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PAPER

Enzymatic synthesis of amoxicillin by penicillin G acylase in the presence of ionic liquids

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A major obstacle for the industrial implementation of the enzymatic synthesis of β-lactam antibiotics is the limited yield of the product, due to undesirable hydrolytic reactions. This drawback can be partially avoided by reducing the water activity in the medium. Ionic liquids (ILs) have emerged as an alternative to conventional organic media due to their high thermal and chemical stability, negligible vapor pressure, non-flammability, and easy recycling. In this context, this paper assesses the catalytic activity of penicillin G acylase (E.C.3.5.1.11) in the synthesis of amoxicillin using different ILs, all based on the 1-butyl-3-methylimidazolium cation. An increase of 400% in selectivity (synthesis/hydrolysis, S/H ratio) was observed for the reactions carried out with BMI·PF_6 as a cosolvent at 75% (v_{IL}/v_{water}) when compared to the totally aqueous medium (using phosphate buffer). This figure reached 350% for BMI·NTf_2, while for BMI·BF_4 there was only a slight increase in selectivity. The highest conversion of the β-lactam nucleus (6-APA) was achieved using BMI·NTf_2 as a cosolvent at 71% (v/v), more than 36% above the one in water. No deactivation of the enzyme after the reactions was observed in any of the ILs, and the physical integrity of the biocatalyst particles was maintained.

Introduction

Penicillin G acylase (PGA, E.C.3.5.1.11), also known as penicillin acyltransferase, penicillin amidase or penicillin amidohydrolase, is an important enzyme for the pharmaceutical industry. It is used for the hydrolysis of penicillin G, producing 6-amino-penicillanic acid (6-APA), a key molecule in the synthesis of semi-synthetic penicillins.[1] Additionally, PGA can also catalyze the enzymatic synthesis of semi-synthetic β-lactam antibiotics, through the condensation of different acyl groups to a β-lactam nucleus (for example, 6-APA).[2]

Bacteria, yeasts and fungi produce PGA, but the most used in industrial processes is the enzyme from *Escherichia coli* ATCC 11105.[3] This enzyme is a heterodimer. The smaller subunit, α, with 209 amino acids, has a molecular mass of 20 500 Da and the larger one, β, has 69 000 Da and 557 amino acids.[4] The role of the α subunit is to recognize the side chain of the substrate, while the terminal serine, essential for the catalytic activity, is in the β subunit.[5] Residues from both subunits are part of the active site of the enzyme.[6]

Among the most routinely used pharmaceuticals worldwide, β-lactam antibiotics occupy a prominent position. Semi-synthetic cephalosporins and penicillins such as cephalexin, cefadroxil, cefazolin, ampicillin, amoxicillin, among many others, represent approximately 65% of the ever-growing production of antibiotics,[7] which outpaced 45 000 tons in 2000.[8] Amoxicillin occupies an important place in this market. It has a broad spectrum of activity, high solubility, a high rate of absorption and is stable under acid conditions, allowing the oral administration of this drug, which resists the gastric pH.[9]

The current industrial process used to produce these antibiotics is a rather drastic chemical route, including the protection and de-protection of reactive groups. It demands low temperatures (−30 °C), the use of toxic organochloride solvents, and generates non-recyclable waste.[10] Presently there is a tendency of concentrating the production of β-lactam antibiotics in a few countries The yields that are achieved by the chemical synthesis are very high, thus contributing to the low market prices of these drugs. This is of course an important feature for public health, especially in non-developed countries. But the tendency towards stricter environmental regulation opens a window of opportunity for other, less harmful, technologies.

The enzymatic synthesis of semi-synthetic β-lactam antibiotics was first proposed by Cole.[11] Working under mild conditions of reaction and generating harmless salts as waste of the purification process, the enzymatic route became a target of intense research as a "cleaner" process. Enzymatic reactions are "environmental-friendly" strategies. Enzymes are biodegradable, and consequently less polluting than chemical catalysts. Nevertheless, the

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enzymatic synthesis of β-lactam antibiotics is still not economically competitive with the fine-tuned conventional chemical process.[2] Indeed, despite the advances in this field, which allowed some optimism with respect to the economic feasibility of the enzymatic process, to the best of our knowledge there is no industry using the enzymatic synthesis of amoxicillin in its production process over the world.[12]

To reduce costs of the enzymatic synthesis, two points must be addressed: increasing the process yield and selectivity; and reducing the cost of the biocatalyst, through its reuse. Soluble enzymes usually exhibit lower stability than chemical catalysts and often cannot be recovered. This fact hinders their application in industrial practice. This problem can be overcome by enzyme immobilization techniques, which enhance thermal and operational stabilities, make the catalyst easy to handle, and prevent enzyme aggregation. Besides, the feasibility of recovery and reuse of enzymes immobilized on solid supports reduces operational costs in industry.

Semi-synthetic antibiotics can be produced using enzymes *via* two approaches: thermodynamic controlled synthesis (TCS), which is the reverse reaction of the antibiotic hydrolysis; or kinetically controlled synthesis (KCS), which requires substrate activation.[13] In the TCS, the yield is constrained by the thermodynamic equilibrium constant of the reaction, while for the KCS the competition between the different catalytic activities (transferase, esterase and amidase) of PGA determines the yield and selectivity of the overall process.

A major problem with the TCS is that the synthesis of amoxicillin is completely shifted towards the hydrolysis of the antibiotic in an aqueous medium, at the conditions where the enzyme is active. Besides, for the enzymatic synthesis of amoxicillin to occur, the amino group of 6-APA and the carboxylic group of *p*-hydroxyphenylglycine must be neutral.[14] However, there is no range of pH where both substrates are predominantly in a non-ionized state, as is requested for the enzyme action.[15]

In the KCS of β-lactam antibiotics, an activated derivative of the acyl donor, an ester or amide, reacts with the β-lactam nucleus (6-APA) and produces the antibiotic, thus circumventing the need for a neutral carboxylic group in the side chain precursor, as is requested in the TCS. In this reaction, PGA acts as a transferase. However, this enzyme is also a hydrolase, thus 6-APA and water molecules compete in the nucleophilic attack to the acyl-enzyme intermediate.[2] As a result, selectivity becomes a problem. Fig. 1 exemplifies the reactions involved in the KCS of amoxicillin when the *p*-hydroxyphenylglycine methyl ester (PHPGME) is used.

In these series–parallel reactions, PHPGME reacts with 6-APA to form amoxicillin and methanol, while its hydrolysis produces *p*-hydroxyphenylglycine (PHPG) and methanol. The hydrolysis of amoxicillin generates 6-APA and PHPG. It should be stressed that *p*-hydroxyphenylglycine ethyl ester (PHPGEE) may also be employed, reducing even further the environmental impact of the process. This substrate was successfully used in our group (results not shown), but since the methyl ester is the standard substrate in the literature, it will be used in this work as well, for the sake of comparison.

Thus, the hydrolytic reactions are the main drawback hindering the industrial implementation of the enzymatic synthesis of semi-synthetic penicillins.[16] Hence, maximization of the

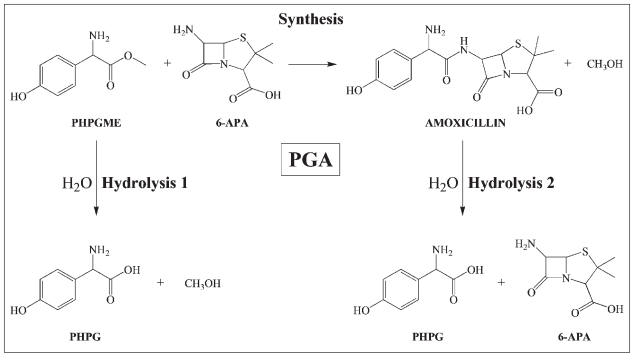

Fig. 1 KCS of amoxicillin catalyzed by PGA. In reactions, PGA acts as transferase (for synthesis) and as hydrolase, promoting two undesired side reactions (hydrolysis 1, of the acyl donor derivative, and hydrolysis 2, of the antibiotic).

selectivity (synthesis/hydrolysis, S/H ratio) towards the antibiotic is a key question for the economics of the enzymatic process.[16] These undesirable side reactions could be partially avoided by reducing the water activity (a_w) in the reaction medium. One way to reduce the hydrolytic reactions is the use of organic cosolvents.

In recent years, much effort has been directed towards the study of the enzymatic synthesis of semi-synthetic penicillins in the presence of organic cosolvents, either for the TCS or for the KCS strategy.[17–25] However, although some good results have been reported with respect to yield and selectivity, organic solvents have the disadvantage of being volatile, flammable, toxic, and harmful to the environment.

In this scenario, ionic liquids (ILs) have emerged as solvents that may replace the traditional organic media in various biotechnological processes. One decade ago, to the best of our knowledge, ILs began to be used as reaction media in biotransformations.[26–28] The hydration reaction of 1,3-cyanobenzene catalyzed by nitrile hydratase employed 1-butyl-3-methylimidazolium hexafluorophosphate (BMI·PF_6).[26] Almost simultaneously, BMI·PF_6 was also used on the synthesis of Z-aspartame, catalyzed by thermolysin.[27] Furthermore, an aqueous solution of Candida antarctica lipase B (CALB) dissolved in pure 1-ethyl-3-methylimidazolium triflimide (EMI·NTf_2) or 1-butyl-3-methylimidazolium triflimide (BMI·NTf_2) was the biocatalyst for both butyl butyrate synthesis and for the kinetic resolution of 1-phenylethanol by transesterification.[28] After these pioneering works, the use of ILs in enzymatic reactions has been widely investigated.

ILs are organic salts that exist as liquids below a threshold temperature (usually around 100 °C).[29] ILs have been considered a promising class of "green solvents" mainly due to their negligible vapor pressure. Moreover, they are non-flammable and have high thermal and chemical stability.[30,31] In addition, ILs have been recognized as "designer solvents", since their physicochemical properties such as hydrophobicity, viscosity, density, and solubility can be tuned by selecting different combinations of cations and anions, and of the attached substituent as well.[32,33]

Typical ILs are based on organic cations paired with a variety of anions that have a strongly delocalized negative charge.[34] The

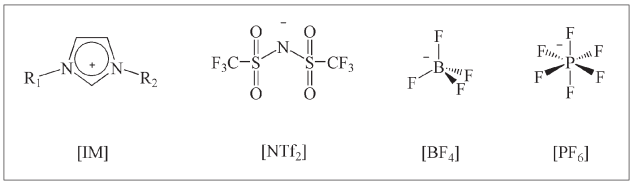

Fig. 2 Chemical structure of the ionic liquids commonly used in biotechnological processes.

imidazolium (IM) based salts are the most investigated ILs in biotechnology.[29] Moreover, bis(trifluoromethylsulfonyl)imide (NTf_2), tetrafluoroborate (BF_4), and hexafluorophosphate (PF_6) are by far the anions most often used.[35,36] Fig. 2 shows the chemical structure of these ions.

In short, enzymatic synthesis of β-lactam antibiotics has not gained industrial feasibility yet due to the low selectivity of the reaction, caused by hydrolysis of substrate and product. Consequently, important antibiotics (amoxicillin among them) continue to be produced by the chemical route, using organochloride solvents. Thus, using the "greenest solvent", water, is not an alternative in practice. With this motivation, this work assessed the catalytic activity of PGA for the KCS of amoxicillin using different ILs, all based on dialkylimidazolium cations. The reaction in a totally aqueous medium (using phosphate buffer) was chosen as a standard for comparison with reactions carried out in increasing proportions of ILs, in order to evaluate the influence of these solvents on the performance indexes of the reactions (6-APA conversion and selectivity).

Results and discussion

The behavior of enzymes in non-aqueous solvents is still not completely understood. Ever since the pioneering studies of enzymatic catalysis in organic solvents, it is questioned if a totally aqueous microenvironment is essential for maintaining the active conformation of enzymes.[37] Water molecules are distributed among the vicinities of the enzyme macromolecule, the solvent itself (since there is no total immiscibility), and the support for immobilization (when used). Thus, one important aspect, more than the bulk concentration of water in the medium, is its availability to the enzyme molecule, in order to sustain the catalytic properties.

The amount of water available for the enzyme in the reaction medium (enzyme hydration), particularly for interaction with the active site, is closely related to the thermodynamic water activity in the medium (a_w).[38] Thus, measurements of a_w were undertaken. Table 1 shows the values of a_w for the three IL–water media used in the kinetic assays, for different bulk compositions of each medium, measured at 25 °C after being equilibrated for 24 h. It is worth noting that the bulk "compositions" of the IL–water mixture are here expressed as volumetric fractions.

According to Table 1, the anions have a great influence on the IL–water interaction. For all volumetric fractions, the lowest values of a_w are found for BMI·BF_4, while higher values are for BMI·NTf_2. Values of a_w for the ILs without addition of water, 100% (v/v), are corroborated by an earlier study (0.17 and 0.30 for BMI·BF_4 and BMI·PF_6, respectively).[39] As expected,

Table 1 Water activity (a_w) for different ionic liquid–water mixtures (IL–water), measures at 25 °C after being equilibrated for 24 h, with standard deviations of triplicate measurements

IL–water (v/v)	BMI·BF_4	BMI·PF_6	BMI·NTf_2
12.5%	0.7950 ± 0.0003	0.9001 ± 0.0006	0.9988 ± 0.0002
25.0%	0.6487 ± 0.0005	0.8979 ± 0.0011	0.9945 ± 0.0003
37.5%	0.5765 ± 0.0006	0.8902 ± 0.0002	0.9902 ± 0.0004
50.0%	0.5321 ± 0.0011	0.8765 ± 0.0014	0.9862 ± 0.0002
62.5%	0.4703 ± 0.0007	0.8434 ± 0.0003	0.9521 ± 0.0003
75.0%	0.3987 ± 0.0010	0.7777 ± 0.0008	0.8904 ± 0.0002
87.5%	0.3467 ± 0.0006	0.6244 ± 0.0012	0.7586 ± 0.0004
100.0%	0.1705 ± 0.0005	0.3710 ± 0.0009	0.5719 ± 0.0008

Table 2 Reichardt's dye polarity (E^N_T) values at 25 °C and water content of the ionic liquids equilibrated at 25 °C in water, both reported in the literature[43,44]

Ionic liquid	E^N_T [43]	Water equilibrated[44]
BMI·BF_4	0.673	Miscible
BMI·PF_6	0.667	11 700 ppm
BMI·NTf_2	0.642	3280 ppm

reducing the volumetric fraction of IL implies higher values of a_w. It is known that a_w depends on the specific interactions between the solvent and water, *i.e.*, a_w is higher for a solvent that contains a more hydrophobic group.[40,41] In short, these interactions are favored in hydrophilic solvents, resulting in lower values of a_w.

The miscibility of ILs with water varies widely and unpredictably, since BMI·BF_4 is water-miscible, but BMI·PF_6 and BMI·NTf_2, which have similar polarity as BMI·BF_4, are only partially miscible in water.[42] It was observed that solvents with similar polarities may have very different values of miscibility in water. Table 2 presents Reichardt's dye polarity (E^N_T) values obtained at 25 °C and water content of the ILs equilibrated at 25 °C in water, both reported in the literature.[43,44]

Although ILs are considered as "nano-structured" with polar and non-polar regions, their polarity is similar to short-chain alcohols.[45,46] However, it is important to distinguish between the concepts of hydrophobicity and polarity because the former is often related to the miscibility with water, *i.e.*, hydrophobicity may be considered as a narrower concept of polarity.[33] These observations endorse that the identity of the anion exerts great influence on the interactions between water and IL molecules.

The E^N_T empirical polarity scale is based on the shift of the charge-transfer absorption band of a solvatochromic probe in the presence of a solvent. Changes in the position of the charge-transfer absorption band within the visible spectrum are due to hydrogen bonding between the solvent and the phenoxide oxygen atom present in Reichardt's dye.[43] An E^N_T value is then determined as a function of the position of the charge-transfer absorption band.[43,45,48] E^N_T values for ILs are dependent on the bonding strength of hydrogen between the cation that composes the IL and the phenoxide group present in Reichardt's dye.[45] High E^N_T values indicate that ILs exhibit strong hydrogen bonding forces, and solvents that demonstrate the ability for hydrogen bonding present the potential to interfere with the enzyme structure.[47,48]

Table 3 Experimental viscosities (η) of the ionic liquids obtained at 25 °C and data reported in the literature at the same temperature for the dried ionic liquids[44]

Ionic liquid	η (cP)	η (cP)[44]
BMI·BF_4	91	219
BMI·PF_6	234	450
BMI·NTf_2	44	69

According to the solvent polarity scale, higher values of E^N_T match higher solvent polarity.[43] Solvatochromic parameters provide a simple method to determine the polarity of a solvent.[48] Therefore, a comparison between the data presented in Tables 1 and 2 shows that the order of miscibility with water (hydrophilicity), BMI·BF_4 > BMI·PF_6 > BMI·NTf_2, follows the polarity of these ILs, thus explaining the values of a_w found in the present work.

Table 3 presents the experimental data of viscosity of the ILs, obtained at 25 °C, together with data reported in the literature.[44] It is observed that the rank of viscosities found in this study is BMI·PF_6 > BMI·BF_4 > BMI·NTf_2. In addition, the higher symmetry of the inorganic anions (PF_6 or BF_4) compared to the organic anion (NTf_2) may play an important role, *i.e.*, the geometry and molar mass of the anions have a strong influence on the viscosity of this class of IL, since BMI combined with either PF_6 or NTf_2 produces ILs with significantly different viscosities.[44] These differences in viscosity are one of the reasons behind the recent development of ILs based in NTf_2, which are relatively less viscous compared to ILs containing other anions.[49]

ILs are more viscous fluids than conventional organic solvents.[50] Nevertheless, the high viscosity of the ILs may slow down conformational changes of proteins, allowing enzymes to maintain their native structures and activity.[51] In industrial production processes, the majority of enzymes are immobilized-stabilized to facilitate handling and to improve their operational stability. These heterogeneous catalyst particles are subject to internal and external mass transport limitations, which are strongly influenced by the viscosity of the reaction medium.[52]

An important aspect that must be observed in research with innovative solvents, as is the case of employing ILs for the process of enzymatic synthesis of amoxicillin, is the experimental reproducibility that can be achieved. Therefore, to illustrate the reproducibility of the reactions that were performed in this work, Fig. 3 shows the evolution of the KCS of amoxicillin catalyzed by PGA immobilized on Sepabeads® in a totally aqueous medium, while Fig. 4 shows some reactions with ILs as cosolvents (in different concentrations of each one of the ILs). These graphics show that the standard deviation (S.D.) from triplicates was small, and in all cases lower than 5%, evidencing the high reproducibility of the experiments. Besides, the profiles of substrates and products follow the expected trend for this system of series–parallel reactions. It is important to note that mass balance calculations were made for all the reactions, always with deviations lower than 5%.

The stoichiometry of the reactions shown in Fig. 1 provides two degrees of freedom for the system, so the conversion of 6-APA and the selectivity (synthesis/hydrolysis, S/H ratio) were

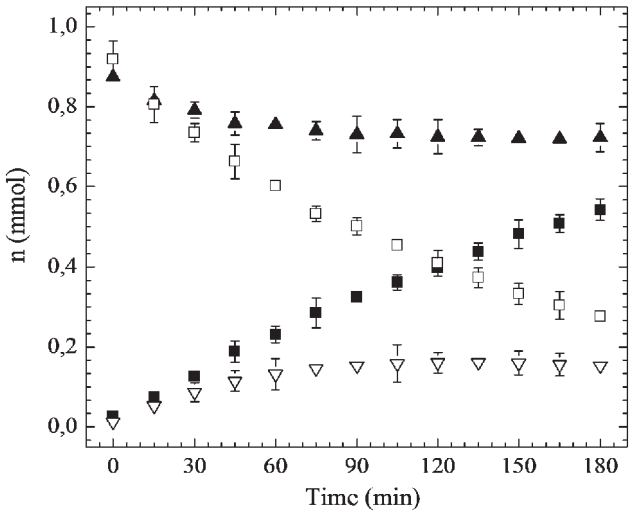

Fig. 3 Amoxicillin synthesis at 25 °C and pH 6.5 with initial bulk concentrations: 50 mM PHPGME, 50 mM 6-APA, and 0.2 g of 260 IU g^{-1} of PGA preparation in a totally aqueous medium (100 mM phosphate buffer pH 6.5). In the graphics: (□) PHPGME, (▲) 6-APA, (■) PHPG and (▽) amoxicillin. Error bars: S.D., estimated from triplicates.

the indices chosen to assess the performance of the enzymatic synthesis of amoxicillin. It should be recalled that the experimental conditions were the same for the reactions carried out in phosphate buffer without the presence of any IL, specifically, 0% (v_{IL}/v_{water}), and for the reactions using ILs as cosolvents. The IL/water volumetric ratios ranged from 12.5 to 87.5% (v_{IL}/v_{water}), besides the assay conducted in the presence of an unmixed IL, namely, 100% (v_{IL}/v_{water}).

Fig. 5 presents a comparison, in terms of selectivity, of all the experiments that were performed in this study. It is observed that the reactions carried out with BMI·BF_4 as a cosolvent showed just a slight increase of selectivity compared to the standard reaction, 0% (v/v), while in the reactions that were conducted with BMI·PF_6 and BMI·NTf_2 there was a significant increase of selectivity. The selectivity profiles were similar for all the ILs employed in these syntheses, differing simply in magnitude, *i.e.*, the selectivity increases with the increase of concentration of the IL until it reaches the maximum value at 75% (v/v) of the IL. At higher concentrations, the selectivity decreases. As previously mentioned, hydrolytic reactions are the main drawback in the industrial implementation of the enzymatic synthesis of semi-synthetic penicillins.[16] Fig. 5 indicates that with the increase of concentration of the IL, and consequently with the reduction of a_w in the reaction medium, it is possible to reduce these undesirable hydrolytic side reactions. In this sense, the results of selectivity for the reactions performed with BMI·PF_6 and with BMI·NTf_2 are promising. It should be noticed that reactors operating in the fed-batch mode would most certainly be the best operational solution for the industrial process of synthesis: the antibiotic would precipitate during the operation, and so it would be retrieved from the hydrolytic action of the enzyme, while the ester would be fed as it is consumed for the synthesis.[53] Consequently, a much higher selectivity can be achieved than the ones reported here. However, in order to compare the different systems using the same basis, the assays presented in this study

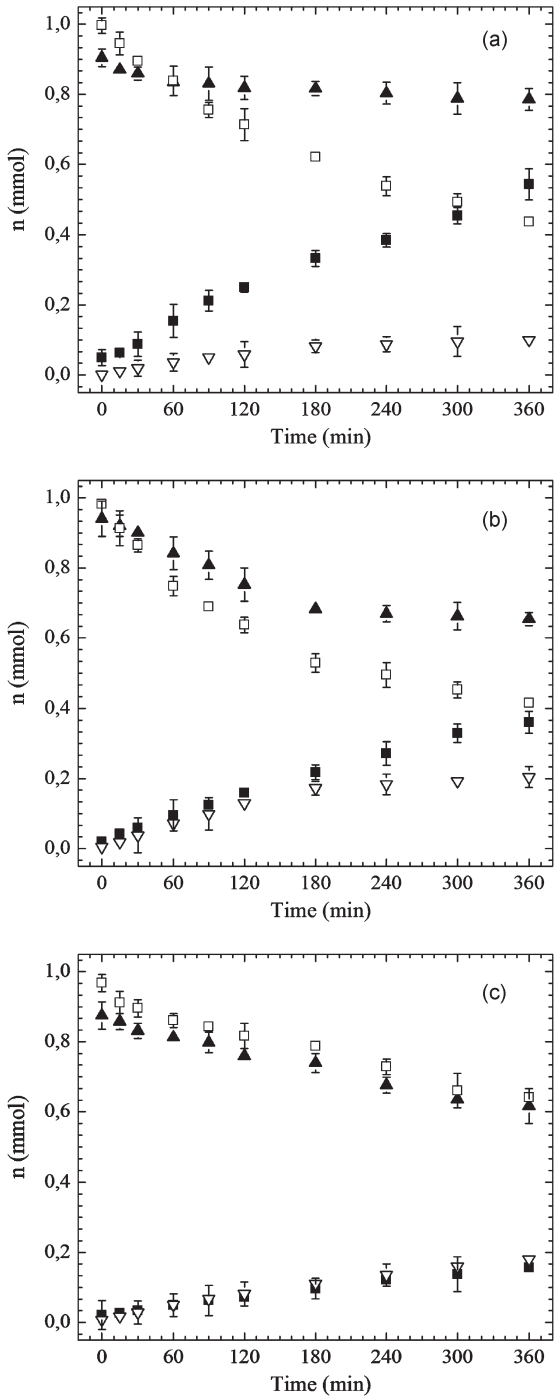

Fig. 4 Amoxicillin syntheses at 25 °C and pH 6.5 with initial bulk concentrations: 50 mM PHPGME, 50 mM 6-APA, and 0.2 g of 260 IU g^{-1} of PGA preparation in (a) 25% (v/v) BMI·BF_4, (b) 50% (v/v) BMI·NTf_2, and (c) 75% (v/v) BMI·PF_6. In the graphics: (□) PHPGME, (▲) 6-APA, (■) PHPG and (▽) amoxicillin. Error bars: S.D., estimated from triplicates.

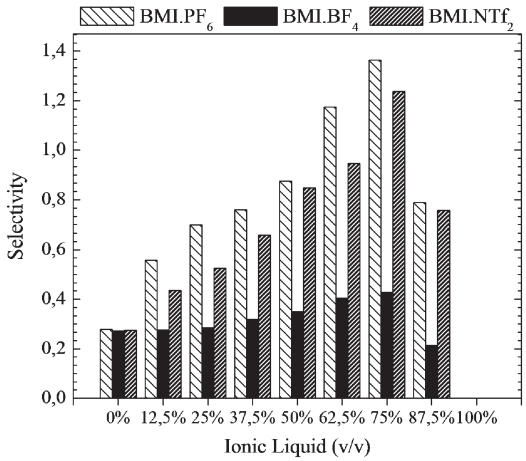

Fig. 5 Selectivity (synthesis/hydrolysis, S/H ratio) after 180 min of reaction. Amoxicillin syntheses at 25 °C and pH 6.5 with initial bulk concentrations: 50 mM PHPGME, 50 mM 6-APA, and 0.2 g of 260 IU g^{-1} of PGA preparation.

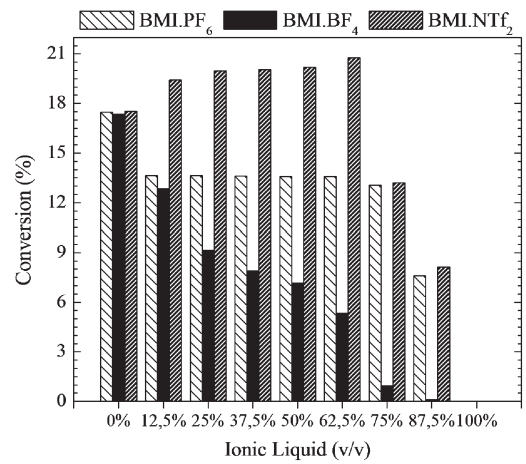

Fig. 6 Conversion of 6-APA (%) after 180 min of reaction. Amoxicillin syntheses at 25 °C and pH 6.5 with initial bulk concentrations: 50 mM PHPGME, 50 mM 6-APA, and 0.2 g of 260 IU g^{-1} of PGA preparation.

were performed in batch reactors, only to standardize the procedure.

An interesting aspect to be highlighted is the comparison of the selectivity obtained in the reactions conducted in BMI·PF_6 and in BMI·NTf_2 with the measurements of a_w for these systems. The analysis of the data in Table 1 shows that all values of a_w were higher for BMI·NTf_2. In addition, BMI·NTf_2 is more hydrophobic than BMI·PF_6 (see Table 2). This means that reactions performed with BMI·NTf_2 as a cosolvent had more water in the reaction medium available for the enzyme, which results in a higher rate of hydrolysis, *i.e.*, lower selectivity, as can be seen in Fig. 5.

On the other hand, BMI·BF_4, the more hydrophilic IL, shows the lowest values for the selectivity. The high amount of water miscible in this IL, in this case, did not hamper the hydrolyses as was observed for BMI·PF_6, showing once again that the phenomena involved in the enzyme–IL–substrates (including water) interactions are very complex and interconnected.[46,47] Thus, the selection of the optimal solvent cannot be based only on its physical–chemical properties: kinetic tests are essential for this assessment.

Conversion is another important variable in the evaluation of the performance of the enzymatic process. Fig. 6 presents a

comparison, in terms of 6-APA conversion (%), of all the experiments that were performed in this work. The graphic shows that the conversion decreases sharply in the reactions with BMI·BF_4 as a cosolvent in comparison to the standard reaction. The reactions in BMI·PF_6 also showed a decrease of conversion with the increasing of concentration of the IL, but much less pronounced. The assays with BMI·NTf_2 showed an increase of conversion until 62.5% (v/v) of IL, and for higher volumetric fractions there was a decrease in conversion.

It is important to mention that for pure ILs, 100% (v/v), there were no detectable reactions taking place. In Fig. 6, an "unexpected" plateau in the range 12.5–62.5% (v/v) of the more hydrophobic ILs, BMI·PF_6 and BMI·NTf_2, is noted. The comparison between the conversion profiles and the values of a_w in Table 1, however, indicates that a_w values do not change significantly in the range 12.5–62.5% (v/v), *i.e.*, the enzyme hydration in this region was sufficient to maintain the enzymatic activity almost unaltered for these two ILs in this region of composition.

PGA immobilized on Sepabeads®, the biocatalyst used in the present study, is a highly stable catalyst, able to withstand adverse reaction conditions.[54] These supports are robust and suitable for industrial purposes, with a very high surface density of epoxide groups, allowing an intense enzyme–support interaction.[55] Additionally, in this work the enzymatic activity of PGA was measured before and after each reaction, and no deactivation was ever observed. Even when no enzymatic activity was noticed during the kinetic essay, after a careful washing this activity was fully restored.

In order to ensure that the particles of PGA immobilized on Sepabeads® were not structurally affected by the presence of ILs in the reaction medium, image analyses were performed through the technique of scanning electron microscopy (SEM). For this purpose, the biocatalyst particles were equally washed and dried under vacuum for posterior fixation and coating with gold. Fig. 7 shows the photomicrographs of the catalyst before being used in the reactions (Fig. 7a and b) and after having been employed in the synthesis of amoxicillin with IL as a cosolvent (Fig. 7c and d). It was observed that the integrity of the particles was perfectly preserved.

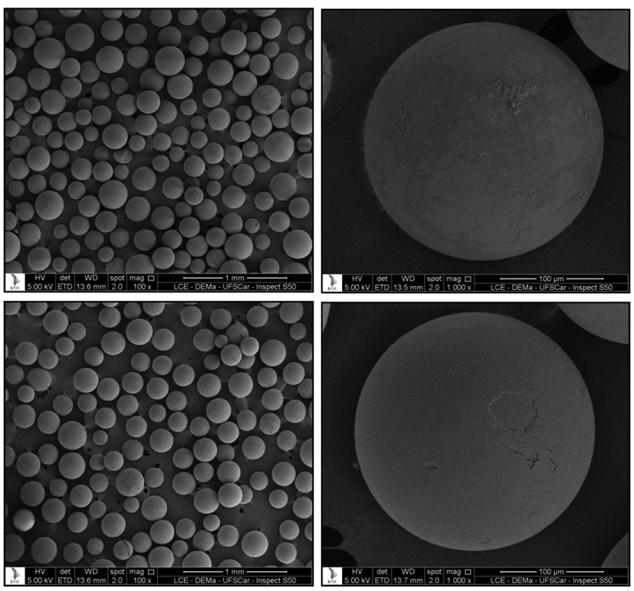

Fig. 7 Photomicrographs of the catalyst (PGA immobilized on Sepabeads®): (a) and (b) SEM of the particles before the reaction, (c) and (d) SEM after the reaction in the presence of an ionic liquid.

The viscosity of the ILs also interferes with the observed activity of the enzyme, mostly because immobilized enzymes were used, *i.e.*, the process is subjected to mass transfer resistances both in the external film and in the pores of the particles.[56] Table 3 shows that the rank of viscosities was BMI·PF_6 > BMI·BF_4 > BMI·NTf_2. This same rank of viscosities was reported in the literature, however, the viscosities for the dried ILs were higher than the values found in this study, showing that the presence of water has an important effect on the viscosity of the medium.[44] Hence, mass transfer resistance may have corroborated for a higher conversion in BMI·NTf_2 than in BMI·PF_6. On the other hand, for up to 62.5% (v/v) of BMI·NTf_2 the conversion was higher than in a totally aqueous medium. This is an example of the complexity of the phenomena involved in the IL–enzyme interaction.

Unlike the other two ILs, there is a systematic decline of 6-APA conversion with increasing volumetric fraction of BMI·BF_4 (see Fig. 6). In this case, rather than the viscosity of the BMI·BF_4 it is the miscibility in water and polarity that contribute in a decisive way to these results, because these latter directly affect a_w. Certain solvents have a tendency to remove water from the vicinity of the enzyme, leading to insufficient hydration and hence to a decrease in enzymatic activity.[57,58] The extent of this phenomenon is greater for hydrophilic solvents, and for this reason hydrophobic solvents usually have better performance in enzymatic reactions.[38]

The great majority of enzymatic processes using ILs reported in the literature involve reactions catalyzed by lipases.[59–65] Little information is found concerning the reactions catalyzed by PGA. Among them, a study reported that a_w should be close to 0.8 to guarantee the enzymatic activity of PGA in BMI·PF_6 and in BMI·BF_4.[39] Fig. 8 cross-references the information provided in Table 1 with Fig. 5 and 6. Fig. 8a shows that there is a moderate increase of enzymatic activity with BMI·NTf_2 for $0.95 < a_w < 1.00$. The other two ILs showed monotonic behavior, with the conversion decreasing systematically with a_w, and the three ILs show a sensible reduction of the enzymatic activity for $a_w < 0.8$. Fig. 8b, in turn, indicates that selectivity towards the synthesis goes through a maximum when a_w is reduced, for all the three systems.

In another study, the stability of PGA from *E. coli* in its native form in the presence of ILs at 40 °C was evaluated, and it was reported that the enzyme half-life in these ILs follows the order BMI·NTf_2 > BMI·PF_6 > BMI·BF_4.[66] These results were later corroborated.[67] Besides, it was not observed any activity for PGA in the presence of various concentrations of BMI·BF_4 (v/v) for the hydrolysis reaction of the penicillin G, while the results were promising in BMI·PF_6, with PGA showing greater stability than in water at 10 °C.[68] Interestingly, in a more recent study, PGA was unable to catalyze the synthesis of β-lactam antibiotics in the presence of *N*-methylimidazole, which is commonly used as a precursor to some ILs.[69]

The obtained results for selectivity and conversion indicate that BMI·BF_4 does not seem to be a suitable solvent for carrying

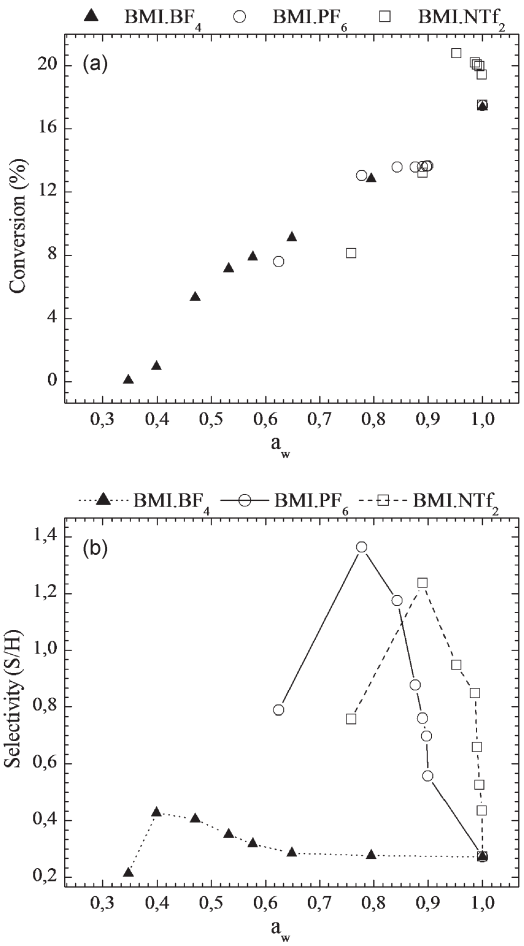

Fig. 8 Conversion of 6-APA (%) in (a) and selectivity (synthesis/hydrolysis, S/H ratio) in (b), both after 180 min of reaction for different water activities (a_w). Amoxicillin syntheses at 25 °C and pH 6.5 with initial bulk concentrations: 50 mM PHPGME, 50 mM 6-APA, and 0.2 g of 260 IU g^{-1} of PGA preparation. In the graphics: (▲) BMI·BF_4, (○) BMI·PF_6 and (□) BMI·NTf_2.

out reactions of synthesis of amoxicillin, since there is not a significant increase in selectivity, and a very sharp fall in conversion occurs with respect to the aqueous system. The most promising solvents were BMI·PF_6 and BMI·NTf_2, which presented a very significant increase in selectivity, reaching a value 400% higher at 75% (v/v) of BMI·PF_6 than in a totally aqueous medium, while BMI·NTf_2 showed an increase of more than 350% for this same volumetric ratio. However, BMI·NTf_2 showed better results in terms of conversion of 6-APA than BMI·PF_6 in the range 12.5–62.5% (v/v).

An increase of selectivity reflects the reduction of the undesirable hydrolytic reactions. Thus, a smaller amount of by-product will be formed, *i.e.*, the cost to recover PHPG and to perform subsequent synthesis of the PHPGME substrate will be lower. Similarly, a greater conversion of the β-lactam nucleus (6-APA) represents lower costs to recycle it. At this point, the comparison between the concentrations 62.5 and 75% (v/v) of BMI·NTf_2 shows a decrease of 36% in the conversion and an increase of 30% in the selectivity. The choice between 62.5 or 75% (v_{IL}/v_{water}) will depend on a cost analysis of this enzymatic process.

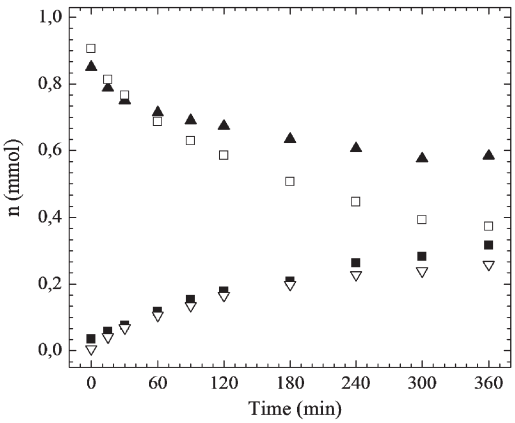

Fig. 9 Amoxicillin synthesis at 25 °C and pH 6.5 with initial bulk concentrations: 50 mM PHPGME, 50 mM 6-APA, and 0.2 g of 260 IU g^{-1} of PGA preparation in 71% (v/v) BMI·NTf_2. In the graphics: (□) PHPGME, (▲) 6-APA, (■) PHPG and (▽) amoxicillin.

The reactions carried out in BMI·PF_6 showed the highest values of selectivity, but this increase was at the expense of conversion. One probable explanation for the reduction of conversion in reactions with BMI·PF_6 could be the high viscosity of this solvent, which may have reduced the apparent rates of reaction. The viscosity of BMI·PF_6 could be reduced by increasing the reaction temperature or by adding some organic solvent that did not prejudice the stability of PGA, which is beyond the purpose of this study. In addition, under certain conditions, the contact of BMI·PF_6 with an aqueous phase may result in hydrolysis of the PF_6 anion generating HF.[44,70,71]

The IL that has shown the best characteristics for use in the enzymatic synthesis of amoxicillin was BMI·NTf_2, which is the least viscous, and does not tend to remove water from the enzyme vicinity, since it is the most hydrophobic of them, without, however, being responsible for any noticeable deformation of the enzyme structure, preserving the integrity of the active site. Therefore, a study in the range of 62.5 to 75% (v/v) of BMI·NTf_2 was conducted to determine the concentration limit that would not reduce the conversion of 6-APA and still preserve the selectivity of the reaction. Fig. 9 shows the evolution of the KCS of amoxicillin with 71% (v_{IL}/v_{water}) of BMI·NTf_2, which showed an increase of 36% in conversion compared to the standard reaction (phosphate buffer), 0% (v/v), and an increase of more than 300% in selectivity.

An important point that must be evaluated after the synthesis of a drug is the feasibility of isolation of the target molecule, amoxicillin, from the reaction mixture. Fig. 10 presents a typical chromatogram of a sample that was withdrawn during a reaction of enzymatic synthesis of amoxicillin using BMI·NTf_2 as a cosolvent.

After the enzymatic synthesis of amoxicillin in the presence of 71% (v_{IL}/v_{water}) of BMI·NTf_2, the result is a mixture of crystals of amoxicillin and PHPG, as can be observed in Fig. 11. A small amount of IL is still retained, probably adhered to the crystal surface (which was not washed yet at this point).

The purification of the antibiotic was successful, as is shown in Fig. 12.

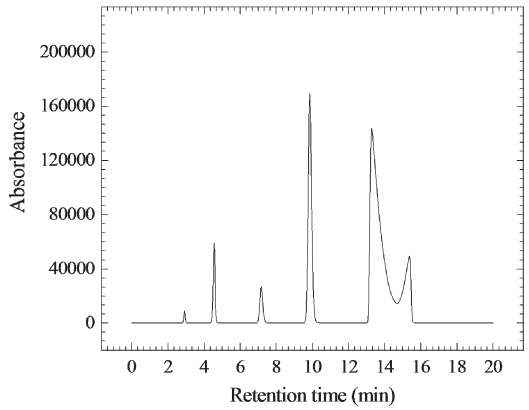

Fig. 10 Typical chromatogram of a sample withdrawn during the enzymatic synthesis of amoxicillin, including bands of PHPG (2.9 min), 6-APA (4.5 min), amoxicillin (7.1 min), PHPGME (9.8 min), and BMI·NTf_2 (13–16 min). The mobile phase was composed of 1.4 g of SDS, 0.6805 g of KH_2PO_4, 650 mL of ultrahigh-purity water, and 350 mL of acetonitrile, at pH 3.0. Analyses were carried out at 25 °C and detection at 225 nm as λ_{max}. Column: Phenomenex Gemini C18, 150 × 4.6 mm, 5 µm.

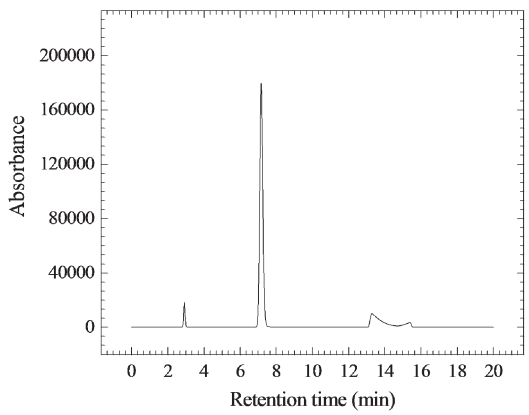

Fig. 11 Typical chromatogram of the crystals formed after the enzymatic synthesis of amoxicillin in the presence of 71% (v/v) of BMI·NTf_2, including bands of PHPG (2.9 min), amoxicillin (7.1 min), and BMI·NTf_2 (13–16 min). The mobile phase was composed of 1.4 g of SDS, 0.6805 g of KH_2PO_4, 650 mL of ultrahigh-purity water, and 350 mL of acetonitrile, at pH 3.0. Analyses were carried out at 25 °C and detection at 225 nm as λ_{max}. Column: Phenomenex Gemini C18, 150 × 4.6 mm, 5 µm.

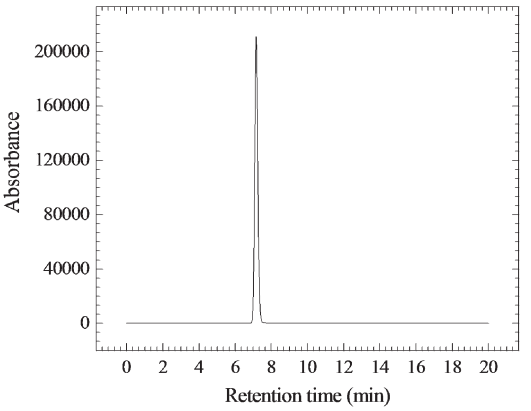

Fig. 12 Typical chromatogram obtained after the purification step (washing, solubilization and re-crystallization) of the crystals resulting from the enzymatic synthesis of amoxicillin in the presence of 71% (v/v) of BMI·NTf_2, with a single band of amoxicillin (7.1 min). The mobile phase was composed of 1.4 g of SDS, 0.6805 g of KH_2PO_4, 650 mL of ultrahigh-purity water, and 350 mL of acetonitrile, at pH 3.0. Analyses were carried out at 25 °C and detection at 225 nm as λ_{max}. Column: Phenomenex Gemini C18, 150 × 4.6 mm, 5 µm.

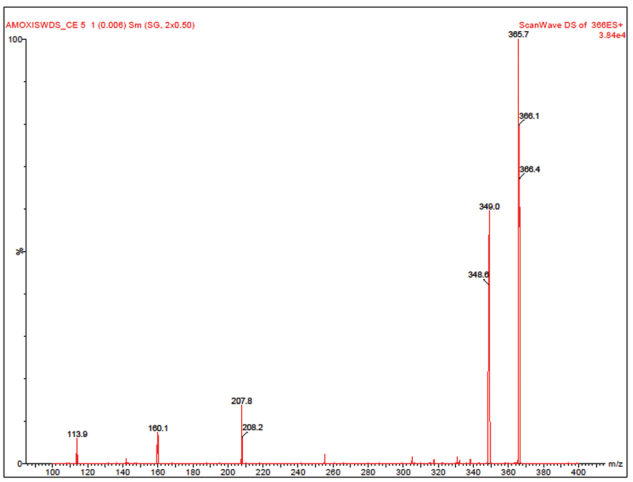

Fig. 13 Mass spectrum of amoxicillin synthesized in 71% (v/v) of BMI·NTf_2 and purified by precipitation in its isoelectric point, obtained using 9 V of cone voltage and 5 eV of collision energy. Spectral data: MS (ESI^+, triple quadrupole) *m/z*: 366 $[M + H]^+$, 349 $[M + H - NH_3]^+$, 208 $[M + H - C_6H_9NO_2S]^+$, 160 $[C_6H_9NO_2S + H]^+$, and 114 $[C_6H_9NO_2S - COOH]^+$.

A mass spectrum after the purification step confirms the identity of the synthesized amoxicillin (see Fig. 13). The precursor ion for amoxicillin was the protonated molecular ion $[M + H]^+$ at 366 *m/z*. One of the main cleavages for amoxicillin produces a product ion at 349 *m/z*, due to the loss of ammonia $[M + H - NH_3]^+$. A typical cleavage among β-lactam antibiotics is the opening of the β-lactam ring, generating a product ion at 160 *m/z* $[C_6H_9NO_2S + H]^+$, and a further loss of the carboxylic group from the thiazolidine ring moiety at 114 *m/z* $[C_6H_9NO_2S - COOH]^+$. The product ion at 208 *m/z* $[M + H - C_6H_9NO_2S]^+$ is also a result from the cleavage of the antibiotic nucleus.

A green industrial process using the IL would yield only a solution of NaCl as a purge stream. The antibiotic would crystallize in the fed-batch reactor, together with the side-product PHPG, and then the IL (containing small amounts of dissolved reactants) would be filtered and recycled for another run of the reactor, together with un-reacted 6-APA and PHPGME. The crystals of amoxicillin and PHPG are then washed, for removing any trace of IL. This stream is then evaporated (providing pure water as outflow) and the retained IL is recycled to the reactor, too. The clean amoxicillin crystals would then be dissolved using NaOH for increasing the pH, the solid PHPG would be collected and recycled to an esterification reactor, where it would react with methanol (or even better, ethanol), to provide the side chain ester substrate. The antibiotic would be finally

re-crystallized, using HCl to lower the pH, thus yielding a NaCl solution as the only residue. It should be noticed that no degradation of the IL was observed after several recycles in lab scale.

Conclusions

The application of ionic liquids (ILs) based on alkyl-substituted imidazolium for the enzymatic synthesis of amoxicillin was assessed. The results clearly showed that the nature of the anion exerts great influence on the interaction of the IL with water molecules.

The lowest values of water activity (a_w) were found for mixtures of water and BMI·BF_4, while the highest values were observed for BMI·NTf_2, followed by BMI·PF_6. This thermodynamic property directly influenced the selectivity of the reactions, which were significantly higher in the presence of the ILs, reaching increases up to 400%.

The viscosity of these solvents also affected the conversion of 6-APA. The overall effect was that conversions in the presence of BMI·PF_6 were lower than in a totally aqueous medium (with 100 mM phosphate buffer pH 6.5, the standard medium for comparison), but for BMI·NTf_2, they were higher than in the standard medium, approximately 36%.

The biocatalyst used, PGA immobilized on a Sepabeads® support, did not deactivate during the reaction and its structure remained unaffected, allowing enzyme reuse and thus reducing the cost of the synthetic process.

The isolation of the target molecule amoxicillin from the reaction mixture proved to be feasible using a simple purification procedure, which generates the harmless salt NaCl.

These results have opened up promising possibilities to follow in trying to make the enzymatic synthesis of semi-synthetic penicillins industrially feasible. However, further research is clearly required to explore the process implications of using ILs.

Experimental

Materials

The chemicals, amoxicillin, 6-aminopenicillanic acid (6-APA), *p*-hydroxyphenylglycine (PHPG), *p*-dimethylaminobenzaldehyde (PDAB), and *p*-hydroxyphenylglycine methyl ester hydrochloride (PHPGME), were from Sigma-Aldrich. Penicillin G acylase (PGA) from *Escherichia coli* was covalently immobilized on a Sepabeads® support (Mitsubishi Chemical Corporation) by the group of Prof. Guisán, from the Department of Enzymatic Biocatalysis, Institute of Catalysis, CSIC, Madrid, Spain, and kindly donated for this work.[55] All the ionic liquids (ILs), 1-butyl-3-methylimidazolium hexafluorophosphate (BMI·PF_6), 1-butyl-3-methylimidazolium tetrafluoroborate (BMI·BF_4), and 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl) imide (BMI·NTf_2), were prepared as previously described.[72] The other chemicals were of laboratory grade obtained from different commercial suppliers.

Enzymatic activity

Enzymatic activity of PGA immobilized on Sepabeads® was measured by the *p*-dimethylaminobenzaldehyde (PDAB) method, based on the formation of a Schiff base after the reaction of 6-APA with PDAB.[73] The 6-APA produced in the enzymatic hydrolysis of penicillin G (Pen G) reacts with PDAB generating a colored compound that absorbs light in the visible region and can be monitored in a spectrophotometer at a wavelength of 415 nm.

A jacketed batch reactor with mechanical stirring was used in all experiments. The temperature and pH of the reaction medium were kept constant during the enzymatic hydrolysis assays. The operational conditions were as follows: a solution of Pen G 5% (w/v) in 100 mM phosphate buffer pH 8.0, and 37 °C. One international unit of enzyme (IU) was defined as the amount of enzyme that hydrolyzes 1 μmol of Pen G per minute at 37 °C and pH 8.0.

Amoxicillin syntheses

Enzymatic reactions for the synthesis of amoxicillin were conducted under kinetic control. In these experiments, the reaction between PHPGME and 6-APA was catalyzed by PGA immobilized on Sepabeads®. This catalyst presented an apparent enzymatic load of 260 IU g^{-1} of catalyst. A jacketed batch reactor with constant mechanical stirring was used in all assays. The temperature of the reaction medium was kept constant during the enzymatic synthesis by a thermostatic bath, while the pH was continuously monitored by a pH meter. The experimental conditions were identical both for the reactions carried out in a totally aqueous medium and for reactions with each ionic liquid as a cosolvent.

Initial substrate concentrations were 50 mM for both PHPGME and 6-APA. In addition, the ratio of enzyme to substrate (E/S) was 52 IU $mmol^{-1}$ of substrate and the total reaction volume was 20 mL; thus, the amount of catalyst used in all the experiments was 0.2 g. Every synthesis was conducted at 25 °C. The standard for comparison was the reaction in a totally aqueous medium (100 mM phosphate buffer pH 6.5), without the presence of any ionic liquid. The ILs employed as cosolvents were BMI·NTf_2, BMI·PF_6, and BMI·BF_4 at (v_{IL}/v_{water}). Throughout the course of the reactions, aliquots were withdrawn and diluted in the mobile phase for HPLC analysis.

Amoxicillin purification

At the end of the enzymatic syntheses of amoxicillin in the presence of 71% (v_{IL}/v_{water}) of BMI·NTf_2, the resulting crystals of amoxicillin and PHPG were removed from the reaction medium by filtration, and washed with a saturated solution of amoxicillin, with the purpose of dragging any trace of IL that could be adhered. Thereafter, the crystals were solubilized at pH 8.5 and 25 °C. After complete solubilization, amoxicillin was re-crystallized in its isoelectric point, 4.9 at 4 °C. All the steps of the purification were monitored by HPLC analyses.

Amoxicillin characterization

Amoxicillin was characterized using a mass spectrometer (Waters Xevo TQ, Massachusetts, U.S.). Direct infusion of amoxicillin solubilized in methanol/water (1 : 1), at 2 μg mL^{-1},

was performed with a syringe pump. The ionization was carried out by an electrospray source in the positive mode (ESI^+). A triple quadrupole mass analyzer was used. The mass spectrum (MS) was obtained using 9 V of cone voltage and 5 eV of collision energy.

Analytical method

Concentrations were determined by high performance liquid chromatography (HPLC) in a Shimadzu LC-6AD with an SPD-10Avp UV-Vis detector and a Phenomenex Gemini C18 column (150 × 4.6 mm, particle size 5 μm). The chromatographic separation was conducted in isocratic elution with the mobile phase composed of 35% acetonitrile, 4.85 mM sodium dodecyl sulfate (SDS), 5 mM potassium phosphate monobasic anhydrous (KH_2PO_4), and correction to pH 3.0 using phosphoric acid (H_3PO_4).

The eluent was filtered through a 0.45 μm membrane and degassed with ultrasound before use. Analyses were at 25 °C and detection at 225 nm with an injection volume of 10 μL and a flow rate of 1 mL min^{-1}. All samples were dissolved in the mobile phase. All solvents were of HPLC grade. The components of the reaction medium were eluted in the following order: PHPG, 6-APA, amoxicillin, PHPGME, and IL.

Performance indices for amoxicillin kinetically-controlled synthesis

The performance of enzymatic synthesis of amoxicillin under the KCS route was assessed using indices of overall selectivity (eqn (1), S, synthesis/hydrolysis ratio) and 6-APA conversion (eqn (2), $X_{6\text{-APA}}$) after 180 min of reaction, as follows:

$$S = \frac{C_{\text{Amoxicillin}} - C^{\text{initial}}_{\text{Amoxicillin}}}{C_{\text{PHPG}} - C^{\text{initial}}_{\text{PHPG}}} \quad (1)$$

$$X_{6\text{-APA}} = \frac{C^{\text{initial}}_{6\text{-APA}} - C_{6\text{-APA}}}{C^{\text{initial}}_{6\text{-APA}}} \quad (2)$$

where C is the concentration of amoxicillin, or PHPG or 6-APA, at the beginning and after 180 min of reaction.

Water activity

Water activity (a_w) was measured using an AquaLab Series 4 TEV hygrometer from Decagon Devices. Measurements were carried out in a sealed dew point sensor at 25 °C, until obtaining constant readings. All samples were previously equilibrated for 24 h. The hygrometer was continuously calibrated with standard solutions (Decagon Devices).

Viscosity

Measurements of viscosities (η) of all the ionic liquids were performed in a Brookfield DV-III Programmable rheometer at 25 °C. The rheometer was previously calibrated with standard oil (Brookfield).

Scanning electron microscopy

Photomicrographs of the catalyst (PGA immobilized on Sepabeads®) were obtained through the technique of scanning electron microscopy (SEM) in a FEI Company Inspect S50 microscope equipped with an EBSD detector. All samples were fixed in aluminium stubs using carbon tape. The coating of the samples was done with gold in a BAL-TEC SCD 005 Sputter Coater system. Prepared samples were maintained in a desiccator until the time of analysis.

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