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Supported ionic liquids to purify microbial L-Asparaginase

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ABSTRACT

L-Asparaginase (ASNase) is a versatile enzyme that converts L-asparagine into ammonia and aspartic acid. This enzyme has applications in the food industry and health sector. However, high purity ASNase is required, resulting in high production costs. Therefore, in this work, two supported ionic liquids (SILs), specifically silica modified with dimethylbutylpropylammonium chloride ([Si][N_{3114}]Cl) or triethylpropylammonium chloride ([Si][N_{3222}]Cl), were investigated as alternative adsorption materials to purify ASNase. Different conditions were evaluated to improve enzyme purity, including total protein content in the cell extract, contact time, and SIL/cell extract ratio (w/v). Under optimal conditions using [Si][N_{3114}]Cl, a maximum ASNase purification of 6.1-fold is achieved in a single step, resulting from the preferential attachment of other proteins on [Si][N_{3114}]Cl SIL. According to the results, hydrophobic interactions rule the selective adsorption of protein impurities from the cell extract by the SIL, thereby increasing the ASNase purification levels. This approach offers a significant advantage, not requiring the desorption and elution of the target enzyme, while envisioning the application of SILs in a flow-through elution approach. The protonation state of protein surface was calculated by computational analysis, revealing that positively charged amino acids such as arginine and lysine block the effective binding of the enzyme to the SILs. Overall, if properly designed, SILs are promising alternative supports for the downstream processing of ASNase from cell extracts.

1. Introduction

Enzymes catalyse their substrates with high specificity, converting them into products with negligible side reactions. Therefore, a variety of enzymes are applied in distinct areas, namely in the food and pharmaceutical industries [1,2]. Among relevant enzymes, bacterial L-Asparaginase (ASNase), specifically ASNase type II (encoded by *ansB* and placed in the periplasmic, with a homotetrameric arrangement) is applied as a biopharmaceutical in the therapy of Acute Lymphoblastic Leukemia (ALL) and other related diseases [3–5], while fungal ASNase is applied as a food agent to reduce the formation of acrylamide [2,6]. ASNases, which are hydrolytic enzymes acting upon amide bonds, catalyse the hydrolytic reaction of L-asparagine to ammonia and L-aspartic acid. Similarly, some ASNases also convert L-glutamine to L-glutamate, however, with a lower effectiveness [7]. Currently, the commercialised recombinant ASNase is utilized as a therapeutic and it is exclusively produced by *Dickeya dadantii* and *Escherichia coli*, with a high therapeutic success rate in paediatric patients [4,8,9]. Contrarily, only fungal ASNase from *Aspergillus niger* and *A. oryzae* is approved for use in the food industry [2]. For both applications, a high purity level of ASNase is required, which leads to a high ASNase production cost [10]. ASNase purification methods comprise low resolution techniques such as precipitation [11,12], liquid-liquid systems [13,14], centrifugation and dialysis [15]. High-resolution methodologies such as chromatography (immobilized metal affinity chromatography – IMAC [16]; anion exchange [11] and gel filtration [12]) are also employed. Due to the high purity levels required for ASNase, while envisioning a reduction in its cost, it is mandatory to develop cost-effective extraction and purification processes.

Supported Ionic Liquids (SILs) have attracted the attention of the

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scientific community due to their improved efficiency in extract and/or purify several (bio)molecules, such as proteins, enzymes, and nucleic acids [17–22]. SILs are typically prepared by the covalent attachment of ionic liquids (ILs) in an appropriate support, such as polymeric materials or silica-based materials [23]. The combination of the unique IL properties, particularly their designer solvent nature, with the support's advantages (e.g., mechanical stability and high surface area) allows the synthesis of a modified material with tailored properties. In this field, Neves et al. [22] described the application of SILs, using macroporous polymeric supports modified with 1-methyl-3-propylimidazolium chloride IL, for purifying nucleic acids. The use of SIL allowed, in a unique step, to purify genomic DNA, ribosomal RNA and small RNAs from bacterial cell extracts with high selectivity and capacity of attachment. Later, Pereira et al. [21] investigated the utilization of silica functionalized with chloride-based ILs to purify RNAs produced by *E. coli*. On the other hand, Capela et al. [18] applied silica-based SILs to capture and purify immunoglobulin G (IgG) antibodies from cell cultures of rabbit and human sera, and Chinese Hamster Ovary (CHO) cells. Based on these reports and their improved results obtained, our research group proposed SILs based on quaternary ammonium ILs with several alkyl chain lengths as cation and chloride as anion as supports for ASNase extraction [24]. However, with the goal of developing a simple one-step purification process with high ASNase purity levels, in this study, silica modified with two ILs were investigated as alternative supports to extract and purify ASNase (Fig. 1). Contrarily to previous works, the goal of this work is to identify the optimal conditions suitable for the flow-through operation mode. This approach is advantageous since it does not require the additional step of desorption and elution of the target enzyme.

2. Materials and methods

2.1. Materials

Reagents for the SIL preparation: silica gel 60 (0.2–0.5 mm; Merck); 3-chloropropyltrimethoxysilane (≥98.0 % pure; Acros Organics); dimethylbutylamine (99.0 % pure; Aldrich); triethylamine (HPLC grade; Carlo Erba); ethanol (≥99.8 % pure; Fluka); hydrochloric acid (HCl) (37.0 % w/w; Honeywell); methanol (HPLC grade; Fisher Scientific) and toluene (99.8 % pure; Fisher Scientific). In the ASNase production and cell lysis, the following reagents were used: Luria-Bertani (LB) broth (for molecular biology; Sigma-Aldrich), erythromycin (for cell culture; Sigma Aldrich), D-(+)-Xylose (≥99 % pure; Merck), and phosphate-buffered saline (PBS) tablets (p.a; Sigma-Aldrich). To determine the activity of ASNase, L-asparagine (99.0 % pure; Acros Organics); tris (hydroxymethyl) aminomethane (Tris) (p.a.; Pronalab); trichloroacetic acid (99.5 % pure; Sigma), and Nessler's reagent (pure; Panreac) were used.

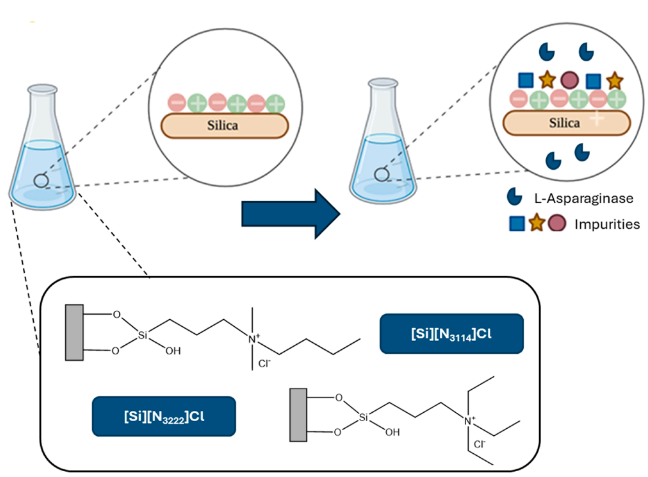

Fig. 1. Schematic strategy for ASNase extraction and purification process using silica-based SILs. Protein impurities from the cell extract are selectively adsorbed on SILs, while ASNase remains in solution. The chemical structures and abbreviations of the SILs used are also provided.

2.2. Production of microbial ASNase

ASNase was produced using the Gram-negative bacterium *Delftia lacustris*. Production was carried out in an orbital shaker. For the preparation of the inoculum, a stock culture maintained at − 80 °C in 20 v/v % glycerol aqueous solution, was activated in 15 mL of LB culture medium. Then, the *D. lacustris* cells were grown overnight in an orbital shaker at 37 °C and 200 rpm (Excella E24, New Brunswick Scientific, USA). The ASNase production initiated by transferring the inoculum to 250 mL of the LB broth (in 1 L Erlenmeyer). The initial optical density ($OD_{600\ nm}$) was adjusted to 0.1. The bioprocess was conducted in an orbital shaker at 200 rpm and 30°C. After 26 h, cells were separated by centrifugation (1605 *g*, 4 °C, 20 min; Neya 16 R, Remi, India) and resuspended in 10 mL PBS (pH = 7.4). To perform the cell lysis, the recovered cells were maintained in an ice bath and sonicated in an ultrasound equipment under a power of 60 % in 40 sets of 5 s pulse with intervals of 10 s (Branson 450 Digital Sonifier, Branson Ultrasonics Corporation, USA). Then, the cell extract of *D. lacustris* was recuperated after centrifugation at 4 °C and 1817 *g* for 10 min.

2.3. Purification of microbial ASNase using SILs

Silica surface was modified with two ILs containing cations with quaternary ammonium and chloride anion [19]. The resulting SILs present the following functional groups: N, N-dimethylbutylpropylammonium chloride ([Si][N_{3114}]Cl) and triethylpropylammonium chloride ([Si][N_{3222}]Cl), as showed in Fig. 1. These SILs were selected according to the results reported by Nunes et al. [19] dealing with the immobilization of pure and commercial *E. coli* ASNase.

To purify ASNase, cell extracts were mixed with [Si][N_{3114}]Cl or [Si][N_{3222}]Cl in a Multi Bio RS-24 rotator (PRS-26 platform) at room temperature (RT). Firstly, four cell extract dilutions were prepared with final total protein concentrations of 10, 7.5, 5, and 3 mg/mL; 1.0 mL of each cell extract dilution was mixed with 10 mg of each SIL and stirred for 60 min at RT. After that, to isolate the supernatant from the SIL, the samples were centrifuged during 15 min at 13800 *g*. Secondly, the effect of contact time was evaluated. 10 mg of SIL were mixed with 1 mL of cell extract (3 mg/mL of total protein) and kept in contact for different times (90, 60, 30, 15, 10 and 5 min). The samples were mixed at RT for 60 min and then centrifuged at 13800 *g* for 15 min. Finally, different amounts of each SIL (100, 50, 30, 20, 10, and 5 mg) were mixed with 1 mL of cell extract (total protein concentration of 3 mg/mL) to study the effect of the SIL/cell extract ratio in the ASNase purification. The samples were mixed at RT for 60 min and then centrifuged for 15 min at 13800 *g*. All purification tests were carried out in triplicate and the total protein content and the activity of ASNase were determined in all supernatants.

2.4. Quantification of the total protein

To quantify the total amount of proteins, the absorbance of cell extracts was measured using a microplate reader (Synergy HT, BioTek, USA) at 280 nm. Based on a calibration curve previously established, using bovine serum albumin (BSA) as standard, the total protein concentration was calculated.

2.5. Quantification of the ASNase activity

The activity of the enzyme in the samples (supernatants or cell extracts after contact with SIL and centrifugation) was quantified using the protocol previous reported [25]. Briefly, 0.5 mL of sample (cell extract or supernatant) was added to 0.5 mL of Tris-HCl 0.05 M pH 8.6 and 50 μL of L-asparagine (189 mM) and incubated at 37 °C for 30 min. Then, 250 μL of trichloroacetic acid 1.5 M was added to stop the enzymatic reaction and the mixture was centrifugated at 13800 *g* for 10 min. After centrifugation, 0.5 mL of sample, 0.5 mL of distilled water, and 0.25 mL of Nessler's reagent were agitated and then incubated during 30 min at RT. The absorbance was read at 436 nm. Ammonium sulphate was used to prepare the calibration curve. The activity of ASNase (A_A) in μmol/mL/min (U/mL) was determined according to Eq. (1):

$$A_A = \frac{[NH_4^+] \times V_R \times V_{Nessler}}{V_S \times R_{Time} \times V_{Ext}} \quad (1)$$

where V_R is the reaction volume (1.05 mL); $[NH_4^+]$ is the concentration (μmol/mL) of ammonium, V_S is used volume of the trichloroacetic acid solution (0.5 mL) to stop the reaction; $V_{Nessler}$ is the Nessler's solution volume (1.25 mL), V_{Ext} is the cell extract volume (0.5 mL) and R_{Time} is the time of reaction. One unit of enzyme activity (U) is defined as the amount of ASNase to produce 1 μmol of ammonium per minute at 37 °C and pH 8.6.

2.6. Quantification of the specific enzyme activity and purification factor

The specific activity of ASNase ($ASNase_{SA}$), U/mg, was determined using Eq. (2):

$$ASNase_{SA} = \frac{ASNase_{activity}}{[Total\ protein]} \quad (2)$$

where $ASNase_{activity}$ is the activity of the enzyme (U/mL) determined by Eq. (1), and [Total protein] is the concentration (mg/mL) of the total protein.

The determination of the enzyme purification factor (PF) was calculated according to Eq. (3),

$$P_F = \frac{ASNase_{AP}}{ASNase_{BP}} \quad (3)$$

where $ASNase_{AP}$ is the specific ASNase activity after purification (U/mg), and $ASNase_{BP}$ is the specific ASNase activity before purification (U/mg).

2.7. Computational analysis

The protonation state of ASNase amino acid residues was predicted using ProteinPrepare (PlayMolecule web server - playmolecule.org) [26]. The 3D structure of ASNase monomer was downloaded from (AlphaFold (https://alphafold.ebi.ac.uk/) UniProt: A0A7T2YVY2) and submitted in the ProteinPrepare. The pK_a of ASNase amino acid residues were calculated at pH 7.4. Then, the protonated ASNase structure and protonation results were downloaded and analysed. The ASNase electrostatic surface was obtained using automatically configured sequential focusing multigrid calculation on an Adaptive Poisson-Boltzmann Solver (APBS). The results visualization (ASNase surface) was performed using PyMOL Molecular Graphics System, Version 2.5.2 Schrödinger, LLC.

3. Results and discussion

ASNase purification from a microbial cell extract was studied using two SILs, namely [Si][N_{3114}]Cl or [Si][N_{3222}]Cl. These SILs were synthetized and characterized to assure the synthesis success, being in accordance with previous results [19,24].

To develop a one-step purification process that could be applied in flow-through mode, aiming to specifically adsorbing the protein impurities while leaving ASNase in solution, different parameters were evaluated. These include the concentration of total protein present in the cell extract, contact time between the cell extract and the SIL, and the material ratio (w/v) (SIL/cell extract). Initially, four total protein cell extract concentrations - 10, 7.5, 5, and 3 mg/mL - were investigated for ASNase purification using 10 mg of each SIL during 60 min. The obtained results are shown in Fig. 2 (detailed data is given in Table S1 in the Supplementary Material (SM)). For both SILs, as the concentration of total protein in the cell extract decreases, the ASNase activity also decreases (0.108–0.046 U/mg for [Si][N_{3114}]Cl; 0.122–0.042 U/mg for [Si][N_{3222}]Cl). Considering our hypothesis of avoiding the specific adsorption of ASNase, this trend is expected since at lower total protein concentrations in the cell extract, there is also a lower concentration of ASNase, and thus, a lower ASNase activity. On the other hand, for [Si][N_{3114}]Cl (Fig. 2 A), there is an increase in the ASNase purity with the decrease in total protein concentration of the cell extract. This suggests that at higher concentrations, this material may have reached its maximum adsorption capacity for protein impurities, leaving other proteins in the supernatant. Using [Si][N_{3114}]Cl, a maximum purification of 4.7-fold was obtained with a cell extract of 3 mg/mL, resultant from a selective and increased adsorption of cell extract protein impurities over ASNase. The sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of the initial cell extract and cell extracts with different total protein concentrations, following purification using [Si][N_{3114}]Cl, are shown in Fig. 3. The profile of the purified cell extract (3 mg/mL) is in accordance with the previous results (Fig. 2) as it shows the ASNase band (around 34 kDa in line with commercial ASNase and the initial cell extract profiles) and a reduction in protein impurities (present in the initial cell extract profile). A similar pattern was obtained for the ASNase purification using [Si][N_{3222}]Cl, although lower purification factors were attained. With this SIL, a maximum purification factor of 1.3 was achieved, indicating the lack of selectivity of this material in adsorbing protein impurities over ASNase. The low purification factor obtained using [Si][N_{3222}]Cl is in accordance with the initial screening of SILs for the purification of ASNase from *Aliivibrio fischeri* [24]. Comparing both materials and specifically the IL ligand chemical structure (cf. Fig. 1), there are difference in the length of the alkyl chains of the cation and the cation symmetry, with [Si][N_{3222}]Cl exhibiting a more symmetric cation.

To elucidate the mechanism of ASNase lack of affinity to the SILs, thus leaving it in solution, the protonation state of the protein surface was calculated for pH 7.4 (pH of the cell extract in which the experiments were carried out). Each monomer of ASNase from *D. lacustris* is composed of 329 amino acids, of which 71 of them are exposed on the protein surface and the others (258 amino acids) are responsible for protein folding and stability. The electrostatic surface with charged amino acids at pH 7.4 in ASNase surface is displayed in Fig. 4. In particular, aspartic acid and glutamic acid are negatively charged, whereas arginine and lysine are positively charged. Therefore, these last two amino acids seem to be responsible to the lack of affinity of ASNase to the SILs, leading to electrostatic repulsion between them and the SIL cations. The positively charged SILs (point of zero charge between 9.0 and 9.3 [19]) may interact however through attractive electrostatic interactions with γ-carboxyl groups and β-carboxyl groups of glutamic and aspartic acids (that are negatively charged). Nevertheless, these complexes show high instability, derived from the simultaneous presence of the positively charged guanidinyl group (arginine) and ε-amino group (lysine), possibly promoting further repulsive interactions with SILs. The hydrophobic and hydrophilic residues on ASNase do not appear to significantly contribute to its adsorption onto the SILs. The ASNase surface displays only two non-charged amino acid residues (cysteine and tyrosine), being not to establish a stable enzyme-SIL complex by hydrophobic interactions. Therefore, there is no significant difference between the two SILs in what regards their adsorption capacity for ASNase. The different purification factors observed are directly correlated with the adsorption capacity of the SILs for the protein impurities present in the cell extract. This hypothesis is confirmed by the similar enzyme activity observed for both SILs at each protein concentration (Fig. 2). Therefore, the impurities of the cell extract (lane 3, Fig. 3), which should

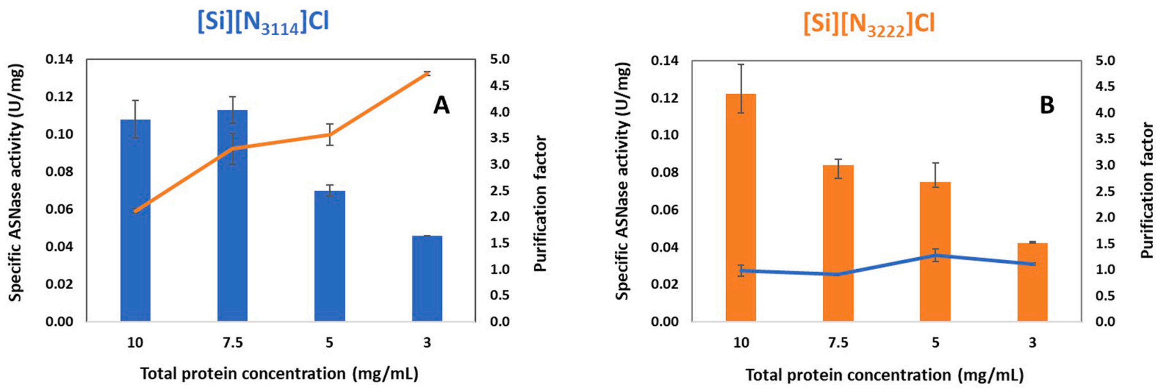

Fig. 2. Purification of ASNase produced by *D. lacustris* using [Si][N_{3114}]Cl (A) or [Si][N_{3222}]Cl (B) at different total protein concentrations from the cell extract. ASNase specific activity (bars) and purification factor (lines).

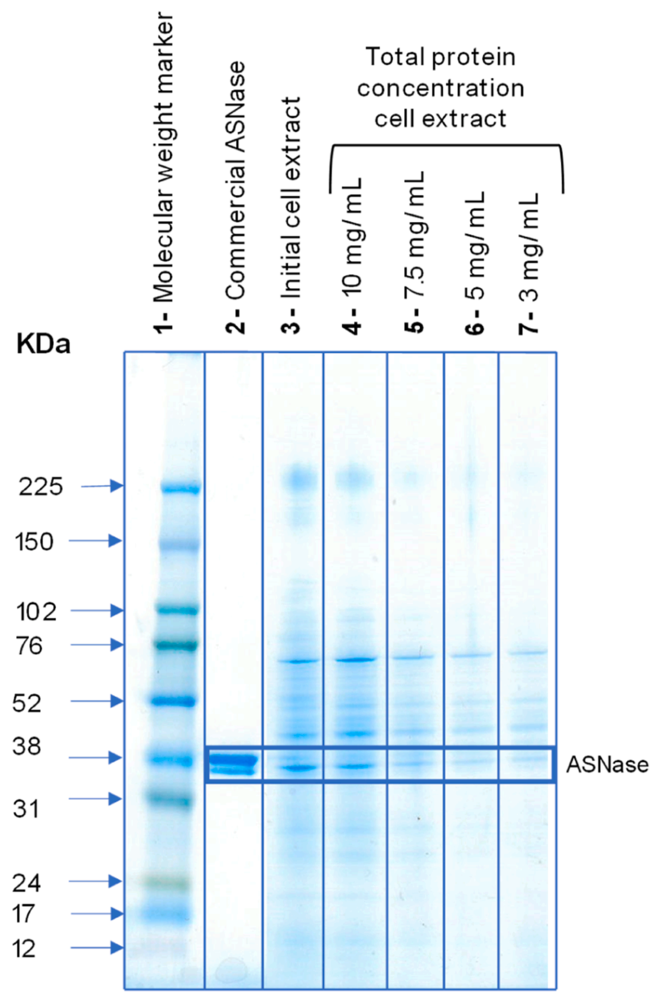

Fig. 3. SDS-PAGE of molecular weight marker (lane 1), commercial ASNase (lane 2), initial cell extract (3), cell extract dilutions prepared to different total protein concentrations (10, 7.5, 5, and 3 mg/mL) after purification using 10 mg of [Si][N_{3114}]Cl for 60 min at RT (lanes 4, 5, 6, and 7).

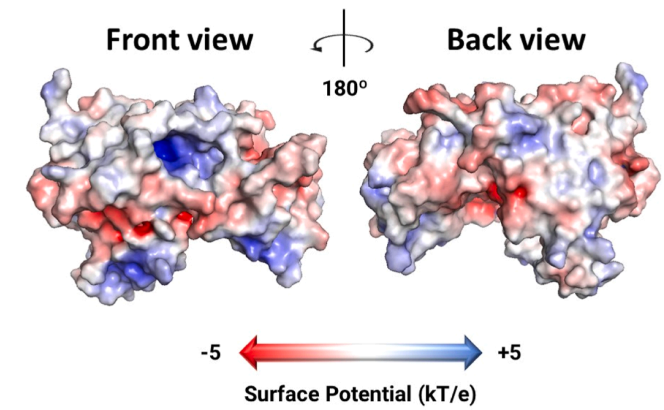

Fig. 4. 3D electrostatic charge surface of ASNase at pH 7.4: minimum (-5 kT/e, red) and maximum (5 kT/e, blue) surface potential.

be more hydrophobic and negatively charged proteins, are preferentially adsorbed onto the SILs. However, since the [Si][N_{3114}]Cl results in a higher purification factor, it is possible to conclude that hydrophobic interactions between the protein impurities and SILs are the main driving force, being in agreement with other studies reporting on the capture and immobilization of antibodies and enzymes using SILs [18, 19].

Based on the results shown in Fig. 2, particularly considering the best purification factor of 4.7 obtained with [Si][N_{3114}]Cl that was achieved with a total concentration of protein of 3 mg/mL, this concentration was chosen for the following tests on the evaluation of the contact time between each SIL and the enzyme. Overall, six contact times (90, 60, 30, 15, 10, and 5 min) were investigated. The results are given in Fig. 5 (complete data is provided in Table S2 in the SM). For both SILs, there is a slight increase in the specific ASNase activity until 60 min of contact between the SIL and the cell extract (0.039–0.046 U/mg for [Si][N_{3114}] Cl and 0.038–0.042 U/mg for [Si][N_{3222}]Cl). After 90 min, the specific ASNase activity decreases to 0.030 and 0.033 U/mg using [Si][N_{3114}]Cl and [Si][N_{3222}]Cl, respectively. A similar trend exhibiting a maximum along time was observed for the purification fold, and for both SILs, the ASNase purification slightly increases until the 60 min of contact (reaching a maximum of 4.7 for [Si][N_{3114}]Cl and 1.1 for [Si][N_{3222}]Cl) and then decreasing after 90 min. The impurities adsorption by the materials should increase with time, but there is a loss of ASNase activity along time, leading to lower purification factors after 90 min. As described by Bento et al. [8], this specific ASNase exhibits decreasing thermal stability over time, which can explain the obtained results. Additionally, the same trend was observed by Nunes et al. [19] for the ASNase immobilization from *E. coli*. Comparing the results of Figs. 2 and 5, the study of the contact time with ASNase did not conduct to better results, being the 60 min used in the previous assays the optimum period of time to allow the maximum adsorption of protein impurities. Therefore, 60 min of contact was applied in the following experiments.

The SIL/cell extract ratio (w/v) effect on enzyme purification was finally investigated. This set of assays was conducted using six amounts

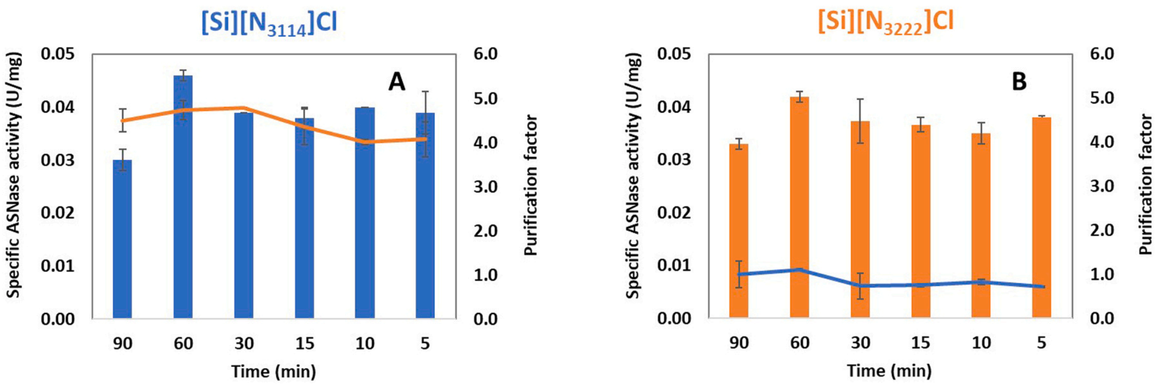

Fig. 5. ASNase purification using [Si][N_{3114}]Cl (A) or [Si][N_{3222}]Cl (B) at different contact times. Specific activity of ASNase (bars) and purification factor (lines).

of both SILs (100, 50, 30, 20, 10, and 3 mg) and 1 mL of cell extract (3 mg/mL), while kept the contact for 60 min. It should be remarked that the previous assays were performed with 10 mg of SIL. The gathered results are shown in Fig. 6 (detailed data is provided in Table S3 of the SM). Remarkably, for both SILs there is an opposite trend for the enzymatic activity and a similar trend for the purification factor. For [Si][N_{3114}]Cl, higher amounts of material lead to higher ASNase activity, but when [Si][N_{3222}]Cl was used, a decrease in the ASNase activity is observed with the increase in the SIL content. On the other hand, when increasing the amount of SIL used, there is an increase in the ASNase purification factor, for both SILs. These results indicate that besides the impurities adsorption, when more material is available, [Si][N_{3222}]Cl is also adsorbing ASNase, explaining the lower ASNase purification values obtained with this material. Regarding the performance of both SILs in the ASNase purification, an increase in the SIL content leads to a higher capacity to adsorb protein impurities. For 100 mg of both materials, a maximum purification factor of 6.1 was achieved with [Si][N_{3114}]Cl, while for [Si][N_{3222}]Cl a maximum purification factor of 3.7 was attained. As with the other parameters studied, the results on the ASNase purification are better with [Si][N_{3114}]Cl, meaning that this SIL has a higher capacity to adsorb protein impurities over ASNase. SDS-PAGE profile of cell extract after purification with higher amounts of [Si][N_{3114}]Cl is in agreement with the previous results since the majority of protein impurities (present in the initial cell extract profile) were removed, while keeping ASNase in the supernatant (as observed in commercial ASNase and initial cell extract profiles) (Fig. 7). Comparing the results obtained in this work with the literature, it is evident that the strategy applied here is highly promising with outstanding results obtained in a single step. Nunes et al. [24] reported a purification factor of 3.4 in one-step using SILs, and to increase the ASNase purification a semi-continuous process was applied, ultimately enabling a purification factor of 5.2. Furthermore, it should be remarked that in the current work we are adsorbing more amount of protein impurities and eliminating the need of an extra desorption step of the target enzyme.

Ameen et al. [27] partially purified ASNase from the crude enzyme extract using a conventional precipitation purification process with ammonium sulphate (85 %), achieving a purification fold of 3. Chityala et al. [28] reported a purification fold of 3.8 using an Ni–NTA affinity chromatography (specific type of IMAC) to purify a His-tagged extracellular recombinant ASNase type II. Although a highly specific/-selective type of chromatography was applied, a lower purification fold was obtained when compared with the results using [Si][N_{3114}]Cl. Furthermore, El-Naggar et al. [11] applied a two-step procedure to purify ASNase enzyme from alkaliphilic *Streptomyces fradiae* NEAE-82: ammonium sulphate precipitation combined with ion exchange chromatography (DEAE Sepharose CL-6B), and still a lower purification factor was attained (3.3). These ASNase purification results are summarized in Table 1. It is important to highlight that this approach eliminates the need of dialysis of IL since the IL was covalently attached to silica, and previous works have reported the non-leaching of IL from SILs [29,30]. Moreover, [Si][N_{3114}]Cl was found to be non-cytotoxic to HepG2 cells [30]. Previous work has demonstrated the use of silica-based-IL in AKTA Avant® chromatography system for RNA purification [31]. Additionally, the viscosity of the sample (i.e., cell extract obtained after cell lysis by sonication in PBS buffer and then centrifugation) is the same as the PBS buffer, a common buffer used in chromatography systems for proteins [32]. Based on these reports and the results obtained in this work, [Si][N_{3114}]Cl is a promising material to consider for ASNase downstream processing. Nevertheless, the removal of the remaining protein impurities from the cell extract using other

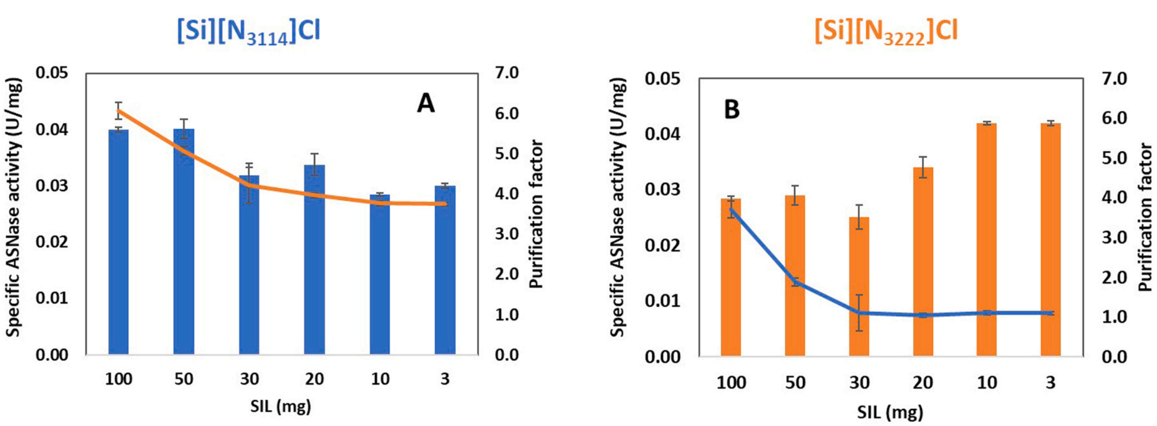

Fig. 6. Purification of *A. fischeri* ASNase produced by *D. lacustris* using different amounts of [Si][N_{3114}]Cl (A) or [Si][N_{3222}]Cl (B). Specific activity of ASNase (bars) and purification factor (lines) from the supernatants after contact with SILs.

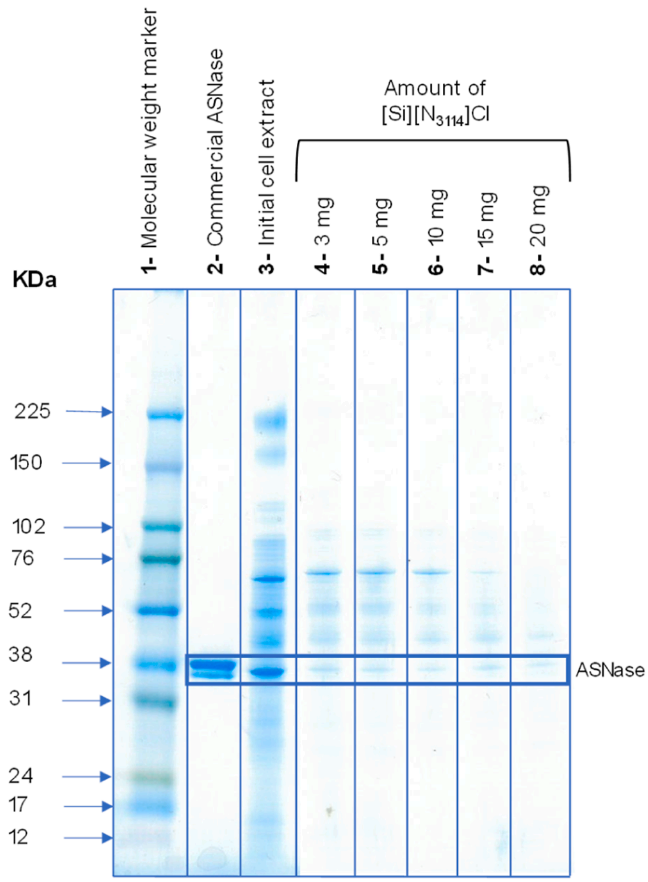

Fig. 7. SDS-PAGE of molecular weight marker (lane 1), commercial ASNase (lane 2), initial cell extract (lane 3), cell extracts after purification with different amounts of [Si][N_{3114}]Cl (3, 5, 10, 15, and 20 mg) (lanes 4, 5, 6, 7, and 8).

Table 1
ASNase purification processes and purification factors.

ASNase purification process	Purification factor	Ref.
SILs	6.1	This work
SILs	5.2	[24]
Ammonium sulphate precipitation	3	[27]
Ni-NTA affinity chromatography	3.8	[28]
Ammonium sulphate precipitation + Ion exchange chromatography	3.3	[11]

techniques, e.g., amicon centrifugal filters may be necessary to meet the high purity pharmaceutical levels [33].

4. Conclusion

Developing cost-effective platforms for ASNase purification is crucial to reduce production costs of this enzyme, which is currently applied in both the food industry and the health sector. In this work, the potential of SILs to purify a bacterial ASNase produced by *D. lacustris* was addressed. Silica-based SILs, namely [Si][N_{3114}]Cl and [Si][N_{3222}]Cl, were investigated as an alternative purification approach for ASNase, particularly focusing on the adsorption of protein impurities from the cell extract while envisioning their potential use in the flow-through mode. Under the best optimized conditions (cell extract concentration of 3 mg/mL, 100 mg of SIL and contact time of 60 min), [Si][N_{3222}]Cl increased the ASNase purity by at least 3.7 times compared to the initial enzymatic extract. Notably, using [Si][N_{3114}]Cl at the best conditions, a maximum ASNase purification of 6.1 was accomplished in a single step. This high purification factor results from the selective adsorption of protein impurities present in the cell extract on the [Si][N_{3114}]Cl, leaving ASNase in the supernatant. Computation analysis identified repulsive electrostatic interactions between the target enzyme and the SILs, avoiding thus the enzyme adsorption. On the contrary, hydrophobic interactions seem to rule the adsorption of the protein impurities. These findings gathered the potential of SILs as novel and alternative materials for the downstream processing of ASNase.

CRediT authorship contribution statement

Heitor B. S. Bento: Validation, Methodology, Investigation, Conceptualization. **Matheus M. Pereira:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **João C. F. Nunes:** Methodology, Investigation, Data curation, Writing – review & editing. **Mafalda R. Almeida:** Writing – original draft, Methodology, Investigation, Data curation. **Ana Paula Mora Tavares:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Márcia C. Neves:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Mara G. Freire:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Danielle B. Pedrolli:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization. **Valéria C. Santos-Ebinuma:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bej.2024.109445.

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