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Review

Multiproduct Microalgae Biorefineries Mediated by Ionic Liquids

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Ionic liquids (ILs) are salts with low melting points that can be used as solvents for mild extraction and selective fractionation of biomolecules (e.g., proteins, carbohydrates, lipids, and pigments), enabling the valorisation of microalgal biomass in a multiproduct biorefinery concept, while maintaining the biomolecules' structural integrity and activity. Aqueous biphasic systems and emulsions stabilised by core-shell particles have been used to fractionate disrupted microalgal biomass into hydrophobic (lipids and pigments) and hydrophilic (proteins and carbohydrates) components. From nondisrupted biomass, the hydrophobic components can be directly extracted using ILs from intact cells, while the most fragile hydrophilic components can be obtained upon further mechanical cell disruption. These multiproduct biorefinery concepts will be discussed in an outlook on future separations using IL-based systems.

Highlights

Hydrophobic components can be extracted from intact microalgae.

Separation of hydrophilic and hydrophobic components is feasible with intact and disrupted microalgae.

Extraction can be performed without compromising the proteins structural integrity.

Multiproduct microalgae biorefining is a feasible approach.

Ionic Liquids as Solvents for the Separation of (Fragile) Biomolecules

Microalgae (see Glossary) are attractive organisms as an alternative feedstock for fossil fuel replacements: they have a high lipid productivity by using sunlight, CO_2, and sea or fresh water as growing conditions and do not compete for arable land [1,2], making them efficient organisms. However, it has recently become clear that the costs for biofuel production alone are economically unfeasible [2–4]; isolating other high-value components in microalgae (e.g., proteins, carbohydrates, and pigments) for the feed, chemical, cosmetic, and food industries [5–11] can create enough value to make the process economically feasible. The main challenge is to isolate these products from the lipids following sequential but sustainable strategies. Unfortunately, lipid extraction mostly occurs by solid–liquid or liquid–liquid extraction processes using volatile organic solvents [12–14], which is straightforward for a multitude of lipids but not always useful for other high-value products like proteins and carbohydrates. To obtain the remaining high-value compounds (proteins and carbohydrates) this strategy is not always feasible, due to the incompatibility between their chemical structures and properties and the conditions imposed by the use of organic solvents. Unlike **hydrophobic** pigments, the **hydrophilic compounds** will denature, precipitate, or degrade in some of the most used organic solvents under the **harsh** conditions they require, namely of temperature (e.g., > 40°C). Proteins are particularly **fragile**, having a compact 3D structure in which hydrogen bounding, ionic interactions combined with the secondary structure elements as β-sheets and α-helices define their native tertiary and/or quaternary structure in aqueous solutions. Organic solvents distort these protein structures irreversibly in a way that these high-value products can no longer be used as active ingredients [9,10]. In this context, more sustainable and **mild** (compatible with the target compounds in terms of media conditions) separation techniques are needed to recover the hydrophilic components (e.g., proteins and carbohydrates) before the hydrophobic components (e.g., lipids and pigments) out of the microalgae cells without compromising the chemical structure and main activities of the target compounds, thus allowing their application as refined products in different sectors of activity (e.g., cosmetics, nutraceuticals, and pharmaceuticals). A few possible

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combinations have been mentioned [9,10], including cell disruption (e.g., bead milling and pulsed electric field), extraction [e.g., **ionic liquids (ILs)** and polymers] and fractionation (e.g., membrane filtration and chromatography) for a **multiproduct biorefinery approach**.

Following this rationale, attractive extraction techniques with ILs have gained a lot of interest in the past couple of years due to their high extraction efficiency and yields of extraction focusing the most interesting bioactive compounds obtained from biomass [15,16]. There is a clear need to develop greener, more sustainable, cost- and energy-effective, and environmentally safer processes by improving extraction and purification technologies to meet the requirements of a viable bioeconomy. With the plethora of available ILs (Box 1), it is possible to develop extraction methods tailored for specific microalgae products. In the past years, ILs have attracted considerable attention as medium for catalysis [17–20] in extraction and separation processes [21–24], and for the dissolution of biomaterials [25].

An extensive review on the past, present, and future trends of IL-mediated extraction and separation of bioactive compounds shows the different possibilities compared with traditional solvents [16]. Future challenges should address process and product life cycle analysis, develop scalable and economical feasible separation processes, and decrease energy and solvent consumption [16]. The efficiency of IL-based systems in the extraction of fragile and complex biomolecules such as proteins, is mostly focused on model proteins (e.g., albumin, cytochrome c, haemoglobin, and lysozyme) [8,16] or on isolating specific proteins from different biomass sources (e.g., algae, yeast, and bacteria) [16]. However, a multiproduct approach focusing on the biorefinery concept for the sequential isolation of several biomolecules with commercial interest (e.g., proteins, lipids, pigments, and carbohydrates) mediated by ILs under mild conditions is so far lacking.

In agriculture, a consistent amount of waste is generated that could account for at least 50% of the fresh harvested crops [26]. In this scenario, the circular economy and the bioeconomy intersect in their common aims to add value to biological wastes and residues through the smart management of agrowastes and their conversion via multipurpose biorefinery platforms into biobased products and biofuels, thus avoiding harmful consequences on soils, water, and air quality. For the agrowaste lignocellulosic biomasses, a multiproduct approach is already further in development not only in theory by assessing their economic evaluations [27–32] but also in practice by developing processes for gasses, sugars, and fatty acids [33,34]. However, these biorefineries operate under harsh conditions, which are acceptable for the particular case of lignocellulosic feedstock-based biorefineries but not for microalgae biorefineries. Regarding microalgae processing, recent reviews have discussed microalgae biorefining by applying a variety of different downstream techniques [7–9,11,35–38]. However, microalgal multiproduct processes are still too expensive. For instance, typical downstream processing for industrial biotechnological bulk products accounts for 20–40% of the total production costs, while for the microalgae multiproduct scenario, these costs are substantially higher: between 50% and 60% of the overall cost. This discrepancy of results is attributed to the lack of cheap, selective, and mild technologies to access the complexity of different product fractions (proteins, carbohydrates, lipids, and pigments) in their native or at least, in their functional state. To reduce the costs, simplified and integrated processes [11,35] under the strategy of a multiproduct scenario need to be developed.

Recently, (mild) multiproduct approaches mediated by ILs have been investigated (Table 1). The focus is still on separating different classes biomolecules (proteins, lipids, pigments, and carbohydrates) and not yet on specific compounds, except for the selective extraction of certain pigments (e.g., lutein and astaxanthin) as indicated in Table 1. Taking into account the state of the art, this

Glossary

Aqueous biphasic system (ABS): the development of two phases for the separation of molecules based on their different partition coefficients between the two phases.
Fragile: biomolecules such as proteins have a complex 3D structure needed to accomplish their physiological functions. Under other conditions, the native structure might be affected by organic solvents (e.g., ethanol, chloroform, and methanol), ionic strength resulting from high salt or ionic liquid concentrations (e.g., >25%) or temperature conditions (>40°C).
Harsh: conditions or process parameters such as high temperature (>40°C) and organic solutions (e.g., ethanol, hexane, chloroform, and methanol) that are desaturating conditions for proteins that could also affect carbohydrate polymers.
Hydrophilic compounds: molecules such as proteins and carbohydrates that dissolve easily in aqueous solutions.
Hydrophobic compounds: molecules such as lipids and pigments that do not dissolve in polar solvents.
Ionic liquids (ILs): salts with disperse charge leading to poor electrostatic interactions between the ions and low melting points.
Microalgae: microscopic unicellular organisms, typically found in freshwater and marine systems, which exist individually, or in chains or groups.
Mild conditions: processing conditions that allow recovery of various biomass fractions without damaging the individual (fragile) compounds present. These conditions or process parameters do not affect the structure or activity of the proteins by denaturation, degradation, or subunit dissociation, as proteins are mostly in their optimal conformation in aqueous buffer solutions with a pH range of 4–9.
Multiproduct biorefinery: production scenario that does not aim at a single (bulk) product, but instead at a full valorisation of all biomass fractions.
RuBisCo: high-molecular-weight multimeric protein (~540 kDa) with eight large and eight smaller subunits noncovalently interacting with each other for functionality.
Water-based emulsion stabilised by microgel particles: combination of ionic liquids and disrupted microalgal cell suspension with poly(N-isopropylacrylamide) microgel particles

review intends to focus on novel IL-based systems for the extraction and separation of multiple biomolecules (proteins, pigments, lipids, and carbohydrates) from microalgae supporting the development of a mild multiproduct approach aligned with the demands of the biorefinery concept.

Product Stability

ILs have been used for different applications, such as crystallization, solubilisation, separation, extraction, enzymatic reactions, and stabilisation of proteins [19,39]. The stability of proteins from microalgae in IL-based systems is one of the primary requirements as their functionality depends

forming a microgel-stabilised emulsion with a hydrophobic environment inside the nanoparticle and a hydrophilic environment outside the nanoparticle. The hydrophobic components (e.g., pigments) are transferred inside the nanoparticle whereas the hydrophilic components (e.g., proteins) remain outside the nanoparticle.

Box 1. Ionic Liquids Have Separation Potential

ILs are salts with low melting temperatures. They are composed, in general, of a large organic cation with dispersed charge, and a small organic or inorganic anion. The dispersed charge minimises the electrostatic interactions between the ions, and the large dimension of its ions make organisation into a crystalline structure difficult, so they are liquids at lower temperatures than conventional salts. The interest in ILs as separation media can be attributed to their interesting chemical and physical properties such as low vapour pressure, high thermal and chemical stability, nonflammability, and good solvation properties [15,51,90,91]. These are also referred to as designer solvents [92], as their properties such as polarity, viscosity, and hydrophobicity can be tuned by the adequate combination of cations and anions. These properties make them a desirable class of solvents for liquid–liquid extraction (Table I). The most commonly used anions and cations are depicted in Figure I, and are separated as toxic and harmless or practically harmless following the categorisation defined by GHS [74]. Recently, ILs have been used for extraction of value-added components from biomass [15,93], in synthesis [94], and in material chemistry as electrolytes for the electrochemical industry and liquid crystals. Moreover, ILs also demonstrate good performance in biocatalysis [18], while providing a nondenaturing environment for biomolecules and maintaining the protein structure and enzymatic activity [16].

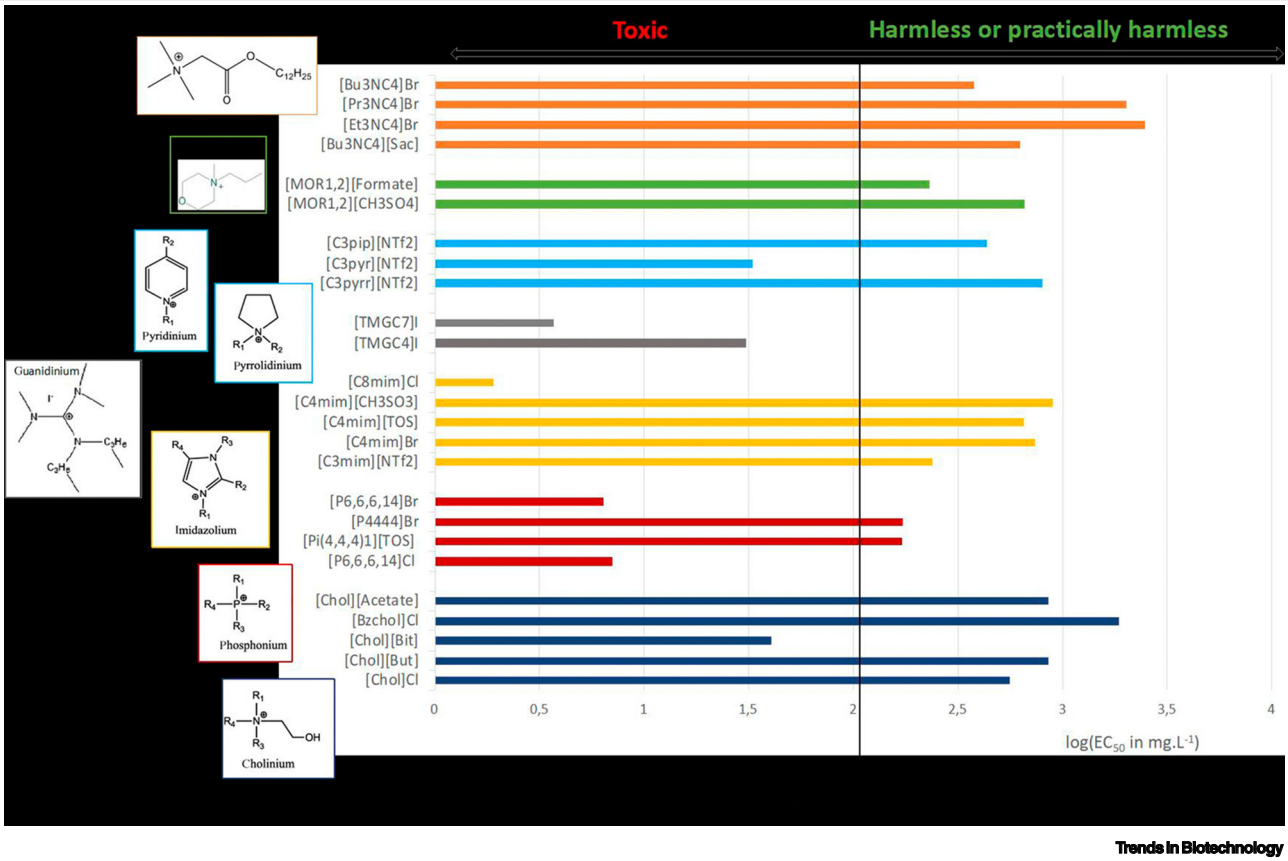

Figure I. Illustration of the Toxic and Nontoxic Nature of Different ILs Following the Classification Defined by GHS [75]. Various cations and anions were selected and the respective example of each family of IL is chemically represented as well. The results depicted represent the (logarithmic function) EC_{50} data obtained by Microtox (toxicity against *Allivibrio fischeri*) at 15 min of exposure. Data were collected from literature for imidazolium [95], pyridinium, pyrrolidinium, and piperidinium [95], phosphonium [96], morpholinium [97], guanidinium [96], cholinium, [98], and betainium [99]. Abbreviations: GHS, Globally Harmonized System of Classification and Labelling Chemicals; IL, ionic liquid.

Table I. Differences in Properties for Organic Solvents and ILs

Property	Organic solvent	IL
Possibilities	Numerous	Much more as organic solvents
Applicability	Single function	Multifunction
Catalytic ability	Rare	Common and tuneable
Chirality	Rare	Common and tuneable
Flammability	Usually flammable	Usually nonflammable
Solvation	Weakly solvating	Strong solvating
Cost	Normally cheap	Typically between 2 and 100 times cost of organic solvents (reusable)
Refractive index	1.3–1.6	1.5–2.2

on noncovalently stabilised 3D structures that can be disrupted by elevated temperatures and various solvents. In the past decade, the stability and activity of many proteins were tested in ILs [19,39,40]. The potential of some ILs to stabilise proteins by maintaining their 3D structure because of the complex interactions they establish (e.g., electrostatic interaction, H-bonds, and dispersion forces) was demonstrated [19,40]. Improved stability (longer storage at room temperature in the presence of ILs) when compared with the same biomolecules in an aqueous buffer was shown for proteins such as lysozyme, bovine serum albumin (BSA), cytochrome c,

Table 1. IL-Mediated (Mild) Extraction of Microalgal Components

IL	Organism	Extraction conditions	Valorisation	Yield[a] (~%)	Refs
Trihexyl(tetradecyl)phosphonium bis (trifluoromethanesulfonyl)amide	*Haematococcus pluvialis*	Disrupted cells RT[b] Ionic liquids	Proteins Pigments (astaxanthin)	68 82	[57]
1-methyl-3-octylimidazolium chloride	*Isochrysis galbana*	Disrupted cells organic solvents 70°C	Proteins, carbohydrates (arabinose/glucose-rich polysaccharides)	100 71	[24]
1-Butyl-3-methylimidazolium dibutylphosphate	*Neochloris oleoabundans*	Intact and disrupted cells RT ILs	Lipids Proteins Carbohydrates	44 77 49	[64]
Tributylmethylphosphonium methyl sulfate	*N. oleoabundans*	Intact and disrupted cells RT ILs	Lipids Proteins Carbohydrates	68 80 77	[64]
Iolilyte 221PG	*N. oleoabundans/ Tetraselmis suecica*	Disrupted cells RT ILs	Proteins Carbohydrates	43–48 22–37	[47]
Choline dihydrogenphosphate	*N. oleoabundans*	Disrupted cells RT ILs	Proteins Pigments (lutein)	92 97	[55]
Tributyl-1-tetradecylphosphonium	*N. oleoabundans*	Intact cells RT ILs	Proteins Carbohydrates Pigments (lutein)	100 80 98	[65]
Choline dihydrogenphosphate	*N. oleoabundans*	Disrupted cells RT ILs	Lipids Proteins Pigments (lutein, chlorophyll) Carbohydrates	71 82 98 93	[56]

[a]Recovery mainly based on biomolecules (proteins and carbohydrates) available in cytoplasm or biomolecules (pigments and lipids) available in cytoplasm/cell wall.
[b]Abbreviation: RT, room temperature (15–25°C).

ribonuclease A, and enzymes such as lipases, alcohol dehydrogenase, and various proteases in IL structures [8,19,39–41]. Due to the diverse nature of proteins and ILs, not all proteins are stable in ILs [8,19,39,40,42]. Depending on the IL and its concentration, the structural integrity of proteins may be affected with regard to unfolding or protein aggregation [43–46]. Some ILs consisting of large organic cations and organic or inorganic anions (Box 1) are able to interact strongly with the amino acids that comprise proteins, consequently affecting the integrity of the proteins. Protein/IL interactions depend on the size and complexity of these macromolecules. BSA, for example, is a compact tertiary globular folded protein with a molecular weight of ~66 kDa consisting of one subunit and is stable in an IL solution of up to 50% lolilyte 221PG (w/w) in water. At higher concentrations of lolilyte 221PG, BSA starts to aggregate [46], due to the interaction of the IL with the surface amino acids, resulting in BSA unfolding and, consequently, interactions inducing aggregation. Ribulose-1,5-biphosphate carboxylase/oxygenase (**RuBisCo**) present in microalgae is a large ternary multimeric protein with a molecular weight of ~540 kDa consisting of eight large and eight smaller subunits noncovalently interacting with each other. This protein, in contrast to BSA, forms aggregates at lower concentrations (up to 25%) of lolilyte 221PG (w/w) in water. Moreover, the enzymatic activity of RuBisCo drastically diminishes due to the enzyme inactivation promoted by the IL [46,47] due the competition of the IL with the noncovalently interacting subunits (e.g., surface exposed amino acids) of RuBisCo , in addition to the subunit dissociation and/or aggregation that may occur. Furthermore, it is concluded that protein destabilization may occur at different IL concentrations depending on the size, structure, hydrophobicity, and surface charges of the protein.

In conclusion, IL protein interactions are crucial for the maintenance of protein functionality during extraction of high value biomolecules (proteins, carbohydrates, lipids, and pigments) in a microalgae multiproduct biorefinery scenario.

The next section discusses IL-based systems for the (back) extraction of microalgae components within the biorefinery concept, and the fractionation of the different components by manipulating the affinity between target products and the different phases investigated. The recovery of biobased compounds dissolved in ILs is an important aspect that demands more research.

IL-Based Systems

This section discusses two innovative IL-based systems for the separation of (fragile) biomolecules from microalgae: IL-based biorefineries of disrupted microalgae biomass (Figure 1) and nondisrupted microalgae biomass (Figure 2).

IL-Based Biorefinery of Disrupted Microalgal Biomass

IL-based **aqueous biphasic systems (ABSs)** have an advantage over traditional ABSs in terms of the process conditions (e.g., lower viscosity and fewer diffusional and mass and heat transfer problems) and process outputs [16,48]. The range of variability on the hydrophobicity and polarities differences between the phases in traditional ABSs is limited whereas with IL-based ABSs this range can be extended [16,48,49]. Using IL-based ABSs, specific interactions can be introduced in the system allowing for a much better extraction performance (in terms of extraction efficiency, recovery, and/or purity) or instead to design systems with tuneable selectivity for the different biomolecules of interest, thus improving the process performance. For example, the extraction of RuBisCO using IL-based ABSs shows a higher partition coefficient for RuBisCO, a major microalgal protein, towards the IL-rich phase in comparison to the conventional polyethylene glycol-based ABS [8,46,50,51]. While IL-based ABSs are widely studied for extraction of biomolecules such as lipids, amino acids, pigments, proteins, nucleic acids, etc. [16] using model systems, their potential for extracting biomolecules like proteins from raw materials has only been described

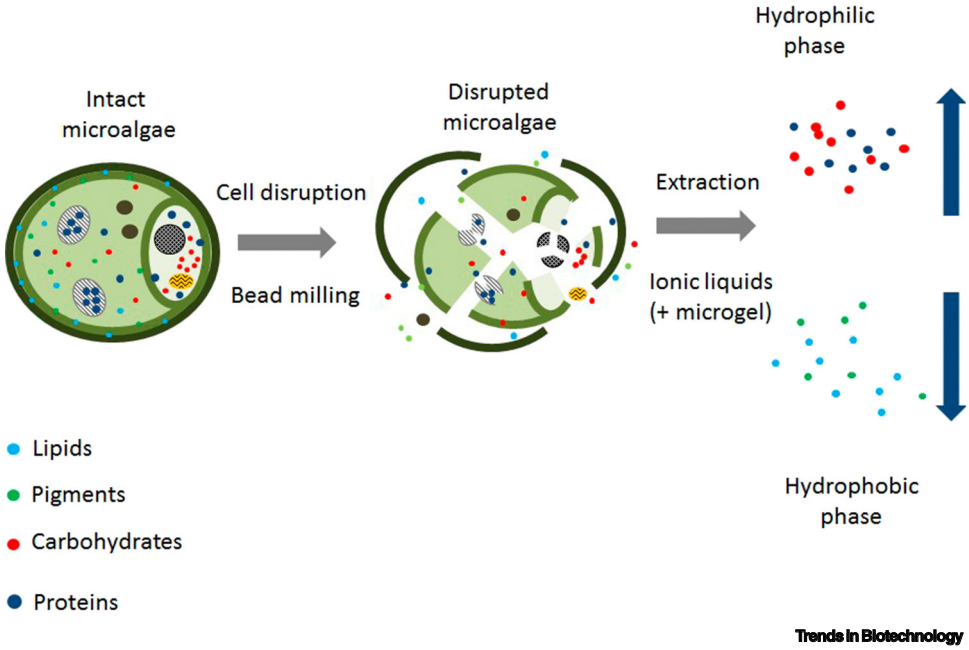

Figure 1. Ionic-Liquid-Based Separation of Disrupted Microalgal Biomass.

in a few cases [40,52–54]. This ability of IL-based ABSs holds a huge prospect in extracting fragile biomolecules from raw materials such as microalgae, which contain molecules of different polarity and solubility (proteins, pigments, lipids, and carbohydrates). Beyond one study [24] that used harsh extraction conditions, only a few studies reported the separation of multiple (fragile) microalgae biomolecules using IL-based ABSs under mild conditions

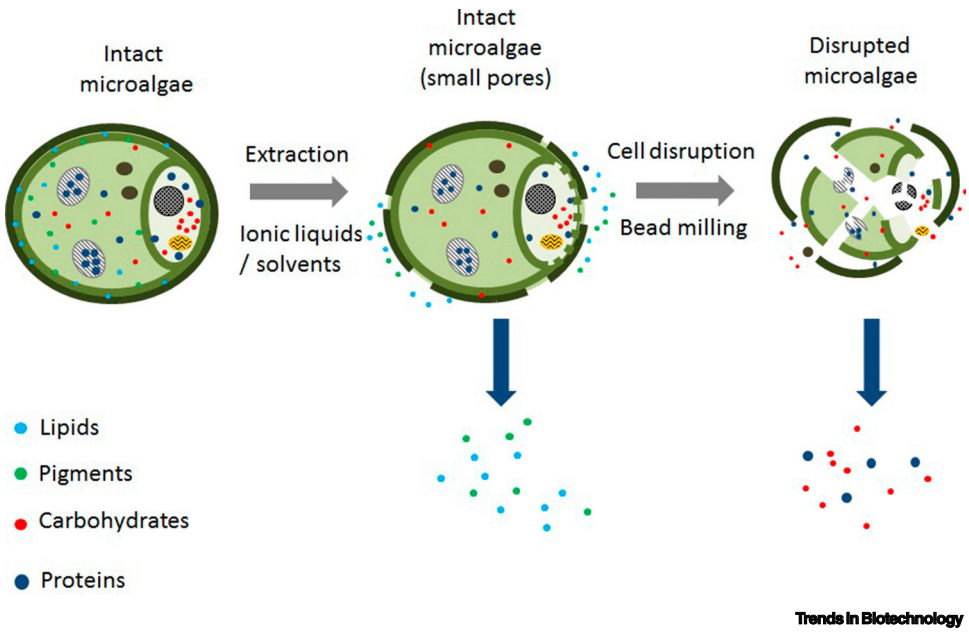

Figure 2. Ionic-Liquid-Based Separation of Nondisrupted Microalgal Biomass.

[55,56]. Although these studies showed promising results, additional work is required before this approach renders suitable strategies for a microalgal multiproduct biorefinery. More extensive studies using different ILs and the proper, careful design of IL-based ABSs should be performed, in which the role of IL recycling to reduce costs and environmental impact needs to be scrutinised.

Another strategy recently proposed in this field was the use of Cyphos 109-based systems in **water-based emulsion stabilised by microgel particles** to simultaneously separate fragile biomolecules of different polarities from microalgae [57]. Earlier studies were analytically focused [58–60]. More recently, preparative applications have shown promising results. When mixing with microalgae biomass (disrupted cells), the proteins remain in the aqueous phase outside the microgel particles whereas astaxanthin (the most abundant pigment present in the microalgae cells) partitions to the most hydrophobic medium in the microgel particles. The most interesting aspect of these systems is that they allow the proteins to retain their structural integrity. The microgel particles used to stabilise the emulsions are porous, allowing the transfer of small molecules (e.g., astaxanthin) and prevent direct contact between proteins and the IL, thus assisting with the maintenance of the structural integrity of the proteins. A proof of concept for novel continuous separation was recently reported [57]. The microgel particles were thermoresponsive, a property which was unexplored in this work. However, when the temperature was raised above 32°C, the particles collapsed and the emulsion broke [61]. This property of the emulsion could be explored in the separation process in the future for the development of formulations to be applied in other fields of application, namely in cosmetics, food ingredients, or pharmaceuticals [62], given the innovative profile of this approach towards the microalgae field. Although promising, this technique is still in its infancy and should be tested with different ILs and smaller IL droplets to improve mass transfer phenomena. Additionally, although the emulsion facilitates the simultaneous separation of different components from the biomass, the process still needs to be optimised in terms of efficiency and reusability of the IL and microgel particles. Future research should focus not only on the optimization of the process parameters (e.g., selection of the IL, ratio of IL to microgel particles, and ratio of IL to water content) and scalability, but also on finding newer applications for this emulsion, which provides a complete toolbox for the creation of high performant downstream processes.

IL-Based Biorefinery of Nondisrupted Microalgal Biomass

The performance of IL solutions on the extraction of biological compounds from nondisrupted microalgal cells has also been investigated. Microalgae have a rigid cell wall that requires mechanical disruption to release the intracellular components. However, a more recent study [63] showed that with 40% of an aqueous solution of 1-ethyl-3-methylimidazolium dibutylphosphate it was possible to permeabilise vegetative cells of *Haematococcus pluvialis* under mild conditions, allowing the release of astaxanthin that could subsequently be extracted with ethyl acetate. Microscopic studies confirmed that the cells were not ruptured. Based on these observations, a possible mechanism was postulated wherein the permeabilisation of the microalgal cells occurs by the action of the ILs. However, it remains unclear how this effect takes place and what specific action of the IL makes the cell wall porous. A similar observation was also made when *Neochloris oleoabundans* cells were treated with imidazolium-based ILs [64,65] and a phosphonium-based IL, Cyphos 108. Despite the good solubility achieved with Cyphos 108 towards the *N. oleoabundans* cells, the same effect was not observed in the *H. pluvialis* cell wall, which indicates the relevant role played by the cell wall composition. Of course, while the results seem to be promising, in-depth knowledge and understanding of the effect of ILs on cell walls, which appears to be microalgae-specific, is required. When the more hydrophobic components (lipids and pigments) are extracted out of intact cells by cell wall permeabilisation with ILs and mediated

by organic solvents (e.g., ethylacetate), the more hydrophilic components (proteins and carbohydrates) remain in the cells and can be extracted by mechanical disruption to recover these components in suitable aqueous buffer solutions maintaining the structural integrity of the components (i.e., keeping their native form). These results show the potential of ILs to fractionate and separate the microalgal biomass components in a multiproduct biorefinery approach.

The most logical approach would be to start by studying the cell wall composition and then to use this characterisation to select the most appropriate solvent and technique to proceed. No less relevant is the need for advanced analytical methods to properly identify and quantify the cell components that can cope with IL solutions. This is not straightforward since often the ILs cause massive interference with the methods of quantification used. These studies would help in selecting and designing ILs that are better suited to the cells' morphology and biochemistry and the target compounds' dissolution.

Overall, extraction of proteins with ILs shows good recovery [16,46,55,64]. However, future work should focus on the back-extraction of proteins from ILs and investigate the protein–IL interactions to understand the solvation of proteins in IL solutions.

Concluding Remarks and Future Perspectives

ILs have become popular as remarkable solvents in various fields of application, such as biocatalysts for bioactive compounds and for separation processes. Different combinations of ILs can be prepared (Box 1) and used in separation techniques as mobile or stationary phase modifiers in HPLC, as running buffer additives in capillary electrophoresis, as crystallising agent in protein crystallisation, protein solubilisation in formulation studies, or as new extraction solvents [39,66]. Most of the IL-based separation techniques reported focus on analytical separation and sample preparation [66], whereas the extraction and separation of bioactive compounds from complex matrices, such as biomass, at preparative scale is step by step and focuses on specific components [16]. Recently, more multiproduct biorefinery approaches are coming up as well (Table 1), but will take more time to implement as their complexity increases. The expectation is that this will further improve if multiple products can be recovered with simple IL-mediated extraction methods. However, more research and development is needed to achieve large scale production processes (see Outstanding Questions), not only for microalgae but also for other organisms (e.g., yeast, macroalgae, bacteria, and fungi).

Novel IL-based systems for the extraction of fragile molecules from complex matrices are herein discussed, showing new directions towards multiproduct biorefinery approaches of microalgae [56,64]. Figures 1 and 2 summarise the possibilities to selectively extract hydrophobic components (lipids and pigments) and hydrophilic components (proteins and carbohydrates) from (non-)disrupted microalgae (R. Desai, PhD thesis, Wageningen University, 2016). Unfortunately, as previously mentioned, the exact mechanism of extracting the hydrophobic biomolecules (lipids and pigments) from intact cells, and which interactions play major roles to explain the results obtained when using ILs and/or organic solvents, remain unknown. The same holds true when disintegrating microalgae cells (e.g., bead milling) and recovering the biomolecules with IL-based extractions. However, despite the potential of ILs in the pretreatment and separation of biomolecules from microalgal cells, some bottlenecks need to be overcome before their successful use at larger scale. For instance, the cost and environmental impact (e.g., biodegradability and toxicity) of using ILs as extractants or solvents (for biomass pretreatment) needs to be considered. Despite the overgeneralised idea that ILs are toxic [67–73], this is not necessarily correct. The toxicity of ILs mainly depends on the length of their alkyl chains, and generalisations that all ILs are toxic are neither straightforward nor supported by recent literature data. The European

Outstanding Questions

Recycling of ILs is challenging. How can we develop efficient recycling technologies for ILs in microalgal biorefineries?

The multiproduct biorefinery is important to develop cost-effective processes for microalgae, but how can we scale-up the reported bench scale processes?

Will it be possible to improve thermoresponsive solvents, or microgel particles, for a multiproduct biorefinery approach?

Will the development of cost-effective, biobased, biodegradable, and non-toxic ILs be feasible?

What is the mechanism of interaction, and what interactions play a role, between ILs and microalgal cell wall components that allow the extraction of intercellular biomolecules?

legislation from Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) [74] requires ecotoxicological data for all chemicals produced or imported into the EU above 1 ton/year. In accordance with the United Nations Globally Harmonized System of Classification and Labelling Chemicals (GHS) [75], these data should be classified in categories [76], for which compounds with median effective concentration (EC_{50}) values above 100 mg/l should be considered as practically harmless, and compounds with EC_{50} values below this value should be considered hazardous. Imidazolium ILs are not toxic *per se* (the same is true of ILs with some other head groups), with some already having been registered (e.g., 1-ethyl-3-methylimidazolium acetate) by REACH and industrially applied (e.g., by 525 Solutions). However, simultaneously considering biocompatibility, low toxicity, price, and availability, some structures that can be considered as more advantageous, are those based on cholinium [47,56,73,77–80] and betainium cations [81]. In this context, Box 1 depicts several IL chemical structures and demonstrates their toxic or nontoxic nature with respect to the GHS categorisation, allowing researchers to select the most appropriate solvents focusing on the target products and their final applications (Table 1).

Beyond the appropriate selection of both the process conditions and IL chemical structure, the environmental impact of ILs could also be lowered by their efficient recycling and reuse in the process so that the recovered biomolecules contain no, or only traces of, ILs, allowing the potential application of the final products as ingredients in cosmetic, nutraceutical, or pharmaceutical formulations. Therefore, the final preparation step should be a formulation in which the different biomolecules remain stable for specific periods of time, at room temperature (15–25°C) or, if necessary, refrigerated (2–8°C).

The most common strategies to recycle ILs used as solvents were recently reviewed [82]. These include ultrafiltration/diafiltration (mainly applied to isolate proteins and carbohydrates), back-extraction with sustainable organic solvents followed by the evaporation of the organic solvents if needed (a protocol normally applied to lipids and hydrophobic pigments), and precipitation of the compounds extracted followed by the precipitate redissolution in the appropriate media for further application, a strategy commonly applied to proteins but also small compounds.

In addition to these standard techniques, recycling and reuse of ILs may be defined considering the particularities of the optimised downstream process. In the case of processes using IL-based emulsions, from the approaches reported, the thermoresponsive properties of the IL emulsions can easily break the emulsions to recycle ILs [57]. However, and as previously discussed, this technology needs further improvement, such as the use of more biocompatible (less toxic and more biodegradable) ILs, which is the case for cholinium- [47,56,79,80] or betainium [81]-based structures, mainly considering their good scalable potential observed from millilitre to litre scales.

Table 2 gives an overview of the advantages and disadvantages of IL-based multiproduct approaches under the scope of the biorefinery concept. The main benefits include the capacity to recover multiple biomolecules (proteins, carbohydrates, pigments, and lipids), implying a high economic return, more products for more applications and less waste (more products recovered means less biomass residues wasted). By contrast, the process complexity increases to improve the separation of the different biomolecules.

The sustainable nature of the industry is a crucial concern. There is no commonly agreed upon definition of sustainability, but we believe that it should be the direct result of the balance between the process performance, environmental, and economic impacts. Process performance and the

Table 2. Advantages and Disadvantages of a Future Microalgal Biorefinery with ILs

Future microalgal biorefinery	Advantages	Disadvantages
Product functionality	Functional multiple biomolecules	Each biomolecule needs specific conditions
Complexity	Development of new biorefinery concepts	Complex processing
High value products	Recovery of different biomolecules (proteins, lipids, carbohydrates, and pigments)	—
Temperature	Room temperature	—
ILs	Recycling	Toxicity
Yield	Medium–high yield, extraction of biomolecules from complete cells	—
Selectivity	Specific for different biomolecules	Complex processing
Stability/storage	Room/refrigerator temperature	Storage at -20°C is required for less-stable compounds
Energy costs	Low temperature	Multiple steps
Targeted application	Cosmetics, nutraceuticals, pharmaceuticals	—
Costs	High value Economically competitive	—

environmental impact are already under discussion, but the economic impact analysis is still in its infancy. In the past year, a study focusing on analysing different industrial scenarios for multiproduct versus single-product approaches, considering the biomolecule selection, yields and economic performance, and considering the biological characteristics of algae species [4] was published. In this work, the authors showed the most promising economic gains of working under the multiproduct biorefinery approach [4].

Despite the increased enthusiasm for using ILs as solvents, some others, such as deep eutectic solvents (DESs) have emerged as alternative solvents for the extraction of biomolecules. DESs have been explored as potential alternatives to ILs [73,82–84]. Some components like choline can form deep eutectic solvents [73,85]. These mixtures may possess similar characteristics to ILs (e.g., low melting temperatures, low volatility, nonflammability, tunability, high solubility, and chemical and thermal stability), while benefitting from the availability and low cost of the starting materials. Moreover, they are easy to store and prepare, which consequently allows them to have lower costs. DESs result from the combination of a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD) whereby the interactions between HBA and HBD mostly involve hydrogen bonding and, to a lesser extent, electrostatic forces and van der Waals interactions [86]. An interesting approach was reported [85] using a switchable-hydrophilicity solvent system consisting of a fatty-acid-based DES, and complemented by a biofriendly dilute amine solution. This study demonstrated that a DES can be made to reversibly switch from a hydrophobic phase to a hydrophilic phase, thereby being able to extract various biological fractions (proteins, carbohydrates, and lipids) making up the biomass [85,87]. This work was carried out according to a circular extraction paradigm [88] in two subsequent steps and in both the forward and backward mode. A synthetic matrix representing the microalgal biomass, made of selected proteins, lipids, carbohydrates, and water, was extracted by DES and the extraction streams were characterised. Despite their sustainable character, new developments in DES are required to demonstrate if they could become attractive alternatives for the recovery of multibiomolecules including proteins. Moreover, and considering the monopoly of chromatographic techniques to

purify proteins, the use of IL-based ABSs should be focused on compounds for which the use of chromatography is not an option. ABSs, as separation techniques, have several advantages over chromatography when implemented properly [10,35]. They allow the development of multiproduct downstream processes, can be easily integrated with other techniques, and have high potential to be conjugated with miniaturised systems whenever needed [89]. Taking this into account, they have good potential for (micro)algal biorefinery, wherein there is a need to develop efficient processes to ensure the complete fractionation of the algal biomass, allowing the creation of more sustainable processes, which will be needed as the world moves towards a biobased economy.

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Declaration of Interests

No interests are declared by the authors.

References

1. Wijffels, R.H. *et al.* (2010) An outlook on microalgal biofuels. *Science* 329, 796–799
2. Wijffels, R.H. *et al.* (2010) Microalgae for the production of bulk chemicals and biofuels. *Biofuels Bioprod. Biorefin.* 4, 287–295
3. Ruiz, J. *et al.* (2016) Towards industrial products from microalgae. *Energy Environ. Sci.* 9, 3036–3043
4. Slegers, P.M. *et al.* (2020) Design of value chains for microalgal biorefinery at industrial scale: process integration and techno-economic analysis. *Front. Bioeng. Biotechnol.* 8, 1–17
5. Dixon, C. and Wilken, L.R. (2018) Green microalgae biomolecule separations and recovery. *Bioresour. Bioprocess.* 5, 1–24
6. Cuellar-Bermudez, S.P. *et al.* (2015) Extraction and purification of high-value metabolites from microalgae: essential lipids, astaxanthin and phycobiliproteins. *Microbial Biotechnol.* 8, 190–229
7. Khan, M.I. *et al.* (2018) The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. *Microb. Cell. Fact.* 17, 36 1-21
8. Lee, S.Y. *et al.* (2017) Recent advances in protein extraction using ionic liquid-based aqueous two-phase systems. *Sep. Pur. Rev.* 1–14
9. Vanthoor-Koopmans, M. *et al.* (2012) Biorefinery of microalgae for food and fuel. *Bioresour. Technol.* 135, 142–149
10. Eppink, M.H.M. *et al.* (2019) From current algae products to future biorefinery practices: A Review. *Adv. Biochem. Eng.* 166, 99–123
11. Khoo, C.G. *et al.* (2019) Algae biorefinery: review on a broad spectrum of downstream processes and products. *Bioresour. Technol.* 292, 121964
12. Roux, J.M. *et al.* (2017) An overview of microalgae lipid extraction in a biorefinery framework. *Energy Procedia* 112, 680–688
13. Orr, V.C. *et al.* (2015) Disruption and wet extraction of the microalgae *Chlorella vulgaris* using room-temperature ionic liquids. *ACS Sustainable Chem. Eng.* 4, 591–600
14. Orr, V.C. *et al.* (2016) Ionic liquids for the fractionation of microalgae biomass. *Curr. Op. Green Sustain. Chem.* 2, 22–27
15. Passos, H. *et al.* (2014) Ionic liquid solutions as extractive solvents for value-added compounds from biomass. *Green Chem.* 16, 4786–4815
16. Ventura, S.P.M. *et al.* (2017) Ionic liquid-mediated extraction and separation processes for bioactive compounds: past, present and future trends. *Chem. Rev.* 117, 6984–7052
17. Wasserscheid, P. and Keim, W. (2000) Ionic liquids – new "solutions" for transition metal catalysis. *Angew. Chem. Int. Ed.* 39, 3772–3789
18. Sheldon, R.A. *et al.* (2002) Biocatalysis in ionic liquids. *Green Chem.* 4, 147–151
19. Sivapragasam, M. *et al.* (2016) Recent advances in exploiting ionic liquids for biomolecules: Solubility, stability and applications. *Biotech. J.* 11, 1000–1013
20. Schindl, A. *et al.* (2019) Proteins in ionic liquids: reactions, applications, and futures. *Front. Chem.* 7, 347
21. Huddleston, J.G. *et al.* (1998) Room temperature ionic liquids as novel media for 'clean' liquid-liquid extraction. *Chem. Commun.* 16, 1765–1766
22. Gutowski, K.E. *et al.* (2003) Controlling the aqueous miscibility of ionic liquids: aqueous biphasic systems of water-miscible ionic liquids and water-structuring salts for recycle, metathesis, and separations. *J. Am. Chem. Soc.* 125, 6632–6633
23. Lee, S.Y. *et al.* (2017) Cell disruption and lipid extraction for microalgal biorefineries: A review. *Bioresour. Technol.* 244, 1317–1328
24. Santos, J.H.P.M. *et al.* (2018) Fractionation of *Isochrysis galbana* proteins, arabinans, and glucans using ionic-liquid-based aqueous biphasic systems. *ACS Sustain. Chem. Eng.* 6, 14042–14053
25. Swatloski, R.P. *et al.* (2002) Dissolution of cellose with ionic liquids. *J. Am. Chem. Soc.* 124, 4974–4975
26. Gontard, N. *et al.* (2018) A research challenge vision regarding management of agricultural waste in a circular bio-based economy. *Crit. Rev. Environ. Sci. Technol.* 48, 614–654
27. Farzad, S. *et al.* (2018) Multi-product biorefineries from lignocelluloses: a pathway to revitalisation of the sugar industry? *Biotechnol. Biofuels* 10, 1–24
28. Ulonska, K. *et al.* (2018) Optimization of multiproduct biorefinery processes under consideration of biomass supply chain management and market developments. *Ind. Eng. Chem. Res.* 57, 6980–6991
29. Nieder-Heitmann, M. *et al.* (2019) Process design and economic evaluation of integrated, multi-product biorefineries for the co-production of bio-energy, succinic acid, and polyhydroxybutyrate (PHB) from sugarcane bagasse and trash lignocelluloses. *Biofuels Bioprod. Bioref.* 13, 599–617
30. Obydenka, S.V. *et al.* (2019) Industrial lignin from 2G biorefineries – assessment of availability and pricing strategies. *Bioresour. Technol.* 291, 121805
31. Giuliano, A. *et al.* (2015) Process design of a multi-product lignocellulosic biorefinery. *Comput. Aided Chem. Eng.* 37, 1313–1318

32. Serna-Loaiza, S. *et al.* (2019) A review on the feedstocks for the sustainable production of bioactive compounds in biorefineries. *Sustainability* 11, 1–24
33. Righetti, E. *et al.* (2020) A multiproduct biorefinery approach for the production of hydrogen, methane and volatile fatty acids from agricultural waste. *Waste Biomass Valorization* 11, 5239–5246
34. Fava, F. *et al.* (2015) Biowaste biorefinery in Europe: opportunities and research & development needs. *New Biotechnol.* 32, 100–108
35. 't Lam, G. *et al.* (2017) Multi-Product microalgae biorefineries: from concept towards reality. *TIBS* 36, 216–227
36. Wilken, L.R. *et al.* (2018) Ionic liquids: a brief history. *Biophys. Rev.* 10, 691–706
37. Phong, W.N. *et al.* (2018) Mild cell disruption methods for bio-functional proteins recovery from microalgae – recent developments and future perspectives. *Algal Res.* 31, 506–516
38. Soto-Sierra, L. *et al.* (2018) Extraction and fractionation of microalgae-based protein products. *Algal Res.* 36, 175–192
39. Patel, R. *et al.* (2014) Recent advances in the applications of ionic liquids in protein stability and activity: a review. *Appl. Biochem. Biotechnol.* 172, 3701–3720
40. Zhao, H. *et al.* (2016) Protein stabilization and enzyme activation in ionic liquids: specific ion effects. *Chem. Technol. Biotechnol.* 91, 25–50
41. Lin, X. *et al.* (2013) Extraction and separation of proteins by ionic liquid aqueous two-phase system. *Analyst* 138, 6445–6453
42. Heller, W.T. *et al.* (2010) Characterization of the influence of the ionic liquid 1-butyl-3-methylimidazolium chloride on the structure and thermal stability of green fluorescent protein. *J. Phys. Chem. B* 114, 13866–13871
43. De Gonzalo, G. *et al.* (2007) Asymmetric biocatalytic reduction of ketones using hydroxyl-functionalized water-miscible ionic liquids as solvents. *Tetrahedron* 18, 2541–2546
44. Dreyer, S. *et al.* (2008) Ionic liquids for aqueous two-phase extraction and stabilization of enzymes. *Biotech. Bioeng.* 99, 1416–1424
45. Kohlmann, C. *et al.* (2011) Ionic liquid facilitates biocatalytic conversion of hardly water soluble ketones. *J. Mol. Catal.* 68, 147–153
46. Desai, R. *et al.* (2014) Extraction and stability of selected proteins in ionic liquid based aqueous two phase systems. *Green Chem.* 16, 2670–2679
47. Suarez Garcia, E. *et al.* (2018) Fractionation of proteins and carbohydrates from crude microalgae extracts using an ionic liquid based-aqueous two phase system. *Sep. Pur. Tech.* 204, 56–65
48. Freire, M. (2016) *Ionic-Liquid Based Aqueous Biphasic Systems: Fundamentals and Applications*, Springer-Verlag
49. Pereira, J.F.B. *et al.* (2013) Combining ionic liquids and polyethylene glycols to boost the hydrophobic-hydrophilic range of aqueous biphasic systems. *Phys. Chem. Chem. Phys.* 15, 19580–19583
50. Suarez Ruiz, C. *et al.* (2018) Rubisco separation using biocompatible aqueous two-phase systems. *Sep. Pur. Technol.* 196, 254–261
51. Sequeira, R.A. *et al.* (2020) One-step selective partition of the ε-polylysine present in broth cultures in ionic liquid-based aqueous biphasic systems. *Sep. Sci. Technol.* 15, 1–9
52. Martins, M. *et al.* (2016) Recovery of phycobiliproteins from the red macroalgae *Gracilaria sp.* Using ionic liquid aqueous solutions. *Green Chem.* 18, 4287–4296
53. Shu, Y. *et al.* (2010) Ionic liquid-polyvinyl chloride ionomer for highly selective isolation of basic proteins. *Talanta* 81, 637–642
54. Plowman, J.E. *et al.* (2014) Ionic Liquid-assisted extraction of wool keratin proteins as an aid to MS identification. *Anal. Methods* 6, 7305–7311
55. Suarez Ruiz, C. *et al.* (2018) Selective and mild fractionation of microalgal proteins and pigments using aqueous two-phase systems. *J. Chem. Technol. Biotechnol.* 93, 2774–2783
56. Suarez-Ruiz *et al.* (2020) A Multistep fractionation of microalgal biomolecules using selective aqueous two-phase system. *Chem. Eng. J.* 8, 2441–2452
57. Desai, R. *et al.* (2017) One step biorefinery of functional biomolecules from microalgae extracts. *React. Chem. Eng.* 2, 182–187
58. Burguera, J.L. *et al.* (2012) Analytical applications of emulsions and microemulsions. *Talanta* 96, 11–20
59. Chen, J. *et al.* (2013) Environmentally friendly ionic liquid-in-water microemulsions for extraction of hydrophilic and lipophylic components from Flos Chrysanthemi. *Analyst* 138, 5933–5941
60. Mao, X. *et al.* (2014) A dual-ionic liquid microemulsion system for the selective isolation of haemoglobin. *RSC Adv.* 4, 8177–8182
61. Agrawal, G. *et al.* (2018) Stimuli-responsive microgels and microgel-based systems: advances in the exploitation of microgel colloidal properties and their interfacial activity. *Polymers* 10, 418
62. Hashemnejad, S.M. *et al.* (2019) Thermoresponsive nanoemulsion-based gel synthesized through a low-energy process. *Nat. Commun.* 10, 2749
63. Desai, R. *et al.* (2016) Novel astaxanthin extraction from *Haematococcus pluvialis* using cell permeabilising ionic liquids. *Green Chem.* 18, 1261–1267
64. Desai, R. *et al.* (2019) Mild fractionation of hydrophilic and hydrophobic components from *Neochloris oleoabundans* using ionic liquids. *Front. Bioeng. Biotech.* 7, 1–9
65. Suarez Ruiz, C. *et al.* (2020) *Neochloris Oleoabundans* biorefinery: integration of cell disruption and purification steps using aqueous biphasic systems-based in surface-active ionic liquids. *Chem. Eng. J.* 399, 125683
66. Han, D. and Row, K.H. (2010) Recent applications of ionic liquids in separation technology. *Molecules* 15, 2405
67. Zhao, D. *et al.* (2007) Toxicity of ionic liquids. *Clean Soil Air Water* 35, 42–48
68. Pretti, C. *et al.* (2011) Acute toxicity and biodegradability of N-alkyl-N-methylmorpholinium and N-alkyl-DABCO based ionic liquids. *Ecotoxicol. Environ. Saf.* 74, 748–753
69. Flieger, J. and Flieger, M. (2020) Ionic liquid toxicity – benefits and threats. *Int. J. Mol. Sci. (MDPI)* 21, 6267
70. Gouveia, W. *et al.* (2014) Toxicity of ionic liquids prepared from biomaterials. *Chemosphere* 104, 51–56
71. Sardar, S. *et al.* (2018) Physicochemical properties, Brönsted acidity and ecotoxicity of imidazolium-based organic salts: non-toxic variants of protic ionic liquids. *J. Mol. Liq.* 269, 178–186
72. Gore, R.G. and Gathergood, N. (2013) *Safer and Greener Catalysts – Design and High Performance, Biodegradable and Low Toxic Ionic Liquids*, InTechOpen
73. Sequeira, R.A. *et al.* (2020) Recent trends in processing of proteins and DNA in alternative solvents: a sustainable approach. *Sustain. Chem.* 1, 116–137
74. European Commission (2006) Regulation (EC) No 1907/2006 of the European Parliament and of the Council. *Off. J. Eur. Union* 49, L396
75. United Nations (2013) *Globally Harmonized System of Classification and Labelling of Chemicals (GHS)*. United Nations
76. Official Journal of the European Union, Commission Regulation (EU) No. 286/2011. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32011R0286&from=DE.
77. Gadilohar, B.L. and Shankarling, G.S. (2016) Choline based ionic liquids and their applications in organic transformation. *J. Mol. Liq.* 227, 234–261
78. Kunz, W. *et al.* (2011) Low toxic ionic liquids, liquid cationics, and ionic liquid microemulsions. *J. Dispers. Sci. Technol.* 32, 1694–1699
79. Quental, M.V. *et al.* (2015) Enhanced extraction of proteins using cholinium-based ionic liquids as phase-forming components of aqueous biphasic systems. *Biotechnol. J.* 10, 1457–1466
80. Crespo, J.G. *et al.* (2017) Characterization and modeling of the liquid phase of deep eutectic solvents based on fatty acids/alcohols and choline chloride. *I EC Res.* 56, 12192–12202
81. Deferm, C. *et al.* (2018) Purification of crude In(OH)3 using the functionalized ionic liquid betainium bis(trisfluoromethylsulfonyl) imide. *Green Chem.* 20, 412–424
82. Vanda, H. *et al.* (2018) Green solvents from ionic liquids and deep eutectic solvents to natural deep eutectic solvents. *C. R. Chim.* 21, 628–638
83. Mbous, Y.P. *et al.* (2016) Applications of deep eutectic solvents in biotechnology and bioengineering - promises and challenges. *Biotechnol. Adv.* 35, 105–134
84. Roehrer, S. *et al.* (2016) Deep eutectic solvents in countercurrent and centrifugal partition chromatography. *Chromatogr. A* 1434, 102–110

85. Sed, G. *et al.* (2018) A novel switchable-hydrophilicity, natural deep eutectic solvent (NaDES)-based system for bio-safe biorefinery. *RSC Adv.* 8, 37092
86. Zhang, Q. *et al.* (2012) Deep eutectic solvents: synthesis, properties and applications. *Chem. Soc. Rev.* 41, 7108–7146
87. Chen, Q. *et al.* (2017) A fatty acid solvent of switchable miscibility. *J. Colloid Interface Sci.* 504, 645–651
88. Cicci, A. *et al.* (2018) Circular extraction: an innovative use of switchable solvent for the biomass biorefinery. *Green Chem.* 20, 3908–3911
89. Vicente, A.F. *et al.* (2020) Separation and purification of biomacromolecules based on microfluidics. *Green Chem.* 22, 4391–4410
90. Hallett, J.P. and Welton, T. (2011) Room-temperature ionic liquids: solvents for synthesis and catalysis. 2. *Chem Rev.* 111, 3508–3576
91. Cassity, C.G. *et al.* (2013) Ionic liquids of superior thermal stability. *Chem. Commun.* 49, 7590–7592
92. Freemantle, M. (1998) Designer solvents. *Chem. Eng. News Arch.* 76, 32–37
93. Louros, C.L. *et al.* (2010) Extraction of biomolecules using phosphonium-based ionic liquids + K(3)PO(4) aqueous biphasic systems. *Int. J. Mol. Sci.* 11, 1777–1791
94. Wasserscheid, P.W. and Welton, T. (2003) *Ionic Liquid in Synthesis*, Wiley-VCH
95. Ventura, S.P.M. *et al.* (2010) Assessing the toxicity on [C_3mim][Tf_2N] to aquatic organisms of different trophic levels. *Aquat. Toxicol.* 96, 290–297
96. Ventura, S.P.M. *et al.* (2012) Toxicity assessment of various ionic liquid families towards *Vibrio fischeri* marine bacteria. *Ecotoxicol. Environ. Saf.* 76, 162–168
97. Das, R.N. *et al.* (2016) Development of predictive QSAR models for *Vibrio fischeri* toxicity of ionic liquids and their true external and experimental validation tests. *Toxicol. Res.* 5, 1388–1399
98. Ventura, S.P.M. *et al.* (2014) Ecotoxicity analysis of cholinium-based ionic liquids to *Vibrio fischeri* marine bacteria. *Ecotoxicol. Environ. Saf.* 102, 48–54
99. Parajó, J.J. *et al.* (2019) Glycine-betaine-derived ionic liquids: synthesis, characterization and ecotoxicological evaluation. *Ecotoxicol. Environ. Saf.* 184, 109580