Enhanced Activity of Immobilized Lipase by Phosphonium-Based Ionic Liquids Used in the Support Preparation and Immobilization Process

Milson S. Barbosa,[†] Adriana J. Santos,[‡] Nayara B. Carvalho,[†] Renan T. Figueiredo,[†,‡] Matheus M. Pereira,[†] Álvaro S. Lima,[†,‡] Mara G. Freire,[§] Rebeca Y. Cabrera-Padilla,[∥] and Cleide M. F. Soares*,[†,‡]

[†]Universidade Tiradentes, Av. Murilo Dantas 300, Farolândia, 49032-490 Aracaju, SE Brazil

[‡]Instituto de Tecnologia e Pesquisa, Av. Murilo Dantas 300, Prédio do ITP, Farolândia, 49032-490 Aracaju, SE, Brazil

[§]CICECO − Instituto de Materiais de Aveiro, Departamento de Química, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[∥]Instituto de Química, Universidade Federal de Mato Grosso do Sul, Av. Senador Filinto Müller 1555, 79074-460 Campo Grande, Mato Grosso do Sul, Brazil

S Supporting Information

ABSTRACT: Biocatalysis has significant advantages over chemocatalysis in the context of sustainability since environmentally compatible catalysts (enzymes) and mild reaction conditions are used. However, enzymes are labile macromolecules, and strategies such as the addition of cosolvents or additives or their immobilization in solid supports are the target of intensive investigation to improve the catalytic performance and recyclability potential. In this work, we investigated the use of phosphonium-based ionic liquids (ILs) on the activity of immobilized *Burkholderia cepacia* lipase (BCL) by two approaches: (i) use of ILs to prepare silica used as support and (ii) use of ILs during the enzyme immobilization process. Several phosphonium-based ILs were investigated, allowing us to address the cation alkyl side chain and anion nature effects. The enzymatic performance depends on the IL employed to prepare silica, with a positive effect observed when employing ILs comprising cations with longer alkyl side chains and more hydrophobic anions. The best identified IL, namely, $[P_{666(14)}][NTf_2]$, allows for a relative activity of 209.8% and immobilization yield of 77.3% and is capable of being recycled eight times (keeping more than 50% of the enzyme initial activity). When ILs are added during the BCL immobilization process, similar negative and beneficial effects are observed. With IL $[P_{666(14)}][NTf_2]$, the immobilized biocatalyst has a relative activity of 322.7%, a total activity recovery yield of 91.1%, and can be recycled 17 times (down to 50% of the enzyme initial activity). Finally, both approaches were combined; i.e., IL $[P_{666(14)}][NTf_2]$ was used both in the material preparation and immobilization process of the enzyme. This strategy allows for an increase in the relative activity up to 231%, an immobilization yield of 98%, and an increase of 9% in the enzyme relative activity. Although BCL activity is not significantly enhanced by this strategy, the combined use of the IL in silica preparation and during the enzyme immobilization process increased the recyclability potential of the immobilized biocatalyst material, capable of being recycled 26 times, while keeping more than 50% of the enzyme initial activity (equivalent to a half-life of 13 h). The results obtained in this work open the path to the efficient use of ILs, and particularly of the less explored phosphonium-based ILs, in the biocatalysis field.

KEYWORDS: *Phosphonium-based ionic liquids, Lipase, Immobilization, Physical adsorption, Biocatalysis*
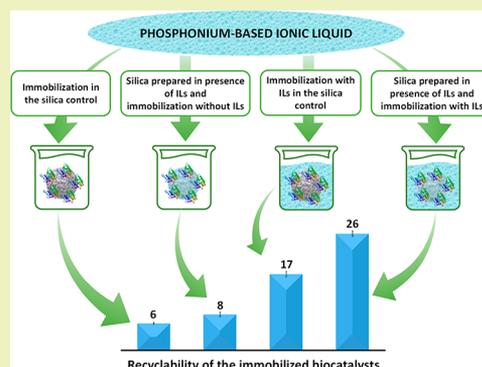
INTRODUCTION

Biocatalysis has significant advantages over chemocatalysis in the context of green chemistry due to the use of environmentally compatible catalysts (enzymes) and mild reaction conditions (temperature, pH) and solvents (usually water). Biocatalysis may also lead to high catalytic activity and enhanced regio- and chemo-selectivities for multifunctional molecules, while avoiding the need of functional groups activation or unnecessary steps. As a result, biocatalytic transformations often result in less time-consuming, and more environmentally and economically attractive, processes when compared to conventional chemical syntheses.[1−3]

Being highly available, and with high selectivity and specificity, lipases (glycerol ester hydrolase EC 3.1.1.3) appear as one of the most used biocatalysts in various industrial fields, such as in food, textile, pulp and paper, cosmetic and

pharmaceutical industries.[4] However, to be economically viable and feasible to use at a large scale, it is mandatory to have a stable, efficient, and reusable biocatalyst. The immobilization of enzymes on solid supports is a promising alternative to fulfill these requirements.[5] Aiming at keeping the enzymes activity, previous studies have been reported either on the use of additives as enzyme stabilizers or on the use of novel materials as supports.[6−8] Among these, the use of ionic liquids (ILs) is gaining relevant attention in the biocatalysis field. ILs have attracted increasing academic and industrial interest due to their physicochemical properties, e.g., negligible vapor pressure, nonflammability, high chemical and thermal stabilities, and enhanced ability to dissolve a wide range of organic, inorganic, and polymeric compounds.[9−14] However, one of the major characteristics behind the interest in ILs is the ability to manipulate their physicochemical properties by combining different cation and anion chemical structures.[15−17] Several literature works reported that enzymes in the presence of some ILs have a higher catalytic activity;[18−22] however, the effects of ILs on enzymes can also be negative, depending on the characteristics of the IL and on the enzyme under study.[23,24] In addition to the use of ILs and related mixtures as solvents, there is also evidence that the use of ILs as modifying agents of solid supports may lead to an increase in the materials surface area and pore volume and size, thus favoring the adsorption of enzymes onto the support and biocatalytic performance.[25,26] Furthermore, ILs have also been used as additives during the immobilization process, improving the activity and stability of the immobilized biocatalysts.[27−29]

In addition to the well-studied imidazolium-based ILs in the field of biocatalysis, there are few reports in the literature regarding the application of phosphonium-based ILs.[20,30,31] Itoh and co-workers[31] carried out transesterification reactions by *Burkholderia cepacia* lipase (BCL) using phosphonium-based ILs. The authors demonstrated that the lipase-catalyzed transesterification reaction using the IL 2-methoxyethoxymethyl(tri-*n*-butyl)phosphonium bis-(trifluoromethanesulfonyl)amide ($[P_{444MEM}][NTf_2]$) is faster than when using a conventional organic solvent, such as diisopropyl ether.[31] The same authors[30] then focused on phosphonium-based salts with an alkyl ether group, demonstrating that the introduction of an alkyl ether moiety allows for the design of improved ILs for transesterification reactions. In particular, IL 2-methoxyethoxymethyl(tri-*n*-butyl)-phosphonium bis(trifluoromethanesulfonyl)amide ($[P_{444MEM}].[NTf_2]$) is an excellent solvent while keeping enantioselectivity.[30] More recently, a series of phosphonium-based ILs were investigated as coating materials of BCL. The resulting IL-coated biocatalysts have different reaction rates toward alcohols as substrates, where tributyl ([2-methoxy]-ethoxymethyl)phosphonium cetyl(PEG)$_{10}$ sulfate ($[P_{444MEM}].[C_{16}(PEG)_{10}SO_4]$) was identified as the best IL, by promoting higher enantioselectivity and by increasing the transesterification reaction rates.[20] In addition to these evidences, phosphonium-based ILs can be considered relevant in this field due to their higher thermal stability, lower viscosity, and higher stability to strongly alkaline or strongly reducing conditions.[32−35] In a previous work,[36] we analyzed the effect of phosphonium-based ILs used as solvents on the activity of BCL. It was found that both the alkyl chain length of the cation and type of anion influence the BCL activity. Due to the remarkable results obtained in terms of enzyme activity, in this work, we investigate the use of phosphonium-based ILs for

BCL immobilization aiming at extending the ILs application in the biocatalysis research field. Silica was investigated as the enzyme support, and the effect of ILs was studied by two approaches: (i) use of ILs to tailor the surface of silica produced by the sol−gel technique, which was then used to immobilize BCL, and (ii) addition of ILs during the BCL immobilization process in silica (prepared with no ILs present). Both approaches were then combined with the best identified IL.

■ EXPERIMENTAL SECTION

Chemicals. BCL was purchased from Sigma Co, St. Louis, MO, USA (≥2.900 U g^{-1}, pH 7.0, 50 °C). The silane precursor used in the preparation of the support was 98% pure tetraethylorthossilicate (TEOS), purchased from Sigma-Aldrich Co (Milwaukee, WI, USA). Hexane (>99% pure), ethanol (>99% pure), ammonia (at 28%), hydrochloric acid (at 36%), and arabic gum were obtained from Synth (Sao Paulo, Brazil). Olive oil was purchased in a local market. The investigated phosphonium-based ILs were kindly provided by Cytec Industries, Inc. These ILs correspond to tetrabutylphosphonium chloride $[P_{4444}]Cl$ (mass fraction purity >96%), tributyltetradecyl-phosphonium chloride $[P_{444(14)}]Cl$ (mass fraction purity >98%), trihexyltetradecylphosphonium chloride $[P_{666(14)}]Cl$ (mass fraction purity >98%), trihexyltetradecylphosphonium bromide $[P_{666(14)}]Br$ (mass fraction purity >98%), trihexyltetradecylphosphonium dec-anoate $[P_{666(14)}][Deca]$ (mass fraction purity >97%), trihexyltetrade-cylphosphonium bis(2,4,4-trimethylpentyl)phosphinate $[P_{666(14)}]-[Phosp]$ (mass fraction purity >97%), and trihexyltetradecylphospho-nium bis(trifluoromethylsulfonyl)amide $[P_{666(14)}][NTf_2]$ (mass fraction purity >98%). Their chemical structures are given in the Supporting Information (Figure S1).

Activity of Lipase in Hydrolysis of Emulsified Olive Oil. The hydrolytic activity, relative activity, and total activity recovery yield of lipase in the hydrolysis of olive oil was determined according to the method described by Soares et al.[37] The substrate was prepared by emulsifying 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. 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, and 1 mL of enzyme solution (0.1 g of BCL in 1 mL of Milli-Q water) for the free enzyme studies or 0.1 g of enzyme for the immobilized studies. The reaction temperature was maintained at 37 °C under constant agitation (200 rpm) for 10 min. The reaction was stopped by adding 2 mL of a solution of acetone:ethanol:water (1:1:1). The fatty acids release was titrated with potassium hydroxide solution (0.04 mol L^{-1}), using phenolphthalein as the indicator. All reactions were performed in triplicate. One unit (U) of enzyme activity is defined as the amount of enzyme that originates 1 μmol of free fatty acid per min (μmol min^{-1}) under the assay conditions (37 °C, pH 7.0, 200 rpm). The hydrolytic activity (U g^{-1}) of lipase was determined by eq 1

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

where V_{TS} is the volume of titrated sample, V_{TB} is the volume of titrated blank, M is the molarity of the KOH, R_T is the reaction time, and S_{MR} is the sample mass used in the reaction.

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

$$\text{Relative activity} = \frac{\text{Hydrolytic activity}}{\text{Initial enzymatic activity}} \times 100 \tag{2}$$

Analysis of hydrolytic activity performed on the free and immobilized lipases was used to determine the total activity recovery yield (%), according to eq 3
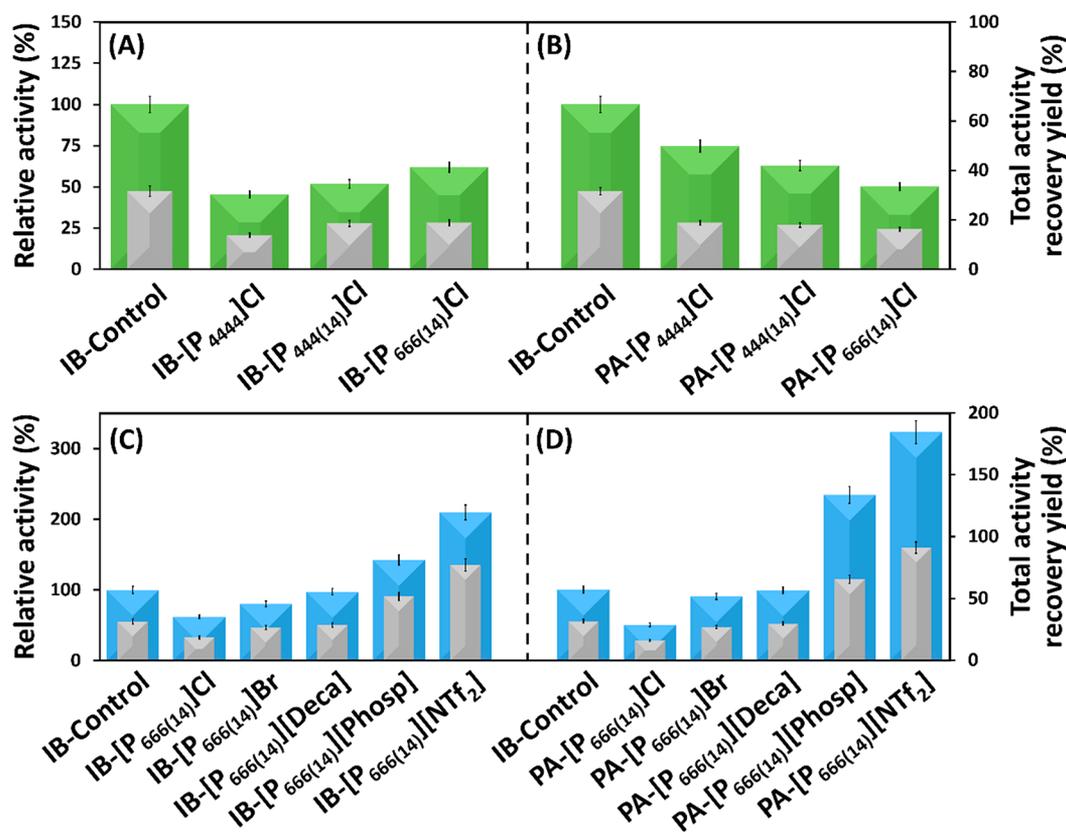
Figure 1. Effects of phosphonium-based ILs on the relative activity and total activity recovery yield of BCL. ILs were used in the material preparation to modify the morphological characteristics of silica (A and C) or added during immobilization of BCL onto silica (B and D). ILs comprise the same anion (A and B), and ILs comprise the same cation (C and D). Inner bars correspond to total activity recovery yield (gray), while outer bars correspond to relative activity (green, blue).

Total activity recovery yield

$$= \frac{\text{Total enzymatic activity present in the support}}{\text{Initial activity of the free enzyme}} \times 100 \quad (3)$$

Support Preparation. The methodology established by de Souza et al.[28] was used to prepare silica in the presence of ILs, leading to eight types of silica supports: Silica-Control (no IL added during the synthesis), Silica-$[P_{4444}]Cl$, Silica-$[P_{444(14)}]Cl$, Silica-$[P_{666(14)}]Cl$, Silica-$[_{P666(14)}][Deca]$, Silica-$[P_{666(14)}]Br$, Silica-$[P_{666(14)}][Phosp]$, and Silica-$[P_{666(14)}][NTf_2]$. It should be noted that there are no chemical functionalizations of silica by ILs; ILs are only used in the sol−gel procedure to modify the morphological characteristics of silica, such as surface area, pore size, and volume. To this end, 15 mL of TEOS was dissolved in 18 mL of absolute ethanol under an inert nitrogen atmosphere and kept under stirring for 5 min. A volume of 0.11 mL of hydrochloric acid was slowly dissolved in 2.5 mL of ultrapure water (prehydrolyzing solution), and the mixture was agitated (200 rpm) for 90 min at 35 °C. The silica control was prepared without additives, while the modified supports were prepared in the presence of 1% (v/v) of each IL. Then, 0.5 mL of ammonium hydroxide dissolved in 3 mL of ethanol (hydrolysis solution) was added, and the mixture was kept at 35 °C for 60 min and under low temperature conditions for 24 h to complete the polycondensation. After this period, silica was washed with hexane under Soxhlet to remove ILs. All samples were kept in a desiccator for at least 72 h or up to use.

BCL Immobilization. The effect of ILs on BCL activity was evaluated by two approaches: (i) BCL immobilization onto silica prepared in the presence of ILs to tailor the support morphological properties and (ii) BCL immobilization in the presence of ILs onto silica (prepared with no ILs present). In both approaches, BCL was physically immobilized on silica closely following the method

described by Cabrera-Padilla et al.[38] Here, 10 mL of hexane was added to 1 g of each support under vigorous stirring at room temperature for 15 min. Then, 10 mL of enzyme solution (0.3 g of BCL in 10 mL of Milli-Q water) was added. For the study involving the addition of ILs during the enzyme immobilization process, 1% (v/v) of each phosphonium-based IL was added. The mixture was stirred for 3 h, and the material was left for 24 h. The support containing the immobilized BCL was washed three times with 30 mL of hexane, recovered by filtration under vacuum, and maintained for 48 h in a desiccator. The immobilized biocatalysts (IB) in the silica control without the presence of IL additives (IB-Control) and in materials prepared in the presence of ILs (IB-$[P_{4444}]Cl$, IB-$[P_{444(14)}]Cl$, IB-$[P_{666(14)}]Cl$, IB-$[P_{666(14)}][Deca]$, IB-$[P_{666(14)}]Br$, IB-$[P_{666(14)}][Phosp]$, IB-$[P_{666(14)}][NTf_2]$), and the enzyme immobilized in the presence of additives in silica prepared with no ILs (PA-$[P_{4444}]Cl$, PA-$[P_{444(14)}]Cl$, PA-$[P_{666(14)}]Cl$, PA-$[P_{666(14)}][Deca]$, PA-$[P_{666(14)}]Br$, PA-$[P_{666(14)}]$-$[Phosp]$, PA-$[P_{666(14)}][NTf_2]$), were stored at 4 °C.

Morphological and Physicochemical Properties. The surface areas of silica and immobilized biocatalyst samples were determined using the Brunauer−Emmett−Teller (BET) method.[39] The volume and average pore diameter were determined based on the model developed by Barret, Joyner, and Halenda (BJH).[40] Surface areas were determined according to the materials' N_2 adsorption at −196 °C using the BET apparatus and software (Model NOVA 1200e, surface areaand pore size analyzer, Quantschrome Instruments, version 11.0). Before performing such analyses, samples were subjected to a heat pretreatment at 120 °C for 48 h in order to remove water or other volatile solvents. Scanning electron microscopy (SEM; JEOL JSM-6510LV) was used to characterize the surface morphology of the samples.

Thermogravimetric analysis (TGA) assays were performed for the supports and for supports with the immobilized biocatalyst using a Shimadzu DTG-60H simultaneous DTA-TG apparatus, under a

nitrogen atmosphere and from room temperature up to 1000 °C, at a heating rate of 20 °C min^{-1}. Samples of the supports and supports with immobilized lipase were additionally analyzed by Fourier transform infrared spectroscopy (FTIR) analysis (BOMEMMB-100 FTIR spectrophotometer), with a resolution of 4 cm^{-1}, from 500 to 4000 cm^{-1}. For the analysis of the enzyme secondary structure, spectra of free BCL and immobilized biocatalysts were recorded from 1200 to 1800 cm^{-1}. The deconvolutions of the amide I region (1600−1700 cm^{-1}) were performed using the Origin 8.5 software.

Operational Stability. The operational stability or recycling capability of the immobilized biocatalysts was determined by their hydrolysis reactions performance in consecutive reactions. At the end of each cycle, immobilized biocatalysts were collected by filtration and washed with hexane to remove residual reactants or product molecules retained and resuspended in a fresh reaction mixture. The relative enzymatic activity was determined at the end of each recycle. Each reaction corresponds to a hydrolysis reaction for 30 min at 37 °C and pH 7.0. Experiments were carried out in triplicate, with a respective standard deviation below 4%. The rates of denaturation and half-life were calculated using eqs 4 and 5, respectively.

$$A = A_0 \exp(-k_d \times t) \tag{4}$$

$$t_{1/2} = \ln(0.5)/-k_d \tag{5}$$

where A is the final activity, A_0 is the initial activity, k_d is the denaturation constant, t is time, and $t_{1/2}$ is the time of half-life.

■ RESULTS AND DISCUSSION

Effect of IL Chemical Structure on Activity of BCL. As previously highlighted, silica was investigated as the enzyme support, and the effect of ILs was studied by two approaches: (i) use of ILs to tailor the surface of silica produced by the sol−gel technique, which was then used to immobilize BCL, and (ii) addition of ILs during the BCL immobilization process in silica (silica prepared with no ILs present). All detailed results of relative activity and total activity recovery yield are given in the Supporting Information (Table S1).

Figure 1A shows the relative activity and total activity recovery yield of immobilized BCL in silica (IB-Control) and silica prepared in the presence of ILs comprising different cations and a common anion (IB-[P$_{4444}$]Cl, IB-[P$_{444(14)}$]Cl and IB-[P$_{666(14)}$]Cl). Overall, the application of these ILs as modifying agents in the synthesis of silica leads to a negative effect on BCL activity (values ranging between 45% and 62%) and total activity recovery yield (values ranging between 13% and 19%) when compared to the control (silica prepared with no ILs present). The use of chloride-based ILs with longer cation alkyl chain length leads however to a less negative impact on the BCL activity. The catalytic yield of the immobilized biocatalyst is dependent on the characteristics of the support, such as its surface area, pore volume, and size. Therefore, it seems that ILs comprising cations with longer alkyl side chain lengths have a positive impact on changing the pore volume and size, with a consequent impact on the substrate diffusion process.

In Figure 1B, the effect of the addition of chloride-based ILs during the immobilization process of BCL onto silica (PA-[P$_{4444}$]Cl, PA-[P$_{444(14)}$]Cl and PA-[P$_{666(14)}$]Cl) is shown. A contrary effect was observed when compared to the results previously described. The relative activity and total activity recovery yield increase according to the following IL cations rank (with chloride as counterion): [P$_{666(14)}$]$^+$ < [P$_{444(14)}$]$^+$ < [P$_{4444}$]$^+$. Overall, it seems that ILs comprising cations with longer alkyl side chains ([P$_{666(14)}$]$^+$ and [P$_{444(14)}$]$^+$) combined with the chloride anion are less beneficial to improve the

relative activity and total activity recovery yield of BCL. Relative activities and total activity recovery yields ranging between 50% and 75% and 16% and 19%, respectively, have been obtained in the presence of the studied ILs. According to the literature,[41−43] an increase in the cation alkyl side chain length favors hydrophobic interactions with the nonpolar residues of the enzyme, and depending on the physicochemical properties of the anion associated with the IL cation, it can lead to enzyme structural modification, obstruction of the active site, and inactivation.

The influence of phosphonium-based ILs with a common cation and different anions, namely, [P$_{666(14)}$]Cl, [P$_{666(14)}$]Br, [P$_{666(14)}$][Deca], [P$_{666(14)}$][Phosp], and [P$_{666(14)}$][NTf$_2$], was additionally addressed on the relative activity and total activity recovery yield of BCL. Figure 1C depicts the results obtained with BCL immobilized by physical adsorption onto silica (IB-Control) and silica prepared in the presence of ILs (IB-[P$_{666(14)}$]Cl, IB-[P$_{666(14)}$]Br, IB-[P$_{666(14)}$][Deca], IB-[P$_{666(14)}$]-[Phosp], and IB-[P$_{666(14)}$][NTf$_2$]). The use of [P$_{666(14)}$]-based ILs to prepare silica influence both positively and negatively the enzymatic performance. The relative activity and total activity recovery yield of BCL range between 62% and 210% and between 19% and 77%, respectively, in respect to the control (silica prepared with no IL present). When using smaller ILs anions, namely, [P$_{666(14)}$]Br and [P$_{666(14)}$]Cl, a decrease in both the relative activity and total activity recovery yield of lipase was observed. However, the BCL immobilized onto the silica modified with ILs of a more hydrophobic nature or higher size, namely, [P$_{666(14)}$][Phosp] and [P$_{666(14)}$][NTf$_2$], displays a higher enzymatic activity and total activity recovery yield. Remarkably, for the biocatalyst immobilized onto silica synthesized in the presence of [P$_{666(14)}$][NTf$_2$], an increase in the relative activity up to 210% was observed when compared to the control. With this IL, there is an increase in the lipase activity from 594 to 975 U g^{-1}. It seems thus that the morphological modification of silica caused by the IL comprising the most hydrophobic anion ([P$_{666(14)}$][NTf$_2$]) is favorable to enhance the catalytic performance of lipase. The results found in this work are superior to those found by Zou et al.,[44] where porcine pancreas lipase (PPL) was immobilized by physical adsorption onto silica modified with IL 1-methyl-3-(3-trimethoxysilyl-propyl) imidazolium tetrafluoroborate. The better results obtained in this work seem thus to be a direct consequence of the use of a larger hydrophobic fluorinated anion, which is beneficial to change the silica morphological characteristics and to improve lipase activity.

The effect of the IL anion during the lipase immobilization process onto silica is shown in Figure 1D (PA-[P$_{666(14)}$]Cl, PA-[P$_{666(14)}$]Br, PA-[P$_{666(14)}$][Deca], PA-[P$_{666(14)}$][Phosp], and PA-[P$_{666(14)}$][NTf$_2$] in respect to IB-Control). As verified before, both positive and negative effects on the lipase activity were obtained. ILs such as [P$_{666(14)}$]Cl, [P$_{666(14)}$]Br and [P$_{666(14)}$][Deca] lead to a decrease in the enzymatic activity and total activity recovery yield (values ranging between 50% and 99% and between 16% and 30%, respectively). On the other hand, the relative activity and total activity recovery yield of immobilized lipase in the presence of ILs [P$_{666(14)}$][Phosp] and [P$_{666(14)}$][NTf$_2$] remarkably increase in respect to the control. Values up to 323% in the relative activity and up to 91% in the total activity recovery yield are achieved with [P$_{666(14)}$][NTf$_2$]. This strategy of adding ILs during the immobilization process also leads to better catalytic performance when compared with the use of the same ILs to tailor the

characteristics of the support. Anions can establish strong interactions with enzymes, leading to conformational changes and directly influencing their activity and stability.[45] These changes and interactions can affect the active site and accessibility of the substrate and thus can be beneficial or not. For instance, the anion Cl$^-$ has a strong ability to form hydrogen bonds and may interact with polar clusters of the enzyme and destabilize its structure.[43,46−48] In a previous work, we investigated the enzymatic activity of BCL in the presence of phosphonium-based ILs and applied molecular docking to better understand the chemical features of ILs required to improve the enzymatic activity.[36] The highest activity of BCL was identified with IL $[P_{666(14)}][NTf_2]$, in agreement with the findings of the current work. According to the interactions appraised by molecular docking, IL cations preferentially interact with the Leu17 residue (amino acid present in the BCL oxyanion hole). However, contrarily to the majority of the IL anions that interact by hydrogen-bonding with Ser87, an amino acid residue which constitutes the catalytic triad of BCL, $[NTf_2]^-$ preferentially interacts with the side chain amino acids of the enzyme and not with residues of the active site.[36] Therefore, IL $[P_{666(14)}][NTf_2]$ may lead to some conformational changes of the enzyme and to an easier access of the substrate to the BCL active site.

Aiming at better identifying if interactions of the IL anion with the enzyme may play a role, the relative activity and total activity recovery yield were correlated against the experimentally obtained relative cation−anion interaction energies.[49] The ILs studied comprise $[P_{666(14)}]$-based ILs, in which their relative cation−anion interaction energies decrease according to the following IL anions rank: Cl$^-$ > Br$^-$ > $[Deca]^-$ > $[Phosp]^-$ > $[NTf_2]^-$. As shown in Figure S2 of the Supporting Information, there is a close correlation between the enzyme activity and the IL cation−anion interactions strength. The higher the IL cation−anion interaction strength is, the lower the lipase activity is. These results and correlation indicate that IL anions that do not strongly interact with the IL cation are more "free" to participate in favorable interactions with the enzyme. This tendency is in accordance with the findings by Mateo et al.,[50] in which the authors stated that more hydrophobic microenvironments are beneficial for increasing lipase activity, with subsequent positive effects on the hydrolysis reaction. Overall, weaker cation−anion interactions in ILs, as is the case with $[P_{666(14)}][NTf_2]$, allows stronger hydrophobic interactions between the IL anion and lipase.

The results obtained are consistent with those found by Wu et al.,[51] in which an increase in the enzymatic activity of Candida rugosa lipase immobilized on vesicular silica in the presence of a hydrophobic additive was observed. Zarcula et al.[52] studied the immobilization of Pseudomonas fluorescens lipase in a hybrid sol−gel matrix using imidazolium-based ILs as additives and compared the gathered results with those obtained with common organic solvents. The authors demonstrated that the presence of hydrophobic groups or more hydrophobic ILs during the immobilization process leads to an optimal microenvironment to improve the enzyme activity. In the same line, Cabrera-Padilla et al.[53] studied several ILs as additives during the immobilization of Candida rugosa lipase on a poly(3-hydroxybutyrate-cohydroxyvalerate) (PHBV) support. The results obtained demonstrate that the most efficient biocatalyst is the one immobilized in the presence of the most hydrophobic IL, namely, 1-butyl-3-

methylimidazolium bis(trifluoromethylsulfonyl)amide ($[C_4mim][NTf_2]$), with a 2-fold increase in the total activity recovery yield (78%) compared to the control (30%). By the combination of an even more hydrophobic and larger cation in our work, namely, $[P_{666(14)}]^+$, we were able to improve 3-fold the total activity recovery yield (91% vs 32% of the control), thus overcoming the best results obtained with imidazolium-based ILs. Therefore, if properly designed and selected, phosphonium-based ILs are promising IL alternatives in the field of biocatalysis.

Morphological and Physicochemical Characterization of Supports. From the data gathered regarding the relative activity and total activity recovery yield, the immobilized biocatalyst in silica (IB-Control) and the most efficient immobilized biocatalysts using ILs (IB-$[P_{666(14)}]$-$[NTf_2]$ and PA-$[P_{666(14)}][NTf_2]$) were chosen for morphological (BET and SEM) and physicochemical (TGA and FTIR) characterizations. The materials Silica-Control and Silica-$[P_{666(14)}][NTf_2]$, with no enzyme supported, were also analyzed by the same techniques.

The morphological characteristics of the support, namely, the surface area, pore diameter, and volume, play important roles on the enzyme immobilization process and enzyme catalytic performance.[54,55] The results shown in Table 1
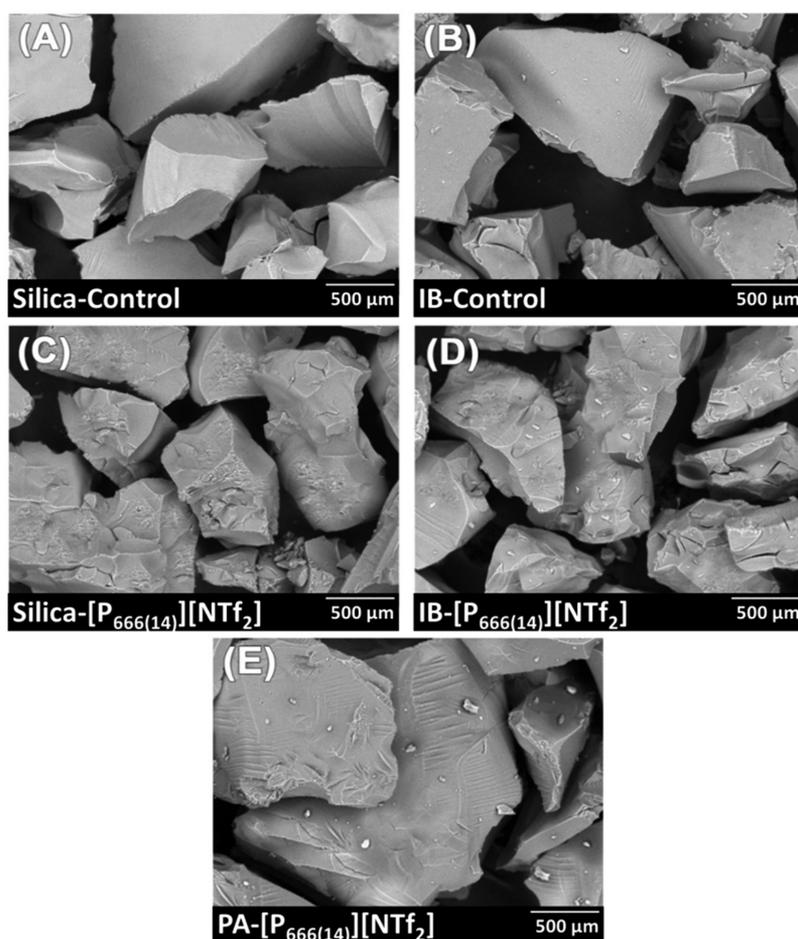
Table 1. Surface Area, Pore Volume, and Pore Diameter of Supports (Silica-Control and Silica-$[P_{666(14)}][NTf_2]$) and Supports with Immobilized Biocatalyst (IB-Control, IB-$[P_{666(14)}][NTf_2]$ and PA-$[P_{666(14)}][NTf_2]$)^a

Samples	Surface area (m^2 g^{-1})	Pore volume (cm^3 g^{-1})	Pore diameter (nm)
Silica-Control	802.7 ± 5.4	0.9 ± 0.1	1.9 ± 0.2
Silica-$[P_{666(14)}][NTf_2]$	733.6 ± 4.8	4.1 ± 0.6	6.4 ± 0.8
IB-Control	701.2 ± 4.6	0.9 ± 0.1	1.8 ± 0.2
IB-$[P_{666(14)}][NTf_2]$	378.4 ± 2.1	1.4 ± 0.1	3.7 ± 0.4
PA-$[P_{666(14)}][NTf_2]$	392.4 ± 2.0	0.6 ± 0.1	1.6 ± 0.3

^{a}All analyses were conducted in triplicate.

indicate a decrease in the surface area of silica prepared in the presence of the IL, from 802.7 to 733.6 m^2 g^{-1}. This reduction can be attributed to changes in the support domains and surface promoted by the presence of additives.[25,26] However, a significant increase in both the pore volume (from 0.9 to 4.1 cm^3 g^{-1}) and diameter (from 1.9 to 6.4 nm) was noticed, which is a main result of the presence of an IL with a high molar volume (716.6 cm^3 mol^{-1} at 25 °C).[56] These results confirm the positive effect of $[P_{666(14)}][NTf_2]$ on the modification of the morphological characteristics of silica, further enhancing lipase adsorption and activity. From the comparison with Silica-$[P_{666(14)}][NTf_2]$, a noticeable decrease in all the parameters of samples IB-$[P_{666(14)}][NTf_2]$ and PA-$[P_{666(14)}][NTf_2]$ is verified, meaning that lipase is successfully immobilized in the materials pores. These results are in accordance with the literature,[41,44] indicating a higher number of biocatalyst molecules inside the silica pores, thus supporting a higher efficiency of immobilized BCL.

The differences in the materials surface morphology before and after the enzyme adsorption were investigated by SEM. Figure 2 shows the micrographs of the silica control (Figure 2A), silica control containing the immobilized biocatalyst (Figure 2B), silica prepared in the presence of $[P_{666(14)}][NTf_2]$ (Figure 2C), and silica prepared in the presence of $[P_{666(14)}]$-

Figure 2. SEM micrographs of (A) silica control (Silica-Control), (B) silica control with immobilized BCL (IB-Control), (C) support prepared in the presence of $[P_{666(14)}][NTf_2]$ (Silica-$[P_{666(14)}][NTf_2]$), (D) support prepared in the presence of $[P_{666(14)}][NTf_2]$ with immobilized BCL (IB-$[P_{666(14)}][NTf_2]$), and (E) support with immobilized BCL in the presence of $[P_{666(14)}][NTf_2]$ (PA-$[P_{666(14)}][NTf_2]$).

Table 2. Total and Partial Weight Loss Observed for Free BCL, in Supports (Silica-Control and Silica-$[P_{666(14)}][NTf_2]$) and Supports with Immobilized Biocatalyst (IB-Control, IB-$[P_{666(14)}][NTf_2]$ and PA-$[P_{666(14)}][NTf_2]$)[a]

Samples	0–200 °C	200–400 °C	400–1000 °C	Total weight loss (%)
Free BCL	16.8 ± 0.9	82.4 ± 1.6	0.2 ± 0.1	99.4 ± 0.9
Silica-Control	17.7 ± 1.0	2.1 ± 0.1	0.6 ± 0.2	20.6 ± 1.2
Silica-$[P_{666(14)}][NTf_2]$	16.0 ± 1.0	2.0 ± 0.2	1.0 ± 0.1	19.1 ± 1.1
IB-Control	18.2 ± 1.3	4.4 ± 0.2	1.6 ± 0.1	24.3 ± 1.6
IB-$[P_{666(14)}][NTf_2]$	17.9 ± 1.1	4.9 ± 0.3	2.0 ± 0.1	24.9 ± 1.5
PA-$[P_{666(14)}][NTf_2]$	18.4 ± 1.4	4.7 ± 0.4	2.1 ± 0.1	25.2 ± 1.9

[a]All analyses were conducted in triplicate.

$[NTf_2]$ with the immobilized enzyme (Figure 2D). It is clear that the presence of ILs during silica preparation leads to a more fissured surface (Figure 2A and C), facilitating the enzyme adsorption. These observations are in agreement with those described by Zou et al.,[6] who reported that the modification caused by additives does not destroy the structure of silica but yet promotes small changes in its porous surface relevant for improving enzyme activity and total activity recovery yield. In summary, these results demonstrate that use of ILs during the preparation of silica beneficially influences its morphology and, subsequently, the lipase immobilization efficiency. When the biocatalyst is immobilized in the presence of IL (PA-$[P_{666(14)}][NTf_2]$) (Figure 2E), a more irregular

surface is seen, being an indication of the preferential adsorption of lipase onto the support.

TGA was carried out to provide information on the thermal stability and weight loss of the enzyme and related materials acting as enzymatic supports, namely, BCL, silica control (Silica-Control), silica prepared in the presence of IL (Silica-$[P_{666(14)}][NTf_2]$), and supports containing the immobilized biocatalyst (IB-Control and IB-$[P_{666(14)}][NTf_2]$). The thermographs were divided into three ranges, with the respective results given in Table 2. The respective thermographs are shown in Figure S3 of the Supporting Information. In Range I, whose temperature is between 25 and 200 °C, the weight loss is especially associated with the decomposition of amino groups in the protein and loss of water molecules. It should be
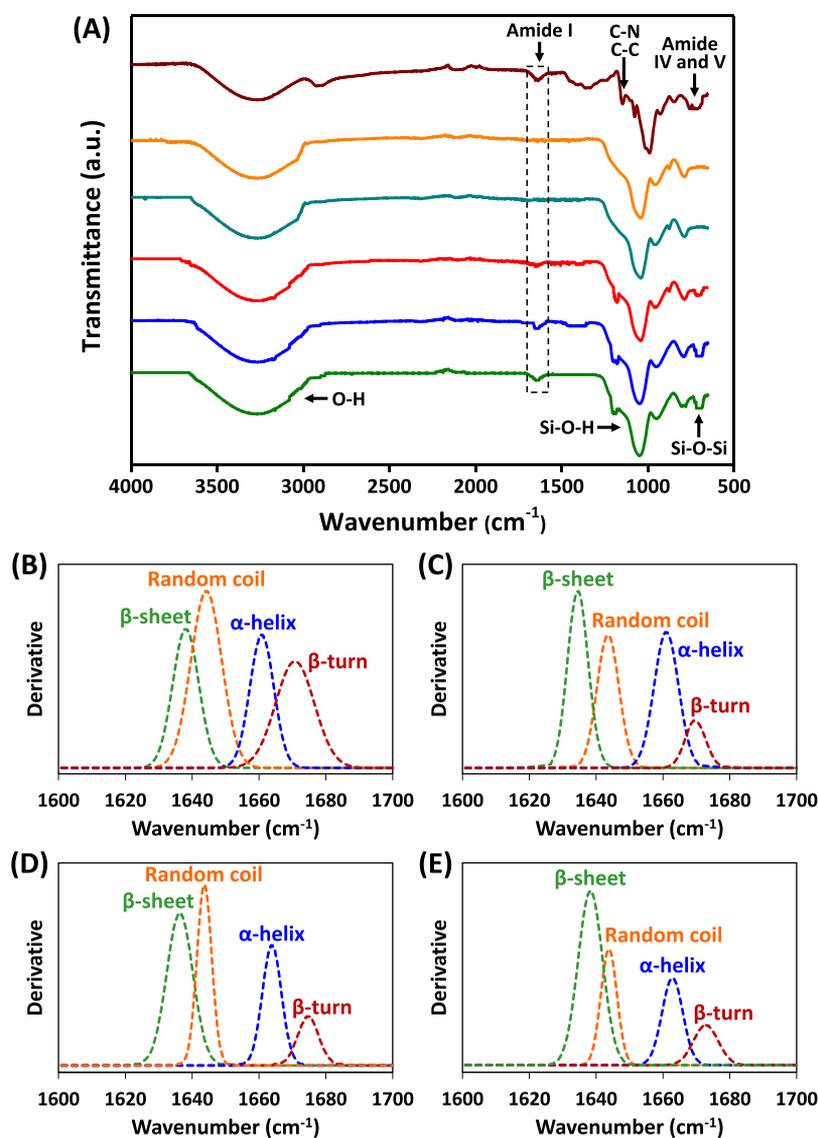
Figure 3. FT-IR spectra: (A) free enzyme (BCL, brown), silica control (Silica-Control, orange), support prepared in the presence of $[P_{666(14)}][NTf_2]$ (Silica$[P_{666(14)}][NTf_2]$, greenish blue), silica control with immobilized BCL (IB-Control, red), support prepared in the presence of $[P_{666(14)}][NTf_2]$ with immobilized BCL (IB-$[P_{666(14)}][NTf_2]$, blue) and silica control with immobilized BCL in the presence of $[P_{666(14)}][NTf_2]$ (PA-$[P_{666(14)}][NTf_2]$, green). Amide I region of the secondary derivative spectra: (B) BCL, (C) IB-Control, (D) IB-$[P_{666(14)}][NTf_2]$, and (E) PA-$[P_{666(14)}][NTf_2]$.

remarked that silica obtained by the sol−gel technique contains a significant number of Si−O−H groups that easily absorb water.[51] In Range II, which covers the range between 200 and 400 °C, the weight loss is associated with the decomposition of organic compounds, including lipase, and of silanol groups of TEOS present in the silica that do not react. In Range III, between 400 and 1000 °C, weight loss is associated with the final dehydroxylation reactions and final carbonization of organic compounds, leading to the complete degradation of the material.[57,58] From the analysis of Table 2, the total weight loss of the silica control is 20.60%, attributed to the presence of unreacted silanol groups of TEOS and water molecules present in the silica matrix. For the silica prepared in the presence of IL $[P_{666(14)}][NTf_2]$, a total mass loss lower than 19.14% was observed. Mateo et al.[50] observed similar lower weight losses (increase in the thermal stability of the material) of supports prepared in the presence of additives. Accordingly, the corresponding weight loss is higher in Range I

for the Silica-Control (17.77%) compared to Silica-$[P_{666(14)}]$-$[NTf_2]$ (16.07%). Regarding the supports with the immobilized biocatalysts, an increase in the total weight loss of both samples was noticed, about 24.32% for IB-Control and 24.89% for IB-$[P_{666(14)}][NTf_2]$. These results are due to the presence of immobilized lipase since a higher weight loss was observed in Range II, being a result of the decomposition of organic compounds.

For the materials in which ILs were used on the BCL immobilization process, in Range I, the thermographs indicate weight losses of 18.25% and 18.42% for IB-Control and PA-$[P_{666(14)}][NTf_2]$, respectively, associated mainly with the removal of water present on the surface of the immobilized biocatalyst and to the decomposition of amino groups. In Range II, lower weight losses were observed compared to Range I, namely, 4.42% for IB-Control and 4.70% for PA-$[P_{666(14)}][NTf_2]$, which are related with the thermal decomposition of silanol groups of silica and organic constituents,

including lipase. In Range III, the weight loss is associated with the loss of residual water, dihydroxylation, and final carbonization of organic compounds, including lipase. Also in this region, more specifically in the range above 750 °C, the complete degradation of the material is achieved. Overall, these results suggest that the lower values obtained for the weight loss associated with the immobilized biocatalysts result from an increased thermal stability due to the interactions occurring between silane precursors and organic components (lipase and ionic liquids).

The materials chemical composition and presence of BCL were monitored by FTIR. In Figure 3A, the presence of bands characteristic of silica produced by the sol−gel method, such as Si−O−Si groups (between 800 and 1000 cm^{-1}), Si−OH groups (between 900 and 1100 cm^{-1}), and OH groups (between 3400 and 3500 cm^{-1}) is shown, indicating the formation of silica bonds and the polymerization of TEOS alkoxide during the formation of silica.[28,58] Regarding the efficiency of lipase immobilization in the control and support prepared in the presence of [P$_{666(14)}$][NTf$_2$], bands characteristic of proteins are noticed, with amide characteristic bands, groups IV and V (695 cm^{-1}), amide I (between 1600 and 1700 cm^{-1}), and C−C and C−N (between 1000 and 1100 cm^{-1}). These are observed in the spectra of IB-Control and IB-[P$_{666(14)}$][NTf$_2$], confirming the immobilization of BCL onto the supports. BCL immobilized on the silica control in the presence of [P$_{666(14)}$][NTf$_2$] (PA-[P$_{666(14)}$][NTf$_2$]) confirms the presence of immobilized lipase by bands characteristic of the protein in three different regions: 695 cm^{-1} (groups C−C and C−N), 1000 and 1100 cm^{-1} (amide IV and V), and between 1600−1700 cm^{-1} (amide I). These last bands are more evident in the sample PA-[P$_{666(14)}$][NTf$_2$], probably due to the positive effect of the phosphonium-based IL used as an additive during BCL immobilization.

To address the BCL secondary structure changes, which is particularly relevant to evaluate protein stability, FTIR spectra deconvolutions were performed, particularly to analyze the amide I region (1600−1700 cm^{-1}). The dominant secondary structural components of proteins contributing to the amide I band are β-sheet (1626−1640 cm^{-1}), random coil (1640−1651 cm^{-1}), α-helix (1650−1657 cm^{-1}), and β-turn (1655−1675 cm^{-1}).[59,60] However, it is has been demonstrated that the lid composed of an α-helix is a functional determinant of BCL activity.[36,61,62] The decrease in α-helix contents of lipase affects the lipase active site by stimulating the interfacial activation (open conformation), allowing easier access of the substrate. The deconvolved spectra (dashed lines) of free BCL and supports with immobilized biocatalysts are shown in Figures 3B−E. Compared with free lipase (Figure 3B), BCL immobilized on the silica control (Figure 3C) and BCL immobilized on the support prepared in the presence of [P$_{666(14)}$][NTf$_2$] (Figure 3D) exhibit small decreases in the contents of the α-helix. However, BCL immobilized on the silica control in the presence of [P$_{666(14)}$][NTf$_2$] (Figure 3E) shows a higher decrease in the α-helix content. Therefore, the presence of IL during the immobilization process promotes important conformational changes of BCL, namely, by decreasing the α-helix content, thus facilitating the access of the substrates to the active site and improving the catalytic performance of lipase.

Recyclability of Immobilized Biocatalyst. With the goal of developing a sustainable process feasible of industrial application, the immobilized biocatalysts in IB-Control, IB-

[P$_{666(14)}$][NTf$_2$], and PA-[P$_{666(14)}$][NTf$_2$] were evaluated in concerning their recyclability potential. The gathered results in terms of relative activity for 17 recycles of the immobilized enzyme are shown in Figure 4. Table S2 provides the
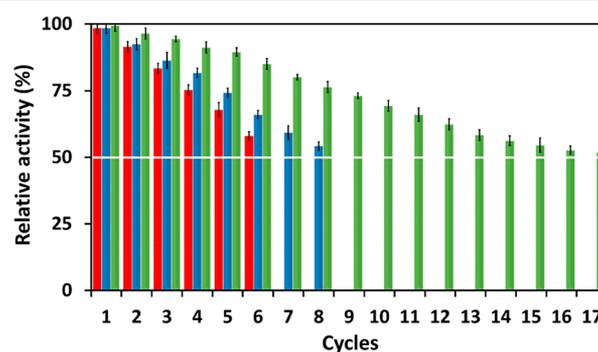

Figure 4. Recyclability tests for silica control with immobilized BCL (IB-Control, red), support prepared in the presence of [P$_{666(14)}$]-[NTf$_2$] with immobilized BCL (IB-[P$_{666(14)}$][NTf$_2$], blue), and silica control with immobilized BCL in the presence of [P$_{666(14)}$][NTf$_2$] (PA-[P$_{666(14)}$][NTf$_2$], green).

denaturation rates and half-life values for the 17 recycles of the enzyme. The relative activity of the immobilized biocatalysts decreases in each recycling step, which may be due to leaching of BCL within the silica pores. However, both supports containing the enzyme in which ILs were used, IB-[P$_{666(14)}$][NTf$_2$] and PA-[P$_{666(14)}$][NTf$_2$], display higher relative activity along time when compared to the control. The support prepared in the presence of IL containing the biocatalyst IB-[P$_{666(14)}$][NTf$_2$] has a higher operational stability than the IB-Control, reaching eight recycles, equivalent to a half-life of 3.5 h, with more than 50% of its initial activity. The results obtained are superior when compared to those shown by Barbosa et al.[63] The authors immobilized BCL in silica aerogel prepared in the presence of N-methylmonoethanolamine pentanoate, where 50% of enzymatic activity was lost after 30 min in the second recycle (half-life of 0.73 h),[63] thus reinforcing the potential of ILs to improve the catalytic performance of immobilized lipase. However, better results are even achieved when using ILs in the lipase immobilization process. The support with the biocatalyst immobilized in the presence of IL PA-[P$_{666(14)}$]-[NTf$_2$] can be recycled for 17 times, equivalent to a half-life of 7.9 h, maintaining 50% of its initial activity. To date, there have been no reports of the use of ILs as additives during the immobilization process by physical adsorption of BCL onto silica for comparison. However, these results are promising since the presence of a low concentration of IL (1%) during the lipase immobilization process promotes a significant increase in the recycling capacity of the immobilized biocatalyst. Overall, the physical, chemical, and morphological modifications caused by the presence of IL additives during the preparation of supports or during the enzyme immobilization process can increase the interactions established between the enzyme and the support and the access to the substrate, thus minimizing leaching during the reactions and improving the catalytic performance of continuous and discontinuous processes.

Use of [P$_{666(14)}$][NTf$_2$] in Both Silica Preparation and Lipase Immobilization. The results found show that the use of the IL [P$_{666(14)}$][NTf$_2$] during the preparation of silica has a

significant influence on the material morphological characteristics, which facilitate the adsorption of the biocatalyst and improve the catalysis performance. In addition, the presence of this IL during the immobilization process allows for a remarkable increase in the enzymatic activity and total activity recovery yield. Based on the favorable results afforded by the two strategies, IL $[P_{666(14)}][NTf_2]$ was first used to tailor the surface of silica produced by the sol−gel technique, and then, BCL was immobilized in the presence of the same IL. The effects of the combined use of $[P_{666(14)}][NTf_2]$ were finally evaluated for the enzymatic activity, total activity recovery yield, and recyclability of the immobilized biocatalysts (IB-Control, IB-$[P_{666(14)}][NTf_2]$, PA-$[P_{666(14)}][NTf_2]$, and IB+PA-$[P_{666(14)}][NTf_2]$), whose results are shown in Figure 5A. This
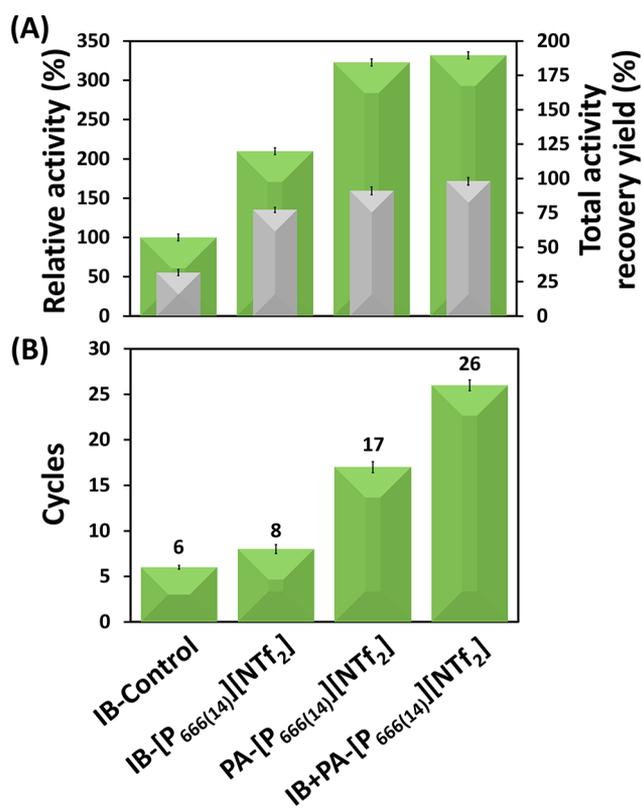
Figure 5. Effects of the combined use of IL $[P_{666(14)}][NTf_2]$ in the preparation of the material and immobilization process: (A) total activity recovery yield (gray) and enzymatic activity of the immobilized biocatalysts (green) and (B) recyclability tests.

combined strategy (IB+PA-$[P_{666(14)}][NTf_2]$) allows for an increase in the relative activity up to 231% and up to 122% when compared to the IB-Control and IB-$[P_{666(14)}][NTf_2]$, respectively. Furthermore, the combined use of the ILs in silica preparation and during the enzyme immobilization process resulted in an immobilization yield around 98%, whereas the relative activity remained similar (increase in 9%) when compared to IB+PA-$[P_{666(14)}][NTf_2]$ and PA-$[P_{666(14)}][NTf_2]$. These results show that the support was saturated with BCL. The saturation of supports by enzymes has been reported in the literature.[64−66] Even so, Figure 5B shows that the combined use of the IL in silica preparation and during the enzyme immobilization process influence positively the recyclability potential of the immobilized biocatalyst. The IB+PA-$[P_{666(14)}][NTf_2]$ material has a higher operational stability

compared to all other immobilized biocatalyst materials (IB-Control, IB-$[P_{666(14)}][NTf_2]$, and PA-$[P_{666(14)}][NTf_2]$), reaching 26 recycles, equivalent to a half-life of 13 h. Although this combined strategy does not promote a substantial improvement in the adsorption efficiency, the combined use of IL $[P_{666(14)}][NTf_2]$ in the two approaches certainly provides a higher operational stability of the immobilized biocatalyst and a higher recyclability potential.

CONCLUSIONS

To the best of our knowledge, this is the first work that reports the effects of the application of ILs in different steps of the enzyme immobilization process, namely, on the use of ILs to prepare materials to modify their morphological characteristics and on the use of ILs during the enzyme immobilization process. A large range of phosphonium-based ILs were investigated, allowing us to address the cation alkyl side chain and anion nature effects. It was found that the enzymatic performance depends on the IL employed to prepare silica, with a negative effect observed for ILs $[P_{4444}]Cl$, $[P_{444(14)}]Cl$, $[P_{666(14)}]Cl$, $[P_{666(14)}]Br$, and $[P_{666(14)}][Deca]$ and a beneficial effect with ILs $[P_{666(14)}][Phosp]$ and $[P_{666(14)}][NTf_2]$. The preparation of silica in the presence of $[P_{666(14)}][NTf_2]$ resulted in an immobilized biocatalyst (IB-$[P_{666(14)}][NTf_2]$) with a relative activity of 209.8% and immobilization yield of 77.26%, capable of being recycled eight times (keeping more than 50% of the enzyme initial activity). When ILs were added during the BCL immobilization process, similar negative and beneficial effects were observed with the same ILs. It was shown that the relative activity of BCL increases when using more hydrophobic ILs or weaker cation−anion interaction strengths. With IL $[P_{666(14)}][NTf_2]$, the immobilized biocatalyst (PA-$[P_{666(14)}][NTf_2]$) allows for a relative activity of 322.7%, a total activity recovery yield of 91.09%, and the capability of being recycled 17 times (keeping more than 50% of the enzyme initial activity). Based on these results, both approaches were finally combined; i.e., IL $[P_{666(14)}][NTf_2]$ was used both in the material preparation and immobilization process of the enzyme. This combined strategy (IB+PA-$[P_{666(14)}][NTf_2]$) allows an increase in the relative activity up to 231%. Furthermore, the combined use of ILs in silica preparation and during the enzyme immobilization process resulted in an immobilization yield around 98%, whereas the relative activity remained similar (increase in 9%). Although the BCL activity is not significantly enhanced by this strategy, the combined use of the IL in silica preparation and during the enzyme immobilization process remarkably increased the recyclability potential of the immobilized biocatalyst material, capable of being recycled 26 times, while keeping more than 50% of the enzyme initial activity, equivalent to a half-life of 13 h. Although scarcely investigated in the biocatalysis field, phosphonium-based ILs are improved candidates to improve the enzyme immobilization process and biocatalytic performance.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.9b03741.

　　　Chemical structures of ionic liquids (cations and anions), thermographs, detailed activity values, correla-

tions between the cation–anion interaction strength, and half-life values. (PDF)

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: cleide18@yahoo.com.br.
ORCID ⊙
Milson S. Barbosa: 0000-0001-5412-7865
Álvaro S. Lima: 0000-0002-0603-8187
Mara G. Freire: 0000-0001-8895-0614
Cleide M. F. Soares: 0000-0002-5932-9842

Author Contributions
All authors have given approval to the final version of the manuscript.

Notes
The authors declare no competing financial interest.

■ ABBREVIATIONS

ILs = ionic liquids
BCL = *Burkholderia cepacia* lipase
$[P_{4444}]Cl$ = tetrabutylphosphonium chloride
$[P_{444(14)}]Cl$ = tributyltetradecylphosphonium chloride
$[P_{666(14)}]Cl$ = trihexyltetradecylphosphonium chloride
$[P_{666(14)}]Br$ = trihexyltetradecylphosphonium bromide
$[P_{666(14)}][Deca]$ = trihexyltetradecylphosphonium decanoate
$[P_{666(14)}][Phosp]$ = trihexyltetradecylphosphonium bis-(2,4,4-trimethylpentyl)phosphinate
$[P_{666(14)}][NTf_2]$ = trihexyltetradecylphosphonium bis-(trifluoromethylsulfonyl)amide
TEOS = tetraethylorthossilicate
IB = immobilized biocatalyst
PA = immobilized biocatalyst in the presence of IL
BET = Brunauer–Emmett–Teller method
TGA = thermogravimetric analysis
SEM = scanning electron microscopy
and FTIR = Fourier transform infrared spectroscopy

■ REFERENCES

(1) Straathof, A. J. J. Transformation of Biomass into Commodity Chemicals Using Enzymes or Cells. *Chem. Rev.* **2014**, *114* (3), 1871–1908.
(2) Sheldon, R. A.; Woodley, J. M. Role of Biocatalysis in Sustainable Chemistry. *Chem. Rev.* **2018**, *118* (2), 801–838.
(3) Lipshutz, B. H.; Gallou, F.; Handa, S. Evolution of Solvents in Organic Chemistry. *ACS Sustainable Chem. Eng.* **2016**, *4* (11), 5838–5849.
(4) Hasan, F.; Shah, A. A.; Hameed, A. Industrial Applications of Microbial Lipases. *Enzyme Microb. Technol.* **2006**, *39* (2), 235–251.
(5) Liese, A.; Hilterhaus, L. Evaluation of Immobilized Enzymes for Industrial Applications. *Chem. Soc. Rev.* **2013**, *42* (15), 6236.

(6) Zou, B.; Song, C.; Xu, X.; Xia, J.; Huo, S.; Cui, F. Enhancing Stabilities of Lipase by Enzyme Aggregate Coating Immobilized onto Ionic Liquid Modified Mesoporous Materials. *Appl. Surf. Sci.* **2014**, *311*, 62–67.
(7) Sheldon, R. A.; van Pelt, S. Enzyme Immobilisation in Biocatalysis: Why, What and How. *Chem. Soc. Rev.* **2013**, *42* (15), 6223–6235.
(8) Thompson, M. P.; Peñafiel, I.; Cosgrove, S. C.; Turner, N. J. Biocatalysis Using Immobilized Enzymes in Continuous Flow for the Synthesis of Fine Chemicals. *Org. Process Res. Dev.* **2019**, *23* (1), 9–18.
(9) Cull, S. G.; Holbrey, J. D.; Vargas-Mora, V.; Seddon, K. R.; Lye, G. J. Room-Temperature Ionic Liquids as Replacements for Organic Solvents in Multiphase Bioprocess Operations. *Biotechnol. Bioeng.* **2000**, *69* (2), 227–233.
(10) Rebelo, L. P. N.; Canongia Lopes, J. N.; Esperanca, J. M. S. S.; Filipe, E. On the Critical Temperature, Normal Boiling Point, and Vapor Pressure of Ionic Liquids. *J. Phys. Chem. B* **2005**, *109* (13), 6040–6043.
(11) Welton, T. Room-Temperature Ionic Liquids. Solvents for Synthesis and Catalysis. *Chem. Rev.* **1999**, *99* (8), 2071–2084.
(12) Toledo Hijo, A. A. C.; Maximo, G. J.; Costa, M. C.; Batista, E. A. C.; Meirelles, A. J. A. Applications of Ionic Liquids in the Food and Bioproducts Industries. *ACS Sustainable Chem. Eng.* **2016**, *4* (10), 5347–5369.
(13) Hulsbosch, J.; De Vos, D. E.; Binnemans, K.; Ameloot, R. Biobased Ionic Liquids: Solvents for a Green Processing Industry? *ACS Sustainable Chem. Eng.* **2016**, *4* (6), 2917–2931.
(14) Chatel, G.; Rogers, R. D. Review: Oxidation of Lignin Using Ionic Liquids-an Innovative Strategy to Produce Renewable Chemicals. *ACS Sustainable Chem. Eng.* **2014**, *2* (3), 322–339.
(15) Crowhurst, L.; Mawdsley, P. R.; Perez-Arlandis, J. M.; Salter, P. A.; Welton, T. Solvent–solute Interactions in Ionic Liquids. *Phys. Chem. Chem. Phys.* **2003**, *5* (13), 2790–2794.
(16) Deetlefs, M.; Seddon, K. R. Assessing the Greenness of Some Typical Laboratory Ionic Liquid Preparations. *Green Chem.* **2010**, *12*, 17–30.
(17) Benedetto, A.; Ballone, P. Room Temperature Ionic Liquids Meet Biomolecules: A Microscopic View of Structure and Dynamics. *ACS Sustainable Chem. Eng.* **2016**, *4* (2), 392–412.
(18) Sintra, T. E.; Ventura, S. P. M.; Coutinho, J. A. P. Superactivity Induced by Micellar Systems as the Key for Boosting the Yield of Enzymatic Reactions. *J. Mol. Catal. B: Enzym.* **2014**, *107*, 140–151.
(19) da Silva, V. G.; de Castro, R. J. S. Biocatalytic Action of Proteases in Ionic Liquids: Improvements on Their Enzymatic Activity, Thermal Stability and Kinetic Parameters. *Int. J. Biol. Macromol.* **2018**, *114*, 124–129.
(20) Matsubara, Y.; Kadotani, S.; Nishihara, T.; Hikino, Y.; Fukaya, Y.; Nokami, T.; Itoh, T. Phosphonium Alkyl PEG Sulfate Ionic Liquids as Coating Materials for Activation of *Burkholderia Cepacia* Lipase. *Biotechnol. J.* **2015**, *10* (12), 1944–1951.
(21) Lee, S. Y.; Vicente, F. A.; E Silva, F. A.; Sintra, T. E.; Taha, M.; Khoiroh, I.; Coutinho, J. A. P.; Show, P. L.; Ventura, S. P. M. Evaluating Self-Buffering Ionic Liquids for Biotechnological Applications. *ACS Sustainable Chem. Eng.* **2015**, *3* (12), 3420–3428.
(22) Itoh, T. Ionic Liquids as Tool to Improve Enzymatic Organic Synthesis. *Chem. Rev.* **2017**, *117* (15), 10567–10607.
(23) Ventura, S. P. M.; Santos, L. D. F.; Saraiva, J. A.; Coutinho, J. A. P. Concentration Effect of Hydrophilic Ionic Liquids on the Enzymatic Activity of *Candida Antarctica* Lipase B. *World J. Microbiol. Biotechnol.* **2012**, *28* (6), 2303–2310.
(24) Dabirmanesh, B.; Daneshjou, S.; Sepahi, A. A.; Ranjbar, B.; Khavari-Nejad, R. A.; Gill, P.; Heydari, A.; Khajeh, K. Effect of Ionic Liquids on the Structure, Stability and Activity of Two Related α-Amylases. *Int. J. Biol. Macromol.* **2011**, *48* (1), 93–97.
(25) Yang, J.; Hu, Y.; Jiang, L.; Zou, B.; Jia, R.; Huang, H. Enhancing the Catalytic Properties of Porcine Pancreatic Lipase by Immobilization on SBA-15 Modified by Functionalized Ionic Liquid. *Biochem. Eng. J.* **2013**, *70*, 46–54.

(26) Hu, Y.; Tang, S.; Jiang, L.; Zou, B.; Yang, J.; Huang, H. Immobilization of *Burkholderia Cepacia* Lipase on Functionalized Ionic Liquids Modified Mesoporous Silica SBA-15. *Process Biochem.* 2012, 47, 2291−2299.

(27) Vila-Real, H.; Alfaia, A. J.; Rosa, J. N.; Gois, P. M. P.; Rosa, M. E.; Calado, A. R. T.; Ribeiro, M. H. α-Rhamnosidase and β-Glucosidase Expressed by Naringinase Immobilized on New Ionic Liquid Sol-Gel Matrices: Activity and Stability Studies. *J. Biotechnol.* 2011, 152 (4), 147−158.

(28) de Souza, R. L.; de Faria, E. L. P.; Figueiredo, R. T.; Freitas, L. d. S.; Iglesias, M.; Mattedi, S.; Zanin, G. M.; dos Santos, O. A. A.; Coutinho, J. A.P.; Lima, A. S.; Soares, C. M. F. Protic Ionic Liquid as Additive on Lipase Immobilization Using Silica Sol-Gel. *Enzyme Microb. Technol.* 2013, 52, 141−150.

(29) Cabrera-Padilla, R. Y.; Melo, E. B.; Pereira, M. M.; Figueiredo, R. T.; Fricks, A. T.; Franceschi, E.; Lima, Á. S.; Silva, D. P.; Soares, C. M. F. Use of Ionic Liquids as Additives for the Immobilization of Lipase from Bacillus Sp. *J. Chem. Technol. Biotechnol.* 2015, 90 (7), 1308−1316.

(30) Abe, Y.; Yoshiyama, K.; Yagi, Y.; Hayase, S.; Kawatsura, M.; Itoh, T. A Rational Design of Phosphonium Salt Type Ionic Liquids for Ionic Liquid Coated-Lipase Catalyzed Reaction. *Green Chem.* 2010, 12 (11), 1976.

(31) Abe, Y.; Kude, K.; Hayase, S.; Kawatsura, M.; Tsunashima, K.; Itoh, T. Design of Phosphonium Ionic Liquids for Lipase-Catalyzed Transesterification. *J. Mol. Catal. B: Enzym.* 2008, 51 (3−4), 81−85.

(32) Cowan, M. G.; Masuda, M.; McDanel, W. M.; Kohno, Y.; Gin, D. L.; Noble, R. D. Phosphonium-Based Poly(Ionic Liquid) Membranes: The Effect of Cation Alkyl Chain Length on Light Gas Separation Properties and Ionic Conductivity. *J. Membr. Sci.* 2016, 498, 408−413.

(33) Del Sesto, R. E.; Corley, C.; Robertson, A.; Wilkes, J. S. Tetraalkylphosphonium-Based Ionic Liquids. *J. Organomet. Chem.* 2005, 690 (10), 2536−2542.

(34) Atefi, F.; Garcia, M. T.; Singer, D.; Scammells, P. J. Phosphonium Ionic Liquids: Design, Synthesis and Evaluation of Biodegradability. *Green Chem.* 2009, 11 (10), 1595−1604.

(35) Yang, Q.; Xu, D.; Zhang, J.; Zhu, Y.; Zhang, Z.; Qian, C.; Ren, Q.; Xing, H. Long-Chain Fatty Acid-Based Phosphonium Ionic Liquids with Strong Hydrogen-Bond Basicity and Good Lipophilicity: Synthesis, Characterization, and Application in Extraction. *ACS Sustainable Chem. Eng.* 2015, 3 (2), 309−316.

(36) Barbosa, M. S.; Freire, C. C. C.; Souza, R. L.; Cabrera-Padilla, R. Y.; Pereira, M. M.; Freire, M. G.; Lima, Á. S.; Soares, C. M. F. Effects of Phosphonium-Based Ionic Liquids on the Lipase Activity Evaluated by Experimental Results and Molecular Docking. *Biotechnol. Prog.* 2019, 35, 1−10.

(37) Soares, C. M. F.; De Castro, H. F.; De Moraes, F. F.; Zanin, G. M. Characterization and Utilization of *Candida Rugosa* Lipase Immobilized on Controlled Pore Silica. *Appl. Biochem. Biotechnol.* 1999, 79, 745−758.

(38) Cabrera-Padilla, R. Y.; Lisboa, M. C.; Fricks, A. T.; Franceschi, E.; Lima, A. S.; Silva, D. P.; Soares, C. M. F. Immobilization of *Candida Rugosa* Lipase on Poly(3-Hydroxybutyrate-Co- Hydroxyvalerate): A New Eco-Friendly Support. *J. Ind. Microbiol. Biotechnol.* 2012, 39 (2), 289−298.

(39) Brunauer, S.; Emmett, P. H.; Teller, E. Adsorption of Gases in Multimolecular Layers. *J. Am. Chem. Soc.* 1938, 60, 309−319.

(40) Barrett, E.; Joyner, L. G.; Halenda, P. P. The Determination of Pore Volume and Area Distributions in Porous Substances. I. Computations from Nitrogen Isotherms. *J. Am. Chem. Soc.* 1951, 73 (1), 373−380.

(41) Zhao, H. Methods for Stabilizing and Activating Enzymes in Ionic Liquids - A Review. *J. Chem. Technol. Biotechnol.* 2010, 85 (7), 891−907.

(42) Attri, P.; Venkatesu, P.; Kumar, A. Activity and Stability of α-Chymotrypsin in Biocompatible Ionic Liquids: Enzyme Refolding by Triethyl Ammonium Acetate. *Phys. Chem. Chem. Phys.* 2011, 13 (7), 2788−2796.

(43) Klähn, M.; Lim, G. S.; Seduraman, A.; Wu, P. On the Different Roles of Anions and Cations in the Solvation of Enzymes in Ionic Liquids. *Phys. Chem. Chem. Phys.* 2011, 13 (4), 1649−1662.

(44) Zou, B.; Hu, Y.; Yu, D.; Xia, J.; Tang, S.; Liu, W.; Huang, H. Immobilization of Porcine Pancreatic Lipase onto Ionic Liquid Modified Mesoporous Silica SBA-15. *Biochem. Eng. J.* 2010, 53 (1), 150−153.

(45) Naushad, M.; ALOthman, Z. A.; Khan, A. B.; Ali, M. Effect of Ionic Liquid on Activity, Stability, and Structure of Enzymes: A Review. *Int. J. Biol. Macromol.* 2012, 51, 555−560.

(46) Cláudio, A. F. M.; Swift, L.; Hallett, J. P.; Welton, T.; Coutinho, J. A. P.; Freire, M. G. Extended Scale for the Hydrogen-Bond Basicity of Ionic Liquids. *Phys. Chem. Chem. Phys.* 2014, 16 (14), 6593.

(47) Ventura, S. P. M.; Santos, L. D. F.; Saraiva, J. A.; Coutinho, J. A. P. Ionic Liquids Microemulsions: The Key to Candida Antarctica Lipase B Superactivity. *Green Chem.* 2012, 14 (6), 1620.

(48) Kumar, A.; Venkatesu, P. Does the Stability of Proteins in Ionic Liquids Obey the Hofmeister Series? *Int. J. Biol. Macromol.* 2014, 63, 244−253.

(49) Neves, C. M. S. S.; Silva, A. M. S.; Fernandes, A. M.; Coutinho, J. A. P.; Freire, M. G. Toward an Understanding of the Mechanisms behind the Formation of Liquid-Liquid Systems Formed by Two Ionic Liquids. *J. Phys. Chem. Lett.* 2017, 8 (13), 3015−3019.

(50) Mateo, C.; Palomo, J. M.; Fernandez-Lorente, G.; Guisan, J. M.; Fernandez-Lafuente, R. Improvement of Enzyme Activity, Stability and Selectivity via Immobilization Techniques. *Enzyme Microb. Technol.* 2007, 40 (6), 1451−1463.

(51) Wu, C.; Zhou, G.; Jiang, X.; Ma, J.; Zhang, H.; Song, H. Active Biocatalysts Based on *Candida Rugosa* Lipase Immobilized in Vesicular Silica. *Process Biochem.* 2012, 47 (6), 953−959.

(52) Zarcula, C.; Corîci, L.; Croitoru, R.; Ursoiu, A.; Peter, F. Preparation and Properties of Xerogels Obtained by Ionic Liquid Incorporation during the Immobilization of Lipase by the Sol-Gel Method. *J. Mol. Catal. B: Enzym.* 2010, 65 (1−4), 79−86.

(53) Cabrera-Padilla, R. Y.; Lisboa, M. C.; Pereira, M. M.; Figueiredo, R. T.; Franceschi, E.; Fricks, A. T.; Lima, Á. S.; Silva, D. P.; Soares, C. M. F. Immobilization of *Candida Rugosa* Lipase onto an Eco-Friendly Support in the Presence of Ionic Liquid. *Bioprocess Biosyst. Eng.* 2015, 38, 805−814.

(54) Collins, K. D.; Neilson, G. W.; Enderby, J. E. Ions in Water: Characterizing the Forces That Control Chemical Processes and Biological Structure. *Biophys. Chem.* 2007, 128 (2−3), 95−104.

(55) Zhou, Z.; Hartmann, M. Progress in Enzyme Immobilization in Ordered Mesoporous Materials and Related Applications. *Chem. Soc. Rev.* 2013, 42 (9), 3894−3912.

(56) Neves, C. M. S. S.; Carvalho, P. J.; Freire, M. G.; Coutinho, J. A. P. Thermophysical Properties of Pure and Water-Saturated Tetradecyltrihexylphosphonium-Based Ionic Liquids. *J. Chem. Thermodyn.* 2011, 43 (6), 948−957.

(57) Mukherjee, I.; Mylonakis, A.; Guo, Y.; Samuel, S. P.; Li, S.; Wei, R. Y.; Kojtari, A.; Wei, Y. Effect of Nonsurfactant Template Content on the Particle Size and Surface Area of Monodisperse Mesoporous Silica Nanospheres. *Microporous Mesoporous Mater.* 2009, 122 (1−3), 168−174.

(58) Soares, C. M. F.; Dos Santos, O. A.; Olivo, J. E.; De Castro, H. F.; De Moraes, F. F.; Zanin, G. M. Influence of the Alkyl-Substituted Silane Precursor on Sol-Gel Encapsulated Lipase Activity. *J. Mol. Catal. B: Enzym.* 2004, 29 (1−6), 69−79.

(59) Kong, J.; Yu, S. Fourier Transform Infrared Spectroscopic Analysis of Protein Secondary Structures. *Acta Biochim. Biophys. Sin.* 2007, 39 (8), 549−559.

(60) Wi, S.; Pancoska, P.; Keiderling, T. A. Predictions of Protein Secondary Structures Using Factor Analysis on Fourier Transform Infrared Spectra: Effect of Fourier Self-Deconvolution of the Amide I and Amide II Bands. *Biospectroscopy* 1998, 4 (2), 93−106.

(61) Liu, Y.; Chen, D.; Yan, Y.; Peng, C.; Xu, L. Biodiesel Synthesis and Conformation of Lipase from *Burkholderia Cepacia* in Room Temperature Ionic Liquids and Organic Solvents. *Bioresour. Technol.* 2011, 102 (22), 10414−10418.

(62) Pan, S.; Liu, X.; Xie, Y.; Yi, Y.; Li, C.; Yan, Y.; Liu, Y. Esterification Activity and Conformation Studies of *Burkholderia Cepacia* Lipase in Conventional Organic Solvents, Ionic Liquids and Their Co-Solvent Mixture Media. *Bioresour. Technol.* **2010**, *101* (24), 9822−9824.

(63) Barbosa, A. S.; Lisboa, J. A.; Silva, M. A. O.; Carvalho, N. B.; Pereira, M. M.; Fricks, A. T.; Mattedi, S.; Lima, Á. S.; Franceschi, E.; Soares, C. M. F. The Novel Mesoporous Silica Aerogel Modified with Protic Ionic Liquid for Lipase Immobilization. *Quim. Nova* **2016**, *39* (4), 415−422.

(64) Alves, M. D.; Aracri, F. M.; Cren, É. C.; Mendes, A. A. Isotherm, Kinetic, Mechanism and Thermodynamic Studies of Adsorption of a Microbial Lipase on a Mesoporous and Hydrophobic Resin. *Chem. Eng. J.* **2017**, *311*, 1−12.

(65) Alves, M. D.; Cren, É. C.; Mendes, A. A. Kinetic, Thermodynamic, Optimization and Reusability Studies for the Enzymatic Synthesis of a Saturated Wax Ester. *J. Mol. Catal. B: Enzym.* **2016**, *133*, S377−S387.

(66) Bolina, I. C. A.; Salviano, A. B.; Tardioli, P. W.; Cren, É. C.; Mendes, A. A. Preparation of Ion-Exchange Supports via Activation of Epoxy-SiO2 with Glycine to Immobilize Microbial Lipase − Use of Biocatalysts in Hydrolysis and Esterification Reactions. *Int. J. Biol. Macromol.* **2018**, *120*, 2354−2365.