



Selective fractionation of free glucose and starch from microalgae using aqueous two-phase systems



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ABSTRACT

Microalgae are a promising source of lipids, pigments, proteins and carbohydrates, which are valuable compounds for many industries. However, optimal fractionation and valorization of all produced compounds is necessary to improve the economic viability of microalgae production. This paper aims to understand the fractionation of microalgae carbohydrates (free glucose and starch) in aqueous two-phase systems. Three aqueous two-phase systems were investigated to efficiently and mildly separate carbohydrates from disrupted *Neochloris oleoabundans*. This strain contains 16 w/w% of proteins, 48 w/w% total fatty acids and 27 w/w% carbohydrates when cultivated under saline water and nitrogen depletion conditions. The protein content decreases and the amount of fatty acids and carbohydrates increases notably under stress conditions and glucose becomes the main carbohydrate in this microalgae. Glucose is present in the disrupted microalgae as part of polymeric carbohydrates (starch) or in monomeric form (free glucose). With the aqueous two-phase system Polyethylene Glycol 400 - Cholinium dihydrogen phosphate (PEG400-ChDHP) microalgal free glucose is fractionated up to a recovery of 99% to the most hydrated bottom phase in a single step. Simultaneously, a recovery of 70% is reached for microalgal starch in the interface after two additional liquid-liquid extractions with PEG400-ChDHP. The final fractions obtained were free of pigments.

1. Introduction

Carbohydrates derived from renewable biomass are a promising and sustainable alternative for the petroleum-based products that are used in diverse applications (e.g. renewable energy, commodity chemicals, bioplastics, food and feed additives). For these purposes, microalgae have received increased attention [1–4]. These microorganisms can accumulate up to 60% carbohydrates, depending on the species, growth and environmental conditions [5]. Compared to plant-based feedstock, microalgae are efficient photosynthetic organisms, have high growth rates and they do not need arable land and fresh water, since they can grow in brackish water, seawater and even wastewater [6]. Currently, microalgal carbohydrates are investigated mainly for the production of biofuels [7,8]. However, carbohydrates are also a promising ingredient for the food and animal feed industries [9] and for the production of new materials such as biopolymers [10] or used as fermentation feedstock to produce hydrocarbons, lower alcohols, diols and carboxylic acids [11].

Eukaryotic microalgae and cyanobacteria accumulate carbohydrates in the plastids and cytosol as storage components (e.g. starch and free glucose) or as part of the cell wall in the form of (hemi)cellulose and polysaccharides [12], also some species can produce extracellular polysaccharides [13]. The type of carbohydrates present in microalgae is species-dependent. Red algae synthesize floridean starch, while green algae synthesize amylopectin-like polysaccharides (starch). The composition of the cell wall depends on the microalgae strain as they are complex and poorly understood. Moreover, growth conditions might affect the carbohydrate composition as well. Lignin is not present in microalgae, and some species lack hemicellulose, cellulose or a cell wall [8]. These facts can be considered advantages for biorefinery, since harsh pretreatments that are normally used for lignocellulosic feedstock are not needed.

Among the carbohydrates present in green microalgae, starch is an important polysaccharide essential in human nutrition and food industry [14] and even in non-food applications. Its composition and structure varies between the sources, influencing its physico-chemical

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properties and functionalities [15]. It is widely used in food industry as thickener, emulsifier, gelling agent and stabilizer. Some non-food industry applications are: paper, adhesives, rubber production, formulation of pharmaceutical and cosmetic products, microcapsules for small molecules and for the production of high quality biodegradable plastic films [15–17]. Limited research has been done on the extraction and use of starch from microalgae for food applications and other industries, which opens up many research opportunities in strain development, biorefinery, starch structure and functionality [18].

Common methods to extract polysaccharides from microalgae cells and convert them into easily fermentable sugars involve: hydrolysis using harsh acidic (H_2SO_4 and H_3PO_4) or alkaline conditions (NaOH and ammonium) or enzymatic methods [19]. Additionally, physical methods include: hot-water treatment, microwave-assisted extraction and ultrasonic-assisted extraction [20]. Organic solvents are generally used for the separation of hydrophobic and hydrophilic components from the disrupted microalgae cells. These conditions, however, could affect the structure and functionality of other microalgae valuable components such as proteins and pigments [21,22]. Therefore, it is preferable to find novel and mild alternatives to the conventional extraction technologies.

Aqueous two-phase systems (ATPS) have emerged as a more biocompatible and more efficient liquid-liquid extraction method for the fractionation and purification of biomolecules [23,24]. ATPS is formed by two immiscible aqueous phases, providing a mild environment for the biomolecules, because their main component is water. ATPS is a technologically simple process, low cost and easy to scale up [25]. The broad collection of phase forming components that exist, make this technology very versatile. Additionally, ionic liquids (ILs) have been studied to enhance the extraction efficiencies of different target molecules. ILs are molten salts with unique characteristics that make them very attractive for separation processes. They possess low vapor pressure, are non-flammable, have good thermal and chemical stabilities and great solvation abilities [26]. They are considered a “green” solvent, are tunable and multipurpose materials due to their ionic character, structure and organization [27].

Aqueous two-phase systems have been studied mainly for the fractionation of proteins and enzymes [28]. The interest in applying this technique to biological mixtures has led to the research of other target molecules like carbohydrates, pigments and small bioactive compounds. ATPSs were employed to purify and concentrate carbohydrates from *Cordyceps sinensis* [29], *Aloe vera* [30], *Brassica oleracea* L. [31] and *beetroots* [32]. This is the first study to investigate the separation of microalgal carbohydrates (free sugars such as glucose as well as starch) using aqueous two-phase systems, and its integration in a multi-product biorefinery concept.

2. Materials and methods

2.1. Materials

The ionic liquids used: IoliLyte 221 PG, > 95% and choline dihydrogen phosphate (ChDHP), > 98% were procured from Iolitec (Ionic Liquid Technologies, Germany). Potassium citrate tribasic monohydrate, polyethylene glycol (PEG) 400, hydrochloric acid, and standard D-(+)-glucose were purchased from Sigma-Aldrich. Citric acid was obtained from Merck and starch from maize was included in the Megazyme® kit (Wicklow, Ireland). The GOPOD kit was also from Megazyme and all other chemicals were from the highest purity.

2.2. Microalgae cultivation, harvesting and cell disruption

To study the carbohydrate content in microalgae, four cultivation conditions were used: Fresh water, artificial seawater, nitrogen depletion (N⁻) and no nitrogen depletion (N⁺).

Neochloris oleoabundans (UTEX 1185, University of Texas Culture

collection of Algae, USA) was cultivated in a fully automated 1300 L vertical stacked tubular photo bioreactor (PBR) located at AlgaePARC, The Netherlands. It was cultivated using Bold's Basal medium [33] 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; MgCl_2 : 9.8 g/L; CaCl_2 : 0.53 g/L; K_2SO_4 : 0.85 g/L; NaSO_4 : 3.2 g/L; NaHCO_3 : 0.8 g/L. The microalgae were harvested (4000 rpm) using a spiral plate centrifuge (Evodos 10, Evodos, The Netherlands) and the biomass obtained was suspended in Milli-Q® water to obtain a biomass concentration of ~90 g/L. The algal cells (approx. 28 ml) were disrupted for 10 min in a horizontal stirred bead mill (Dyno-Mill Research Lab from Willy A. Bachofen AG Maschinenfabrik, Switzerland) with a milling chamber of approx. 79.6 ml using 0.5 mm ZrO_2 beads (approx. 51.6 ml) as described by Postma et al. [34]. Bead milled microalgae suspension was stored at -20 °C until further use.

2.3. Microalgal characterization

2.3.1. Carbohydrates

The total carbohydrate content was determined with the Dubois method [35] whereby glucose was used as standard and analysis made in triplicate and statistically evaluated by including the standard error. The carbohydrate composition of *N. oleoabundans* was determined by High Performance Anion Exchange Chromatography (HPAEC) as described by Gilbert-López et al. [36]. The microalgae were first freeze dried and subsequently acid hydrolyzed before their composition was determined by HPAEC. Deoxy-galactose was used as internal standard and experiments were carried out in single.

2.3.2. Protein

Protein analysis was done according to Gilbert-López et al. [36] using a FlashEA 1112 nitrogen analyzer (Thermo Fisher Scientific, 337 Waltham, MA, USA). D-methionine was used as standard and a N-to-protein conversion factor of 5.5 was used to calculate total protein from total nitrogen. Analyses were made in triplicate and statistically evaluated by including the standard error.

2.3.3. Lipids

Samples were freeze dried before analysis of the total fatty acid concentration. The analysis consisted of a sequence of mechanical cell disruption, solvent-based lipid extraction, transesterification of fatty acids to fatty acid methyl esters (FAMES), and quantification of FAMES using gas chromatography (GC-FID) as described by Breuer et al. [37]. The triacylglycerides (TAGs) and polar acyl lipids (PLs) were fractionated using solid phase extraction. TAGs were eluted from the column using 10 mL 7:1 (v/v) hexane:diethylether. Subsequently, PLs were eluted using 10 mL 2:2:1 (v/v/v) methanol:acetone:hexane [38]. Total fatty acid (TFA) composition and content were calculated by taking the sum of all fatty acids in both fractions [37]. Analyses were made in triplicate and statistically evaluated by including the standard error.

2.4. Preparation of aqueous two-phase systems

Based on the total carbohydrate content *N. oleoabundans* cultivated in saline water and nitrogen depleted conditions were used for the fractionation experiments. Three ATPSs were selected for the fractionation of microalgae biomolecules [39]. This selection was based on biocompatibility, low toxicity and their interaction with the protein Rubisco (Ribulose-1,5-biphosphate Carboxylase Oxygenase), which is present in microalgae and able to lose its native conformation under non-mild conditions. Mixtures were prepared gravimetrically $\pm 10^{-4}$ g with a volume ratio (V_r) between top and bottom of 1. D-glucose and starch from maize were selected as standard molecules to study their partitioning behavior. The total concentration of the standard molecules in the mixture was 0.05 g/L. We prepared the ATPS mixtures with

Table 1
Concentration (w/w%) of phase forming components used for the separation experiments.

Tie line	Polymer - ionic liquid		Polymer - salt		Ionic liquid - salt	
	PEG400	ChDHp	PEG400	Citrate	lilolyte 221PG	Citrate
1	29.4	30.4	21.3	31.3	18.7	26.8
2	33.3	31.2	21.8	32.6	20.7	28.9
3	33.9	35.0	23.9	35.7	25.3	28.4
4	35.8	36.8	25.2	39.0	28.6	31.3

increasing the phase forming component concentrations along four tie lines previously constructed by Suarez Ruiz et al. [39] (Table 1).

To study the fractionation of microalgae carbohydrates, 1 g of bead milled *N. oleoabundans* suspension was added to each system and Milli-Q® water was finally added to complete 10 g in each system. Experiments were made in duplicate and blanks without microalgae or standard molecules were prepared as control for the analysis methods. All mixtures were mixed for 1 h at room temperature in a rotary shaker at 50 rpm and centrifuged at 2500 rpm (1200 × g) for 10 min at room temperature to ensure phase separation. Afterwards, the phases were separated and the weights and volumes were noted.

To perform the second and third fractionation steps, the interface was gently separated from the aqueous phases and weighted. Subsequently, the same amount of phase forming components as in the first extraction was added to the interface.

2.5. Quantification of glucose

Two methods were used to quantify glucose before and after the fractionation experiments: a YSI 2700® biochemistry analyzer (Yellow Springs Instruments) and the Megazyme® kit (Wicklow, Ireland): Soluble sugars in ATPS were quantified by the reaction with a solution containing *p*-hydroxybenzoic acid, sodium azide (0.095% w/v), glucose oxidase plus peroxidase and 4-aminoantipyrine (GOPOD reagent) from Megazyme®. Samples were mixed with the reagent at a ratio 0.1:3 (v/v) and incubated at 50 °C for 30 min. After cooling down to room temperature, quantification was conducted by measuring absorbance at 510 nm using a spectrophotometer (Hach Lange DR6000). GOPOD reagent and glucose were used as blank and standard, respectively. Analyses were made in triplicate and statistically evaluated by including the standard error.

2.6. Starch analysis

Starch content was quantified before and after fractionation experiments by the total starch protocol of Megazyme® kit (Wicklow, Ireland) adapted from Dragone et al. [40]. Maize starch was used as a positive control and calibration curves were made from D-glucose. The quantification was performed by absorbance at 510 nm using a spectrophotometer (Hach Lange DR6000). Analyses were made in triplicate and statistically evaluated by including the standard error.

To describe the distribution of glucose and starch in the ATPS, partition coefficients (Kp) and extraction efficiencies (EE%) were calculated. For glucose, for example, Eq. (1) and Eq. (2) were used to calculate Kp and EE%, respectively. $C_{glucose}$ is the concentration of glucose in the phase, V the volume and $m_{glucose, initial}$ the initial mass of glucose in the microalgae added to the systems.

$$K_{p_{glucose}} = \frac{C_{glucose, top}}{C_{glucose, bottom}} \quad (1)$$

$$EE_{glucose} \% = \frac{C_{glucose, top} * V_{top}}{m_{glucose, initial}} \quad (2)$$

In the separation of carbohydrates from microalgae using ATPS an

Table 2

(Bio)chemical composition (w/w%) of *N. oleoabundans*. All measurements were performed in triplicate and standard error included.

Growth condition		Carbohydrates	Protein	Total fatty acids (TFA)
Fresh water	N+	10.7 ± 0.7	44.7 ± 0.4	12.4 ± 0.0
	N-	17.4 ± 0.5	18.7 ± 1.4	44.1 ± 0.8
Artificial seawater	N+	13.3 ± 0.2	49.6 ± 0.6	7.7 ± 0.0
	N-	27.1 ± 0.1	16.0 ± 0.4	48.0 ± 6.2

interface was formed. This interface was carefully separated from the aqueous phases and its glucose content and starch were analysed to complete the mass balance Eq. (3). $m_{glucose, interface}$ represents the mass of glucose in the interface and $m_{glucose, initial}$ the initial mass of glucose in the microalgae added to the systems.

$$EE_{glucose} \% = \frac{m_{glucose, interface}}{m_{glucose, initial}} \quad (3)$$

3. Results and discussion

3.1. Carbohydrate composition of *Neochloris oleoabundans*

Table 2. presents the biochemical composition (carbohydrates, proteins and total fatty acids) of *N. oleoabundans* cultivated under four different conditions. These conditions were: artificial sea water or fresh water under nitrogen depletion (N-) or nitrogen repletion (N+). It can be observed that the growth conditions notably influence the biochemical composition of the microalgae. Under saline and nitrogen depletion conditions *N. oleoabundans* accumulates more carbohydrates and lipids (TFA), while the protein content decreases. The increase of carbohydrates and lipids in this microalgae has previously been used as a strategy to provide a more economically feasible scenario for microalgal-based biofuels [38,41]. Furthermore, the use of salt water can reduce production costs and the fresh water footprint of large scale microalgae production [42].

The industrial application of carbohydrates depends on the chemical and physical properties of these biomolecules. Therefore, the carbohydrates accumulated in *N. oleoabundans* cultivated under different conditions were characterized. The total carbohydrate content in *N. oleoabundans* was ~10.7 w/w% dry matter when it is cultivated in fresh water and an excess of nitrogen (N+). When the microalgae grow in artificial saline water and under nitrogen depletion (N-), the content of total carbohydrates increases up to ~27.1 w/w% dry matter.

The carbohydrate composition (mol%) of *N. oleoabundans* is presented in Table 3 which were performed in duplicate with the main focus showing large changes in the composition and not having the intention to know the exact value. Whereas for the total carbohydrate content the exact values are important and performed in triplicate and statistically evaluated. It is clear that depending on the cultivation conditions, not only the total carbohydrate content changes, but also the carbohydrate profile. The amount of galactose, for example, is higher than glucose when *N. oleoabundans* is grown in fresh water under no nitrogen depletion (N+). Instead, when microalgae are grown in artificial saline water, more glucose is accumulated, surpassing galactose as the main carbohydrate in the algal cells. Additionally, when the microalgae grow under nitrogen depletion (N-), the content of glucose increases. Microalgae cultivated in artificial saline water under nitrogen depletion (N-) the content of total carbohydrates is 27.1 w/w %, with glucose as the most abundant monosaccharide after hydrolysis. This glucose is stored in the microalgae as part of a polysaccharide (starch) or as monomer (free glucose). Microalgae contains under these cultivation conditions 14.3 ± 1.4 w/w% of starch and 9.8 ± 1.3 w/w % of free glucose.

The effect of the cultivation conditions on the carbohydrate profile has been addressed in different studies and this knowledge has been

Table 3
Carbohydrate composition in (mol%) of *Neochloris oleoabundans* in different cultivation conditions performed in duplicate.

Growth condition	Fresh		Artificial saline	
	N +	N –	N +	N –
Carbohydrate				
Fucose	0.0	0.0	0.0	0.0
Arabinose	3.8	2.9	2.7	0.6
Rhamnose	20.0	10.6	11.8	2.2
Galactosamine	0.0	0.0	0.0	0.0
Galactose	30.0	11.6	23.1	10.3
Glucosamine	7.6	5.2	4.0	0.2
Glucose	22.0	55.3	50.2	76.9
Xylose	4.3	5.1	2.2	0.9
Mannose	8.7	3.3	3.8	3.5
N-Acetyl-galactosamine	0.0	0.0	0.0	0.0
N-Acetyl-glucosamine	0.0	0.0	0.0	0.0
Galacturonic acid	0.0	0.0	0.0	0.0
Glucuronic acid	3.7	6.1	2.1	5.4
Total carbohydrate content ^a	10.7 ± 0.7	17.4 ± 0.5	13.3 ± 0.2	27.1 ± 0.1

^a Total carbohydrate content (w/w% dry matter) carried out in triplicate and standard error included.

used to accumulate metabolites of interest in microalgae cells [38,43,44]. Nitrogen depletion is widely used for this purpose, especially to enhance the production of fatty acids. In the absence of a nitrogen source, the flow of fixed carbon is diverted from proteins to energy and carbon storage compounds. Thus, nitrogen depletion leads to the accumulation of lipids and/or carbohydrates (mainly starch). Salt stress also causes the accumulation of carbohydrates in different microalgae strains [43]. As NaCl is a commonly encountered inorganic nutrient in microalgae grown in salty water. High salt water stress generates reactive oxygen species that inhibit Rubisco activity and mediates photo-inhibition which concomitantly decreases biomass growth and accumulation of carbohydrates takes place as a response to an immediate NaCl shock. It seems that the sucrose pathway in the metabolism is most affected by this cultivation condition [45].

The high content of total glucose (as monomer as well as part of polysaccharides) in microalgae has also been reported for other microalgae species [46,47]. *N. oleoabundans* with a high content of carbohydrates represents a promising feedstock to produce biochemicals, biofuels such as bioethanol [41] and other valuable products [48].

3.2. Carbohydrates separation in aqueous two-phase systems

Aqueous two-phase system (ATPS) was studied as a fractionation method for microalgal carbohydrates. D-Glucose and starch from maize were used as standard molecules and the bead milled suspension of *N. oleoabundans* cultivated in artificial saline water under nitrogen depletion (N –) was used as a complex mixture. This cultivation condition was selected due to the high amount of carbohydrates accumulated by the microalgae. Three ATPSs: polyethylene glycol (PEG)400-potassium citrate, Iolilyte 221PG-potassium citrate and PEG400-cholinium dihydrogen phosphate (ChDHP) were selected based on the screening described by Suarez Ruiz et al. [39]. The ionic liquids, polymers and salt used in these experiments were carefully selected for their biocompatibility, low toxicity and ability to form ATPS without affecting the native conformation of the proteins. A schematic summary of the process performed and a description of the systems phases is shown in Fig. 1.

3.3. Pure D-glucose and glucose from microalgae

D-Glucose was selected as a standard monosaccharide to study its partitioning behavior in the three ATPSs. The separation of this standard molecule was compared with free glucose from microalgae. Both,

the standard monosaccharide (D-glucose) and the free sugar from the microalgae migrate preferentially to the most hydrated phase (bottom phase). This is due to the strong molecular interactions between monomeric sugars and water [49,50]. Recoveries of 82 to 99% of D-glucose were obtained in the bottom phase after a single-step ATPS. The highest extraction efficiencies (%w/w) obtained for D-glucose and microalgae free glucose are shown in Fig. 2. PEG400-ChDHP concentrated the highest amount of free sugars from microalgae in the bottom phase (99%), followed by PEG400-citrate (93%), while Iolilyte221PG-citrate concentrated 82%. Cholinium-based ionic liquids are highly hydrophilic due to the polar hydroxyl group of one of the cation side chains [51]. The hydrophilicity of the IL-rich phase in the PEG400-ChDHP (bottom) phase caused by the amount of water may enhance the separation of carbohydrates in this system.

Carbohydrates (also called saccharides) are polar biomolecules that possess many hydroxyl groups. Monosaccharides as well as some polysaccharides have high water solubility as a result of their ability to establish hydrogen bonds with water [52]. This high affinity of carbohydrates for water seems to be responsible for their preference to migrate to the most hydrated phase (bottom phase). The polymeric carbohydrate component starch is insoluble in water and although containing many –OH functional groups on the surface, water binds to starch forming a gel and partition towards the interface. This polymer presents low solubility in almost any solvent [53]. Starch partitioning in ATPS is discussed in the next section.

The effect of the ATPS phase forming components was studied by increasing the tie line length (TLL), which represents the composition and thermodynamic difference of the two phases. A positive effect was found while increasing the TLL, increasing the concentration of the phase forming components more free sugars are accumulated in the bottom phase (Supplementary data).

Pei et al. [49] describes how the structure of the saccharides seems to influence the partitioning behavior of other molecules (e.g. proteins) present in a solution. A higher amount of hydroxyl groups in the saccharides leads to a higher kosmotropic behavior, enhancing both, hydrogen bonding and hydrophobic interactions. These interactions increase the extraction efficiency of proteins. The hydrogen bond formation between carbohydrates and water reduces the number of free water in the bottom phase, forcing the proteins to migrate to the opposite phase than the carbohydrates. The ability of carbohydrates to form hydrogen bonds is considered an advantage, because carbohydrates migrate to the bottom phase reaching a recovery of 99% while other biomolecules (e.g. proteins) migrate to the opposite phase. Due to this reason ATPS is a very promising technology in the microalgae biorefinery.

3.4. Maize starch and starch from microalgae

The partitioning behavior of starch from microalgae was studied in the three ATPSs. This behavior was compared with the partitioning of starch from maize, selected as a standard polysaccharide. The disrupted microalgal suspension added to the ATPSs forms three phases: top, bottom and a third phase (interface) between the two aqueous phases (top and bottom).

In Fig. 3a) we show the distribution of microalgae starch in the three phases formed. Up to 86% of the microalgae starch was concentrated in the interface in a single-step Iolilyte 221PG-citrate ATPS. Although the polymer/ionic liquid (PEG 400-ChDHP) ATPS fractionates a high amount of starch in the bottom phase (18%) compared with the other two ATPSs, the partitioning preference of starch is clearly to the interface. Unlike the case of D-glucose and free sugars, starch does not prefer to migrate to the most hydrated phase (bottom phase). Some ionic liquids are able to solubilize up to 20% starch, however, the studies found used high temperature (60–100 °C) [54,55]. It seems that the origin of the starch influences the solubility, due to granule form and size differences.

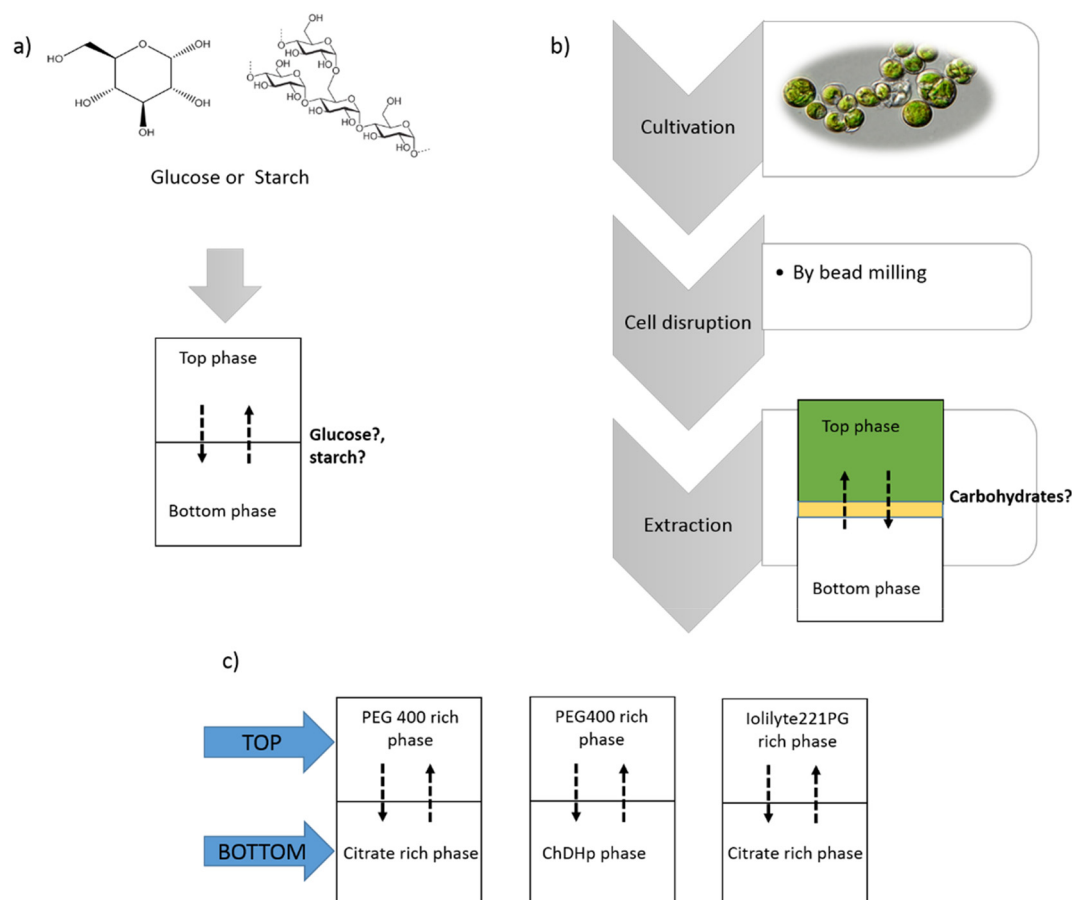


Fig. 1. Schematic representation of the partitioning experiments. a) Partitioning of standard molecules in ATPS: D-glucose and starch from maize; b) Partitioning of carbohydrates from *Neochloris oleoabundans* (bead milled suspension) cultivated in artificial saline conditions under nitrogen depletion (N⁻); c) ATPSs used and the description of the two aqueous phases.

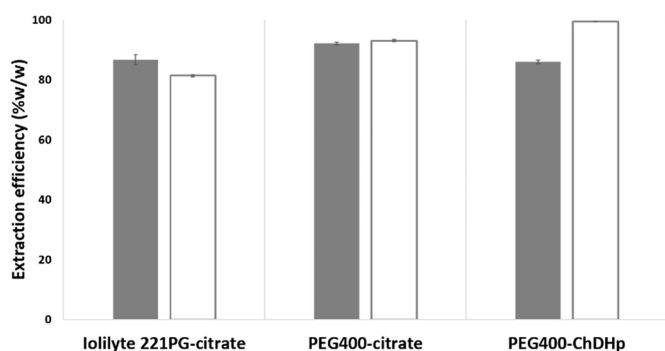


Fig. 2. Glucose partitioning to the bottom phase: Extraction efficiencies (%w/w) using three different ATPSs; Standard glucose (filled bars) and free glucose from *Neochloris oleoabundans* (bead milled suspension) cultivated in artificial saline conditions under nitrogen depletion (N⁻) (open bars). Experiments performed in triplicate and error bars indicate standard deviations.

The concentration of starch in the interface seems to be a consequence of its low solubility in the aqueous solutions used. Fig. 3b) shows the partitioning behavior of purified starch from maize and a bead milled microalgae suspension. The maize starch added to the ATPSs precipitated below the bottom phase and additionally a small layer in the interface was observed. In the aqueous phases (top and bottom) starch was not detected after quantification by the colorimetric method. Starch from maize is not soluble in water at room temperature and it seems that it is neither soluble in the aqueous phases of the ATPSs used. The solubility of starch depends on the amylose and lipid content

and granule organization [56], which may explain the partitioning behavior difference between microalgae starch and maize starch. However, limited information is available in literature about the physico-chemical properties of starch granules in microalgae. Other components in the microalgae suspension (e.g. lipids) may influence the partitioning behavior of microalgae starch, compared to purified starch from maize.

Microalgae suspension forms a thick like-emulsion layer at the interface of the ATPSs, where starch is concentrated together with other microalgae biomolecules. The bead milled microalgae suspension used in the fractionation experiments has soluble and non-soluble components such as cell debris. Furthermore, *N. oleoabundans* cultivated under nitrogen depletion (N⁻) accumulates a high amount of lipids, which may lead to the formation of a stable emulsion after bead milling [57]. In fact, several authors have reported emulsion formation in an attempt of extracting lipids from wet biomass [58]. Thus, the interface layer formed may be caused by other non-soluble components in the microalgae such as lipids and cell debris.

To further improve the purification of microalgae carbohydrates (starch and free glucose), two extra steps of fractionation with ATPS were performed. Fig. 4 shows the distribution of carbohydrates among the three phases in three fractionation steps by PEG400-ChDHp. It was observed that free sugars are separated almost completely in a single-step ATPS (Fig. 4a). Free glucose migrates to the bottom phase due to their high ability to form hydrogen bonding, and only ~5% remains in the interface after the first fractionation step. On the other hand, a low amount of starch (~8%) migrates to the aqueous phases after three fractionation steps. The microalgae starch remains in the interface (~70%) after three fractionation steps (Fig. 4b). The purification of

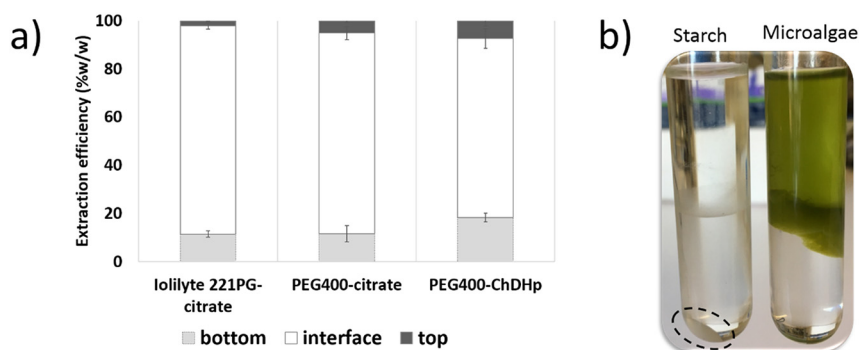


Fig. 3. Purified starch from maize and microalgae partitioning in three ATPS. a) Distribution of starch from *Neochloris oleoabundans* (bead milled suspension) cultivated in artificial saline conditions under nitrogen depletion (N⁻) among the three phases in the ATPS. Experiments were carried out in triplicate and error bars indicate standard deviations; b) Picture of purified starch from maize and microalgae in PEG400-ChDHP ATPS.

starch increases with the number of steps, due to the fractionation of other components of the microalgae (e.g. pigments) to the aqueous phases. After the third ATPS step a whitish emulsion-like solid was observed in the interface (Fig. 4c). The ATPS phase forming components do not have a big influence in the fractionation and purification of microalgae free sugars and starch (Supplementary data), however, they have a great influence in the separation of other microalgae components such as pigments and proteins [37].

3.5. Perspectives on ATPS to separate carbohydrates from microalgae

The integration of a microalgae biorefinery to simultaneously fractionate several valuable components could improve the economics of microalgae production. *N. oleoabundans* cultivated under saline conditions and nitrogen depletion (N⁻) contains free sugars (e.g. free glucose) and polysaccharides such as starch. Microalgae starch, is an important biopolymer that can be used for different industries such as the

food industry. The purification of starch from microalgae with a green technology able to concentrate starch and separate other microalgae biomolecules (e.g. free sugars and pigments) at the same time opens great opportunities in the biorefinery field.

Based on the experimental results, Fig. 5 shows a possible application scenario for the fractionation of microalgal carbohydrates by ATPS. Free glucose (representing free sugars) is separated in the bottom phase with outstanding yields (82–99%) in the first fractionation step. Free sugars can be used as a carbon source in fermentation as a feedstock for alcohols, acids and chemicals. It was previously demonstrated that proteins partition to the bottom phase when using PEG 400-ChDHP ATPS [59]. Since the first step separates most of the free glucose from the microalgal suspension to the bottom phase, the proteins can be separated from the free glucose using ultrafiltration or another ATPS [60], while the salt can be recycled and reused in the following fractionation steps.

Although pigment quantification data was not presented in this

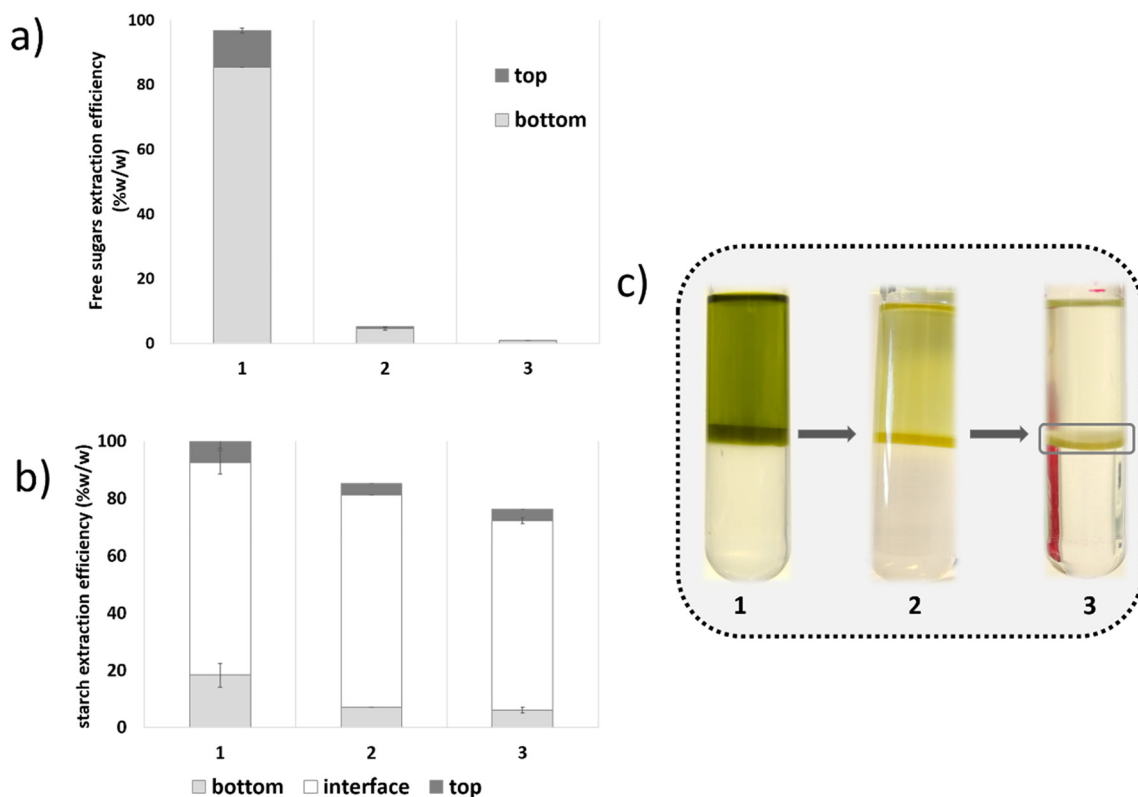


Fig. 4. Extraction efficiency of starch and glucose from *N. oleoabundans* (bead milled suspension) cultivated in artificial saline conditions under nitrogen depletion (N⁻) in three fractionation steps by PEG400-ChDHP ATPS: a) distribution of glucose among the two aqueous phases formed in the three fractionation steps; b) distribution of starch among the three phases formed in three fractionation steps. Experiments were carried out in triplicate and error bars indicate standard deviations; c) Picture of carbohydrates purification in three fractionation steps.

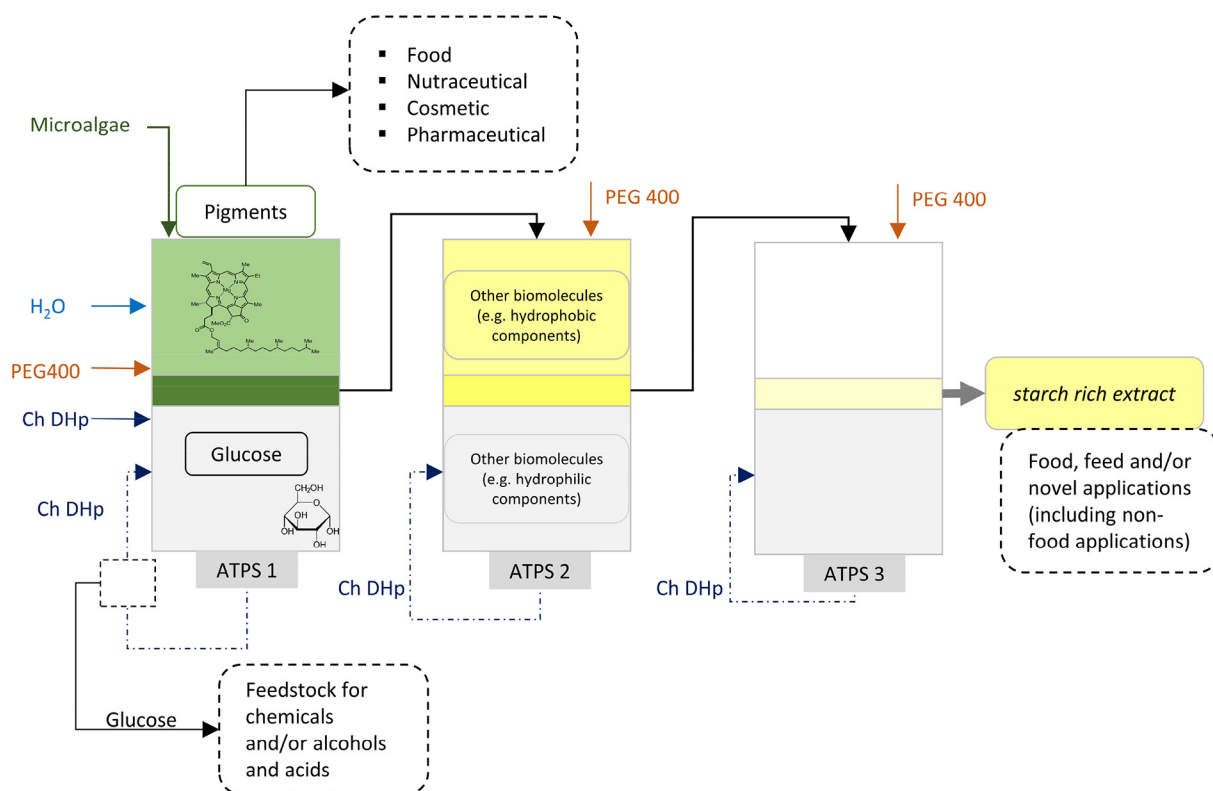


Fig. 5. Scheme of the possible application scenario for the fractionation of microalgae components in ATPS.

study, these high value biomolecules preferentially migrate to the top phase. After a third ATPS separation step, the green characteristic color disappeared from the interface (Fig. 4c). *N. oleoabundans* contains chlorophyll *a* and *b* and it also accumulates lutein, cantaxanthin, zeaxanthin, and astaxanthin monoesters and diesters, which are promising ingredients with pharmaceutical and nutraceutical applications [61]. No recycle step was proposed for the top phase, because its design depends on the biomolecules fractionated in this phase, which might be different for each system. However, other authors have confirmed the recyclability of PEG from aqueous solutions by suitable solvents or by direct distillation [62].

About 70% of the total starch content in *N. oleoabundans* (bead milled suspension) cultivated in artificial saline conditions under nitrogen depletion (N⁻) was concentrated at the interface free of pigments after three ATPSs steps. Moreover, the recovery of starch in the interface makes the process easier, because extra unit operations and/or solvents are not needed to recover the starch fraction. Microalgae for starch production is considered highly efficient and full of advantages in comparison with traditional crops (corn, rice, potato, oat), because they do not compete for (arable) land space and they do not need fresh water for their cultivation. Starch is an important biopolymer widely used in the food and other industries. Novel applications of starch are: in free-fat food formulation; novel material in pharmaceuticals and cosmetics; encapsulation material for flavor essences; coatings in paper and textile industry and in biodegradable plastic film production [14]. The evaluation of the physico-chemical properties of starch from *N. oleoabundans* is necessary to confirm its potential in the different applications.

Carbohydrate partitioning in ATPS is not notably affected by the phase forming components. Since ionic liquid-based ATPS does not alter the yield of extraction of neither free sugar nor starch, the inexpensive PEG400-citrate seems to be a good option to separate carbohydrates from microalgae. However, the partitioning behavior of proteins can be tuned depending on the phase forming components.

Previous results indicate that an ionic liquid-salt ATPS concentrates a higher amount of protein in the top phase, while the polymer-based ATPSs concentrate the protein at the interface [59]. Thus, depending on the desired final product purity, the ATPS can be selected and designed. The cholinium-based ionic liquids are considered to be biodegradable and in addition these ionic liquids enhance the stability and activity of enzymes and proteins [63]. Thus, in the development of a greener separation method, this group of ionic liquids is desired.

4. Conclusions

In this article, the separation of microalgae carbohydrates (free glucose and starch) in three biocompatible ATPSs is reported. Bead milled *N. oleoabundans*, cultivated under saline and nitrogen depleted conditions, was used as carbohydrate source. We demonstrated that an ATPS is a promising separation method for carbohydrates and other components from microalgae. Up to 99% of microalgal free glucose was recovered in the bottom phase in a single ATPS with PEG400-ChDHp, which was explained by the ability of these biomolecules to form hydrogen bonds with water. Starch, an abundant polysaccharide present in the microalgae, was simultaneously separated and concentrated at the interface after two additional ATPS steps up to 70% resulting in a pigment-free starch rich extract.

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.

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Statement of Informed Consent, Human/Animal Rights, if any, for the work described, informed consent, human or animal rights applicable.

Declaration of authors' agreement to authorship and submission of the manuscript for peer review. The authors agree to their contribution, authorship and submission of the manuscript for peer review.

Authors' contributions

Catalina Suarez Ruiz made a proposal for setup, performed all the experiments together with Santiago Zarate Baca including the ATPS studies and the associated analytics. Carbohydrate composition analysis was carried out by Catalina Suarez Ruiz and Lambertus van den Broek and critically analysed. Michel Eppink and Corjan van den Berg were deeply involved in the setup of the various experiments and performed a critical analysis of the data obtained. Results were also discussed with Rene Wijffels who critically analysed the data. Catalina Suarez Ruiz drafted the manuscript which was critically reviewed by Corjan van den Berg, Lambertus van den Broek, Rene Wijffels and Michel Eppink. Catalina Suarez Ruiz finalized the manuscript and Michel Eppink adapted it so that it could be submitted to Algal Research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.algal.2020.101801>.

References

- R.A. Sheldon, Green and sustainable manufacture of chemicals from biomass: state of the art, *Green Chem.* 16 (2014) 950–963, <https://doi.org/10.1039/C3GC41935E>.
- Y. Tang, J.N. Rosenberg, P. Bohutskyi, G. Yu, M.J. Betenbaugh, F. Wang, Microalgae as a feedstock for biofuel precursors and value-added products: green fuels and golden opportunities, *BioResources* 11 (2016) 2850–2885.
- S.R. Chia, H.C. Ong, K.W. Chew, P.L. Show, S.-M. Phang, T.C. Ling, D. Nagarajan, D.-J. Lee, J.-S. Chang, Sustainable approaches for algae utilisation in bioenergy production, *Renew. Energy* 129 (2018) 838–852.
- G.Y. Yew, S.Y. Lee, P.L. Show, Y. Tao, C.L. Law, T.T.C. Nguyen, J.-S. Chang, Recent advances in algae biodiesel production: from upstream cultivation to downstream processing, *Bioresource Technology Reports* 7 (2019) 100227.
- R.B. Draaisma, R.H. Wijffels, P.M. Slegers, L.B. Brentner, A. Roy, M.J. Barbosa, Food commodities from microalgae, *Curr. Opin. Biotechnol.* 24 (2013) 169–177, <https://doi.org/10.1016/j.copbio.2012.09.012>.
- Y. Wang, S.-H. Ho, H.-W. Yen, D. Nagarajan, N.-Q. Ren, S. Li, Z. Hu, D.-J. Lee, A. Kondo, J.-S. Chang, Current advances on fermentative biobutanol production using third generation feedstock, *Biotechnol. Adv.* 35 (2017) 1049–1059, <https://doi.org/10.1016/j.biotechadv.2017.06.001>.
- S.A. Jambo, R. Abdulla, S.H. Mohd Azhar, H. Marbawi, J.A. Gansau, P. Ravindra, A review on third generation bioethanol feedstock, *Renew. Sust. Energy Rev.* 65 (2016) 756–769 <http://EconPapers.repec.org/RePEc:eee:rensus:v:65:y:2016:i:c:p:756-769>.
- G. Markou, I. Angelidaki, D. Georgakakis, Microalgal carbohydrates: an overview of the factors influencing carbohydrates production, and of main bioconversion technologies for production of biofuels, *Appl. Microbiol. Biotechnol.* 96 (2012) 631–645, <https://doi.org/10.1007/s00253-012-4398-0>.
- J. Benemann, Microalgae for biofuels and animal feeds, *Energies* 6 (2013) 5869–5886.
- N. Wu, Y. Li, C.Q. Lan, Production and rheological studies of microalgal extracellular biopolymer from lactose using the green algae *Neochloris oleoabundans*, *J. Polym. Environ.* 19 (2011) 935–942.
- B. Maršáliková, M. Širmerová, M. Kuřec, T. Brányik, I. Brányiková, K. Melzoch, V. Zachleder, Microalgae *Chlorella sp.* as an alternative source of fermentable sugars, *Chem. Eng. Trans.* 21 (2010) 1279–1284 Italian Association of Chemical Engineering - AIDIC.
- C.Y. Chen, X.Q. Zhao, H.W. Yen, S.H. Ho, C.L. Cheng, D.J. Lee, F.W. Bai, J.S. Chang, Microalgal-based carbohydrates for biofuel production, *Biochem. Eng. J.* 78 (2013) 1–10.
- J.D. Gouveia, J. Ruiz, L.A. van den Broek, T. Hesselink, S. Peters, D.M. Kleinegris, A.G. Smith, D. van der Veen, M.J. Barbosa, R.H. Wijffels, *Botryococcus braunii* strains compared for biomass productivity, hydrocarbon and carbohydrate content, *J. Biotechnol.* 248 (2017) 77–86, <https://doi.org/10.1016/j.jbiotec.2017.03.008>.
- L. Copeland, J. Blazek, H. Salman, M.C. Tang, Form and functionality of starch, *Food Hydrocoll.* 23 (2009) 1527–1534 (<http://doi.org/10.1016/j.foodhyd.2008.09.016>).
- S. Jobling, Improving starch for food and industrial applications, *Curr. Opin. Plant Biol.* 7 (2004) 210–218, <https://doi.org/10.1016/j.pbi.2003.12.001>.
- I. Gifuni, G. Olivieri, I.R. Krauss, G. D'Errico, A. Pollio, A. Marzocchella, Microalgae as new sources of starch: Isolation and characterization of microalgal starch granules, in: S. Bakalis, L. Piazza, S. Pierucci, J.J. Klemes (Eds.), *Chemical Engineering Transactions*, 57 Italian Association of Chemical Engineering - AIDIC, 2017, pp. 1423–1428, <https://doi.org/10.3303/CET1757238>.
- L.A.B. Perez, E. Agama-Acevedo, Starch, *Starch-Based Materials in Food Packaging*, Elsevier London Academic Press, 2018, pp. 1–18.
- I. Gifuni, G. Olivieri, A. Pollio, A. Marzocchella, Identification of an industrial microalgal strain for starch production in biorefinery context: the effect of nitrogen and carbon concentration on starch accumulation, *New Biotechnol.* 41 (2018) 46–54, <https://doi.org/10.1016/j.nbt.2017.12.003>.
- D. Hernández, B. Riaño, M. Coca, M. García-González, Saccharification of carbohydrates in microalgal biomass by physical, chemical and enzymatic pre-treatments as a previous step for bioethanol production, *Chem. Eng. J.* 262 (2015) 939–945.
- G. Zhao, X. Chen, L. Wang, S. Zhou, H. Feng, W.N. Chen, R. Lau, Ultrasound assisted extraction of carbohydrates from microalgae as feedstock for yeast fermentation, *Bioresour. Technol.* 128 (2013) 337–344, <https://doi.org/10.1016/j.biortech.2012.10.038>.
- F. Di Caprio, P. Altamari, L. Toro, F. Pagnanelli, Effect of lipids and carbohydrates extraction on astaxanthin stability in *Scenedesmus sp.*, in: S. Pierucci, J.J. Klemes (Eds.), *Chemical Engineering Transactions*, 43 Italian Association of Chemical Engineering - AIDIC, 2015, pp. 205–210, <https://doi.org/10.3303/CET1543035>.
- G.P. t Lam, M.H. Vermuë, M.H.M. Eppink, R.H. Wijffels, C. van den Berg, Multi-product microalgae biorefineries: from concept towards reality, *Trends Biotechnol.* 36 (2018) 216–227, <https://doi.org/10.1016/j.tibtech.2017.10.011>.
- M. Iqbal, Y. Tao, S. Xie, Y. Zhu, D. Chen, X. Wang, L. Huang, D. Peng, A. Sattar, M.A.B. Shabbir, H.I. Hussain, S. Ahmed, Z. Yuan, Aqueous two-phase system (ATPS): an overview and advances in its applications, *Biological Procedures Online* 18 (2016) 1–18, <https://doi.org/10.1186/s12575-016-0048-8>.
- S.P.M. Ventura, F.A. e Silva, M.V. Quental, D. Mondal, M.G. Freire, J.A.P. Coutinho, Ionic-liquid-mediated extraction and separation processes for bioactive compounds: past, present and future trends, *Chem. Rev.* 117 (2017) 6984–7052 (<https://doi.org/10.1021/acs.chemrev.6b00550>).
- R. Hatti-Kaul, *Applied Biochemistry and Biotechnology - Part B Molecular Biotechnology* 19 (2001) 269–277.
- A.P. De Los Ríos, F.J.H. Fernández, *Ionic Liquids in Separation Technology*, Elsevier Inc, 2014.
- H. Olivier-Bourbigou, L. Magna, D. Morvan, Ionic liquids and catalysis: recent progress from knowledge to applications, *Appl. Catal. A Gen.* 373 (2010) 1–56, <https://doi.org/10.1016/j.apcata.2009.10.008>.
- R.K. Desai, M. Streefland, R.H. Wijffels, M.H. Eppink, *Extraction of proteins with ABS, Ionic-Liquid-Based Aqueous Biphasic Systems*, Springer, 2016, pp. 123–134.
- J.K. Yan, H.L. Ma, J.J. Pei, Z.B. Wang, J.Y. Wu, Facile and effective separation of polysaccharides and proteins from *Cordyceps sinensis* mycelia by ionic liquid aqueous two-phase system, *Sep. Purif. Technol.* 135 (2014) 278–284, <https://doi.org/10.1016/j.seppur.2014.03.020>.
- Z.-j. Tan, F.-f. Li, X.-l. Xu, J.-m. Xing, Simultaneous extraction and purification of aloe polysaccharides and proteins using ionic liquid based aqueous two-phase system coupled with dialysis membrane, *Desalination* 286 (2012) 389–393.
- C. Jampani, K. Raghavarao, Differential partitioning for purification of anthocyanins from *Brassica oleracea*, *Sep. Purif. Technol.* 151 (2015) 57–65, <https://doi.org/10.1016/j.seppur.2015.07.030>.
- S. Chethana, C.A. Nayak, K. Raghavarao, Aqueous two phase extraction for purification and concentration of betalains, *J. Food Eng.* 81 (2007) 679–687, <https://doi.org/10.1016/j.jfoodeng.2006.12.021>.
- C. C. o. A. P. CCAP, Bold's basal medium (BB), <https://www.ccap.ac.uk/pdfrecipes.htm>.
- P.R. Postma, T.L. Miron, G. Olivieri, M.J. Barbosa, R.H. Wijffels, M.H.M. Eppink, Mild disintegration of the green microalgae *Chlorella vulgaris* using bead milling, *Bioresour. Technol.* 184 (2015) 297–304, <https://doi.org/10.1016/j.biortech.2014.09.033>.
- M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, *Anal. Chem.* 28 (1956) 350–356.
- B. Gilbert-López, J.A. Mendiola, J. Fontecha, L.A.M. Van Den Broek, L. Sjitsma, A. Cifuentes, M. Herrero, E. Ibáñez, Downstream processing of *Isochrysis galbana*: a step towards microalgal biorefinery, *Green Chem.* 17 (2015) 4599–4609.
- G. Breuer, W.A.C. Evers, J.H. de Vree, D.M.M. Kleinegris, D.E. Martens, R.H. Wijffels, P.P. Lamers, Analysis of fatty acid content and composition in microalgae, *J. Vis. Exp.* 1 (2013) 50628, <https://doi.org/10.3791/50628>.
- G. Breuer, P.P. Lamers, D.E. Martens, R.B. Draaisma, R.H. Wijffels, The impact of nitrogen starvation on the dynamics of triacylglycerol accumulation in nine microalgal strains, *Bioresour. Technol.* 124 (2012) 217–226, <https://doi.org/10.1016/j.biortech.2012.08.003>.
- C.A. Suarez Ruiz, C. van den Berg, R.H. Wijffels, M.H.M. Eppink, Rubisco separation using biocompatible aqueous two-phase systems, *Sep. Purif. Technol.* 196 (2017) 254–261, <https://doi.org/10.1016/j.seppur.2017.05.001>.
- G. Dragone, B.D. Fernandes, A.P. Abreu, A.A. Vicente, J.A. Teixeira, Nutrient limitation as a strategy for increasing starch accumulation in microalgae, *Appl. Energy* 88 (2011) 3331–3335.
- X. Sun, Y. Cao, H. Xu, Y. Liu, J. Sun, D. Qiao, Y. Cao, Effect of nitrogen-starvation, light intensity and iron on triacylglyceride/carbohydrate production and fatty acid profile of *Neochloris oleoabundans* HK-129 by a two-stage process, *Bioresour.*

- Technol. 155 (2014) 204–212, <https://doi.org/10.1016/j.biortech.2013.12.109>.
- [42] L.D. Jaeger, B.M. Carreres, J. Springer, P.J. Schaap, G. Eggink, V.A.P. Martins Dos Santos, R.H. Wijffels, D.E. Martens, *Neochloris oleoabundans* in worth its salt: Transcriptomic analysis under salt and nitrogen stress, *PLoS One* 13 (2018) 1–21, <https://doi.org/10.1371/journal.pone.0194834>.
- [43] C. González-Fernández, M. Ballesteros, Linking microalgae and cyanobacteria culture conditions and key-enzymes for carbohydrate accumulation, *Biotechnol. Adv.* 30 (2012) 1655–1661, <https://doi.org/10.1016/j.biotechadv.2012.07.003>.
- [44] A. Juneja, R.M. Ceballos, G.S. Murthy, Effects of environmental factors and nutrient availability on the biochemical composition of algae for biofuels production: a review, *Energies* 6 (2013) 4607–4638, <https://doi.org/10.3390/en6094607>.
- [45] P. Chiranjeevi, S. Venkata Mohan, Optimizing the critical factors for lipid productivity during stress phased heterotrophic microalgae cultivation, *Frontiers in Energy Research* 4 (2016) 26, <https://doi.org/10.3389/fenrg.2016.00026>.
- [46] M.R. Brown, S.W. Jeffrey, J.K. Volkman, G.A. Dunstan, Nutritional properties of microalgae for mariculture, *Aquaculture* 151 (1997) 315–333.
- [47] T. Fernandes, I. Fernandes, C.A.P. Andrade, A. Ferreira, N. Cordeiro, Marine microalgae monosaccharide fluctuations as a stress response to nutrients inputs, *Algal Res.* 24 (2017) 340–346, <https://doi.org/10.1016/j.algal.2017.04.023>.
- [48] R. Pezoa-Conte, A. Leyton, I. Anugwom, S. von Schoultz, J. Paranko, P. Mäki-Arvela, S. Willför, M. Muszyński, J. Nowicki, M.E. Lienqueo, J.P. Mikkola, Deconstruction of the green alga *Ulva rigida* in ionic liquids: closing the mass balance, *Algal Res.* 12 (2015) 262–273, <https://doi.org/10.1016/j.algal.2015.09.011>.
- [49] Y. Pei, Z. Li, L. Liu, J. Wang, H. Wang, Selective separation of protein and saccharides by ionic liquids aqueous two-phase systems, *SCIENCE CHINA Chem.* 53 (2010) 1554–1560.
- [50] Z. Chen, W. Zhang, X. Tang, H. Fan, X. Xie, Q. Wan, X. Wu, J.Z. Tang, Extraction and characterization of polysaccharides from semen Cassiae by microwave-assisted aqueous two-phase extraction coupled with spectroscopy and HPLC, *Carbohydr. Polym.* 144 (2016) 263–270, <https://doi.org/10.1016/j.carbpol.2016.02.063>.
- [51] S.Y. Lee, I. Khoiroh, C.W. Ooi, T.C. Ling, P.L. Show, Recent advances in protein extraction using ionic liquid-based aqueous two-phase systems, *Sep. Purif. Rev.* 46 (2017) 291–304, <https://doi.org/10.1080/15422119.2017.1279628>.
- [52] R. Xu, Y. Queneau, How the polarity of carbohydrates can be used in chemistry, in: A.R. Rauter, Y.Q. Queneau, T.K. Lindhorst (Eds.), *Carbohydr. Chem, Royal Society of Chemistry*, 40 2014, pp. 31–50.
- [53] M.E. Zakrzewska, E. Bogel-Yukasik, R. Bogel-Yukasik, Solubility of carbohydrates in ionic liquids, *Energy Fuel* 24 (2010) 737–745, <https://doi.org/10.1021/ef901215m>.
- [54] K. Wilpiszewska, T. Spychaj, Ionic liquids: media for starch dissolution, plasticization and modification, *Carbohydr. Polym.* 86 (2011) 424–428, <https://doi.org/10.1016/j.carbpol.2011.06.001>.
- [55] A. Farrán, C. Cai, M. Sandoval, Y. Xu, J. Liu, M.J. Hernáiz, R.J. Linhardt, Green solvents in carbohydrate chemistry: from raw materials to fine chemicals, *Chem. Rev.* 115 (2015) 6811–6853, <https://doi.org/10.1021/cr500719h>.
- [56] N. Singh, J. Singh, L. Kaur, N.S. Sodhi, B.S. Gill, Morphological, thermal and rheological properties of starches from different botanical sources, *Food Chem.* 81 (2003) 219–231, [https://doi.org/10.1016/S0308-8146\(02\)00416-8](https://doi.org/10.1016/S0308-8146(02)00416-8).
- [57] R. Halim, P.A. Webley, G.J.O. Martin, The CIDES process: fractionation of concentrated microalgal past for co-production of biofuel, nutraceuticals, and high-grade protein feed, *Algal Res.* 19 (2016) 299–306, <https://doi.org/10.1016/j.algal.2015.09.018>.
- [58] T. Dong, E.P. Knoshaug, P.T. Pienkos, L.M.L. Laurens, Lipid recovery from wet oleaginous microbial biomass for biofuel production: a critical review, *Appl. Energy* 177 (2016) 879–895, <https://doi.org/10.1016/j.apenergy.2016.06.002>.
- [59] C.A. Suarez Ruiz, D.P. Emmerly, R.H. Wijffels, M.H. Eppink, C. van den Berg, Selective and mild fractionation of microalgal proteins and pigments using aqueous two-phase systems, *J. Chem. Technol. Biotechnol.* 93 (2018) 2774–2783, <https://doi.org/10.1002/jctb.5711>.
- [60] E. Suarez Garcia, C.A. Suarez Ruiz, T. Tilaye, M.H.M. Eppink, R.H. Wijffels, C. van den Berg, Fractionation of proteins and carbohydrates from crude microalgae extracts using an ionic liquid based-aqueous two phase system, *Sep. Purif. Technol.* 204 (2018) 56–65, <https://doi.org/10.1016/j.seppur.2018.04.043>.
- [61] M. Castro-Puyana, M. Herrero, I. Urreta, J.A. Mendiola, A. Cifuentes, E. Ibáñez, S. Suárez-Alvarez, Optimization of clean extraction methods to isolate carotenoids from the microalgae *Neochloris oleoabundans* and subsequent chemical characterization using liquid chromatography tandem mass spectrometry, *Anal. Bioanal. Chem.* 405 (2013) 4607–4616.
- [62] J. Chen, S.K. Spear, J.G. Huddleston, R.D. Rogers, Polyethylene glycol and solutions of polyethylene glycol as green reaction media, *Green Chem.* 7 (2005) 64–82, <https://doi.org/10.1039/B413546F>.
- [63] M. Bisht, D. Mondal, M.M. Pereira, M.G. Freire, P. Venkatesu, J.A.P. Coutinho, Long-term protein packaging in cholinium-based ionic liquids: improved catalytic activity and enhanced stability of cytochrome c against multiple stresses, *Green Chem.* 19 (2017) 4900–4911, <https://doi.org/10.1039/C7GC02011B>.