# IONIC LIQUIDS – BASED BIPHASIC SYSTEMS FOR ENZYME EXTRACTION: PRELIMINARY DATA FROM IONIC LIQUIDS' SCREENING

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**Abstract**

*Ionic liquids consisting of pairs of imidazolium or phosphonium cation and saccharinate or chloride anion were synthesized and examined for extraction efficiency towards the hydrolytic enzyme, α-amylase. The quantification of the enzyme partitioning in the equilibrated biphasic systems of ionic liquid and water was based on the α-amylase activity assay, performed where possible in both phases. The water transfer from the aqueous phase into the ionic liquid-rich phase was also followed. The effect of pH, which determines the electrostatic charge of the protein aminoacid chain, was studied. Backward extractions were also performed in order to evaluate the activity yield resulted from the enzyme transfer though the ionic liquid.*

**Key words:** *ionic liquids, biphasic system, extraction, enzyme*

## 1. INTRODUCTION

Using enzymes in the industrial and household catalysis becomes more and more advantageous over the conventional chemical pathway. As a matter of fact, enzymes are able to catalyze all kinds of chemical reactions, even those which are hard to perform chemically, like regio- or enantioselective hydrolysis or synthesis. The enzymes tend to catalyze the substrates to relatively pure products, thus minimizing waste accumulation. The enzyme-catalyzed conversions proceed under mild conditions at room temperatures. Wide variety of enzyme types allows selection between aqueous medium-acting or organic solvent-tolerant biocatalysts.

The field of enzymes' application is vast, including numerous technical uses, animal feed, food processing, cosmetics, pharmaceuticals, and scientific research and development. At the present, almost 4000 enzymes are known, but among them *ca.* 200 representatives of microbial origin are used commercially, and finally about 20 enzymes are produced on truly industrial scale (Li *et al.*, 2012). Currently, only 5% of the chemical products are manufactured in biotechnological route (Binod *et al.*, 2013). The "white biotechnology" is challenged to compete economically with the well-established chemical processes that have been optimized for years (Sarrouh *et al.*, 2012).

The enzyme production process can be divided into following phases (Leisola *et al.*, 2002): *1)* selection of a production strain; *2)* construction of an overproducing strain by genetic engineering; *3)* optimization of the culture medium and the production conditions; *4)* optimization of the recovery process and the purification; and *5)* formulation of a stable enzyme product. In the fermentation section, the large-volume industrial enzymes are produced in 50-500 $m^3$ fermentors. On the fourth stage, the enzyme recovery and purification require more sophisticated technological scheme. Equipment units for microfiltration, centrifugation and ultrafiltration are compulsory for the primary removal of the extracellular enzymes. If some further purification is needed, diafiltration, ion-exchange chromatography or gel-filtration techniques are applied. Usually, at the step of the enzyme purification, due to the numerous and laborious operations, losses of active enzyme occur (Vaňková *et al.*, 2005). In view of large scale enzyme purification, new methods need to be developed. In the scope of easily scaled-up techniques, liquid-liquid extraction has been always considered as an appropriate alternative.

The liquid-liquid extraction has already become conventional in the inorganic technology (ex. metal solvent extraction), as well for some organics (acids and antibiotics). Concerning the proteins/enzymes, however, the classical organic solvents satisfy neither the selectivity nor the preservation of the biomolecule functional properties. This has evoked the development of new generations of organic solvents suitable to meet the demands of the proteins produced in fermentation way.

Surfactant-based reversed micelle solvents, which represent nano-sized hydrophilic droplets dispersed in a hydrophobic solvent continuum, face this need (Tonova and Lazarova, 2008a). The most promising potential of the reversed micelle solvents is pointed at protein separation and/or purification from the aqueous medium (Tonova and Lazarova, 2005, 2008b; Lazarova and Tonova, 1999). Together with the low cost and the efficient large-scale separation, the reversed micelle liquid-liquid extraction has been demonstrated high extent of the intracellular enzyme purification, 6-7 fold just in two quick and simple steps: cell lysis with reversed micelle solvent followed by product-selective re-extraction (Giovenco *et al.*, 1987). Direct extraction from the original fermentation broth in the presence of cells has been also shown for extracellular enzymes (Krei *et al.*, 1995; Pessoa Jr. and Vitolo, 1997) where with the scaling-up the recovered active enzyme and the purification factor remain intact.

The novel ecological and safety rules impose the replacement of the pollutant hydrocarbons, used in the reversed micelle solvents, by environmentally benign “green solvents”. Ionic liquids (ILs) are compounds consisting entirely of ions, usually a charge-stabilized asymmetric organic cation and an inorganic or organic anion. They represent liquids at ambient temperature and display a wide range of unique properties, such as negligible vapor pressure (practically non-volatile), low chemical reactivity, finely tunable viscosity, polarity, and miscibility with other common solvents (Mallakpour and Dinari, 2012). Structurally, ILs can be regarded as a union of ionic surfactant with organic solvent thus giving both the electric and the hydrophobic forces for the transfer and separation of the electrostatically charged protein molecules. These green solvents succeed the reversed micelle solvents to be used in the liquid-liquid extraction of enzymes.

The ILs have already proved their advantages as solvents in the non-aqueous biocatalysis (Lozano *et al.*, 2015). The possible chelation between the enzymes and the solvent ions or the ions ability to modify the water structure, thus influencing the biocatalyst hydration microenvironment, have been reported to enhance the enzyme activity (Ventura *et al.*, 2012; Naushad *et al.*, 2012; Deive *et al.*, 2015), stability (Ulbert *et al.*, 2005; Kumar and Venkatesu, 2014) and enantioselectivity (Ulbert *et al.*, 2004).

Regarding the ionic liquids as media for protein/enzyme recovery and purification, two kinds of systems seem more explored: ionic liquid-based aqueous two-phase systems (Wu *et al.*, 2013; Yan *et al.*, 2014; Ng *et al.*, 2014) and ionic liquid-supported aqueous micellar two-phase systems (Vicente *et al.*, 2014).

In the former systems, the protein/enzyme migration towards the IL-rich phase is enforced in two manners: electrostatically, by a strong repulsion exerted by the salt-rich phase, which is usually alkaline enough above the protein isoelectric point (Wu *et al.*, 2013; Ng *et al.*, 2014) or as a result of a surface phenomenon, the formation of IL aggregates-protein complexes (Pei *et al.*, 2010; Zeng *et al.*, 2013).

The most used ILs in the aqueous two-phase systems– imidazolium halides, however, appear inappropriate to be applied to hydrophilic hydrolytic enzymes mainly due to the possible coordination of the strongly nucleophilic anion, which unfolds the enzyme active structure (Gorke *et al.*, 2010). Also, the imidazolium halides are hydrophilic ILs and similar to hydrophilic organic solvents they tend to strip the essential water molecules from the enzymes, thus inactivating them (Wahlström and Suurnäkki, 2015). Hydrophobic ILs express more tolerant behavior toward the hydrophilic hydrolytic enzymes (Dabirmanesh *et al.*, 2011; Karbalaei-Heidari *et al.*, 2013; Wahlström and Suurnäkki, 2015), but there is still a lack of studies dealing with the hydrophobic ILs as enzyme extracting media.

In the scope of the present study we synthesize some hydrophobic ILs based on the combinations of low-toxic ions, namely saccharinate anion and phosphonium cation, and screen them along with their imidazolium and chloride analogues in relation to their ability to extract the α-amylase enzyme in biphasic IL-based systems. As the paramount object in the whole extraction process is to preserve more amylolytic activity at each stage, backward extractions were also performed in order to evaluate the activity loss in the enzyme transfer through the IL. The effect of pH and the IL water content, as crucial factors for the hydrolytic activity, were studied.

## 2. EXPERIMENTAL SECTION

### *2.1. Materials*

Tributyltetradecylphosphonium chloride, $[P_{444,14}]Cl$ (>95%) was purchased from IoLiTec (Heilbronn, Germany). The saccharinate-based ILs were synthesized from their chloride-precursors, purified and characterized by us according to recently published protocols for the synthesis of saccharinate-based ILs (Bogdanov *et al.*, 2010; 2012). The abbreviations here used for the synthesized saccharinate-based ILs mean the following: $[P_{444,14}][Sac]$ is tributyltetradecylphosphonium saccharinate and $[C_8C_1im][Sac]$ is 1-methyl-3-octylimidazolium saccharinate.

The α-amylase enzyme (EC 3.2.1.1 from *Bacillus licheniformis*) was a product (Type XII-A) of Sigma. An inorganic salt, KBr (Fluka Chemie AG, p.a.), was added to some systems.

The α-amylase aqueous source was always a buffering solution of 0.15 mol $dm^{-3}$ of the buffering component (acetate or carbonate) and the pH-values set boarded the enzyme stability range at 4.54 and 10.47. The salts ($CH_3COONa \times 3H_2O$ and $Na_2CO_3$) and the acids (acetic acid and HCl) used for the buffers' preparation were puriss. p.a., and all other substances needed for the α-amylase activity assay ($I_2$, KI, starch) were of analytical grade and were used as received.

### *2.2. Equilibrium experiments*

The extractions were performed at 22 °C by vigorous shaking of 3.2047 mmoles dried IL and 2 $cm^3$ aqueous source of both certain initial α-amylase activity and pH. The contact time was set to five minutes since it was found that prolonged shaking (up to 3 h) gave similar equilibrium results. The mixture was allowed to separate overnight at the same temperature, the two phases formed were set aside and their volumes and pH values were measured. Samples from the residual aqueous phases, as well as $[C_8C_1im][Sac]$-rich phases were then withdrawn and subjected to analysis as described in part 2.3. Backward extractions were all performed at initial volume ratio of the IL-rich phase to the stripping solution of 1:1. The stripping composition represented an aqueous buffer of 0.15 mol $dm^{-3}$ $Na_2CO_3$/HCl at pH = 10.62.

A salting-out reagent was needed in order to form $[P_{4,4,4,14}]Cl$/water biphasic system, and KBr was added in quantity comparable to IL, 3 mmoles. KBr is a neutral inorganic salt which function in the CTAB-reversed micelle extraction of α-amylase was well studied (Tonova and Lazarova, 2005, 2008a, 2008b; Lazarova and Tonova, 1999). As far as KBr enhances the interfacial tension of the aqueous phase and thus decreases the emulsification, the system $[P_{4,4,4,14}]Sac$+KBr was also tested.

### *2.3. Analysis and calculations*

The measurements of the α-amylase volume-specific activity (U $cm^{-3}$) were based on the iodine-starch protocol as originally described by Pantchev *et al.* (1981), as well as in Tonova and Lazarova (2005) and Lazarova and Tonova (1999). The activity quantifications in the $[C_8C_1im]Sac$-rich phases required somewhat prolonged period for color reading in order to settle down the precipitate formed caused by iodine-IL complexation (Yan and Mu, 2014). It was established that the sample was ready to be read four hours after the enzyme reaction stopped versus a blank prepared in the same manner except for the starch participation.

The activity of the positively charged α-amylase is extremely sensitive to the pH-value, increasing from 5 to 100% in the pH-range ~4 ÷ 6. That is why, in order to overcome the differences in the activity deriving from the different pH of the source solution, all the samples were beforehand diluted in acetate buffer (pH = 6), 0.067 mol $dm^{-3}$ for all aqueous samples and 0.1 mol $dm^{-3}$ for the samples of the $[C_8C_1im]$Sac-rich phase. These analytical dilutions ranged from 1:3 to 1:59 and in all cases ensured optimal pH for the enzymatic starch conversion.

The percentages of the α-amylase transferred (*acc. to* the activity units, U) in the stages of the extraction/re-extraction process were evaluated with reference to the initial activity units of the given α-amylase aqueous source (buffer type and pH) according to Eq. (1):

$$(\alpha-\text{amylase activity})\,\% = \frac{[\text{U cm}^{-3}]^{*}_{\text{AQ/IL/STR}} \times \text{V}^{*}_{\text{AQ/IL/STR}}}{[\text{U cm}^{-3}]^{0}_{\text{AQ}} \times \text{V}^{0}_{\text{AQ}}} \times 100 \qquad (1)$$

where "$^{*}_{\text{AQ}}$", "$^{*}_{\text{IL}}$" and "$^{*}_{\text{STR}}$" refer to the measurements of the α-amylase activity into the residual aqueous, IL-rich and stripping phases, "$^{0}_{\text{AQ}}$" – in the initial aqueous source, and "V" is the volume of the respective phase.

In the forward extractions, similar to the α-amylase (activity) transfer, the water transfer (*W%*) from the aqueous source into the IL-rich phase at equilibrium was calculated according to Eq. (2):

$$\text{W}\% = \frac{[\text{H}_2\text{O}]^{*}_{\text{IL}} \times \text{V}^{*}_{\text{IL}}}{[\text{H}_2\text{O}]^{0}_{\text{AQ}} \times \text{V}^{0}_{\text{AQ}}} \times 100 \qquad (2)$$

where $[\text{H}_2\text{O}]^{*}_{\text{IL}}$ refers to the water concentration of the IL-rich phase at equilibrium which was determined by Karl Fischer titration on 870 KF Titrino plus (Metrohm, CH).

Based on the measurements of the water concentration of the IL-rich phases, the water content defined as molar ratio of the water to the IL ($W_0$ = mol $H_2O$ / mol IL) was calculated.

## 3. RESULTS AND DISCUSSION

### *3.1. Effect of pH and water content of the IL-rich phase on the α-amylase extraction*

Each protein molecule takes an overall positive charge if the pH–value of the medium is below the protein isoelectric point (pH < pI) and a negative net charge at pH > pI. The α-amylases reach isoelectric state (zero net charge) at the pH–range 5.0 ÷ 5.5. At the same time, the pH–area of the α-amylases' stability lies between 4 ÷ 11, beginning from 4.42 to 10.70 for the specific type of α-amylase under this study (Tonova, 2009). Obviously, the stability range of the positively charged enzyme is rather tiny. For the study below pI, the initial pH of the α-amylase aqueous source was adjusted to 4.54 in the case of using $[C_8C_1im][Sac]$. In the case of $[P_{444,14}][Sac]$ and $[P_{444,14}]Cl$, since both ILs gave additional acidity of the equilibrium aqueous phase, the initial pH of the α-amylase aqueous source was set to 5.88 in order to prevent the pH–drop below the stability limit. Thus, the pH of all the equilibrium phases resulted in similar values, pH* = 4.76 ±0.11, and conclusions about the extraction of the positively charged α-amylase could be drawn.

On Figure 1(a) it is seen that different ILs and systems display different capacity to entrain the α-amylase, the measured residual activity in the equilibrium aqueous phases varies between *ca.* 21 ÷ 81% in reference to the initial one. As already mentioned, it is well known from the reversed micelle extraction of α-amylase (Tonova, 2009; Tonova and Lazarova, 2005, 2008a,b) that the water content of the receiving phase is the crucial parameter determining the ability of the medium to accommodate

the biomolecule safely and without conformational changes. Here, the potential extraction of the α-amylase into the IL-rich phase is also dependent on the water transfer (the parameter *W%*). The extracting capacity of the studied systems follows the increase in *W%* and can be ordered as $[C_8C_1im][Sac] > [P_{444,14}][Sac]+KBr > [P_{444,14}]Cl+KBr \approx [P_{444,14}][Sac]$. As shown on Figure 1(b), the water content $W_0$ = [mol $H_2O$ / mol IL] of the [Sac]-based systems correlates with the transferred α-amylase activity in a similar manner as it was found for the reversed micelle extraction at acidic pH (Tonova and Lazarova, 2008a; Tonova, 2009).

The function of the inorganic KBr (added equally to the IL mol quantity) should be specially emphasized. The high KBr–concentration in the α-amylase aqueous solution helps to gain an extra-activity as the halogen anions stabilize the α-amylase structure (Worthigton Enzyme Manual, 1972). Further, the high content of KBr increases the system interfacial tension thus reducing substantially the emulsification that occurred in the system of only $[P_{444,14}][Sac]$/water. Third, KBr salts-out $[P_{444,14}]Cl$ since anyway a biphasic system can not be formed (Tonova *et al.*, 2015).

Regarding the extraction efficiency, the presence of KBr facilitates the process in $[P_{444,14}][Sac]+KBr$ system, probably due to the enhanced water solubilization and strengthened nucleophilicity of the IL-rich phase. Despite the equality in the water content of $[P_{444,14}][Sac]+KBr$ and $[P_{444,14}]Cl+KBr$, the latter system displays an essential deficiency in the extracting capacity. Further studies are needed to argue the reason.

The lowest residual α-amylase activity remained after the $[C_8C_1im][Sac]$-supported extraction. Recently, studying the mechanism of the lactic acid extraction in the same $[C_8C_1im][Sac]$ aqueous biphasic system, we assumed a secondary structure of the water saturated long side chain imidazolium saccharinates (Tonova *et al.*, 2014). Polar channels separated by non-polar domains were visualized, which channels are composed of polarized water molecules able to host (through H-bonding) the undissociated lactic acid. Here, the same model vision could be applied for the α-amylase extraction. Alike COOH-group of the lactic acid, the carboxylic groups in the protein aminoacids' chain are undissociated at pH < pI and can be embedded in the polar channels by H-bonding. The amino acids' COOH-groups become dissociated at pH > pI. The latter could be considered as a back-extraction condition and it was first validated by performing extractions at pH > pI.

The results obtained from the "alkaline" extractions of the α-amylase are represented on Figure 2. Only the imidazolium saccharinate shows a very slight extraction of the negatively charged enzyme which could probably be attributed to the great water uptake in this IL.

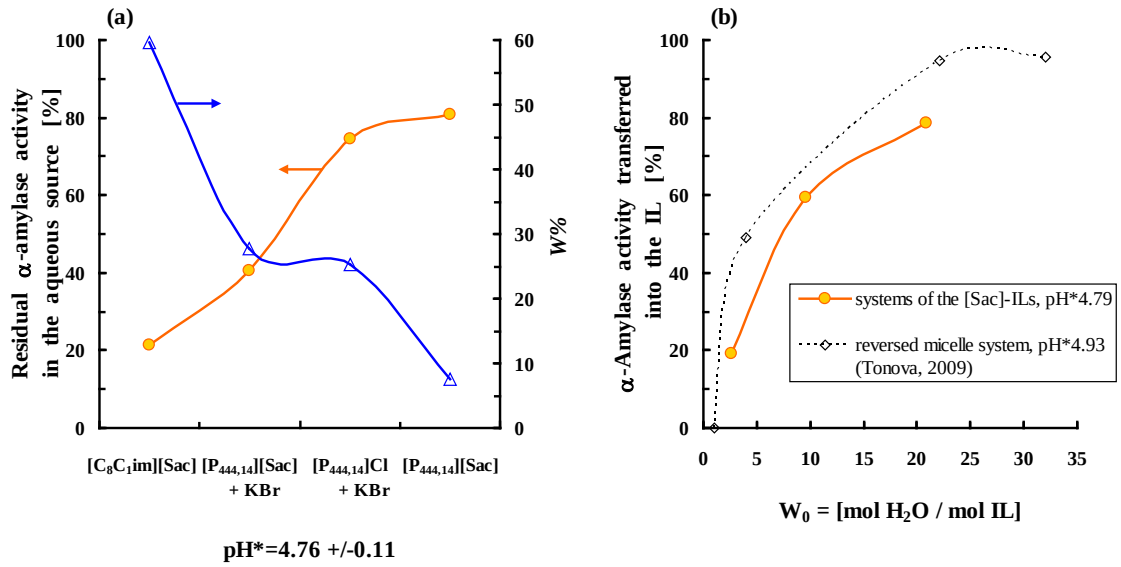



**Figure 1**. Extraction of α-amylase by different ILs at pH < pI.

(a) Residual activity in the aqueous source and water transfer (*W%*) into the IL-rich phase.

(b) Dependence of the α-amylase (activity) transfer on the water content ($W_0$).

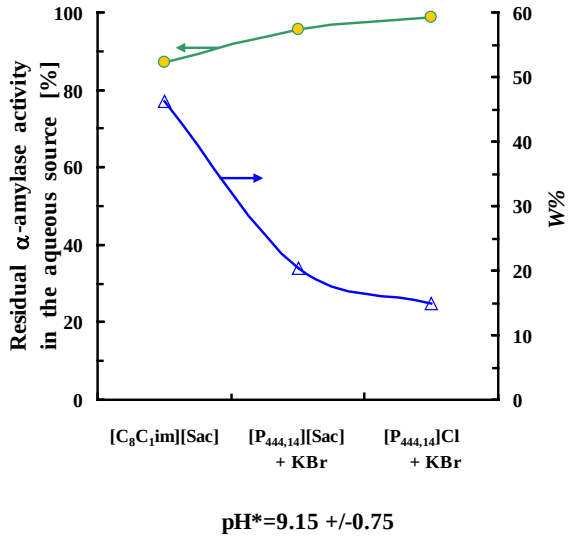



**Figure 2**. Extraction of α-amylase by different ILs at pH > pI.

Residual activity in the aqueous source and water transfer (*W%*) into the IL-rich phase.

Summarizing, the α-amylase extraction through the [Sac]-based ILs is governed by the enzyme electrostatic charge and the water uptake into the IL-rich phase. The best conditions for the α-amylase transfer comprise medium pH ≤ pI, *i.e.* undissociated carboxylic groups of the protein aminoacid chain, and water content of the IL-rich phase corresponding to the value of $W_0$ = [mol $H_2O$ / mol IL] ≥ 21.

### *3.2. Backward extraction of the α-amylase from the IL-rich phase and evaluation of the overall activity yield*

In order to estimate the mass balance of the active enzyme through the whole forward-backward transfer process, successive extractions and re-extractions were carried out under the conditions revealed on Figures 1 and 2. The data about the normalized α-amylase activity in both solutions, the source and the stripping one, are plotted on Figure 3, where also the sum of the activity restored in aqueous media "residual + stripped" is shown.

It can be seen that the sum fulfills the initial α-amylase activity only in the case of $[P_{444,14}][Sac]$ where unfortunately the lowest extraction was registered. In the other [Sac]-based systems, the bigger the amount of enzyme transferred the bigger the loss of activity recuperated.

Special attention should be paid on the system of $[C_8C_1im][Sac]$. From a practical viewpoint, it is the most promising IL among the others studied. $[C_8C_1im][Sac]$-supported system does not suffer from embarrassing emulsification occurring in the phosphonium ILs and does not require an inorganic salt addition either to reduce emulsification or to reach the appropriate extent of the water solubilization into the IL-rich phase. The highest degree of α-amylase extraction was attained by $[C_8C_1im][Sac]$, *ca.* 75% of the initial activity was retained and 53%– yielded in the stripping solution. These preliminary results represent an encouraging start for further process optimization.

Regarding the loss of active enzyme in the mass transfer it should be emphasized that the activity recovered in the stripping solution exactly matches the activity measured in the $[C_8C_1im][Sac]$-rich phase. In the extraction at pH < pI, 54% of the initial activity was analyzed in the IL-rich phase and 98% of it was further detected in the stripping aqueous solution. Similar dependence about the "free" α-amylase, *i.e.* enzyme available to catalyze the starch conversion (in the analysis of the IL-rich phase) and transferable between the phases source-IL-stripping, was also found by using a source of 3-fold lower enzyme concentration. There, 69% of the initial α-amylase was ascertained to be present in the IL-rich phase, and almost 100% of it was further registered in the stripping solution. Applying both initial enzyme loadings and pH < pI, the same amount of the initial activity, *ca.* 25%, remained unrevealed in either phase during the extraction/re-extraction process.

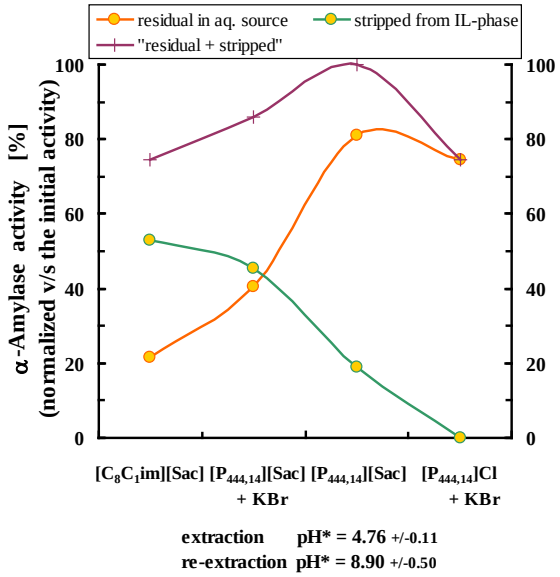



**Figure 3**. Successive extraction and re-extraction of α-amylase by different ILs.

Residual activity in the aqueous source and stripped activity from the IL-rich phase, both normalized in reference to the initial activity.

## 4. CONCLUSIONS

A set of imidazolium and phosphonium saccharinates or chloride were for the first time studied as extracting media for enzyme (α-amylase) recovery from its aqueous sources. Among the studied ILs, imidazolium saccharinate was shown to be the most promising IL based on *i)* the highest extraction degree achieved and *ii)* the handling-friendly system that means: short extraction/re-extraction times; fast phases' separation; clear phases obtained. Two terms were found to be obligatory for successful α-amylase extraction into $[C_8C_1im][Sac]$-rich phase: *i)* aqueous source pH < pI, *i.e.* undissociated carboxylic groups of the protein aminoacid chain and *ii)* water content of the IL-rich phase corresponding to the value of $W_0$ = [mol $H_2O$ / mol IL] ≥ 21. The latter condition correlates with the findings previously established for the CTAB-reversed micelle extraction of the same α-amylase type. In terms of both conditions completed, more than a half of the initial α-amylase activity was recovered in the second stripping solution, and using diluted enzyme start solution, the activity yield could be altered to 69%. These preliminary results represent an encouraging start for further process optimization. Attention might also be paid to the systems of $[P_{444,14}][Sac]$ because of their property of self-adjusting to the desired acidic pH which allows direct implementation to the α-amylase culture medium (pH ~ 6). For the practical application, however, the system emulsification drawback should be solved beforehand.

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