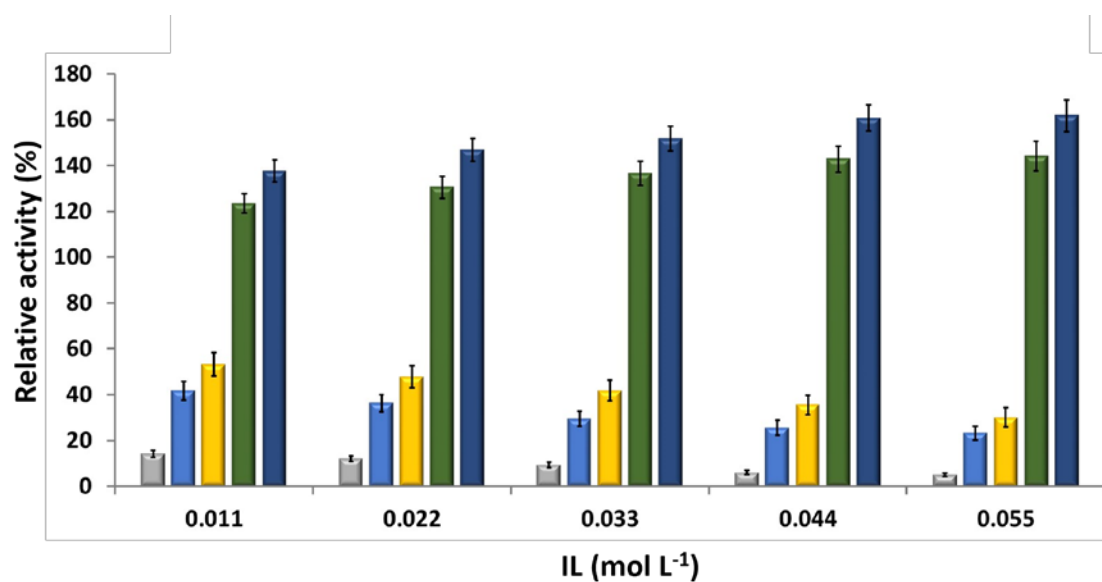


Figure 5. BCL activity in presence of trihexyltetradecylphosphonium-based ILs composed of different anions at different concentrations: [P₆₆₆₍₁₄₎]Cl (■) [P₆₆₆₍₁₄₎]Br (■), [P₆₆₆₍₁₄₎][Deca] (■), [P₆₆₆₍₁₄₎][Phosp] (■) and [P₆₆₆₍₁₄₎][NTf₂] (■).

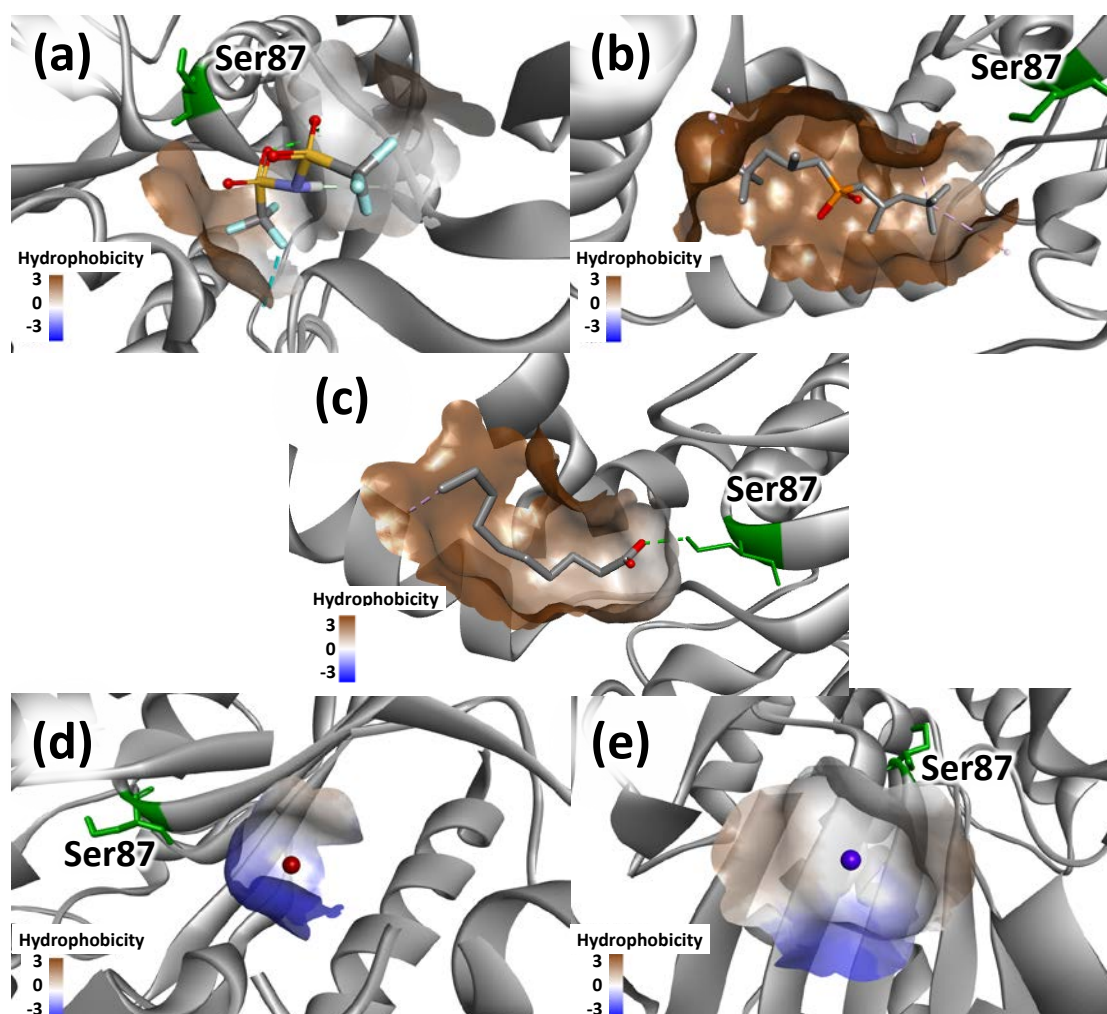


Figure 6. Molecular docking with tetraalkylphosphonium-based ILs anions and BCL: (A) [NTf₂]⁻, (B) [Phosp]⁻, (C) [Deca]⁻, (D) Br⁻, and (E) Cl⁻. Ser87 is shown in green.

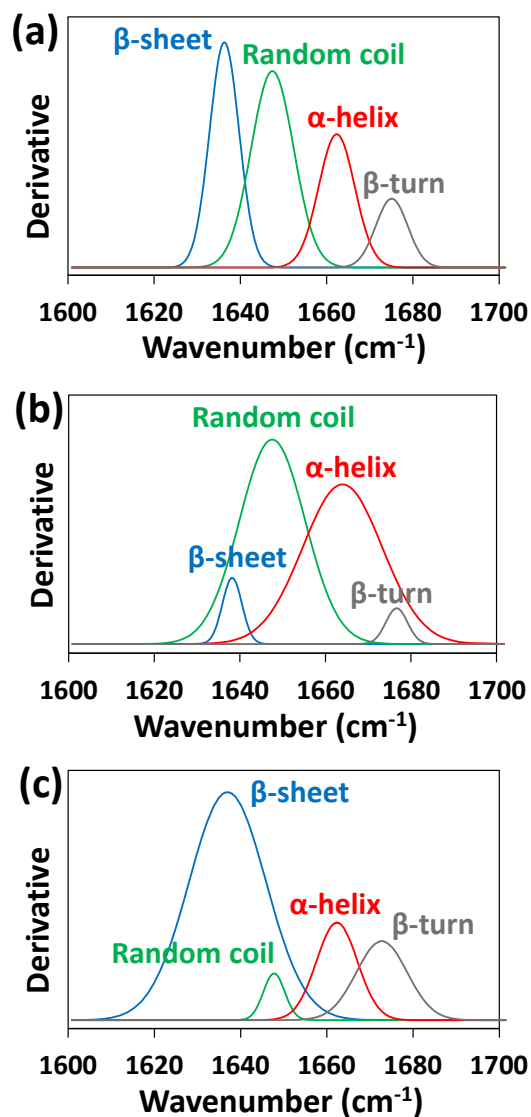


Figure 7. Amide I region (1,700 – 1,600 cm⁻¹) of the secondary-derivative spectra of BCL: (a) without IL, (b) in presence of the IL [P₆₆₆(14)]Cl at 0.055 mol·L⁻¹, and (c) in presence of the IL [P₆₆₆(14)][NTf₂] at 0.055 mol·L⁻¹. β-sheet is shown in blue, random coil in green, α-helix in red and β-turn in gray.