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Separation of polysaccharide and protein by ionic liquid-based extraction techniques

Evelyn C. Antunes[a,c], Felipe Oliveira[a], Hardy Temmink[a,b,†], Boelo Schuur[c,*]

[a] Wetsus – European Centre of Excellence for Sustainable Water Technology, Oostergoweg 9, 8911MA Leeuwarden, the Netherlands
[b] Department of Environmental Technology, Wageningen University and Research, Bornse Weilanden 9, 6708 WG, Wageningen, the Netherlands
[c] Sustainable Process Technology Group, Process and Catalysis Engineering Cluster, Faculty of Science and Technology, University of Twente, Drienerlolaan 5, 7522 NB Enschede, the Netherlands

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ABSTRACT

Biopolymers are natural macromolecules obtained from animal, plant and microbial sources, with the potential to be used in a wide range of applications. A key process step, which is still underdeveloped, is the downstream processing. In this work, water immiscible and water miscible ionic liquids (ILs) were investigated regarding their ability to fractionate a mixture of polysaccharide and proteins. Alginate and bovine serum albumin (BSA) were used as model compounds to mimic natural polymer crude extract. Phosphonium ILs composed of different anions (bromide, dicyanamide and phosphinate) were used as water immiscible ILs while imidazolium ILs, combined with phosphate salts to form biphasic system, were selected as water miscible ILs. In water immiscible IL systems, the partitioning behavior of biopolymers depended on IL's anions and there was formation of insoluble precipitate. The insolubility of precipitate in diverse aqueous and organic solvents hindered the processibility of water immiscible phosphonium IL for fractionation of biopolymers. The partitioning of biopolymers in water miscible ILs systems also depended on the IL's anion, as well the concentration of IL. Separation of alginate (yield = 90% and purity = 99%) from BSA (yield = 89% and purity = 99%) was best achieved by the [C_4mim]Cl-based extraction system. After fractionation, regeneration of IL and salt used was carried out by ultrafiltration, with recovery yields up to 100%. The high extraction yields and recyclability of phase-forming compounds confirm the potential of water miscible ILs systems to fractionate polysaccharide and protein.

Introduction

Natural polymers, also known as biopolymers, are synthesized during the life cycle of all living systems and are considered the building blocks of nature. Polysaccharides, proteins and polyesters are some examples of types of biopolymers (Silva et al., 2022; Verma, 2020). They are formed by linking several repeating units, for example sugars, amino acids or hydroxy fatty acids, leading to the formation of high molecular weight molecules (Silva et al., 2022; Verma, 2020; Silva et al., 2014). The use of natural polymers to replace fossil-based polymers is highly desirable for promotion of sustainability, as they come from renewable sources and can reduce environmental impact, for instance by reducing carbon dioxide emissions through reducing the dependence on fossil fuels.

When it comes to their origin, biopolymers can be obtained from a large variety of sources, including animals, plants and microorganisms. Cellulose (Klemm et al., 2005), chitin (El Knidri et al., 2020), collagen (Silva et al., 2014) and keratin (Silva et al., 2014; Shah, 2019) are some examples of plant-based and animal-based biopolymers. Plant-based and animal-based natural polymer have received significant attention due to their abundant availability and they are already used for paper manufacturing, textiles, adhesives, films, wound dressings and fabrication of blends and composites (Silva et al., 2014; Klemm et al., 2005; El Knidri et al., 2020; Shah, 2019). Additionally, Natural Fiber Welding (Peoria, Illinois) is currently commercializing plant-based materials for production of textile and soft-goods, including fashion accessories and footwear (Natural Fiber 2023).

In contrary to plant and animal-based biopolymers, microbial biopolymers have a higher structural diversity, their production is not easily affected by changes in regional and climatic conditions and presents high growth rates (N. Jindal and Singh Khattar, 2018). Xanthan, alginate, pullulan, dextran are some examples of micro-

* Corresponding author at: Sustainable Process Technology Group, Process and Catalysis Engineering Cluster, Faculty of Science and Technology, University of Twente, Drienerlolaan 5, Meander 221, 7522 NB Enschede, the Netherlands.
E-mail address: b.schuur@utwente.nl (B. Schuur).
† Hardy Temmink sadly passed away on 8 April 2022

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bial biopolymers (Verma, 2020; N. Jindal and Singh Khattar, 2018; Freitas et al., 2017). Microbial biopolymers are generally water-soluble and are mostly used as emulsion stabilizer, hydrogel and gelling and thickener agent (Verma, 2020; Freitas et al., 2017). They also can form micro- and nanosized particles, allowing their usage as carrier in encapsulation purposes (Silva et al., 2022; Goh et al., 2012). Some of them also possess biological properties (such as, antioxidant and anti-inflammatory), which allows the development of novel pharmaceuticals and cosmetic products (N. Jindal and Singh Khattar, 2018; Kojić, 2016; Wu et al., 2017).

Many microbial biopolymers have been reported in literature that possess properties that could be beneficial for a variety of applications. However, only a few (mostly polysaccharide) have found widespread industrial use (Verma, 2020; Freitas et al., 2017). In order to expand the current industrial applications of microbial polysaccharide and allow commercialization of other classes of microbial biopolymers, a key process step, which is currently still underdeveloped is the downstream processing (Goh et al., 2012; Feng et al., 2021; Shi, 2016; Labrou, 2021). The crude biopolymer extract mostly contains polysaccharides and proteins, and a minor amount of other small molecular weight compounds (Ajao, 2019). Generally, the main steps in recovery of biopolymers from their broths are separation, concentration and purification (Freitas et al., 2017; Elhami et al., 2022). Separation refers to a heterogeneous mechanical separation and aims at removing cells and other insoluble large particles by means of centrifugation or microfiltration (Freitas et al., 2017; Elhami et al., 2022). Concentration is also required as these macromolecules are typically present in relatively low concentrations in culture broths, ranging from 1 to 5 g/L depending on the operational conditions (Ajao, 2019; More et al., 2014). Finally, liquid-liquid extraction (LLX), precipitation and chromatography are most often used as the first techniques to purify the target biopolymer from other compounds present in broth (Freitas et al., 2017; N. Jindal and Singh Khattar, 2018).

Column chromatography is the most used technique for purification of natural polymers (Shi, 2016; Liu et al., 2020). Ion exchange chromatography relies on a stationary phase composed of cross linked polymers, such as divinylbenzene (Dowex), dextran (Sephadex) and agarose (Sepharose) to separate neutral and acidic biopolymers (Miller, 2019). Other chromatography techniques (i.e., gel permeation chromatography and affinity chromatography) have also successfully been employed (Shi, 2016; Liu et al., 2020; Kelley et al., 1986). Even though high purity biopolymers can be obtained by chromatography, relatively low throughput (that is, time-consuming) and product dilution are some of their drawbacks (Shi, 2016; Kelley et al., 1986; Warrand et al., 2003; Liu et al., 2015). Precipitation allows both concentration and purification. Trichloroacetic acid (TCA), chloroform and ammonium sulfate are examples of commonly used precipitation agents for proteins (Sivaraman et al., 1997; Koontz, 2014), while polysaccharides can be precipitated by alcohol and acetone (Zou et al., 2013). However, the use of harsh chemicals such as chloroform and trichloroacetic acid in relatively large amounts is undesired and often yields the proteins in denatured state (Koontz, 2014; Peng et al., 2016). Besides, the selectivity of these techniques is often hindered by co-precipitation of different types of biopolymers. For instance, Patel (Patel et al., 2013) et al. fractionated polysaccharide and protein from an algae extract by alcohol precipitation, and obtained a product with purity of 33%. Co-precipitation was also reported by Peng et al. (Peng et al., 2016) as precipitation yields of polysaccharide and protein were 31% and 41%, respectively, when ammonium sulfate precipitation was employed.

Liquid-liquid extraction (LLX) seems an interesting alternative technique since it allows both concentration and purification in one single stage. A promising class of solvent for LLX is that of ionic liquids (ILs). By definition, ILs are organic salts with a melting point below 100 °C (Welton, 1999). Their ionic nature is responsible for some of their beneficial features, such as low flammability and negligible vapor emission (Ohno and Nakanishi, 2019; Chiappe and Pieraccini, 2005). ILs can be divided into two categories: hydrophilic ILs, which are miscible with water, and hydrophobic ILs, which split into a distinct phase when mixed with water. Each class of ILs has its own benefits and drawbacks (Freire et al., 2012; Fukaya and Ohno, 2013).

Water-miscible ionic liquids can be used in an extractive platform known as aqueous two-phase systems (ATPS) (Freire et al., 2012; McQueen and Lai, 2019), which requires an additional agent in order to create a biphasic system. This type of extraction media has a relatively high water content in the co-existing phases, which promotes solubilization and prevents denaturation of biomolecules. ATPS can be created by combining ILs with either salts (phosphate, sulfate or citrate) or polymers (polyethylene glycol or polypropylene glycol) (Elhami et al., 2022). Ionic liquid/salt ATPS seems a more attractive alternative as it presents lower viscosity and higher difference of density between the phases (Freire et al., 2012; McQueen and Lai, 2019). That implies enhanced mass transfer and faster phase separation when compared to other types of ATPS. Previous researchers have shown that IL-based ATPS, in particular imidazolium-based ones, represents a potential separation technique for model biopolymers, as well real crude extracts of natural polymers (Pei et al., 2010; Tan et al., 2012; Yan et al., 2014). For instance, the work of Pei et al. (Pei et al., 2010) demonstrated the separation of BSA from dextran by $[C_4mim][N(CN)_2]/K_2HPO_4$ ATPS. 100% of BSA partitioned to the IL-rich phase while more than 90% of dextran migrated to the salt-rich phase. Tan et al. (Tan et al., 2012) used $[C_4mim][BF_4]/NaH_2PO_4$ ATPS to fractionate neutral polysaccharides and proteins from Aloe leaves crude extract. 96% of proteins partitioned into the IL-rich phase and 93% of polysaccharides were found in the salt-rich phase. Yan et al. (Yan et al., 2014) fractionated a biopolymer mixture obtained from a fermentation broth using a $[C_4mim][Cl]/K_3PO_4$ ATPS. Extraction yield of neutral polysaccharides and proteins were up to 89% and 88%, respectively.

Despite the significant number of publications demonstrating the feasibility of water-miscible ionic liquids for fractionation of biopolymers, the use of this class of IL requires large amounts of salt/polymer to form a biphasic system, which may hinder the recyclability of IL and purity of the target molecule. Considering that the concentration of biopolymer in crude extract is typically very low, alternative options are of interest to be investigated. Not much attention has been paid to the potential of the other class of ILs (water-immiscible) for fractionation of biopolymers. Water-immiscible IL can be highly advantageous from a process engineering perspective, as it does not require additional chemicals. In particular, phosphonium-based ILs are less expensive (Liu et al., 2010; Marcus, 2015) and have been largely investigated for the extraction of smaller molecules with similar functional groups, namely carboxylic acid (Oliveira et al., 2012; Reyhanitash et al., 2016), amino acids (Wang et al., 2005; Absalan et al., 2010) and alcohols (Garcia-Chavez et al., 2012; Ha et al., 2010). Since LLX is an affinity separation, it is anticipated that IL's affinity for such functional groups would enhance biopolymer's extractability to IL-phase. Another key aspect to be investigated for the phosphonium ILs is whether they can be regenerated and recycled properly, which is one of the aspects studied in this contribution.

Recyclability is an aspect of utmost importance for the use of ILs for biopolymer fractionation. Regeneration of ILs is often overlooked by published studies, as highlighted in recent review papers in the field (Elhami et al., 2022; Lee et al., 2017; Ventura et al., 2017; Saha et al., 2022). Because most ionic liquids are costly and sometimes toxic, high recovery rates are imperative for economic and environmental viability of the process. Some attempts have been reported on regeneration of ILs, mostly by precipitation and/or membrane processes. The published works do not report high IL recovery yields (Suarez Ruiz et al., 2020; Yang et al., 2022) or do not quantitatively report the performance of such regeneration process (Tan et al., 2012; Capela et al., 2019; Santos et al., 2018).

Considering the highly interesting nature of the IL-based biphasic systems with their interesting extraction behavior (Reyhanitash et al., 2016; Garcia-Chavez et al., 2012; Lee et al., 2017; Ventura et al., 2017),

Table 1
Molecular weight (MW), isoeletric point (pI) of the proteins investigated and acid dissociation constant (pKa) of the charged polysaccharide investigated.

Biopolymer	MW (kDa)	p.I./pKa
BSA	65 (Forciniti et al., 1991)	4.7 (Forciniti et al., 1991)
Sodium Alginate	300*	3.4 - 4.4 (Shinde and Nagarsenker, 2009)
Dextran	450–650	*Not applicable*

*Measured.

but still missing information on the processability of these ILs, the present study investigates the processability of ionic liquid (IL) biphasic systems, including not only a primary separation, but also focusing on the regeneration. After first evaluating the hypothesis that indeed these IL-based biphasic systems can separate polysaccharides from proteins in aqueous solutions, studies on regeneration of the solvents are presented. For this purpose, two distinctive, IL-based, extraction platforms were investigated: water-miscible imidazolium ILs and water-immiscible phosphonium ILs. Solutions of model compounds were used to mimic crude extract of natural polymers. Alginate was selected as a model polysaccharide as it consists of a commercially relevant biopolymer. This anionic polysaccharide is isolated from plant and microbial sources and its crude extract has considerable amount of protein, which hampers its applicability in diverse fields (Torres et al., 2019; Fertah et al., 2017; Rashedy et al., 2021; Dusseault et al., 2006; Cagla, 2021). The model biopolymer solution also contained bovine serum albumin (BSA) in order to investigate the ability of the studied ILs to separate polysaccharide from protein. The effect of the type and amount of IL as well as feed composition were some of the parameters investigated. Besides that, precipitation and ultrafiltration were used to separate fractionated biopolymer from IL used for extraction. This study offers relevant insights into fractionation of polysaccharide and proteins using ionic liquids, which can contribute to designing cost-effective downstream processing of natural polymers.

Materials and methods

Chemicals

The water immiscible ionic liquids (ILs) used were trihexyl(tetradecyl)phosphonium bromide (purity > 95%), trihexyl(tetradecyl)phosphonium dicyanamide (purity > 95%) and trihexyl(tetradecyl)phosphonium bis(2,4,4-trimethylpentyl)phosphinate) (purity > 95%), purchased from Iolitec. The ILs used to form an aqueous two phase system (ATPS) were 1-butyl-3-methylimidazolium acetate ([C_4mim] CH_3CO_2, purity = 98%, Iolitec), 1-butyl-3-methylimidazolium bromide ([C_4mim]Br, purity = 99%, Alfa Aesar) and 1-butyl-3-methylimidazolium chloride ([C_4mim]Cl, purity = 98%, Acros Organics). The inorganic salt used in the ATPS formation was di-potassium hydrogen phosphate (K_2HPO_4, VWR). The biopolymers used as model compounds were dextran (from *Leuconostoc spp*., Sigma-Aldrich), bovine serum albumin (BSA, purity ≥ 98%, Sigma-Aldrich) and sodium alginate (Sigma-Aldrich). The reagents required for analytical analysis were sodium tetraborate decahydrate ($Na_2B_4O_7$, purity = 99%), m-phenylphenol ($C_{12}H_{10}O$, purity = 85%, Sigma-Aldrich), dimethyl sulfoxide (DMSO, VWR), sulfuric acid (H_2SO_4, 95%, VWR). Protein concentration in the samples was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fischer™).

Extraction procedure

The model biopolymer solution was prepared in ultrapure water, combining model polysaccharide (alginate or dextran) and model protein (BSA). The concentration of biopolymer was 2.5 g/L for each model compound, as such concentration is representative of real systems based on literature (Ajao, 2019; More et al., 2014). Table 1 shows the main properties of the model biopolymers investigated. Experiments using water immiscible ionic liquids were carried out in 15 mL polypropylene tubes. 2 mL of model solution was added to tubes using calibrated pipettes, followed by addition of the same volume of ionic liquid using syringes and a mass check as due to the high viscosity the pipettes were not reliable for ILs. To reach equilibrium, tubes were stirred overnight using a rotary mixer. For experiments using water miscible ionic liquids, each ATPS was prepared by weighting appropriate amount of the phase-forming solutes and biopolymer model solution in 15 mL tubes, mixing thoroughly by a vortex mixer and then left equilibrating for 1 hour. This time has been reported as sufficient for achieving equilibrium in ATPS (Pei et al., 2010; Tan et al., 2012; Yang et al., 2022; Capela et al., 2019; Santos et al., 2018) .

For both types of system, complete phase separation was achieved by centrifugation (4500 rpm for 10 min) using graduated centrifuge tubes and formation of precipitate was observed at interface. Using such tubes, the volumes of the top and bottom phases could be noted with an accuracy of +/−0.13 mL (half the smallest division) and the phases were carefully separated using a syringe. The two separated phase samples were collected and analyzed for protein and polysaccharide concentration. The analytical methods used to quantify polysaccharide and protein concentration are described in detail in section 2.3. All experiments were carried out at room temperature (21 ± 1 °C). The experiments were performed in duplicate, and the results were reported as the average of two independent assays with their respective standard deviation.

Phase composition of IL-based ATPS

For experiments using water-miscible ionic liquids, the selection of different aqueous two-phase systems was done based on the data of phase diagrams available in the literature (Mourão et al., 2012). It is to be noted that the data reported in literature was obtained at 25 °C while the experiments in this work were carried out at room temperature (21 ± 1 °C). Diverse authors have reported that the binodal curve of ionic liquid-salt systems is not significatively sensitive to changes of temperature (Pei et al., 2007; Ruiz et al., 2018; Tanimura et al., 2019). Therefore, it is anticipated that these data are valid to represent the systems investigated in this work with acceptable accuracy.

The composition of each phase in an ATPS was determined by gravimetric method as described by Merchuk et al. (Merchuk et al., 1998). The method consists of weighing appropriate amounts of salt, ionic liquid and water and mixing it thoroughly. Complete phase separation was achieved by centrifugation (4500 rpm for 10 min) and the phases were carefully separated and individually weighed. The mass fraction percentage of the ionic liquid and salt in the top and bottom phases were then calculated by mass balances, expressed by the system of four equations (Eqs. (1)–(4)):

$$[IL]_T = A \exp\left[\left(B \text{ x } [Salt]_T^{0.5}\right) - \left(C \text{ x } [Salt]_T^{3}\right)\right] \quad (1)$$

$$[IL]_B = A \exp\left[\left(B \text{ x } [Salt]_B^{0.5}\right) - \left(C \text{ x } [Salt]_B^{3}\right)\right] \quad (2)$$

$$[IL]_T = \frac{[IL]_M}{\alpha} - \frac{1-\alpha}{\alpha} \text{ x } [IL]_B \quad (3)$$

$$[Salt]_T = \frac{[Salt]_M}{\alpha} - \frac{1-\alpha}{\alpha} \text{ x } [Salt]_B \quad (4)$$

where "T", "B", and "M" stands by top phase, bottom phase and mixture, respectively. [Salt] and [IL] are the weight fraction of salt and ionic liquid, and α is the ratio between the mass of the top phase and the total mass of the mixture. The constants A, B and C were obtained from literature (Mourão et al., 2012). By solving the system, the four unknown values ($[IL]_T$, $[IL]_B$, $[Salt]_T$ and $[Salt]_B$) are calculated.

Regeneration procedure

Precipitate was formed after fractionation of model biopolymers using water immiscible ILs. Attempts to dissolve this precipitate were carried out by adding 10 mL of solvent to 0.5 g of wet precipitate, stirring overnight at room temperature (21 ± 1 °C).

Regeneration of water-immiscible ionic liquid was carried out using solvent precipitation. Different ethanol volumes (2, 4 and 8 mL) were added to the IL phase, which was obtained after extraction, and left overnight at 4 °C. In order to collect precipitate, samples were centrifuged at 4 °C (4500 rpm for 10 min).

Regeneration of water-miscible ionic liquid and salt used in ATPS was conducted by ultrafiltration. Dead-end filtration was carried out using polysulfone membrane in a stirred cell module (10 mL) at 3 bar and room temperature (21 ± 1 °C). For the salt-rich phase, a membrane of 100 kDa molecular weight cut-off (MWCO) (Mycrodin Nadir) was used while a membrane of 20 kDa MWCO (Alfa Laval) was used for the ionic liquid-rich phase. Diavolume (DV) was defined as the total water volume added to the cell during diafiltration divided by the initial feed volume. The permeate flux could not be determined due to practical constraints (i.e., equipment limitations). It is to note that a progressive decline of flux with time was observed. The flux decline was most likely due to concentration polarization considering the relatively short duration of filtration. In addition, no significant indication of fouling could be identified by microscopy analysis and mass balance.

Analytical methods

The protein concentration in the samples was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fischer™). The absorbance of the mixture was measured at 562 nm using a microplate spectrophotometer (Victor3 1420 Multilabel Counter, Perkin Elmer). The alginate concentration was determined by m-phenylphenol uronic acid assay (Miller, 2019), using alginate as standard for the calibration curve. The dextran concentration was determined by phenol-sulfuric acid assay (Kelley et al., 1986), using glucose as standard for the calibration curve. The absorbance of the mixture was measured at 490 nm using a microplate spectrophotometer (Victor3 1420 Multilabel Counter, Perkin Elmer). Due to interference caused by phase-forming compounds, samples were submitted to ultrafiltration prior to analysis, using amicon ultra centrifugal filters (MWCO 50 kDa, Merck Millipore) in order to remove those compounds.

To estimate the concentration of IL and salt in the retentate and concentrate stream in regeneration experiments, the concentration of chloride (Cl^-), and phosphate (PO_4^{3-}), respectively, were determinate by ion chromatography using a Metrohm Compact IC 761 with an anion column (Metrosep A Supp 5–150/4.0).

Calculation methods

For experiments using water-immiscible ILs, the concentration of biopolymer could only be measured in one (aqueous) of the three phases (aqueous, precipitate and IL), due to limitations in the analytical technique. Therefore, the extraction performance was evaluated by calculating the ratio between the mass fraction of biopolymer in raffinate ($X_{raffinate}$) and the mass fraction of biopolymer in the feed (X_{feed}). For water-miscible IL systems, the performance was evaluated by calculating the yield (Eq. (5)), which represents the ratio between the mass of biopolymer (polysaccharide or protein) in one of the phases (top, bottom or precipitation) and the initial mass of biopolymer. The concentration of biopolymer in both bottom and top phases were analyzed and the concentration of biopolymers in the precipitate phase was obtained by mass balance.

Purity was defined as the ratio between the mass of the target biopolymer and the total mass of biopolymer present in that phase (Eq. (6)). For experiments using water-miscible ILs, the purity of polysaccharide was calculated for the salt-rich phase while the purity of protein was calculated considering the ionic liquid-rich phase.

For regeneration experiments, the recovery yields of IL and salt were calculated as the ratios of Cl^- and PO_4^{3-} mass in the permeate in relation to initial mass.

$$Yield\% = \frac{m_{phase} \ / \ g}{m_{initial} \ / \ g} \times 100\% \quad (5)$$

$$Purity\% = \frac{m_{target\ biopolymer} \ / \ g}{m_{total\ biopolymer} \ / \ g} \times 100\% \quad (6)$$

Results and discussion

Fractionation by water immiscible IL

Screening of ILs

The partition behavior of model biopolymers was investigated using three systems composed of phosphonium water-immiscible ionic liquid. Model solutions containing either BSA or alginate (single) and both biopolymers (mixture) were used to mimic crude extracts of natural polymers.

After extraction, all systems presented precipitate at the interface, meaning that biopolymers were extracted to the IL-phase or/and precipitate. The concentration of alginate and BSA in IL-phase and precipitate could not be determined due to limitations of the analytical technique. The performance of such systems was expressed as a function of biopolymer's mass ratio in raffinate ($X_{raffinate}$) and feed (X_{feed}), as shown in Fig. 1. For single compound systems, the extraction of alginate, from the aqueous phase, was as follows: $[P_{666,14}][Br]$ > $[P_{666,14}][DCA]$ > $[P_{666,14}]$[Phosphinate]. $[P_{666,14}][Br]$ also had the highest extraction of BSA, similarly followed by $[P_{666,14}][DCA]$ and a much smaller extraction of BSA by $[P_{666,14}]$[Phosphinate]. $[P_{666,14}][Br]$ and$[P_{666,14}]$[Phosphinate] had higher extraction of alginate while $[P_{666,14}][DCA]$ preferentially extracted BSA from aqueous phase. It is to be noted that for $[P_{666,14}]$[Phosphinate] a mass ratio higher than 1 was observed due to the reduction of the volume of aqueous phase after extraction. As reported in literature (Marták and Schlosser, 2007), the formation of reverse micelles is responsible for the high water uptake by $[P_{666,14}]$[Phosphinate]. When both biopolymers were present in model solution (mixture), $[P_{666,14}][Br]$ and $[P_{666,14}]$[Phosphinate] maintained a similar performance as for single compound solutions. On the other hand, the extraction of BSA was significantly higher for $[P_{666,14}][DCA]$ as solvent and in presence of alginate.

The differences observed in the experiments where mixtures of alginate and BSA were present show that the driving forces for the extraction of alginate and BSA depend on the nature of IL. Alginate was negatively charged at the pH of experiments (~ 7) so it was anticipated that electrostatic interactions play a relevant role on partitioning behavior of this biopolymer. In addition, it has been reported that for extraction of molecules with similar functional groups, ion-exchange played an important role for $[P_{666,14}][Br]$ while hydrogen bonding was the main interaction driving extraction by $[P_{666,14}][DCA]$ and $[P_{666,14}]$[Phosphinate] (Reyhanitash et al., 2016, Marták and Schlosser, 2007). Leaching of Br^- from $[P_{666,14}][Br]$ phase into aqueous phase (3.5% of the initial Br^- content) was also observed. These reported findings supports our hypothesis of ion-exchange as main mechanism for extraction of alginate as $[P_{666,14}][Br]$ had the highest extraction yield while the lowest extraction yield was by $[P_{666,14}]$[phosphinate].

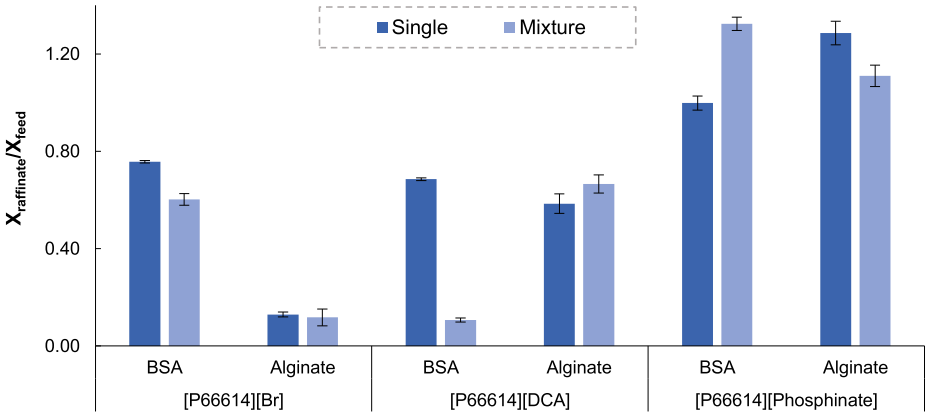

Fig. 1. Partitioning behavior of alginate and BSA, in single or mixture solutions, extracted by $[P_{666,14}][Br]$, $[P_{666,14}][DCA]$ and $[P_{666,14}][Phosp]$ (pH = 7, volumetric S/*F* = 1:1, *T* = 21 °C).

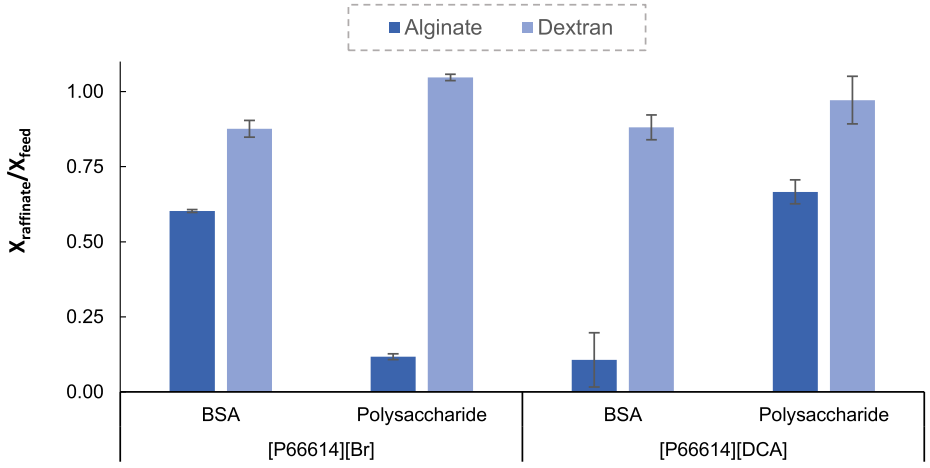

Fig. 2. Partitioning behavior of model polysaccharide (alginate or dextran) and protein (BSA) extracted by $[P_{666,14}][Br]$ and $[P_{666,14}][DCA]$ ($pH_{initial}$ = 7, volumetric S/*F* = 1:1, *T* = 21 ± 1 °C).

The extraction of BSA in single compound systems was significatively lower than when both biopolymers were present in the model solution. The reason might be due to salting-out of BSA, caused by the presence of anionic alginate in the aqueous phase. For the extraction of BSA in mixture compound systems, a correlation between IL's water uptake and extraction yield was observed. As reported in literature (Reyhanitash et al., 2016), after extraction, the water uptake by the studied ILs was as follows: $[P_{666,14}][DCA] < [P_{666,14}][Br] < [P_{666,14}][Phosphinate]$. Opposite trend was observed for the extraction of BSA. In other words, the higher the IL's water uptake, the lower the extraction yield of BSA. In literature, it is suggested that other interactions may also play a role in extraction of BSA by the investigated ILs. For instance, the work of Wang et al (Wang et al., 2005) showed that the partition coefficients of amino acids (monomer units of protein), using several imidazolium hydrophobic ILs, correlates with their hydrophobicity. In addition, Alvarez-Guerra (Alvarez-Guerra and Irabien, 2012) reported low extraction yield of BSA (2%) when using a significatively less hydrophobic ionic liquid than used in this study (1-butyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]imide).

Effect of feed composition

The results from **section 3.1.1** showed that ion-exchange and salting-out seemed to play a role on the fractionation of alginate and BSA. To confirm such hypothesis, extraction using neutral polysaccharide with comparable molecular weight (dextran) was also investigated for the best ILs ($[P_{66614}][Br]$ and $[P_{66614}][DCA]$). Fig. 2 shows that, for $[P_{66614}][Br]$, the extraction of BSA was similar in presence of dextran and alginate. Conversely, the extraction of dextran was considerably lower compared to alginate. $[P_{66614}][DCA]$ as solvent also led to lower extraction of dextran and, for this IL, partitioning behavior of BSA significatively depended on the type of polysaccharide in mixture. In the presence of dextran, extraction of BSA was much lower than that of alginate.

The lower extraction of dextran by $[P_{66614}][Br]$ and $[P_{66614}][DCA]$, in relation to alginate, reinforces the hypothesis that anion exchange plays a relevant role on the extraction of polysaccharides. Dextran is most likely to interact with ILs via hydrogen bonding and it reduces its extraction yields in relation to alginate as alginate can establish both hydrogen bonding interactions and ion exchange. In addition, for extraction using $[P_{66614}][DCA]$, the decrease of BSA extraction in presence of dextran also supports that alginate promotes migration of BSA to IL-phase (salting-out).

Fractionation by water miscible IL

Phase composition in IL-based ATPS

The phase composition of each system studied for separation of alginate and BSA is present in table 2. The composition of the top phase (IL-rich phase) was similar using different ILs ($[C_4mim][CH_3CO_2]$, $[C_4mim][Br]$ and $[C_4mim][Cl]$) at the same concentration. There was a difference in the composition of the bottom phase (salt-rich phase) using different ILs. The mass fraction of K_2HPO_4 in the bottom phase was as follows: $[C_4mim][Br] < [C_4mim][Cl] < [C_4mim][CH_3CO_2]$. It is anticipated that this difference in the composition of the bottom phase

Table 2
Phase composition (mixture, top and bottom phases, in weight fraction) of different IL-based ATPS (composed of K_2HPO_4 and [C_4mim][CH_3CO_2], [C_4mim][Br] or [C_4mim][*Cl*]) used to study separation of alginate and BSA.

System	Mixture		Top phase		Bottom phase	
	IL	Salt	IL	Salt	IL	Salt
C_4mim [CH_3CO_2]/K_2HPO_4	0.200	0.200	0.337	0.081	0.016	0.360
C_4mim [Br]/K_2HPO_4	0.200	0.200	0.347	0.072	0.087	0.298
C_4mim [Cl]/K_2HPO_4	0.200	0.200	0.340	0.069	0.047	0.343
C_4mim [Cl]/K_2HPO_4	0.150	0.250	0.328	0.074	0.039	0.360
C_4mim [Cl]/K_2HPO_4	0.250	0.150	0.334	0.072	0.050	0.378

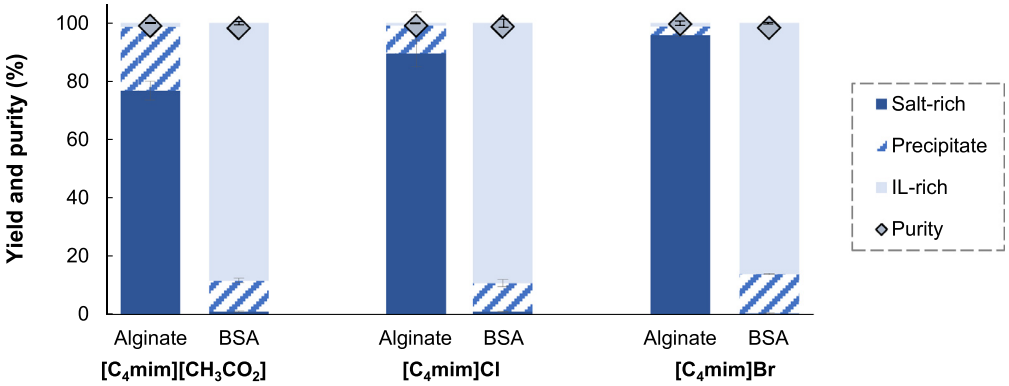

Fig. 3. Partitioning behavior of alginate and BSA in IL-based ATPS composed of K_2HPO_4 and C_4mim[CH_3CO_2], C_4mim[Cl] or C_4mim[Br] (20% IL + 20% salt + 60% alginate and BSA aq. solution (2.5 g/L each), $T = 21 \pm 1$ °C).

will lead to different extraction yields of polysaccharide depending on the IL, as this biopolymer is often mostly extracted to the bottom phase.

In addition, it was observed that using different concentrations of [C_4mim][Cl] and K_2HPO_4 to form ATPS led to different phase composition. In the top phase (IL-rich), the concentration of [C_4mim][Cl] was the highest at mixture of 20% IL + 20% salt and the lowest one using relatively more salt than IL (15% IL + 25% salt). In the bottom phase (salt-rich), the highest concentration of K_2HPO_4 was obtained using relatively more IL than salt (25% IL + 15% salt) and the lowest for the system using equal amounts of IL and salt (20% IL + 20% salt).

Screening of IL-based ATPS

Three distinctive water-miscible ILs were investigated regarding the partition behavior of model biopolymers in IL-based ATPS. Fig. 3 shows that all the investigated systems were able to separate alginate from BSA and that precipitation was observed at interface. The amount of precipitate was obtained by mass balance, after analyzing the IL- and salt-rich phases. The system composed of [C_4mim]CH_3CO_2 had the largest amount of precipitate, as 17% of initial biopolymer mass was found as precipitate after extraction. Less than 10% of alginate and BSA mass was retrieved as precipitate for the systems composed of [C_4mim]Cl and [C_4mim]Br. BSA mostly partitioned into the most hydrophobic phase in the system, namely IL-rich phase. In addition, there was no relevant difference on the extraction yield of BSA among the investigated ILs, ranging from 86 to 89%. High purity of BSA (≥ 98%), in the IL-rich phase, was achieved for the studied ATPSs. It is also shown in Fig. 3 that alginate had a higher affinity to the most hydrophilic phase, namely salt-rich phase. The yield of alginate in that phase ranged from 76%–96% and was as follows: [C_4mim]CH_3CO_2 < [C_4mim]Cl ≈ [C_4mim]Br.

The mechanism governing the partition of proteins in ATPS is still not yet fully understood. However, it is well accepted that driving forces for the partitioning of proteins in ATPS results of a combination of salting-out effect, hydrogen bond and hydrophobic interaction (Dreyer et al., 2009; Pei et al., 2009; Pereira et al., 2015). At the pH of experiments (≈ 10), BSA is negatively charged so electrostatic interactions may also play a role. The preferential partition of alginate to salt-rich phase (most hydrated phase) is explained by dipole-ion/hydrogen bonding interactions between the polysaccharide's carboxyl groups and water. The formation of precipitate at interface after extraction is related to the reduced solubility of biopolymers in the ATPS. Solubilization of biopolymers is achieved by the formation of a hydration layer, which prevents aggregation of the biopolymer's molecules. The presence of IL and salt might disrupt such layer and, therefore, enhance intramolecular interactions of biopolymers, leading to aggregation and precipitation (Guo et al., 2017). [C_4mim]CH_3CO_2 has the lowest Gibbs energy of hydration (−374 kJ.mol^{-1}), followed by [C_4mim]Cl (−334 kJ.mol^{-1}) and [C_4mim]Br (−318 kJ.mol^{-1}) (Marcus, 2015). Therefore, the anion $CH_3CO_2^-$ is more hydrated than Cl^- and Br^- and, consequently, precipitation is the greatest for [C_4mim]CH_3CO_2 -based ATPS.

The extraction yields and selectivity obtained in this work was superior when compared to other types of ATPS, in particular polymer-based ATPS (Farruggia et al., 2004; Dallora et al., 2007; Chow et al., 2015; Saravanan et al., 2008). Flores-Gatica et al. (Gatica, 2021) employed PEG/citrate ATPS for the separation of anionic polysaccharide (hyaluronic acid) and protein and obtained nearly 80% of extraction yield of polysaccharide to the salt-rich phase, with purity of 75%.These findings suggest that IL-based ATPS seems to be a better platform for fractionation of polysaccharides and protein than the polymer-based counterparts. In this study, the best separation of alginate and BSA was achieved by the [C_4mim]Br ATPS. The [C_4mim]Cl system had a similar performance, in terms of yield and selectivity, and was used in the subsequent studies as it is a less expensive IL.

Effect of phase-forming compounds concentration

The effect of phase composition of ATPS on partitioning behavior of alginate and BSA was studied by changing the concentrations of [C_4mim]Cl and K_2HPO_4 used to form the biphasic system. The concentration varied in such a way that the initial mass of model biopolymer solution used was equal in all the systems. Fig. 4 shows that the selectivity was similar for the investigated systems as the purity ranged from 95 to 99%. The extraction yield of alginate and BSA was affected by the concentration of phase-forming compounds. The highest extraction yield of alginate (90%) and BSA (89%) was obtained for the system composed of 20% IL + 20% salt. The lowest yield of alginate in the salt-rich (bottom) phase was obtained when lower concentration of salt, compared to IL, was used (25% IL + 15% salt). Conversely, the lowest yield of BSA was obtained in the IL-rich (top) phase when higher concentration of salt, compared to IL, was used (15% IL + 25% salt).

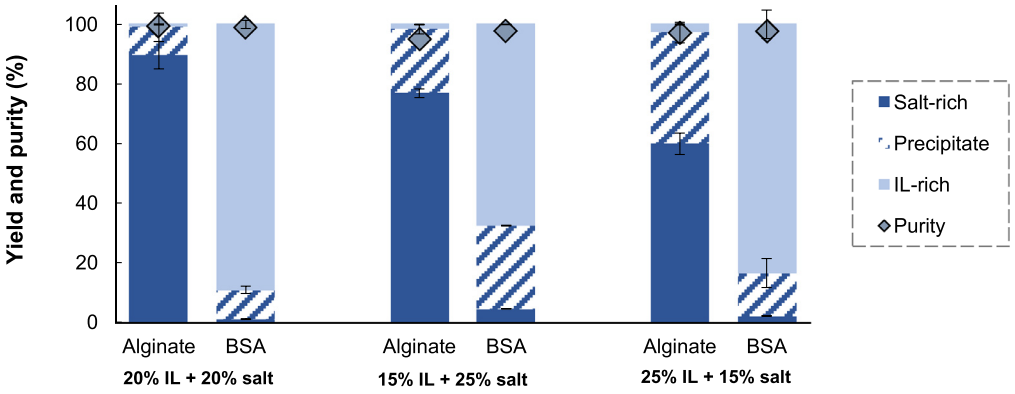

Fig. 4. Partitioning behavior of alginate and BSA in IL-based ATPS composed of K_2HPO_4 and [C_4mim]Cl in different concentrations (alginate and BSA concentration = 2.5 g/L each).

The differences in extraction yield can be explained based on the phase composition of the system (mixture, top and bottom phase), shown in table 2. The highest extraction yield of BSA was achieved for the ATPS with highest concentration of IL in top phase (20% IL + 20% salt), while the lowest yield was obtained for the system with the lowest concentration of IL in top phase (15% IL + 25% salt). π- π interaction between the imidazolium cation and the aromatic residues of BSA is a possible driving force for partitioning of BSA (Pei et al., 2009) to IL phase so this explains the increase in extraction yield of BSA with increase of IL concentration in top phase. In addition, the partitioning behavior of alginate seems to be related to the concentration of salt in the bottom phase. Mixture points with higher concentration of IL (25% IL + 15% salt) led to higher concentration of salt in the salt-rich phase. As result, the higher amount of salt in the bottom phase competed with water molecules, causing a reduction in the solubility of polysaccharides in that phase and, consequently, precipitation. Increase of precipitation caused the decrease on extraction yield of alginate in the salt phase.

These findings are in agreement with the study carried by Tan et al. (Tan et al., 2012), in which the authors reported a decrease of extraction yield of polysaccharide by increasing salt concentration, for fractionation of *aloe vera* crude extract by [C_4mim]BF_4/NaH_2PO_4 ATPS. Similar trend was also reported by Yang et al. (Yang et al., 2022), as the authors showed that increasing IL's concentration increased the protein extraction yield to [C_4mim]Cl-phase for fractionation of real biopolymer crude extract. On the other hand, other authors did not observe significant performance changes by changing the concentration of phase-forming compounds. For instance, Pei et al. (Pei et al., 2009) did not observed significant changes of extraction yield of BSA in [C_4mimBr]/K_2HPO_4 by increasing IL concentration and it might be due to the lower concentration of BSA (1.0 g/L) when compared to this study (2.5 g/L). Similar trend was reported by Santos et al. (Santos et al., 2018), when fractionating neutral polysaccharide and proteins from microalgae extract, and the concentration of biopolymer (0.8 g/L) in the feed was also lower than this study.

Regeneration of ionic liquids

Considering that ILs have significant impact on the water ecosystems and ILs remain very expensive in comparison with the conventional solvents, it is important to recycle ILs. Attempts on regenerating hydrophobic and hydrophilic ILs are presented in the following subsections.

Water immiscible ILs

After extraction, all systems exhibited precipitate at the interface, meaning that biopolymers were extracted to IL phase and/or precipitate. It was not possible to determinate the exact amount present in each one of the phases. This happened because the precipitate was not soluble in diverse solvents. Solubilization of precipitate was only possible in dimethyl sulfoxide (DMSO), as shown in table 3.

Table 3
Solubility of 0.5 g of wet precipitate, obtained using water immiscible ILs, in 10 mL of diverse solvents at 21 ± 1 °C.

Solvent	Soluble?
Acetone	✗
Ethanol	✗
Dimethyl sulfoxide	√
Methanol	✗
Sodium bromide (5 M)	✗
Sodium chloride (5 M)	✗
Sodium hydroxide (5 M)	✗
Hydrochloride acid (6 M)	✗
Water	✗

Table 4
Yield and purity of alginate and BSA in raffinate after fractionation by [$P_{666,14}$][Br] and [$P_{666,14}$][DCA].

IL	Biopolymer	Yield (%)	Purity (%)
[$P_{666,14}$][Br]	BSA	53	86
[$P_{666,14}$][DCA]	Alginate	62	88

In addition, precipitation by ethanol was used as an attempt to regenerate IL and recover biopolymer loaded in IL phase. The formation of precipitate was not observed using different amounts of ethanol. Therefore, only biopolymers in aqueous phase (raffinate) could be retrieved, causing a significant yield loss. As seen in table 4, for [$P_{666,14}$][Br], BSA was the biopolymer majorly found in raffinate. In this case, 53% of BSA could be recovered with a purity of 86%. Alginate was the biopolymer mostly found in aqueous phase after extraction using [$P_{666,14}$][DCA], resulting in a yield and purity of 62% and 88%, respectively.

The insolubility of precipitate in aqueous solvents might indicate denaturation of BSA and/or formation of a complex between alginate (anionic) and the IL's cation (hardly soluble in water). In addition, the lack of precipitation in presence of ethanol suggests that the concentration of biopolymer in IL phase was relatively low. Wang et al. (Wang et al., 2007) also reported relatively low concentration of biopolymer (20 mg/L) in IL phase after extraction using hydrophobic IL ([C_4*mim*]PF_6). Efficient regeneration of IL was also not achieved as low back-extraction yields of protein (30%) were obtained. Similar results were obtained by Alvarez-Guerra (Alvarez-Guerra and Irabien, 2012) as back-extraction of lactoferrin (protein) with water, from [C_4mim]NTf_2 phase, was not achieved at all. In fact, literature suggests that the regeneration of one of the studied ILs ([$P_{666,14}$][Phosphinate]) can only be effectively accomplished using harsh chemicals, namely NaOH and trimethylamine (Reyhanitash et al., 2019). Since mild regeneration techniques were not able to regenerate the studied hydrophobic ILs, the feasibility of fractionation of biopolymers by the studied hydrophobic ILs is hindered.

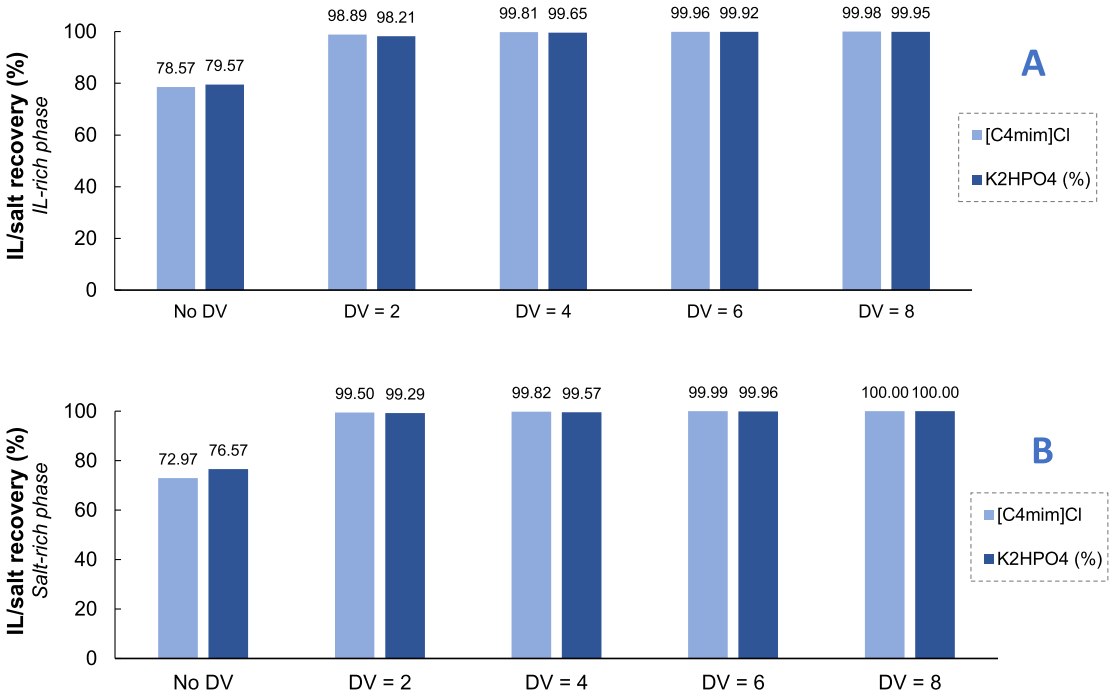

Fig. 5. Ultrafiltration using diverse diafiltration volumes (DV) for separation of fractionated alginate and BSA from [C_4mim]Cl-rich and K_2HPO_4-rich phases from ATPS (a) IL-rich phase, (b) Salt-rich phase (T = 21 ± 1 °C, P = 3 bar).

Water miscible IL

Ultrafiltration was employed to isolate fractionated biopolymer (alginate or BSA) from chemicals used during extraction (C_4mimCl and K_2HPO_4). Relatively low recovery of phase-forming compounds was obtained when the IL-rich and salt-rich phases were concentrated by membrane filtration (4-fold) and no additional water was added **(**Fig. 5**).** This finding was expected as IL and salt freely permeate through the membrane (i.e., 100% rejection coefficient) and only 4 out of 5 parts of the stream volume were filtrated to avoid excessive increase of viscosity. To increase the recovery yield, ultrafiltration was operated in diafiltration mode so that IL and salt could be washed away from retentate. In this case, the filtration process consisted of three steps: initial concentration (4-fold), addition of water (volume equal to 2-, 4-, 6- and 8-times initial stream volume) and final concentration step (4-fold). By doing so, more phase-forming compounds could be retrieved in the permeate stream. The highest recovery yields of [C_4mim]Cl and K_2HPO_4 were obtained using diafiltration volume of 8. For the IL-rich phase, the recovery yields were 99.95–99.98%, while 100.00% of IL and salt present in salt-rich phase were recovered.

UF/diafiltration had a significatively better performance than dialysis, which is a more commonly reported technique in the literature (Tan et al., 2012; Pereira et al., 2015) for recovery of phase-forming compounds. Besides being time consuming, dialysis requires a much larger volume of solvent, when compared to UF/diafiltration. Based on the reported findings, a conceptual process design of fractionation of alginate and BSA by IL-based ATPS coupled with UF/diafiltration is illustrated in Fig. 5. Ultrafiltration operated in diafiltration caused the dilution of IL and salt recovered in the permeate so in order to re-use the phase-forming compounds to form a new ATPS, a concentration step is required.

Further investigations are needed to assess the behavior of membrane under longer filtration duration. For larger scale filtration with longer duration, crossflow configuration is more beneficial as slow fouling formation occurs due to the tangential shear on the membrane surface. In terms of module, flat-sheet modules operated at similar pressure are already employed in dairy industry. It is anticipated that this type of module can also be suitable for this application since the streams have similar composition.

Fig. 6.

Conclusions

Ionic liquid-based biphasic systems seem a promising technique for separation of proteins from polysaccharides as they can overcome limitations associated with conventional separation techniques, namely complexity, low yields and purity. In this study, water immiscible and water miscible ionic liquids were investigated for fractionation of model compounds (alginate and BSA).

Fractionation using water-immiscible ILs (phosphonium-based) led to formation of insoluble precipitate at interface and regeneration of IL was not achieved. As result, biopolymers could only be retrieved from one of the phases (aqueous raffinate) after extraction, leading to significantly low yields. Therefore, the use of the investigated phosphonium ILs may be useful for applications in which removal of polysaccharide or protein from a crude extract is crucial, since these ILs were able to remove more than 90% polysaccharide ([$P_{666,14}$][Br]) or protein ([$P_{666,14}$][DCA), resulting in purity ≥ 85% in a single-stage. Yet, further studies on the regeneration of the studied water immiscible ILs is still needed. An effective regeneration strategy requires high recovery yields (>99%) and that there is not loss of performance after multi extraction cycles.

Water-miscible ionic liquids, in ATPS, were more appropriate for fractionation purpose as both phases in the biphasic platform could be recovered. IL-based ATPS present soluble precipitate at interface, as oppositive to water immiscible ILs. Separation of alginate (yield = 90% and purity = 99%) from BSA (yield = 89% and purity = 99%) was best done by the [C_4mim]Cl-based ATPS. In terms of concentration of phase-forming compounds, using equal amounts of IL and salt (20%, each) led to highest yields. In addition, regeneration of IL and salt was performed using ultrafiltration/diafiltration. It was possible to recover 99.99% of [C_4mim]Cl and 99.96% of phosphate salt used to form the biphasic system, with relatively low biopolymer loss (~12%). Overall, this study

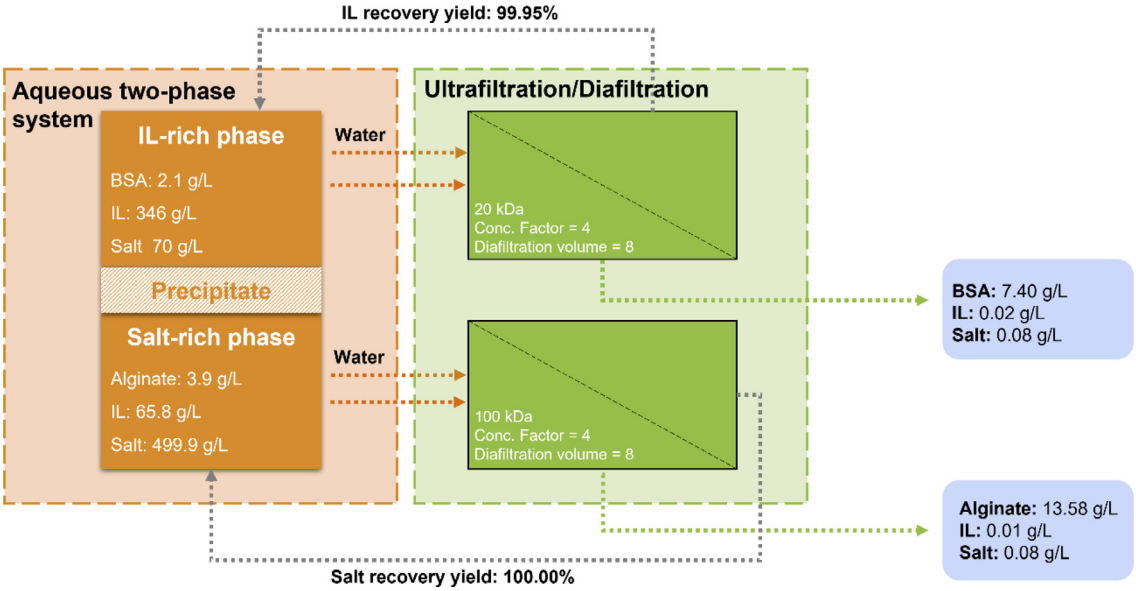

Fig. 6. Scheme of fractionation of alginate and BSA by $[C_4mim]Cl/K_2HPO_4$ ATPS, followed by regeneration of phase-forming compounds using ultrafiltration/diafiltration.

showed that water miscible ILs (IL-ATPS) have superior performance in the field of biopolymer separation.

Addressing the practical applicability of the investigated extraction systems for fractionation of biopolymers, in comparison to traditional methods, is of great relevance. Solvent precipitation is the most used technique to recover biopolymers on industrial scale (Elhami et al., 2022). When compared to precipitation, IL-ATPS can provide a more streamlined fractionation process. This occurs because with this approach, simultaneous separation of polysaccharide from protein can be achieved in one-single stage, with relatively high purity. In addition to the fractionation potential, the low concentrations in the broth direct towards IL-ATPS, because precipitation performs better at relative higher concentration of biopolymer while IL-based extraction systems can fractionate biopolymer mixtures at relative low concentration, which is more representative of real matrices.

Despite its exploratory nature, this study offers valuable insights into fractionation of polysaccharide and proteins using ionic liquids. Such insights can contribute to designing cost-effective downstream processing of natural polymers and, consequently, promote the use of sustainable chemicals.

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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