Selective and mild fractionation of microalgal proteins and pigments using aqueous two-phase systems

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Abstract

BACKGROUND: Microalgal biomass is generally used to produce a single product instead of valorizing all of the cellular components. The biomass production and downstream processes are too expensive if only one product is valorized. A new approach was proposed for the simultaneous and selective partitioning of pigments and proteins from disrupted Neochloris oleoabundans cultivated under saline and freshwater conditions.

RESULTS: An aqueous two-phase system composed of polyethylene glycol and cholinium dihydrogen phosphate selectively separated microalgal pigments from microalgal proteins. 97.3 ± 1.0% of lutein and 51.6 ± 2.3% of chlorophyll were recovered in the polymer-rich phase. Simultaneously, up to 92.2 ± 2.0% of proteins were recovered in a third phase (interface) in between the aqueous phases (interface). The recovered proteins, including Rubisco with a molecular weight of ~560 kDa, seem to be intact and pigments did not suffer degradation, demonstrating the mildness of this system for fractionating microalgal biomolecules.

CONCLUSION: The ability of aqueous two-phase systems (ATPSs) to simultaneously and efficiently fractionate different biomolecules in a mild manner from disrupted microalgae is demonstrated. This is an important step towards the development of a multiproduct microalga biorefinery.

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INTRODUCTION

Microalgae are a promising feedstock for many industries including biopharmaceuticals, biomaterials, bioenergy, nutraceuticals, agriculture, animal health and cosmetics and personal care. Feasibility studies demonstrate that a biorefinery focus on one single product is not cost-efficient and that several microalgal components should be extracted to supply different markets, and thus increase the overall value of microalgal biomass.

Depending on the strain, microalgae can contain chlorophylls (green), carotenoids (red, orange and yellow) and phycobiliproteins (red and blue). Neochloris oleoabundans accumulates mainly lutein, cantaxanthin, zeaxanthin, and astaxanthin monoesters and diesters, which are promising ingredients for pharmaceutical and nutraceutical applications. Moreover, microalgal proteins are recognized for their high quality, showing outstanding nutritional, functional and techno-functional properties that are in some cases superior to conventional protein concentrates.

Although microalgal cells contain several valuable biomolecules, the currently applied separation processes valorize only one specific product (e.g. astaxanthin, phycobiliproteins). The development of efficient, mild and scalable methods/processes, capable of fractionating different microalgal biomolecules, is of particular importance. For the fractionation of hydrophobic molecules such as lipids and pigments from disrupted microalgae, organic solvents are often used, whereas water-soluble components such as proteins and carbohydrates are discarded or undervalued. Proteins are fragile molecules that tend to denature during extraction using organic solvents or other harsh conditions. For a complete valorization of microalgal biomass, it is favourable to use mild separation methods.

Aqueous two-phase systems (ATPSs) have been adopted as a new technology in a microalga biorefinery framework. An ATPS...
Selective and mild fractionation of microalgal biomass using ATPSs and IL-ATPSs are limited. The application of IL-ATPSs provide advantages over conventional ATPSs because: they are highly tuneable through variations in pH, molecular structure, composition and temperature; and the wide variety of potential ions spans the entire hydrophobicity–hydrophilicity range. Thus, IL-ATPSs potentially have a higher selectivity, flexibility and can provide more efficient separations than commonly used ATPSs composed of polymer-polymer or polymer-salt. However, reports on integrated separation processes of biomolecules from biomass using ATPSs and IL-ATPSs are limited. The application of IL-ATPSs is generally studied using pure biomolecules (e.g. proteins), facilitating the understanding of the partitioning behaviour of certain molecules. However, these compounds are normally present in complex biological matrices such as microalgae biomass, which contain a large number of other components (e.g. carbohydrates, pigments, lipids). Therefore, to provide a realistic scenario, investigations using IL-ATPSs need to focus on the fractionation of these compounds from complex matrices.

Studies of ATPSs for the fractionation of biomolecules from microalgae extracts are limited. These studies mainly focus on the recovery of specific molecules such as the fluorescent pigment-protein complex C-phycoeyrin from Spirulina strains, B-phycoerythrin from Porphyridium cruentum and proteins from Chlorella sorokiniana. The simultaneous separation of different molecules from microalgae has not been addressed. To our knowledge, the current study is the first study using IL-ATPSs for the simultaneous fractionation of proteins and pigments from disrupted microalgae.

In this research, Neochloris oleoabundans is used to study the fractionation of pigments and proteins by using one conventional ATPS (polymer/salt) and two IL-ATPSs. Mild and biocompatible phase-forming components were selected based on previous investigations. ATPS was used for the fractionation of pigments and proteins from disrupted N. oleoabundans grown under both freshwater and saline conditions.

**MATERIALS AND METHODS**

**Chemicals**

Potassium citrate tribasic monohydrate, polyethylene glycol (PEG) 400, hydrochloric acid, acetic acid and Bovine serum albumin protein (BSA), >98% were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Citric acid was obtained from Merck. The ILs, IoliLyte 221 PG, >95% and cholinium dihydrogen phosphate (Ch DHp), >98%, were obtained from Iolitec. Acetonitrile and Methanol (HPLC grade) were provided by Biosolve and ethyl acetate by Fisher Scientific.

**Microalgae cultivation, harvesting and cell disruption**

*Neochloris oleoabundans* (UTEX 1185, University of Texas Culture collection of Algae, USA) was cultivated in a fully automated 1300 L vertical stacked tubular photobioreactor (PBR) located at AlgaePARC, The Netherlands. It was cultivated under saline and freshwater conditions, both in Bold’s Basal medium at a pH value of 8.0 and the temperature was controlled at 30 °C. To cultivate microalgae under saline conditions artificial seawater was used: NaCl: 24.5 g L⁻¹; MgCl₂: 9.8 g L⁻¹; CaCl₂: 0.53 g L⁻¹; K₂SO₄: 0.85 g L⁻¹; NaSO₄: 3.2 g L⁻¹; NAHCO₃: 0.8 g L⁻¹. The microalgae were harvested (80 Hz, 3000 g min⁻¹) using a spiral plate centrifuge (Evodos 10, Evodos, The Netherlands). The biomass paste was suspended in MilliQ® water to obtain a solution containing 6% of solids and was disrupted by using a horizontal stirred bead mill (Dyno-Mill ECM-AP) using zirconia beads with bead size of 0.5 mm. Bead-milled microalgae was centrifuged (20 min, 20 000xg) to separate most of the cell debris from the supernatant. The resulting extract (supernatant) was stored at −20 °C until further use.

**Fractionation of pigments and proteins from microalgae using ATPSs**

Previously, three promising ATPSs were selected and characterized for the separation of microalgae components. The selection of the ILs was based on their interaction with the protein Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), a protein present in microalgae with a molecular mass of ~560 kDa consisting of noncovalently bound eight large subunits (~56 kDa) and eight small subunits (~14 kDa). Potassium citrate and PEG 400 were selected due to their biocompatibility and ability to form ATPS with these ILs. The composition of the mixtures prepared for the partitioning of microalgal pigments and proteins is shown in the Table 1 of the supporting information.

The ATPS mixtures were prepared gravimetrically (10⁻⁴ g) by using mixture points along four tie-lines (TLs) with a volume ratio (V_t) of one between the top and bottom phase as described by Suarez Ruiz et al. One gram of microalgal extract was added and MilliQ® water was used to complete 10 g in each system. Mixtures were protected from light during the fractionation process. These were stirred for 1 h in a rotatory shaker (50 rpm) and left to equilibrate at room temperature. To facilitate the separation of the aqueous phases from the interface all systems were centrifuged for 5 min at 1200 xg. The phases (top, bottom and interface) were carefully separated and the volume and weight were noted. The interfaces were resuspended in MilliQ® water to facilitate the quantification of the biomolecules. Possible interferences of phase-forming components on the analytical method were taken into account, and control samples were prepared using water instead of microalgae extract. At least two individual samples for each condition were prepared and the biomolecules were quantified. The results were reported as the average of two independent experiments with the respective standard deviation.

**Pigment analysis**

Pigments in each phase (top, bottom and interface) were analysed by measuring the absorption spectrum between 200 and 750 nm.
and protein content was determined by Bradford’s method using corn software. Samples were collected after purification by FPLC. FPLC chromatograms at 280 nm were integrated using the GE Uni-ductivity and pH monitors were used. For protein quantification, wavelength of 280 nm and to control the purification process detection was conducted by a UV/Vis absorbance detector at a wavelength of 595 nm was measured using a Tecan infinite M200 plate reader.

Reversed-phase high-performance liquid chromatography

RP-HPLC was used to identify and quantify the pigments after separation in ATPS. HPLC analysis was performed in a Shimadzu system coupled with a photo-diode array detector (SPD-M20A) and an Acclaim™ C30 LC reversed-phase column from Thermo Scientific™ was used. Three mobile phases were used: (A) Acetonitrile, (B) Methanol/Ethyl acetate 1:1 (v/v) and (C) 200 mmol L⁻¹ Acetic acid in water with the gradient shown in Table 1. The flow rate was set at 1.5 mL min⁻¹ and the column temperature at 30 °C.

Three pigments were identified and quantified (lutein, chlorophyll a, chlorophyll b) and extraction efficiencies (%w/w) were calculated with Eqn (2), where \( m_{\text{lutein},\text{initial}} \) is the initial mass of lutein in the microalgae.

\[
EE_{\text{Lutein} \%} = \frac{C_{\text{lutein},\text{top}} \times V_{\text{top}}}{m_{\text{lutein},\text{initial}}} \tag{2}
\]

Protein analysis

Proteins in each phase were separated from the phase forming components and quantified by Size Exclusion Chromatography (SEC) on an Äkta pure FPLC system equipped with a Hi Trap 5 mL desalting column with Sephadex G-25 resin (both GE Healthcare), inline detectors and a fraction collector. Samples were injected using a 100 µL injection loop system and were eluted with 0.05 mol L⁻¹ sodium phosphate, 0.15 mol L⁻¹ sodium chloride pH 7 buffer at a flow rate of 1 mL min⁻¹ at room temperature. Peak detection was conducted by a UV/VIS absorbance detector at a wavelength of 280 nm and to control the purification process conductivity and pH monitors were used. For protein quantification, FPLC chromatograms at 280 nm were integrated using the GE Unico software. Samples were collected after purification by FPLC and protein content was determined by Bradford’s method using the Pierce™ Coomassie Plus (Bradford) Assay Kit. Absorbance at 595 nm was measured using a TECAN infinite M200® plate reader. Calibration curves were prepared for both quantification methods with Bovine (BSA) pure protein in MilliQ® water.

Partition coefficient \( (K_p) \) was calculated from the ratio of protein concentrations between top and bottom phase [Eqn (3)]. Protein distribution between the three phases was described by the protein extraction efficiency in each phase \( (EE_{\text{protein} \%}) \) which expresses the ratio of protein amount between the top, interface or bottom phase and the total amount [Eqn (4)]). \( m_{\text{protein},\text{initial}} \) is the initial mass of protein in the microalgae extract added.

\[
K_p \text{ protein} = \frac{C_{\text{protein},\text{top}}}{C_{\text{protein},\text{bottom}}} \tag{3}
\]

\[
EE_{\text{protein} \%} = \frac{C_{\text{protein},\text{top}} \times V_{\text{top}}}{m_{\text{protein},\text{initial}}} \tag{4}
\]

The selectivity of pigments considering the presence of proteins in the top phase was calculated with Eqn (5):

\[
S_{\text{pigment/protein}} = \frac{K_p \text{ pigments}}{K_p \text{ proteins}} \tag{5}
\]

Statistical analysis

All experiments were conducted in duplicate and results were reported as the average of two independent experiments with the respective standard deviation. Statistical analysis was performed using Statistica 10.0 software. One-way ANOVA and Tukey honestly significant difference tests were implemented to assess significant differences among the different treatments.

Electrophoresis

In order to investigate the conformation of the proteins before and after the partitioning step, the samples were analysed by native gel electrophoresis (Native-PAGE). The samples were diluted with native sample buffer in the ratio 1:2 and applied on a 4–20% Criterion TGX (Tris-Glycine eXtended) precast gel. The gel was run in a 10× Tris glycine native buffer at 125 V for 75 min. The native gel was stained with the Pierce Silver Stain Kit from Thermo Scientific. The precast gels and buffers were procured from Bio-Rad.

RESULTS AND DISCUSSION

Three ATPSs – polyethylene glycol (PEG) 400–potassium citrate, loliyite 221PG-potassium citrate and PEG400-Cholinium dihydrogen phosphate (Ch DHp) – were selected to evaluate the partitioning of pigments and proteins from \( N. oleoabundans \) extract. These systems were selected previously based on their interaction with the protein Rubisco as explained by Suarez Ruiz et al. Microalgae cultivated under saline and freshwater conditions were used to investigate the feasibility of this technique in a multiprod biorefinery approach. A brief schematic representation of the process presented in this paper is shown in Fig. 1(a).

Pigment partitioning in ATPSs

As described in Fig. 1(b), loliyite 221PG-citrate consists of a top phase rich in IL (loliyite 221PG) and a bottom phase rich in citrate. PEG400-citrate and PEG400-Ch DHp ATPSs both consist of a top phase rich in polymer (PEG400) and a bottom phase rich in potassium citrate and Ch DHp, respectively. \( Neochlorella oleoabundans \) pigments (carotenoids and chlorophylls) are hydrophobic molecules and therefore tend to partition to the least-hydrated phase (top phase) as shown by the partition coefficient values \( (K_p > 1) \) in Fig. 2(a). The highest partition coefficients were obtained with loliyite221PG-citrate ATPS \( (K_p = 62 \pm 11) \), followed by PEG 400-citrate \( (K_p = 36 \pm 4) \) and PEG 400-Ch DHp \( (K_p = 36 \pm 0.7) \). Partition coefficient values and extraction efficiencies presented in Fig. 2 were obtained using mixture points along the highest tie line lengths (TLLs). Additionally, in the supporting information, Table 1S provides the concentration of phase-forming components for each TLL and Table 2S provides the effect of the TLL on the \( K_p \) of total pigments.

The effect of the phase composition was investigated based on the TLL, which represents the composition and thermodynamic difference of the two phases. The partition coefficient of pigments tends to increase with the TLL and this effect seems to be consistent in the three systems (Table 2S). Increasing the component concentration in the three systems will enhance the salting out...
Selective and mild fractionation of microalgal protein and pigments

Figure 1. (a) Brief diagram of the process followed in the separation of proteins and pigments from microalgae; (b) Description of the main phase-forming components in each aqueous two-phase system (ATPS).

Table 2. Molecular structure of two important pigments in *N. oleoabundans* and the main components of the top phases

| Molecule                 | Chemical structure |
|--------------------------|--------------------|
| Lutein                   | ![Lutein structure](image) |
| Chlorophyll a            | ![Chlorophyll a structure](image) |
| Polyethylene glycol 400 (PEG 400) | ![Polyethylene glycol 400 structure](image) |
| Iolylite 221PG           | ![Iolylite 221PG structure](image) |

Effect, resulting in a less hydrated top phase and an increase of the pigments concentrated in this phase.

Few authors have studied the partitioning of pigments and carotenoids in ATPSs and they all show that the recovery of these highly hydrophobic molecules is higher when the hydrophobic nature of the top phase is increased.20,32,33 Montalvo-Hernández *et al.*34 studied the partitioning of crocins (carotenoids) in four types of ATPSs: polymer–polymer, polymer–salt, alcohol–salt and IL–salt. In the polymer–salt system, increasing molecular weight and TLL leads to higher recovery of the carotenoids due to the higher hydrophobicity of the upper phase. Similarly, for IL–salt, hydrophobic interactions related to the alkyl chain length of the cation seem to be the main driving force for the partitioning of this carotenoid. PEG molecular weight is directly related to PEG hydrophobicity. PEG and Iolylite 221PG were compared previously and the higher ability of Iolylite 221PG to form ATPS is related to its higher hydrophobicity nature as a consequence of its alkyl chain length and molecular weight.29 Comparing the molecular weight of the polymer and the IL, Iolylite 221PG (n = 5–15) is a more hydrophobic molecule in comparison with PEG 400 (n = 9) (Table 2). This could explain the higher recovery of total pigments in Iolylite221PG-citrate.

The hydration of the bottom phase main component also is important in the separation of hydrophobic molecules.33 In the case of PEG400-Ch DHp, the high affinity of the IL for water may lead to a higher partitioning of pigments to the top phase33,35. However, interactions between the polymer and the IL play an important role in the partitioning of biomolecules, making the partitioning of the molecules more difficult to predict.36 Nevertheless, the extraction of hydrophobic molecules in polymer–IL ATPSs could be enhanced by the correct selection of the IL employed based on these observations.

The salinity of the medium did not affect the preference of the pigments to migrate towards the most hydrophobic phase ($K_p > 1$). Microalgae cultivated under saline and freshwater conditions were used to evaluate the feasibility of using ATPSs to fractionate biomolecules from different sources (e.g. microalgae cultivated under different conditions). No significant differences were found ($P > 0.05$) when comparing the partition coefficients of total pigments from microalgae cultivated under saline and freshwater conditions. This is in agreement with the $K_p$ results obtained increasing the concentration of NaCl% in the ATPS mixtures. Figure 1S (supporting information) presents the effect of salt concentration (NaCl, %w/w) on the partitioning of pigments. Although a slight increase trend is observed, only the $K_p$ obtained with 3% NaCl in PEG 400-Ch DHp was significantly higher ($P < 0.05$). The medium to cultivate microalgae under saline conditions was prepared using 2.5 (%w/v) of NaCl. Thus, it does not seem that the salinity in the cultivation medium affects the partitioning of pigments.

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pigments. This is an advantage for the future application of the process, because no washing steps would be necessary before the fractionation process.

Relative partition coefficients were calculated based on the absorbance of total pigments using UV–Vis spectrophotometry at 470 nm. UV-Spectroscopy is used widely to analyse the absorption spectrum of pigments in different solvents. Although it provided information about the overall pigment spectrum, the quantification of specific pigments (e.g. chlorophyll a) was affected by their degradation products. The absorption spectrum of the degradation products of pigments overlapped with the target pigments (e.g. chlorophyll a). Therefore, the quantification of specific chlorophylls and carotenoids (lutein, chlorophyll a and chlorophyll b) was done by HPLC. This method allowed us to identify the individual pigments of interest in the microalgae and to calculate the recovery of these pigments in the top phase. Chromatograms of the pigments present in microalgae under saline conditions and recovered in the top phase using ATPSs are shown in Fig. 25. This figure shows that lutein had the highest peak followed by chlorophyll b and chlorophyll a (quantified at 660 nm). Lutein was efficiently recovered by Iolilyte 221PG-citrate (93.1 ± 0.6%) and PEG400-Ch DHp (97.3 ± 1.0%), whereas PEG400-citrate only recovered 34.8 ± 1.26%. Chlorophyll a was recovered by PEG400-Ch DHp (50.6 ± 2.3%), but it was not detected in the other two systems at 660 nm (Fig. 2(b)). Exceptionally, chlorophyll b was not found in any of the top phases. The lack of chlorophyll a and chlorophyll b in some ATPSs could be due to low extraction efficiencies or a change in the molecule structure by oxidation. Chlorophyll stability is affected by temperature, light irradiance, acids, bases and oxygen, causing the loss of its magnesium ion and/or phytol group and a change in colour to olive-brown. Pigments were not detected in the interfaces and bottom phases by RP-HPLC, due to the lack of or insufficient amount of pigments present in those phases.

The nature, stability and amount of the pigments extracted by ATPSs depend on the phase-forming components. It was observed that the green colour of the top phases was different in each case, being more brownish for Iolilyte-221PG and PEG400-citrate than for PEG-Ch DHp. The brown colour might indicate modification of chlorophyll caused by oxidation, which is in agreement with former studies, where it was established that the quality of chlorophyll could be reduced by some ILs such as propylammonium nitrate yet enhanced by others such as dimethylammonium methanoate.

**Protein partitioning in ATPSs**

Proteins were analysed in the phases after fractionation with ATPSs. Figure 3 shows the partition coefficient of proteins in the three systems using saline- and freshwater-cultivated *N. oleoabundans*. The $K_p$ values show large differences between PEG400-citrate and lollyte 221PG-citrate, in which proteins migrate preferentially to the top phase (high $K_p$ values), and PEG400/Ch DHp, in which $K_p$ values are below 1, showing a preference of the proteins to the opposite phase. The distribution of the proteins among the three phases is shown in Fig. 4(a) using *N. oleoabundans* cultivated under saline conditions and Fig. 4(b) using *N. oleoabundans* cultivated in freshwater. Extraction efficiencies of proteins from microalgae cultivated under saline conditions [Fig. 4(a)] into the top phase was 94.3 ± 2.8% using Iolilyte 221PG-citrate and 61.3 ± 0.9% using PEG400-citrate. These extraction efficiencies (%w/w) are eight to 13 times higher than that found using PEG400-Ch DHp (6.7 ± 0.5%). In previous studies using Rubisco as model protein, results suggested that the high recovery of protein into the IL-rich phase (lollyte 221PG) compared to polymer-based ATPSs is a result of hydrophobic and electrostatic interaction between the...
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Aqueous two-phase systems can generate interfacial partitioning of different molecules, including proteins, and it has gained attention in the last years for large scale-processes. Ionic liquid-based three phase partitioning (ILTPP) has been investigated because it combines the advantages of the IL-ATPS and TPP for the concentration and recovery of different molecules including proteins. ILTPP is capable of inducing the formation of a dense and stable protein layer in the middle with IL-ATPS phase-forming components. Alvarez-Guerra and Irabien suggested that the salt concentration has the greatest influence on the amount of lactoferrin recovered at the interface. However, pH, temperature and protein concentration also influence the partitioning.

The three systems studied in the current research were used previously for the partitioning of different proteins, including Rubisco. No precipitation of these proteins was reported, demonstrating that the studied systems do not always form three phases. Protein partitioning was studied previously using a total concentration of 0.3 mg mL$^{-1}$ of purified Rubisco. In the current study similar microalgal protein amounts were used; 0.4 mg mL$^{-1}$ for freshwater-cultivated *N. oleoabundans* and 0.2 mg mL$^{-1}$ for *N. oleoabundans* cultivated under saline conditions. However, the disrupted microalgae added to the systems contained other biomolecules, such as pigments, lipids, carbohydrates and other proteins apart from Rubisco. The overall composition of microalgae is influenced by cultivation conditions. Microalgae cultivated in freshwater has higher amount of proteins and pigments than microalgae cultivated under saline conditions. When using *N. oleoabundans* cultivated in freshwater more proteins were concentrated in the interface [Fig. 4(b)] than when using *N. oleoabundans* cultivated under saline conditions. This increase reflects a lower solubilizing capacity of the ATPSs on microalgae molecules (e.g. proteins) in these conditions, which may be caused by protein content but also by other molecules present in the microalgae. PEG400-Ch DHp recovered 92.2 ± 1.9% of proteins in the interface, followed by PEG400-citrate (77.2 ± 5.3%) and Iolilyte 221PG-citrate system (59.2 ± 8.2%). We hypothesized that the interfacial precipitation can be caused by the amount of feedstock (including microalgae proteins) added and by the presence of other biomolecules from the microalgae that benefit the emulsification and precipitation of the proteins.

Other authors have reported that ATPSs may form an interface when high protein concentrations are added to the system as well as by increasing the TLL. In Table 3S, the distribution of proteins among the three phases and the influence of the TLL were reported. By increasing the TLL (phase-forming components concentration), more proteins were recovered in the interface instead of the top phase. Temperature, polymer molecular weight, TLL and protein loading affect the solubilizing capacity of an ATPS. In a descriptive model of interfacial partitioning in an ATPS (polymer−salt), Luechau et al. suggested that the interfacial concentration of molecules depends on the phase system, feedstock composition and bioparticles at the interface. Particle size

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**Table 3.** Selectivity results of the separation pigments/proteins from *N. oleoabundans* extract

| ATPS                  | $K_{\text{pigment/protein}}$ |
|-----------------------|-----------------------------|
| PEG 400-citrate       | 0.26                        |
| Iolilyte 221PG-citrate| 0.54                        |
| PEG 400-Ch DHp        | 14.18                       |

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**Figure 4.** Distribution of microalgae proteins among the three phases in the three ATPSs. (a) Saline and (b) freshwater cultivation condition of *N. oleoabundans*. The results represent the average of two independent experiments and error bars indicate standard deviations.
and interfacial tension of the system were used to describe the adsorption of particles in the interface.

In the PEG400-Ch DHp system, proteins were concentrated mainly in the interface and in the bottom phase when using both cultivation conditions. Previous studies have reported the preference of pure proteins to the bottom phase when using ATPS combinations with a more hydrophobic polymer (PPG400) and cholinium-based ILs. Li et al. studied protein partitioning using systems composed of PPG 400 and various cholinium-based-ILs. They reported a decrease in the protein extraction efficiency in the IL-rich phase due to an increase in protein size. The proteins studied were Lysozyme, Papain, Trypsin and BSA. They argued that protein partitioning to the IL-rich phase (bottom phase) requires energy to break the interactions between phase components. Thus, smaller proteins require less energy than larger proteins to migrate into the bottom phase. Previous results reported by us agree with this theory. Rubisco with a higher molecular weight (~560 kDa) than the proteins reported by Li et al. was partitioned preferentially to the PEG-rich phase (top phase) when using PEG400-Ch DHp. This indicates the importance of protein size in this particular ATPS formed by polymer and cholinium based-ILs. Other studies explained the effect of protein structure (e.g. surface amino acid residues) on the partitioning of these molecules using ATPSs formed by polymer and ILs. Concluding that protein partitioning depends on specific interactions (e.g. electrostatic) between the protein and the IL.

Microalgae contain a high amount of proteins with different molecular weights and structures, making the prediction of protein partitioning in PEG400-Ch DHp even more difficult. Molecular interactions between the proteins and the IL (Ch DHp) seems to be a strong driving force for the partitioning of proteins to the bottom phase. However, protein size, feedstock load and the presence of other molecules could trigger the interfacial partitioning of the proteins.

In order to study the conformation of proteins before and after the separation process, Native-PAGE electrophoresis was performed to the initial microalgae added and to the proteins recovered in the interface. Figure 5(a) shows the proteins in the extract of microalgae cultivated under saline and freshwater conditions. Rubisco (~560 kDa) was identified as the most abundant protein known in N. oleoabundans and was therefore used as biomarker. Other proteins present in the extract have not...
been characterized, but other bands were detected. In Fig. 5(b), a Rubisco band appears for the three systems, indicating that this protein remains intact also after interfacing partitioning with the ATPSs. However, the band of Rubisco in the loliylte 221PG-citrate system is faint, indicating the influence that the loliylte221PG has on the protein. The effect of loliylte221PG on Rubisco was studied previously, showing that when increasing the amount of this IL, the protein loses its native conformation and forms aggregates.\textsuperscript{14,29} PEG-Ch DHp and PEG-citrate seem to retain the native conformation not only of Rubisco, but also of other proteins from \textit{N. oleoabundans} (Fig. 5). These systems are therefore considered for the separation of proteins. However, further characterization and techno-functional evaluation of the proteins recovered is needed for their use in different industrial applications.

**Selectivity**

Table 3 shows the selectivity results of ATPSs for the fractionation of pigments and proteins from microalgal extracts. Polymer–IL ATPSs shows higher selectivity between proteins and pigments due to the preference of the pigments for the top phase and of the proteins for the bottom phase. These results suggest that polymer–IL systems do not exclude molecules by salting out, but hydrogen bonding and molecular interactions between the protein and ATPS components allow the molecules to partition, allowing for high selectivity between protein and pigments. As discussed before, this depends on protein concentration as high protein concentrations will cause significant size exclusion effects (interfacial partitioning).

PEG400-Ch DHp partition behaviour is clearly beneficial for the partitioning of the molecules in different phases (Fig. 6). Besides that, proteins recovered in the interface seem to conserve their native conformation based on electrophoresis experiments and pigments did not suffer oxidation in the top phase. Recovery of the proteins in the bottom phase and recycle of the cholinium dihydrogen phosphate for reuse is possible by ultrafiltration as described by Ramalho et al.\textsuperscript{24} A large number of biocompatible ILs such as Ch-DHp are being synthesized,\textsuperscript{26} which opens up research opportunities for polymer–IL ATPSs. This type of ATPS and their recyclability will be studied in the future to separate microalgae biomolecules in an integrated biorefinery approach. The partitioning behaviour of other molecules of industrial interest such as lipids and carbohydrates from microalgae should be investigated to design a correct biorefinery approach.

**CONCLUSIONS**

As a first step towards the development of a multiproduct microalgae biorefinery, three kind of ATPSs were investigated to separate proteins and pigments from microalgal extract. The traditional polymer–salt (PEG400-citrate) and two IL-ATPS: IL–citrate (loliylte221PG-citrate) and polymer–IL (PEG400-Ch DHp). Although loliylte221PG-citrate showed outstanding partition coefficient for pigments, a high amount of proteins also moved to the top phase. This behaviour resulted in low selectivity between pigments and proteins. The quality of pigments and proteins separated by loliylte 221PG-citrate were considered low as pigments suffered degradation (oxidation) and the proteins did not retain their native form.

The PEG400-Ch DHp system was the most selective for separating pigments (top) and proteins (interface and bottom): 97.3 ± 1.0% of the lutein content in \textit{N. oleoabundans} extract was separated into the top phase and a very low amount of proteins moved to the top phase. Proteins moved preferentially to the bottom phase and interface. High protein concentration load resulted in interfacial precipitation, up to 92.2 ± 2.0% of proteins precipitated in the interface. This interfacial partitioning is considered an advantage in the separation of complex matrices such as microalgae, because it combines separation and concentration of proteins. Thus, proteins in the interface can be directly recovered and do not need to be extracted from the phase forming components. This simplifies the recycling of phase forming components. Besides that, proteins recovered in the interface seem to conserve their native conformation based on electrophoresis experiments and pigments did not suffer oxidation in the top phase. We demonstrated the potential of ATPS to separate different biomolecules simultaneously, giving value to different microalgae components for a sustainable multiproduct biorefinery. Furthermore, components from microalgae cultivated in saline water and freshwater can be selectively fractionated using ATPS. Using salt water to cultivate microalgae can reduce costs and the freshwater footprint for large scale production, which is an advantage in view of a sustainable process.

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**Supporting Information**

Supporting information may be found in the online version of this article.

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