SAPIENZA
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Novel biorefining systems for photosynthetic microorganisms

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Summary

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Abstract

This thesis describes research done on novel biorefining systems for microalgae. Following the principles of Green Chemistry, the environmental impact of biomass processing can best be minimized by utilizing many components of the biomass, extracting them with innovative solvents and by getting maximum utility out of any solvent used. Switchable Hydrophilicity Solvents (SHSs) are a new class of solvents able to change their nature from hydrophobic to hydrophilic and vice-versa. In this work a SHS has been used to carry out the fragmentation and extraction of both hydrophobic and hydrophilic molecules from microalgal biomass in the two opposite-hydrophilicity states that the SHS can exhibit, thus bringing about a quite substantial simplification in the biorefining of the matrix and increasing the "useful effect" of the solvent itself that performs a second, complementary biomass extraction task during the regeneration stage. The proposed concept of "Circular Extraction" scheme has been validated with the N,N-dimethyl-cyclohexylamine on the oleaginous microalga *Scenedesmus dimorphus*, showing an extraction efficiency in the backward-mode of 52 % for proteins, 50 % for carbohydrates, and 93 % for lipids. In the second part of this work, the PCH (1,2-propanediol, choline chloride, water 1:1:1) Natural Deep Eutectic Solvent (NaDES) was used to treat microalgal biomass and carry out the extraction of cellular components, such as lipids, proteins, carbohydrates and photosynthetic pigments (chlorophylls and carotenoids) from the biomass itself. NaDES, i.e. mixtures formed by natural primary metabolites present in all organisms form intermolecular hydrogen bonds and, when mixed in a certain ratio, change their state from solid to liquid forming a eutectic system. Experiments were carried out based on different contact time between biomass and PCH: 24 and 72 hours, with and without pre-treatment with ultrasound. Biomass was shaken together with the PCH solvent in the presence of glass beads to promote the extraction efficiency. The analysis of the extract composition was carried out spectrophotometrically for pigments (chlorophylls and carotenoids), with biochemical assays for proteins and carbohydrates and gravimetrically for the determination of lipids. The results showed the ability of PCH, coupled with the mechanical destruction of cell walls, to solubilize a wide range of polar biomolecules at room temperature, precisely the 49 % of proteins, 46 % of carbohydrates, 15 % of neutral lipids, 16 % of chlorophylls and 32 % of carotenoids. In the last part of the experimental work is introduced and demonstrated the first SHS ever described in the open literature which is based on a natural deep eutectic solvent (NaDES). The innovative solvent system that is disclosed therein is based on natural metabolites, that

are intrinsically safe, and on water solution of a hydrophilicity-switching compound whose toxicity is practically nil, therefore also duplicating this as the first finding of a practically bio-safe switchable hydrophilicity solvent. Together, the two findings mark a significant step toward the identification of a destination-neutral biorefining tool for the obtainment of biologic fractions from biomasses with a wide range of potential market applications. We demonstrated the potential of this novel system for fractionating proteins-, carbohydrates- and lipids-containing biomass by following the Circular Extraction biorefining scheme on microalgae. The present finding is quite a substantial step toward intrinsic safety in biomass processing.

Introduction

Biorefining is the sustainable processing of biomass into a spectrum of bio-based products (food, feed, chemicals, and materials) and bioenergy (biofuels, power and/or heat) [Definition IEA Bioenergy Task 42 on Biorefineries]. We can define this concept as "destination neutrality", biomolecules could also be used in industry with greater restrictions on product standard such as food and pharmaceuticals sectors.

The concept of biorefining is intrinsically linked to the concept of solvent, as these chemicals are widely used in downstream processes for the extraction and fractionation of biomolecules of interest. Unfortunately, many solvents are classified as highly problematic substances under the European REACH regulation and equivalent legislation worldwide. Most solvents are volatile, flammable and can be highly toxic. Non-volatile solvents could avoid risks for the operator and the environment, but their use is not common in the chemical industry due to their inability to be distilled and then recovered. Distillation is the standard method for removing solvent from a product, so it is preferred to other separation methods. Unless a new approach to the solvent industry is implemented, regulatory measures will have a huge knock-on effect, reducing growth in the many industries currently dependent on conventional organic solvents. The identification and use of efficient, economical, biodegradable and less toxic solvents, whose applicability can vary in a wide range of markets, is therefore one of the fundamental points of chemical research at the moment. Over the last few decades, potentially safer solvents have emerged: supercritical fluids, ionic liquids, deep eutectic solvents and more recently the switchable hydrophilicity solvents (SHSs). The use of SHSs in combination with water has been proposed as an alternative to distillation for solvent recovery, without requiring the use of volatile compounds. The employment of this class of solvents coupled with the concept of biorefining has been proposed in the literature and we can find some examples in the most recent bibliography. The use of SHS in biorefining could reduce the environmental impact of the use of biomass by operating the "Circular extraction", presented in this thesis and recently published in the journal "Green Chemistry". It proposes the depletion of the microalgal matrix through a switchable solvent in its hydrophobic form and then in its hydrophilic form, or vice-versa, and eventually return to the hydrophobic form to carry out a new extraction cycle.

This work contains studies performed on the synthetic SHS of N,N-dimethylcyclohexylamine (DMCHA), presenting a kinetic model suitable for the description

of the extraction of biocomponents from microalgal biomass, such as carbohydrates and proteins. The model concerns both solvent in its hydrophobic form and in the switched form. The DMCHA achieves its best results in the extraction efficiency of biomolecules when used following the circular extraction protocol, therefore in both its forms, hydrophobic and hydrophilic. As many synthetic solvents, it still presents problems due to its intrinsic toxicity, therefore the extraction performances of two other SHS, dibutylaminoethanol and diisopropylaminoethanol were evaluated. The thesis then proceeds with the study and characterization of a hydrophilic Natural Deep Eutectic Solvent (NaDES), the PCH, investigating its extraction capacity on microalgal biomass, coupled with biomass mechanical pre-treatments such as bead-beating and Ultrasound Assisted Extraction. In the conclusion of this work the formulation of a new invertible system is showed, it combines the bio-friendly characteristics of natural solvents with those of SHS commutability: it is NaDES-Y, the first hydrophobic NaDES presenting switchable characteristic. This "green" solvent was tested on both synthetic and microalgae matrices by applying the circular extraction paradigm, demonstrating its appropriate extraction efficiency and establishing itself as a suitable solvent to maintain the destination neutrality of the biocomponents extracted from microalgae.

Chapter 1. Microalgal Biorefinery

1.1 Biorefinery concept in biobased economy

Since the twentieth century the emerging economy of the developing countries has been characterised by fossil fuels. Petrochemical raw materials were chosen as the main building blocks for the organic synthesis. Global energy consumption has continued to increase, due to improvements in quality of life, industrialization and ongoing expansion of the world population. Fossil inputs are currently used to produce more than 80% of the fuel, energy, materials, chemicals, and products consumed in western European countries [EU Presidency, 2007]. Over time, some undesirable effects of the fossil-fuel-based economic have become apparent such as air pollution, acid rain, and climate change caused by greenhouse gases (GHG) emissions. [Menz et al. 2004, Kampa et al. 2008, National Research Council 2001]. In the twenty-first century the depletion of fossil fuels, the rising oil prices and the growing environmental awareness, push the attention and policy towards a transition from fossil- into bio-based products, which uses biomass as the source for production of energy and raw materials [Sanders et al. 2008]. In a bio-based economy biomass is valorised and used for the sustainable production of food, feed, chemicals, fuels, power and heat [definition of IEA Bioenergy task 42 Biorefinery]. The use of biomass as an input for production is nothing new in traditional food and feed industries, but recently new applications in several fields have been developed. This is encouraged because biomass is renewable and, at least theoretically, inexhaustible. Moreover, the use of biomass offers solutions to many of the problems of the fossil-input-based economy: it ensures both energy diversity and security and is environmentally friendly owing to carbon sequestration and the resulting climate change mitigation [Vandermeulen et al. 2012]. Therefore, several actors will need to work together: the agricultural sector, chemical industries, governments, consumer and user organizations, but are also necessary technology, economic, and policy innovations [Loorbach 2010]. An example of policy incentives are the 20-20-20 goals by the EU that aim for 20% increase in energy efficiency, 20% reduction of CO_2 emissions and 20% renewable energy by 2020 compared to the 1990 levels (European Union 17125/08). Transition towards bio-based products will have consequences on the demand and processing of biomass to enable the development of new markets such as bio-energy, bio-plastics, bio-chemicals, and bio-pharmaceuticals [IEA Bioenergy task 42, King 2010]. To realise a bio-based economy it is important to combine various feedstocks, conversion

techniques and production routes [González Delgado et al. 2011, Carroll et al. 2009]. The integrated process of separating and converting biomass elements is known as biorefinery.

1.2 Biorefinery

The concept of biorefinery is similar to traditional petroleum refinery, the goal is the obtaining of chemicals, fuels and products. The main difference between biorefinery and traditional refinery is in terms of the raw materials (biomass or crude oil) and the technology employed. Biomass contains a mixture of substances: carbohydrates, proteins, lipids and aromatic polymers from which chemicals can be produced by means of biotechnological processes. Ideally, biorefineries use one or several feedstocks to produce a spectrum of low and high value products, thereby balancing the production cost and life cycle impacts between the products [Kamm et al. 2004]. Biorefining biomass for higher value products is expected to improve the overall productivity and efficiency of biomass utilisation. Biorefinery can use all kinds of biomass from forestry, agriculture, aquaculture, and residues from industry and households including wood, agricultural crops, organic residues, forest residues, and aquatic biomass. Industrially, the major domains that can be covered by products derived from biomass refinery could originate from single molecules (any use) or fractions (above all for structural functions, materials and energy). In substitution of petrol derivatives, molecules can be used as solvent surfactants or chemical intermediates. Lipids can be used to produce products like lubricants, pastes or surfactants and fibers can serve as materials like composites. Sugars and oils are currently used to produce biofuels like bioethanol or biodiesel. Industrial biorefinery will be linked to the creation of new processes based on the twelve principles of green chemistry (clean processes, atom economy, renewable feedstocks). Biotechnology, especially white biotechnology, will take a major part into these new processes with bio-transformations and fermentation [de Jong et al. 2015].

To guide future developments on bio-based products, a road map on the biorefinery for bulk chemicals, known as *top twelve chemicals derived from biomass*, has been developed by PNN/NREL (Pacific Northwest National/National Renewable Energy Laboratory) [Werpy et al. 2004]. The target of this roadmap is to produce value-added products from carbohydrates that can substitute petrochemical-based products. Examples of carbohydrate-based products are glycerol, succinic acid, hydroxypropionate, furfural, and sorbitol, which are building blocks for several products that are currently produced via the petrochemical

route. The Nova Institute published a comprehensive overview of the use of biomass and its growth markets. In this report an overview is given of the policies related to the bio-based economy and the required inputs for the EU production of bio-energy, bio-plastics and bio-chemicals [The Nova Institute, 2013]. Figure 1.1 shows a network of possible biorefinery routes using various feedstocks to produce a wide variety of products. The demand of biomass for the production of bioplastics, biochemicals and bioenergy is increasing, according to several reports. In the 2020 the EU will demand for the bio-based economy 50.000 MT of wood pellets for the power and heat sector, 34.000 MT of biomass for biopolymers and biochemicals and in the fuel sector the objective of the Bio-based Industries Consortium (BIC) is to supply 10% of the EU transport energy with sustainable advanced biofuels [FAS Biofuel Annual Report]. On the economic point of view, the value of biomass is driven by capital and operation costs and by the revenue of the products on the market. The size of the market is relevant for the economic feasibility of the biorefining (the higher is the market, the higher are the production costs). The substitution of fossil feedstock by biomass is not always possible because the economic values show large differences, lowest values are attributed to heat production, whereas the highest values are associated with replacement of fossil-derived bulk chemicals. The material costs for the production of 1 GJ of heat will amount to 3 € (assuming a 100% conversion efficiency). The costs to convert biomass feedstock to heat via combustion are 4 €/GJ and are quite low compared to various biorefinery processes. The production of 1 GJ of electric power from coal costs 6 €, from biomass around 22 €. Costs for transportation fuels are comparable (8 €/GJ from oil, 10 €/GJ from biomass. Feedstock costs for bulk chemicals are estimated at 30, but biomass feedstocks could represent values of up to 75 €/GJ if the components are obtained in a pure form. If we could separate biomass into fractions that can be used to produce food, feed, biobased products (chemicals, materials), and/or bioenergy (fuels, power, and/or heat) the financial returns would be greater [Sanders et al. 2007, de Jong et al. 2010].

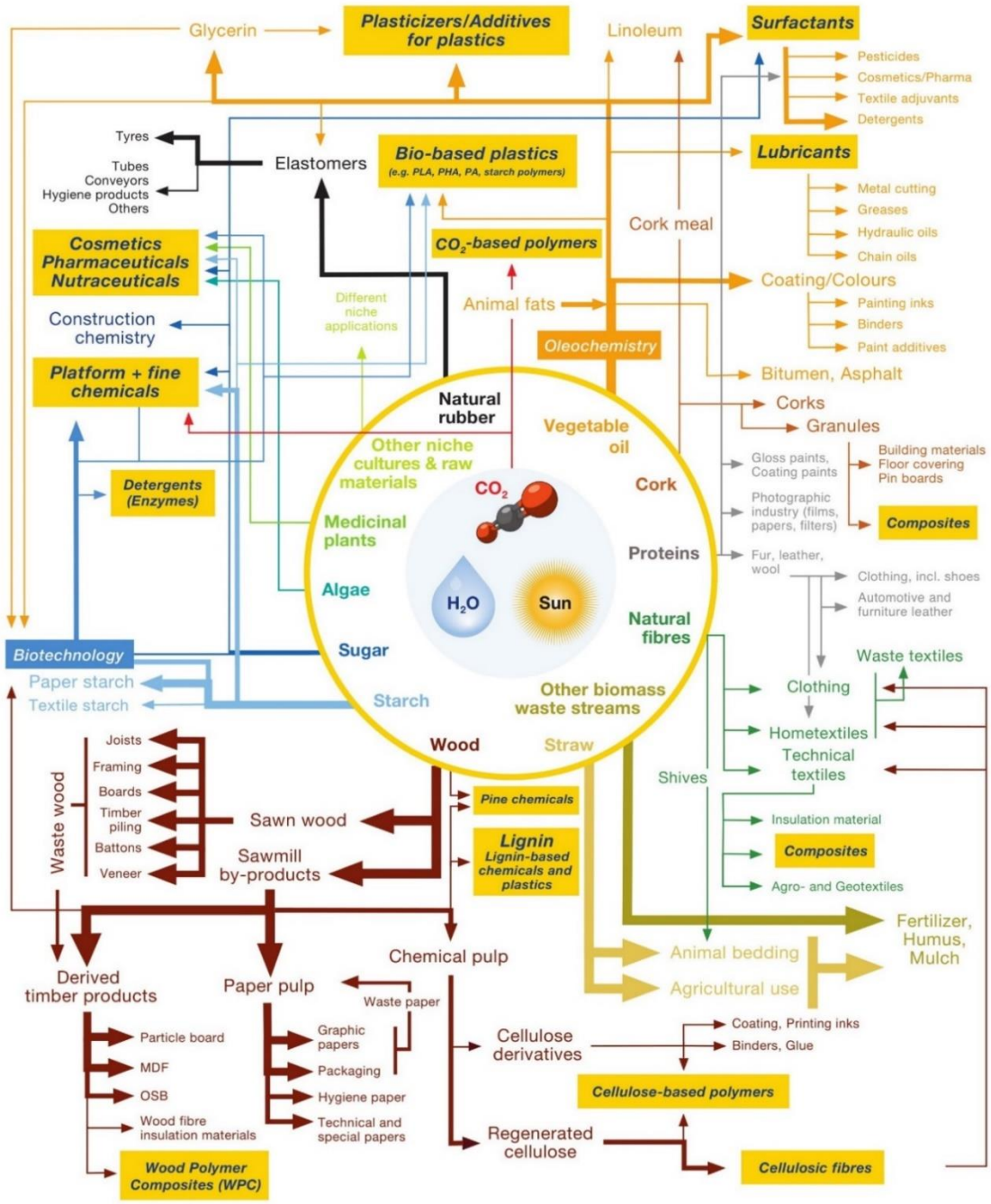

Figure 1.1 Overview of possible biorefinery feedstock and products (The Nova Institute)

Biorefinery Classification

In 2008, IEA Bioenergy Task 42 developed an appropriate biorefinery classification system, it consists of four main features which describe the different biorefinery systems:

- platforms
- products (energy and biobased materials and chemicals)
- feedstocks
- conversion processes

The features are used and combined for the classification of biorefinery systems. The generic biorefinery pathway starts with a feedstock converted to one of the platforms, from which final energy and material products are acquired. The platforms are intermediates which connect different biorefinery systems and their processes, examples are the following: biogas from anaerobic digestion, syngas from gasification, hydrogen from steam reforming or water electrolysis, C5-C6 sugars from hydrolysis of sucrose, starch, cellulose and hemicellulose, lignin from lignocellulosic biomass, pyrolysis liquids, oil from oilseed crops and algae, organic juice from the pressing of wet biomass and electricity and heat. Products can be both energetic and non-energetic. In energy-driven biorefineries the biomass is used for the production of bioethanol, biodiesel, biomethane and biohydrogen, in material-drive biorefineries primarily are generated biobased products such as biomaterials, fertilizers, chemicals and building blocks, food and feed and the process residues can be processed to produce energy. The first generation of biobased feedstocks are wood, grasses and sugar, starch and oil crops. Second generation feedstocks include agricultural residues like straw and corn stover, by-products and waste streams. These feedstocks offer many possibilities to locally produce energy, fuel and chemicals. Microalgae and macroalgae biomass are the third-generation feedstock used for the production of food, feed, chemicals and biofuels [Borines et al. 2011, John et al. 2011, Wijffels 2008]. Different conversion processes are adopted to transform feedstock into final products: mechanical/physical processes perform size reductions or separation of feedstock components, biochemical processes (e.g. anaerobic digestion, fermentation and enzymatic conversion), chemical processes perform changes in the substrate (e.g., hydrolysis, transesterification, hydrogenation, oxidation) and thermochemical processes (e.g., gasification, pyrolysis) [Cherubini et al. 2009].

1.2.1 Microalgae

All microscopic algae, unicellular or filamentous, are called microalgae. The term refers to the eukaryotic unicellular algae and the oxygenic photosynthetic bacteria, the cyanobacteria. These microorganisms are photosynthetic, the cells harvest light energy and produce organic compounds reducing carbon dioxide. A limited number of microalgal species can grow heterotrophically using organic compounds as source of substrates and energy. Mixotrophic growth regime is a possibility, where CO_2 and organic carbon are simultaneously assimilated and both respiratory and photosynthetic metabolism operates concurrently. Microalgae are mainly found both in freshwater and marine environments, but they could live on the surface

of soils in a symbiotic relationship with various organisms such as bacteria and fungi. Aquatic organisms like micro- and macroalgae, depending on species and growing conditions, can accumulate significant amounts of oils, carbohydrates, starch, and vitamins. The most commercially attractive microalgae belong to Chlorella, Arthrospira, Dunaliella and Haematococcus genera (Figure 1.2) [Metting 1996].

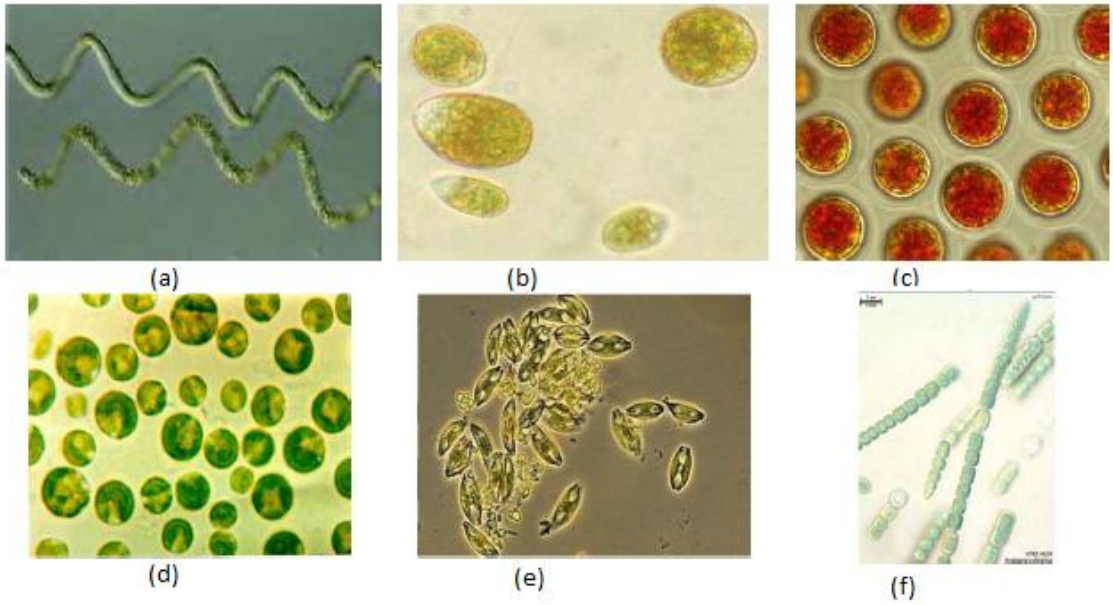

Figure 1.2 Microalgal cells at optical microscope:
(**a**) *Arthrospira platensis* (Spirulina),
(**b**) *Dunaliella*
(**c**) *Haematococcus pluvialis*,
(**d**) *Chlorella vulgaris*,
(e) Amphora sp.
(f) Anabaena cylindrica

Microalgal biomass is interesting for human nutrition due to its high protein content, as "health food", as protein and polyunsaturated fatty acids (PUFAs) source for aquaculture and feeding of cattle, pigs and poultry. *Arthrospira platensis* and *Arthrospira maxima* biomass contain about 60% of crude protein, *Chlorella vulgaris* harbors more than 50% of proteins in its cell mass [Becker 2007]. Indeed, the market for "functional food" surpasses by far all other applications of microalgae. This can be visualised considering that only for Japan, 2400 tons of microalgal biomass is commercialized by year for "health food" purposes [Lee et al. 1997]. Is it possible to take profit of additional biomolecules produced by these organisms, such as pigments, enzymes, sugars, fatty acids, vitamins (vitamins A, B, C, and E) and other bioactive compounds [Borowitzka 1995]. Despite the challenges in producing low value commodities such as biodiesel from microalgae, a few strains have

been commercially exploited for the production of animal feeds and high value products (>US$ 10,000/t), namely, nutraceuticals and cosmeceuticals for human markets including polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [Benemann 2013]. Attractive metabolites with high market values show anti-cancer, antiinflammatory and antibiotic activity and most powerful strains of all result *Haematococcus, Dunaliella and Arthrospira* also famous producers of pigments such as carotenoids (astaxanthin and β-carotene, phycocyanin and phycoerythrin), employed in food industry and for cosmetic purposes (Table 1.1) [Metting 1996, Lorenz et al. 2000]. Value added application of residual algal biomass is the production of green energy carriers like biogas, biohydrogen, or bioethanol [Ruiz et. Al 2016, Vanthoor-Koopmans et al. 2013]. The strain used in this study is Scenedesmus dimorphus 1237 (Figure 1.3), which belongs to the Chlorophyta phylum. Chlorophyta are commonly known as green algae, due to the bright grass-green appearance. Chlorophyta possess chlorophylls a and b, β- and γ-carotene, and several xanthophylls as accessory pigments. *Scenedesmus* is a unicellular microalga usually forming 4- or 8-celled coenobia. Cells are arranged linearly, alternating or in 2-3 rows, touching with the lateral walls or in subpolar region only (Figure 1.3). Cells 3-78 x 2-10 µm, are nearly spherical to ellipsoidal, elongate or fusiform [www.algaebase.org]. *Scenedesmus* was reported world-wide in all climates, mainly in eutrophic freshwater ponds and lakes, with slight acidity but only low salinity. Several strains have hydrogenase and produce secondary carotenoids in nitrogen deficient conditions [Minhas et al. 2016]. Optimal growth temperature is at 28-30 °C. *Scenedesmus* is produced in mass culture for its use as biodiesel feedstock; moreover, it could be employed for municipal wastewaters treatment and to remove metal cations from aqueous solutions [Mandal et al. 2009, Zhang et al. 2008, Harris et al. 1990].

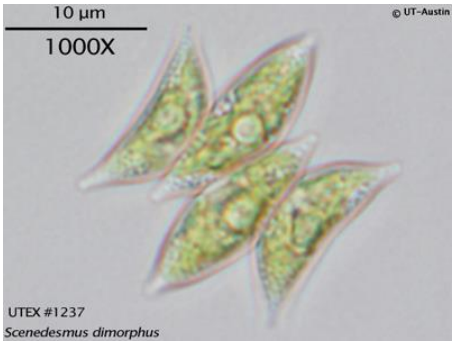

Figure 1.3 S. dimorphus strain 1237 UTEX Culture Collection of Algae

Product	Source organism	Current or potential use
Amphidinolides and amphidinins	*Amphidinium* sp.	Antitumor agent
Astaxanthin	*Haematococcus pluvialis*, *Chlorella* sp., *Dunaliella*	Pigment
β-carotene	*Dunaliella*	Colorant, food supplement
Docosahexanoic acid	*Isochrysis galbana*	Essential fatty acid
γ – Linolenic acid	*Spirulina* sp.	Essential fatty acid
Other PUFAs	*Phaeodactylum tricornutum*, *Isochrysis galbana*	Health care, food supplement
Fucoxanthin	*Phaeodactylum tricornutum*	Antioxidant
Goniodomins	*Alexandrium hiranoi*	Antifungal agent
Oscillapeptin	*Oscillatoria agardhii*	Elastase inhibitor
Phycobiliproteins	Red algae, cyanobacteria	Colorants
Phycocyanin	*Spirulina platensis*	Colorant

Table 1.1 High value products from microalgae and cyanobacteria species [Yamaguchi 1997, Benemann 1989]

Algae offer numerous advantages compared to terrestrial biomasses, thanks to their active ingredients, their rapid growth and their high degree of energy effectiveness. Microalgal biomass has several advantages as compared to grasses and oil crops: they can be cultivated utilising only water and atmospheric CO_2, which may be available at minimum cost and have been recognised for their Green House Gases (GHGs) abatement potential [Campbell et al. 2009]. Due to the ability to grow on degraded land or desertic zones, microalgae production does not create competition for arable land and food crops. According to Khoo and coworkers, high photosynthetic efficiencies coupled with bio-energy production systems, in the future will make these microorganisms the source of sustainable pathways to obtain renewable energy [Khoo et al. 2013].

A multi-product biorefinery could enhance the economic viability of large scale microalgal processes, valorising all biomass components. The only way to increase the total revenue from the process is to produce both bulk and specialty co-products from the biomass. In this way the production of bulk commodities can become economically viable compared to oil refinery [Vermue et al. 2017]. Analogous to an oil/petrochemical refinery, complete fractionation and valorization of the microalgal biomass should be implemented. This concept is referred as a multi-product biorefinery for microalgae [Kamm et al. 2007]. Such a facility typically consists of harvesting, cell disruption, and product extraction steps. To further fractionate or purify the product fractions, additional fractionation must be carried out. Main stages of the microalgal biorefineries are Upstream processing (USP) and downstream processing (DSP).

1.2.2 Upstream processing (USP)

According to the literature, the efficiency of the USP depends on several criteria, among the most important: microalgae strain, light distribution (influenced by illumination and culture optical density), temperature range, supply of carbon dioxide, pH of the culture media and nutrient sources such as nitrogen and phosphorus.

Light distribution

The main energy source for photoautotrophic cells is sunlight (or artificial light) and its availability is determinant for the cultivation. Light input is almost the most important variable in algae growth. Only a specific bandwidth: 400-700 nm is photosynthetically active radiation (PAR) and therefore useful to the algae. This is 43% of the incoming radiation [Geider 1987]. Incoming radiation depends on the location of the cultivation and the season. Algae growth (and photosynthetic activity) is proportional to light intensity until reaching a treshold value, the light saturation point, where further increases in light intensity no longer increases photosynthesis. Intensities higher than this point can damage light receptors in the chloroplasts of the cells and decrease the photosynthetic rate, which is known as photoinhibition (Figure 1.4) [Richmond 2004]. Some plankton species grow with optimal rates at 50 $\mu E/(m^2s)$ and are photoinhibited at 130 $\mu E/(m^2s)$ [Pulz 2001]. In high density culture cells closest to the surface of the liquid catch the majority of the available radiation and cells below reiceve very little light, this problem is called “mutual shading”. Mutual shading is common in high cell density culture because the cells closest to the surface of the liquid receive the majority of the available light and the cells below are left with very little

available light. Extensive research has been conducted to create culture conditions where light can be evenly distributed, taking into account parameters with key roles such as liquid depth and mixing [Grobbelaar 1989, Grobbelaar 1994].

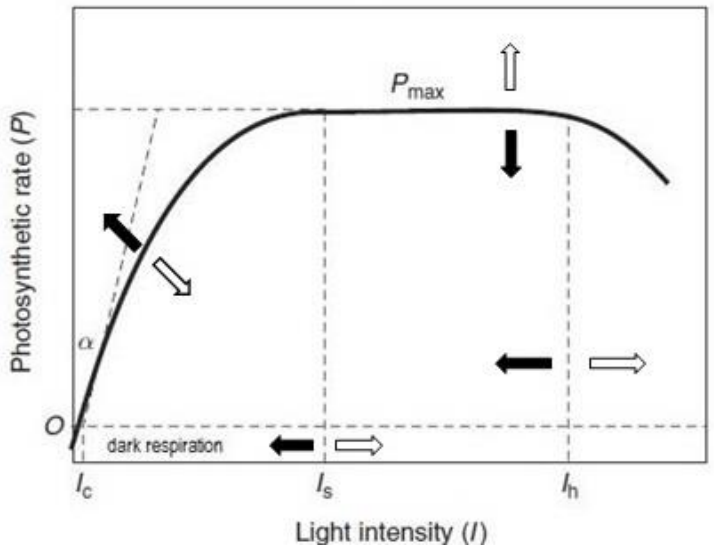

Figure 1.4 Light response curve of photosynthesis versus light intensity (P/I curve). P_{max}, the maximum photosynthetic rate measured ad saturated I; α, maximum photosynthetic efficiency, the intercept on the vertical axis is the measure of O_2 uptake due to dark respiration. I_c, light compensation point; I_s, light saturation intensity; I_h, light intensity value at which photoinhibition occurs. Solid arrows indicate the effect of dark acclimation and open arrows the response to light acclimation (adapted from Richmond 2004 with inclusions from Grobbelaar 2006).

Temperature

Microalgae often have an optimal temperature where growth is most effective, mostly depending on the strain. For Dunaliella salina the optimal temperature of growth could be 22 °C, Scenedesmus almeriensis prefers warmer temperatures around 35 °C, several species such as Chlorella vulgaris, Tetraselmis sp. and Chaetoceros calcitrans reach the maximum growth rate at the intermediate temperature of 30 °C [Wu et al. 2016, Sanchez et al. 2008, Chinnasamy et al. 2009, Adenan et al. 2013, Gong et al. 2014]. Like many microorganisms, growth rate of microalgae generally increases exponentially with increasing temperatures until it reaches an optimum and then the growth rate declines with further temperature increases [Dauta et al. 1990]. In outdoor culture the ability to control temperature is often limited and is dictated by ambient temperatures and solar irradiance. In general, temperatures below the optimal range will not kill the algae until the water freezes, but productivity declines rapidly. However, temperatures above the optimum can kill the algae, that are particularly fragile to high temperatures in times of darkness [Weissman et al 1985]. Due to the impact that temperature variation has on algae culture, it is important to select an appropriate species for the environmental conditions it will be growing in.

Gas exchange

Approximately half of the dry weight of algae cells is made up of carbon, thus algae require continual intake of carbon [Becker 1994]. In autotrophic cultures, carbon is most commonly added in the form of CO_2, which is bubbled though sparging stones or perforated pipes, but in some cases, CO_2 can be supplemented through floating gas exchangers or hollow fiber membranes [Carvalho et al. 2001]. During photosynthesis, CO_2 is consumed and O_2 is released into the liquid. High O_2 concentrations will cause photo-oxidative damage to chlorophyll which will inhibit photosynthesis and reduces productivity [Molina Grima et al. 2001]. In open ponds the large surface area for O_2 mass transfer reduce the dissolved O_2 levels, in closed photobioreactors, there are additional compartments known as gas exchange chambers that aid the oxygen removal.

pH

All strains of microalgae seem to have a limited range of pH where the growth rate reaches the optimum [Lutzu et al. 2012]. Algae respire carbon dioxide during photosynthesis, and at optimal pH, the bicarbonate present in the medium is converted into carbon with the release of hydroxyl ions that tend to increase the pH [Gerardi 2015]. CO_2 is also often used to maintain a steady pH in the culture system. As CO_2 is absorbed into the liquid phase it is converted into carbonic acid, which effectively lowers the pH. As algae consume the carbonic acid the pH rises. By controlling CO_2 levels, the pH can be controlled very effectively. Extremes of pH reduce the rate of photosynthesis. At high pH, the trend of absorption of the trace metals and nutrients might get altered. Similarly, at low pH, enzyme inhibition occurs in the photosynthetic process and there is a high possibility of the growth medium getting contaminated by micro-organisms [Bakuei et al. 2015]. The optimal pH range for photosynthesis to occur in most of the microalgae is in between 6 and 10, wherein the bicarbonate form is considered to be dominant [Rastogi et al. 2017].

Nutrients

Microalgae use inorganic nutrients for their growth and the production of certain specific fatty acids, pigments or vitamins. These nutrients include macronutrients and trace elements. In most cultures the macronutrients required are nitrogen (in the form of nitrogen oxide, ammonia or urea) and phosphorus (phosphorus oxide). Nitrogen is an essential element in proteins and for the cell division. Lack of nitrogen results in a build-up of lipids in the cell [Thompson 1996]. Phosphorus is also essential for almost all cellular processes of the algae. A limitation in phosphorus results in a lower growth rate. Similar to nitrogen starvation, a

phosphorus deficiency causes lipid accumulation [Wymer et al. 60]. For some elements small amounts are needed for optimal growth. The most important trace elements are sulphur and magnesium for building amino acids and chlorophyll, and calcium for nitrogen metabolism. Other elements that show a direct effect on algae growth are manganese, nickel, zinc, boron, vanadium, cobalt, copper and molybdenum [Song et al. 2012].

1.2.2.1 Microalgae culture systems

For autotrophic growth regimes it is necessary to establish a balance between light availability and biomass concentrations. Mixing is essential for light availability as well as eliminating nutritional, thermal, and gaseous gradients. The various types of radiation differ in their wavelengths, and consequently in the amounts of energy carried by their individual quanta. Between 380 and 750 nm (visible light), the energy content is sufficient to produce chemical changes in the absorbing molecules, as happens throughout the photosynthetic pathways prevailing in microalgae [Kommareddy et al. 2003]. Photobioreactors (PBRs) are reactors in which phototrophs are grown or used to carry out a photobiological reaction. In a broad sense, the open shallow basins widely used for microalgae cultivation could also be viewed as photobioreactors [Tredici 2010]. The microalgal cultivation systems can be "open" or "closed" (to the atmosphere) pieces of equipment. In every case it is necessary to minimize the contaminations of the crop, to furnish suitable CO_2, nutrients and illumination, and maintain the required cultural conditions (pH, temperature, salinity etc.), to reduce the capital and maintenance costs [Eriksen 2008].

Open ponds

For commodities the costs of production, both capital and operating, must be very low and for this reason the main configuration for large scale microalgae cultivation is represented by the open pond reactors (Fig. 1.5). Between closed and open cultivation systems, open ponds have the advantage of being cheaper to operate. They require less energy, are easier to clean and maintenance is less expensive on average [Jorquera et al 2010]. Regardless of the end product, for an operation to be feasible, costs must be kept as low as possible in order for the operation to be sustainable. This configuration is extremely simple and the CO_2 necessary for microalgae growth is taken directly from the surface air as well as enlightenment supplied by the sun light. The most common technical design for open pond systems are raceway paddle wheel mixed ponds but are used also unmixed open ponds and circular open ponds mixed with a central pivot mixer. Commonly these reactors operate at

water depths of 15–20 cm, where the biomass concentrations can be of up to 1,000 mg/l and productivities of 60–100 mg/l day^{-1} are possible, because a single open raceway ponds can be scaled well over one hectare (>10,000 m^2) in size (Pulz 2001, Weissman 1988].

One of the issues presented by this kind of cultivation is the threat of contamination and pollution of the mono-culture, for this reason the species need to have high growth rates or have to be adapted to grow in a specific culture medium. Moreover, when algae are grown in regions with warm temperatures and high light intensities, the water evaporation in open reactors is huge. Furthermore, when cell density increases, the light penetration decreases. As a consequence, algae far from the top of the reactor do not have sufficient light and the growth and productivity decrease [Becker 1994]. Harvesting processes cost could be high due to the huge volume of these reactors [Jorquera et al. 2010]. Comparative analysis of microalgal biomass and bio-oil production using three different cultivation systems: raceway ponds, tubular photobioreactors and flat plate photobioreactors.

Figure 1.5 (a-e) Commercial microalgae production ponds
(**a**) Cyanotech Co., Hawaii, producing *Haematococcus pluvialis* and Spirulina;
(**b**) Close-up of Cyanotech Spirulina ponds, ~0.3 ha;
(**c**) Earthrise Nutritionals LLC, California, ~0.4 and 0.8 ha;
(**d**) Chlorella Industries, Japan, circular ponds, each ~500 m^2;
(**e**) Betatene (Cognis), W. Australia, unmixed ponds, *Dunaliella* production ~500 hectares;

Photobioreactors (PBRs)

PBRs are closed environment that can be implemented indoor or outdoor. They allow to control important parameters as well as the contamination risk, CO_2 losses, temperature, pH and other cultivation conditions and light distribution. Constructing and operating PBRs is costly due to high energy requirements for growth and harvesting and high cost of materials to build the systems [Jorquera et al. 2010]. Other challenges exist in their operation from factors such as overheating, biofouling and oxygen accumulation. While closed systems present these and other challenges, they also offer the ability to provide higher biomass productivities compared to open pond systems [Moheimani 2013]. Essential criteria for a successful cultivation in a PBR include: surface-to-volume ratio, orientation and inclination, mixing and degassing, systems for cleaning and temperature regulation and durability of materials [Tredici 2004]. The goal for PBRs design is achieving the maximum photosynthetic efficiency (PE), and for microalgae this is possible using light with a wavelength between 400-700 nm, the photosynthetically active radiation (PAR), that represent between 43-45% of total solar radiation. The light effectively available for each cell in the reactor, the average irradiance (I_{av}) depends on both the irradiance at the culture surface (I_0) and the biomass concentration [Tredici 2004]. Surface to Volume ratio (S/V) is the ratio between illuminated surface area of a reactor and its volume. It determines the amount of light that enters the system per unit volume. High S/V provides high cell concentration at which the reactor can be operated and high volumetric productivity. The S/V also influences productivity per unit of illuminated surface area. There is an optimal S/V at which maximum productivity per unit of illuminated surface area is obtained, and it changes with the cultivated species [Wang et al. 2012]. Various orientations and inclination angles to the sun can be attempted to vary irradiances at the culture surface. The higher productivities are reached by sun-oriented systems. Vertical systems that intercept sun rays at large angles, and thus dilute sunlight, attain higher efficiencies of solar energy conversion. [Tredici 2010]. Efficient mixing should be provided in order to produce a uniform dispersion of microalgae within the culture medium, thus eliminating gradients of light, nutrient concentration (including CO_2) and temperature. In a dense culture, the region where microalgae receive enough light for photosynthesis can be quite shallow (2-5 cm), so vigorous mixing is necessary to provide cells with a uniform average exposure to light [Becker 1994]. CO_2 supply and oxygen removal are related to mixing and to the mass transfer capacity of the system. Nearly 50% of the dry algal biomass is made up of carbon;

consequently, carbon nutrition is a major component of the production cost of autotrophic biomass. Automatic pH control, in which CO_2 is injected when pH is above a desired value, is the most common system employed to both regulate pH and replenish CO_2. Dissolved oxygen concentrations equivalent to more than four times saturation with respect to air (~30 mg O_2/L) are toxic to many microalgae. The maintenance of oxygen levels below the toxic concentration requires frequent degassing and very high flow rates [Wang et al. 2012]. The optimal temperature for the growth must be maintained to achieve the maximum productivity. Materials for PBRs must have transparency, high mechanical strength, durability, chemical stability and low cost. Ease of cleaning and lack of toxicity are important requisites. Commonly glass and different types of transparent plastic and resin (polyethylene, polyvinylchloride, polymethyl metacrilate, Teflon, polycarbonate, fiberglass) can be used for the construction [Tredici 2004]. Main advantages and disadvantages of PBRs and Open Ponds are reported in Table 1.2.

According to the criteria the main configurations adopted are (1) large bags which are commonly used by the aquaculture industry, (2) tubular reactors; (3) flat plate reactors, (3) vertical column reactors.

Reactor Type	**Advantages**	**Disadvantages**
Photobioreactor	- highly efficient method	- Expensive for mass cultivation
	- highly biomass production	- O_2 accumulation
	- Fixing high [CO_2] efficiently	- Problems in treating acidic gases
	- Simple operation	- High pressure drops
	- Efficiently control nutrients, light and [CO_2]	- High investment and maintenance costs
	- increasing CO_2 residence time	
	- Capable of removing all types of pollutants	
	- No waste production	
Raceway pond	- Low investment	- Enormous land area required
	- Natural mass transfer mechanism	- Difficult operation
	- Cost-effective vessel for large scale growing	- Unwanted algae growth
	- High efficiency	- Limited CO_2 transfusion
	- No wastewater production	- Large cultivation areas and difficulty in controlling cultivation conditions
		- Reduced light intensity with increased depth
		- Low productivity

Table 1.2 Advantages and disadvantages of microalgal cultivation in PBRs and Raceway open ponds [Yun et al. 1997].

Plastic bags

Plastic bag PBRs (Figure 1.6) are attractive due to their low cost [Wang et al. 2012]. These bags can be installed with an aerator to promote cell yield and can be arranged in different patterns according to their respective volume. Some pilot plants have been set up, the most promising PBR consist in 20 bags (20 cm width, 2 m length and 0.2 mm thickness) made from polyethylene fixed on a stand for the cultivation of S. obliquus [Abomohra et al. 2014]. Plastic bag PBRs with greater volumes can be immersed in a water pool to reduce cost and to control the temperature in summer. Although plastic bag PBRs have been favoured by many researchers due to their affordable prices in the short run, they have disadvantages as photolimitation due to distortion of the bags by gravity, inadequate mixing, and cell growth inhibition in some zones. Bags are fragile, occasionally leaks could occour and it is not suitable for mass cultivation. Finally, the disposal of large quantities of plastic bags presents a potential problem [Wang et al. 2012].

Figure 1.6 Plastic bag Photobioreactors

Tubular photobioreactors

The tubular reactor is one of the most appropriate and feasible configurations for outdoor mass cultivation because it has a relatively large available surface area for illumination (Figure 1.7). It consists in transparent tubes in glass or plastic orientated in horizontal, vertical, inclined or helical direction (maximising sunlight capture), that can be arranged in patterns e.g. straight, bent or spiral. These transparent tubes can be arranged in different patterns (e.g., straight, bent, or spiral) and orientations (e.g., horizontal, inclined, vertical, or helical) in order to maximize the sunlight capture, Biomass requires the aid of a pump or an airlift system to be circulated. The diameters of tubular PBRs are generally 10 mm to a maximum of 60 mm, and the length can be as long as several hundred meters. A small diameter of 10 mm or less may also be designed in some cases for the production of high

cell concentrations. Liquid velocities ranging from 0.2 m · s^{-1} to 0.5 m · s^{-1} are usually adopted for mass cultivation [Posten 2009]. The main advantage is the possibility for good temperature control, enabling for the use of high concentrations and very high photosynthetic efficiencies compared to open ponds. A good size-length ratio is important to avoid oxygen build up at the end of the tube. Disadvantages are the photolimitation in tubes with large diameters, energy needed for pumping and mass transfer problems that lead to the establishment of gradients in CO_2, O_2 and pH. Finally, cleaning the walls of tubular PBRs is an annoying problem that is closely connected to light permeability. At present, mechanical cleaning is the procedure most often used to do this washing [Junying et al. 2013]. Hence, photolimitation, O_2 accumulation, CO_2 depletion, high temperature, land requirement, and power consumption are the principal problems when using tubular PBRs for mass cultivation [Vasumathi et al. 2012].

Figure 1.7 Tubular PBRs in operation of Varicon Aqua Solutions Ltd, UK.

Flat panel reactors

Flat panel reactors consist of a rectangular transparent box with a depth of only 1 to 5 cm, height and width can be varied (Figure 1.8). Usually, the panels are illuminated from one side by direct sunlight and are positioned vertically or inclined facing the sun [Janssen et al. 2003]. This simple geometry facilitates their placement in the laboratory and the measurement of irradiance at the culture surface. Flat panels can be oriented and tilted at angles that maximize the amount of solar radiation intercepted and thus maximize productivity per reactor. In addition, they can be inclined to reduce the amount of solar radiant energy impinging on the culture and increase the efficiency of light conversion and

the productivity per unit of ground area [Tredici 2010]. At pilot level outdoors have been experimented: alveolar panels, glass and acrylic plastic plates and disposable panels. Among the advantages of the system there is its high S/V, which allows to attain high volumetric productivities at high cell concentrations. A good mixing and high mass transfer capacity can be reached at relatively low power supply. In 2015, de Vree et. al. documented for the microalga Nannochloropsis cultivated continuously a productivity of 19 g m^{-2} day^{-1}, a great achivement when compared with the 12 g m^{-2} day^{-1} of the open pond system [de Vree et al. 2015]. Its temperature can easily be controlled by spraying water onto the irradiated surface of the PBR when the temperature of the culture exceeds a specified value or by submerging the bottom of the PBR in a water pool in summer [Dasgupta et al. 2010]. Among the drawbacks, biofouling and the difficulty of scaling up seem to be the major ones [Ugwu et al. 2008].

Figure 1.8 Flat Panel reactor at Wageningen University

Column photobioreactors

Vertical tubular reactors VTRs are simple system in which mixing is achieved by injecting compressed air at the bottom (airlift system). These systems are made of translucent glass fibre sheets formed into cylinders (Figure 1.9). Illumination is provided either by artificial or natural light usually used for indoor experiments [Tredici 2010]. Diameters are over 20 cm, which implies a dark area in the middle of the column. In addition, the height of the cylinder is limited to about 4 m for structural reasons, due to the strength of the transparent materials employed and in order to reduce mutual shading in large commercial cultivations [Wang et al. 2012, Bitog et al. 2011]. To solve this, an annular column is formed, consisting of two cylinders of different size (e.g. 40 and 50 cm) to form a wrapped flat plate reactor. In

the inside of this annular column lamps could be fitted to increase productivity [Borowitzka 1999]. Airlift PBRs have the substantial advantages of low-operational power consumption and less shear stress exerted on the cells. Furthermore, the airlift reactor has a defined circular fluid flow and relatively better gas-liquid mass transfer performance. Column airlift PBRs are one of the most promising configurations for the industrial production of microalgae [Huang et al. 2016]. However, their high capital cost and cleaning problems are the main obstacles to their large-scale implementation [Soman et al. 2015].

Figure 1.9 Vertical Column Photobioreactor at National University of Singapore

1.2.3 Downstream processing (DSP)

DSP processes are unit processes necessary for the extraction and purification of valuable compounds from microalgae. In the past, were employed conventional methods like bead beating, homogenizers, high pressure heating and organic solvents, but the costs of production were high and the integration of multiple steps was needed to make viable the process [Jacob-Lopes et al. 2015]. The harvesting of biomass followed by biorefinery techniques, preferring the use of mild separation technologies, has assisted much in the production of the desired compounds without damaging other fractions [Vanthoor-Koopmans et al 2013]. Downstream processing of microalgal biomass (harvesting and product extraction) accounts for 70–80% of the total cost and has the most weight in terms of energy consumption. This demonstrates the necessity to identify an optimal process for certain purpose [Stephens et al.2012]. The most important downstream processes, in order of employment, are:

- dewatering: the removal of the extracellular water
- drying: the separation of the intracellular water)

- the disruption of the algal cells
- extraction of valuable components such as lipids, proteins, carbonhydrates, pigments
- hydrothermal treatments

- *Dewatering*

The processing of microalgae demands a high biomass concentration, a dry matter contents of 25% needs to be achieved, separating the extracellular water from the algal suspension [Chisti 2007]. Gravity sedimentation is the simplest method of obtaining biomass, transferring the algal medium to a sedimentation tank, where the algae sink to the bottom. Dry matter contents between 1.5% and 5.0% can be attained in the sump of the tank, depending on the retention time or the alga species [Lundquist et al. 2010]. Sedimentation is possible for microalgae thanks to their ability to form coenobia (colonies with a specific number of cells, four, eight or even sixteen). The sedimentation velocity depends on the properties of the cellular complexes. Coenobias of S. obliquus can sediment within 24 hours [Gutierrez et al. 2015]. Gravity sedimentation does not entail high total cost, but in order to increase the particle size and accelerate sedimentation it is necessary to use flocculants to complete the process in a reasonable period of time [Vunjak-Novakovic et al. 2005]. A disadvantage is the big space covered by this facility, it could be approximately 50% of the volume of the PBRs [Weissman et al. 1987]. Flotation could be a solution, it consists in generate small air bubbles through pressure relief or elecrolysis and introducing them into algal suspension. Algal cells will adhere to the bubbles and will float to the surface of the water and they can be harvested. It takes up a small amount of space and is a rapid method of separation. The yield is high and generally varies from 75% to 98%, however these results are possible only with the aid of coagulants [Sim et al 1988]. A power consumption of at least 1.6 kWh/m³ is required to obtain a suspension with 4% total solid content from a culture medium with just under 0.1% algal biomass per kilogram of water [Shelef et al. 1984]. An alternative is offered by microflotation. The power consumption rate is 0.1 kWh/m³, corresponding to a reduction in energy input of more than 90%. In this case, the use of flocculants can be significantly reduced, or even becomes superfluous [Hanotu et al. 2013]. Filtration is a widely used technique and entails the use of a variety of different methods depending on the properties of the algae. Filters are characterised by their low space take-up compared to sedimentation. The effectiveness of filtration strongly depended on the pore size of the harvester filter and the size of the algal species. The power requirements of the filtration harvester measured averaged between 0.3 and 0.5 kWh/m^3 of pond water treated

with an increase of the slurry concentration from 0.05% to 3% solids [Sim et al. 1988]. The use of centrifuges is possible for algae harvesting. The main advantage of centrifuges is their small space take-up [Carlson et al. 2007]. Unfortunately, they require high energy demand and maintenance commitment. The employment of the centrifuge is justifiable for the alga harvesting for the production of high value products [Molina Grima et al. 2003]. The biomass concentration downstream of the centrifuge is approximately 15–30%. To reduce the energy demand is possible the to utilise the centrifuge as a secondary process, with preconcentrated (by flotation or sedimentation) algal cultures [Sazdanoff 2006].

- *Dehydrating*

The water content in the biomass must be reduced, as otherwise algae will decay rapidly. It may be necessary to dry the biomass almost completely (up to 95% dry matter). The intracellular water remaining in the cells after drainage of the algal suspension can only be removed by thermal treatments. The result could be reached using solar energy (it is simple and economic), but the method takes up a lot of space and time [Greenwell et al. 2009]. With flash driers the process is very rapid. The moist biomass is sprayed into a rising stream of hot gas at the bottom, the water is evaporated and the gas phase and solid matter are separated in a cyclone [Liang et al. 2015]. Biomass harvested with dietary purposes is treated with spray drying because a large number of constituents is retained. Just as in the case of flash dryers, in this continuous process, the paste is dried in a few seconds. The energy demand to dry 200 kg of algae with a moisture content of 80% until the 4% can be estimated at 6.4 kWh/kg [www.trema.de]. Freeze drying has been employed to dry algae only on a laboratory scale, but it is the main technique in the food industry. The method is employed primarily to conserve sensitive materials contained in the biomass.

- *Cell Disruption*

Cell disruption is necessary for the extraction of materials from inside of the cells. Microalgae are eukaryotic cells whose tough cell walls, unlike those of prokaryotic cells, are considerably resistant to mechanical and chemical stresses. The process requires high energy and cost, and it is only convenient with concentrations of feed up to 50 kg/m^3 [Robles-Medina et al. 2009].

It is crucial to recover as much of the desired product as possible from the microalgae, taking into account the economic viability of the process. Cell disruption methods can be divided into two categories: mechanical ones, e.g., bead mill, homogenizers, and ultrasound and non-

mechanicals like organic solvents, osmotic shock, and pulsed electric field (PEF) [Middelberg et al. 1994].

Mechanical Methods

Mechanical disruption requires energy inputs in the forms of shear forces, electrical pulses, waves or heat. In the mechanical methods the disrupting effect is based on tearing apart the cell walls of microorganisms by creating stresses and strains on the cells. They are used mostly when the desired product is intracellular (*inclusion bodies*) and cannot diffuse out into the surrounding medium by damaging the cell wall [Mendes-Pinto et al. 2001]. High-pressure Homogenizers generate shear forces sufficients to disrupt many types of cell. A common apparatus consists of a high-pressure pump and a downstream pressure relief valve. Cells are forced to pass through a narrow gap in the valve at pressures up to 550 atm and after the liquid is rapidly depressurised, which act to comminute the microorganisms [Greenwell et al. 2009]. Homogenizers are widely used in aquaculture to enhance the assimilation of carotenoids from Haematococcus for fish. Cell cracking increases the bioavailability of the pigment. Thus, if the disruption of the homogenised spores is not complete (60%), their bioavailability and assimilation is reduced in the same percentage [Molina Grima et al. 2013].

Bead milling

Cell decomposition by a ball mill is a simple and effective technique. The device consists in a cylindrical chamber with a central shaft supported by agitating elements, filled with the cells and quartz or metal beads [Blecher et al. 1994]. Bead milling has been used for years to disrupt microorganisms and works successfully to disrupt cells such as cyanobacteria, yeast, spores and micro-algae [Hedenskog et al. 1969]. The degree of disruption depends mainly on the collisions between beads and cells. But size, shape, and composition of the beads as well as the strength of the cell walls will also affect the success of disruption. The optimal bead size for microalgae cells is 0.5 mm. This type of cell disruption is usually combined with solvents to recover the released components [Mercer et al. 2011]. Cells of *Chlorella vulgaris*, *Scenedesmus obliquus*, and *Spirulina* sp. were decomposed using ball mills successfully [Hedenskog et al. 1969 and 1972].

Ultrasound Assisted Extraction (UAE)

Ultrasound can be used for cell decomposition. The cell disruption mechanism derives from the intensive shear induced by sonicating the suspension at sound frequencies above 20 kHz [Furuki et al. 2003, Luque-Garcia et al. 2003]. The sound waves alternately generate high-pressure cycles (compression) and low-pressure ones (rarefaction). The sound may be either continuous or pulsed. The sound waves create many micro bubbles at various nucleation sites in the suspension, which collapse implosively during the rarefaction period of the sound waves. This phenomenon of cavitation produces intense local shock waves and shear gradients are generated that cause the cells to deform beyond the limit of elasticity and rupture [Chisti et al. 1986]. Over 90% of the fatty acids and pigments of *Scenedesmus obliquus* could be released in the surrounding medium by this method [Wiltshire et al. 2000]. Extraction of fatty acids is possible and is already being implemented on a laboratory scale, but there is still a lack of information regarding the feasibility or cost of its application on a commercial scale [Harun et al. 2010]. Ultrasonic cell disruption can be applied on *Spirulina platensis* to recover phycocyanin and the purity of the extract depends on the ultrasonic frequency [Kotnik et al. 2015]. UAE is not suitable for all type of pigments, for example Zhao and colleagues (2006) demostrated that ultrasound degrades astaxanthin pigment into colourless compounds because of cavitation produced in the solvent by the propagation of ultrasonic waves [Zhao et al. 2006].

Pulsed Electric Field

Pulsed Electric Field (PEF) is currently discussed as promising technology for mild and scalable cell disintegration of microalgae. By applying short electrical pulses in the order of magnitude of milliseconds (or even microseconds), the cell membrane can be charged sufficiently to cause a rearrangement of the membrane resulting in pore formation [Kotnik et al. 2015] After treatment, both algal species show release of ions, which indicates that PEF treatment resulted in permeabilization of the algal cell [Goettel et al. 2013]. This method has already been applied on microalgae for lipid extraction [Zbinden et al. 2013]. Due to the short electrical pulses applied, this technology requires a low energy input (even lower 1kWh/kgDW) [Postma et al. 2016].

Microwaves

Microwaves applied at a frequency near 2.45 GHz cause the vibration of water and other polar molecules within wet biomass, thereby resulting in temperature increases in the

intracellular liquids which causes the water to evaporate and exert pressure on the cell walls, leading to cell disruption [Hahn et al. 2012].

There are several key operational factors determining the efficiency of MW extraction such as the species of microalga, power of the MW, temperature, and solvent properties and volumes used [Gong et al. 2016]. Choi and colleagues, reported the microwave-assisted extraction of astaxanthin from the yeast *Xanthophyllomyces dendrorhous* and showed that combining a microwave irradiation to destroy cell walls with a solvent extraction allows a good pigment recovery [Virot et al. 2008]. The combination of sonication and microwaves was studied to extract lipids from microalgae sources, both techniques gave excellent extraction efficiencies in term of yields and time and yields increased from 50 to 500 % [Lee et al. 2010]. In a study of 2011, Pasquet et al. reported the solubilization of apolar and polar pigments from the microalgae *Cylindrotheca closterium* and *Dunaliella tertiolecta* with the microwave-assisted extraction [Pasquet et al. 2011].

Steam explosion (SE) process and hydrothermal liquefaction (HTL) are treatments more indicated for bioenergy production. In SE, raw material is exposed to steam at 180–240 °C (ca. 1.03–3.45 MPa) for several minutes, and then subjected to depressurization to ambient condition. The explosion, which occurs as the result of sudden depressurization, causes cell-wall disruption [Nurra et al. 2014]. This process raises questions about the economic feasibilitu for large-scale biorefinements due to the high-temperature and -pressure conditions. HTL allows for conversion of wet biomass directly to biocrude under elevated temperatures and pressures of 280–370 °C and 10–25 MPa, respectively [Chiaramonti et al. 2017]. Water promotes the hydrolysis of macromolecules and polymerization of small molecules into the larger ones that make up biocrude oil. Recently, HTL seems a promising method for obtainment of biocrude from microalgal biomass, as it requires significantly less energy due to the absence of drying and cell disruption processes [Barreiro et al. 2013].

Chemical Methods

Acids, alkalis, and surfactants can be chemicals suitable to induce the hydrolysis of microalgae cell wall. These methods could damage sensitive products such as proteins, instead are effective if the target is the extraction of free fatty acids [Middelberg 1995, Molina Grima et al. 2003]. Alkali treatment for the direct saponification of the moist biomass with a KOH-ethanol mixture was used on *Phaeodactylum tricornatum* and from *Porphyridium cruentum* with good results [Butler et al. 2017, Wu et al. 2016]. Chemical methods consume low energy if compared with mechanical ones, on the other hand they are

not very selective and there is the risk of contamination or disruption of the desired product. When using alkalis, acids, or detergents, there is a risk of permanent contamination of the desired product. Organic solvents such as toluene, alkanes or alcohols are absorbed into the cell envelope lipids, swelling and rupturing the wall. In this way is possible to separate the cell's content and recover target products. Solvents with similar solubility parameters should attack cells in a similar manner. Ideally, we should choose solvents whose solubility parameters match those of the wall lipids but are far from those of desired products locked within cells.

Acids: recently, Park et al. investigated sulfuric-acid catalysed hot-water treatment for efficient extraction of lipid from wet Chlorella vulgaris. Under a 1% sulfuric acid concentration and 120 °C heat treatment for 60 min, the lipid-extraction yield was 88% [Park et al. 2014]. Strong acids such as sulfuric acid, are relatively cheap and effective compared with other chemical agents used for microalgal cell disruption, but their employment raise safety and wastewater treatment issues.

Ionic liquids (ILs): Ionic liquids are liquid salts from 0 to 140 °C, usually large organic cations in combination with smaller inorganic/ organic anions, with the advantage that physical and chemical properties can be tuned to the desired application, just by changing the cation or the anion structures. ILs have been proposed as volatile organic solvent substituents due to their low volatility, high capability to dissolve inorganic and organic compounds, and also due to their chemical stability and peculiar solubility behaviour [Praveenkumar et al. 2015]. 1-butyl-3-methylimidazolium trifluoromethanesulfonate ([Bmim] CF_3SO_3) with methanol as a co-solvent were used to extract lipids from *Chlorella vulgaris* extracting a total lipid content of 19% instead of the 11% obtained with the classic extraction with chloroform/methanol. The ILs broke the hydrogen bonds in the microalgal cell walls, resulting in enhanced microalgal lipid extraction [Kim et al 2012]. 1-ethyl-3-methylimidaxolium ethylsulfate ([Emim] $EtSO_4$) was used by Praveenkumar et al. in the extraction of astaxanthin from *Haematococcus pluvialis* with a yield of 19.5 pg/g cells, about 82% of conventional organic solvent extraction under mechanical methods.

Osmotic shock: Osmotic-shock-based cell disruption can be used to avoid the energy-intensive drying process in a microalgal biorefinery. Yoo et al. demonstrate that the addition of sodium chloride (NaCl; 2% w/v) improves lipid extraction from wet *Chlamydomonas reinhardtii* by approximately a factor of two compared with the NaCl-less control [Yoo et al

2012]. Salt addition is simple and scalable when compared with other disruption methods. Disadvantages are salt's high cost and necessary recovery/clean-up for subsequent downstream processes. The effect of osmotic shock is variable and dependent on microalgal species since they have very different metabolic mechanisms of acclimation/adaptation to osmotic stresses [Ben-Amotz et al. 1973].

Surfactants: Lai et al. observed that cationic surfactants lead to cell disruption because of their ability to bind microalgal cell walls due to their negative charge. anionic surfactant sodium dodecyl benzene sulfonate (SDBS), can help in the extraction of free fatty acid (FFA) from *Chlorella vulgaris* in a sulfuric acid-catalysed hot-water extraction process [Lai et al. 2016]. Under the 1% sulfuric acid and 0.4% SDBS conditions, the lipid-extraction yield and the FFA content of the lipid recovered were improved considerably, respect to the hexane-methanol extraction method [Park et al. 2014]. Some surfactants are biodegradable but issues such as the identification of their respective mechanisms for process-design purposes, minimization of their dosages, and efficient recovery processes need to be considered.

Biological disruption

Biological approach major advantages are their biological specificity, mild operating conditions, and low energy consumption [Gunerken et al. 2015]. To facilitate cell-wall disruption, lysis enzymes such as cellulase, lipase and protease have been used based on their target-specific digestion of cell-wall components (e.g., sugar, cellulose, lipid and protein) [Chen et al. 2016]. Investigation of Wu et al., found that a multiple-enzyme combination (cellulase, protease, lysozyme, and pectinase) showed generally better lipid yields than did single enzyme treatment [Wu et al. 2017]. Although the enzymatic lysis process is considered to be more energy efficient and more environmentally friendly, is difficult to employ in large-scale, due to reaction time are relatively slow, expensiveness, and the difficulty of stability and reuse.

- *Product isolation*

After the insolubles are removed, the following step is usually product isolation. It consists in taking a dilute aqueous solution, removing most of the water and push away materials with divergent properties compared with the target biomolecules. The resulting concentrate can be purified by methods which would not be effective in dilute solution. The two key methods for isolation are extraction (with organic solvents, ILs, switchable hydrophilicity solvents (SHSs), supercritical fluids) and adsorption. Liquid–liquid extraction permits

selective removal of a desired substance from a liquid mixture into a second immiscible liquid. The solute, which is usually in aqueous solution, when contacted with an immiscible solvent, distributes between the two phases. The chemical properties of the solvent and desired molecules are crucial for the selective uptake from the feed phase. The extraction needs to be quick, efficient and gentle to reduce the degradation. Moreover, the solvent should be cheap, volatile and able to form a biphasic system with water and should not extract unwanted components.

Organic Solvents Extraction

Traditional organic solvents like cyclohexane, hexane, chloroform, or ethanol have proven to be effective for extracting algal components, above all lipids. Solvent has to fully penetrate into the cells and completely solubilise the target molecules. (e.g., nonpolar solvents for nonpolar lipids) [Mercer et al. 2011]. Mechanical pre-treatment of the biomass may be helpful to maximise the extraction. The most employed phase for the extraction of lipids is a mixture of methanol, chloroform, and water [Bligh 1959]. Various solvents can be used as extraction agents, for example hexane, ethanol (96%) or hexane/ethanol mixtures (141). Benzene, cyclohexane, acetone, chloroform, and ether are also used [Harun 2010]. The most interesting lipids from the commercial point of view are polyunsaturated fatty acids, such as EPA, DHA and arachidonic acid (AA). Crude extract may be treated with apolar solvents such as chloroform, hexane or diethyl ether, in which the non-lipid contaminants are less soluble and purified extracts were obtained with recovery yields of 80% of EPA and 65% of DHA from *I. galbana* biomass, 98% of EPA from *P. tricornutum* biomass, and 69% of EPA and 68% of AA from *P. cruentum* biomass [Medina et al. 1995, Cartens et al 1996, Giménez et al 1998]. The solvent recovery process takes place in a subsequent distillation stage. Conventional methods for extracting lipids which usually include hexane extraction and vacuum distillation, use flammable and/or toxic solvents and cause adverse health and environmental effects [Liang et al. 2015]. For the extraction of photosynthetic pigments, the employment of acetone is common, because this solvent inhibits the formation of degradation products. Methanol and ethanol showed a better removal compared to acetone and the recovery increases if pigments are stored in ice baths and dark conditions. Dimethyl formamide (DMF) was found to be a superior extraction solvent to methanol, 90% ethanol, 100% ethanol and 90% acetone. Extraction using DMF did not require cell disruption as pigments were completely extracted after a few steps of soaking. Additionally, the pigments remained stable for up to twenty days when stored in the dark at 5°C [Jeffrey 1975]. However, DMF toxic nature decreased its appeal as an

efficient solvent. Sarada and colleagues tested the extractability of carotenoids from *H. pluvialis* cells. Results showed that a treatment with hydrochloric acid (2N) for 10 min at 70°C followed by acetone extraction for 1 hour, extracted 87% (w/w) of astaxanthin in cells without affecting the astaxanthin composition [Sarada et al. 2006].

Since the late 1990s, greater awareness of the impacts of chemical refining processes on the environment, has led to the development of greener processes. As the goal of biorefinery is to use a renewable resource for the production of chemicals and fuels, it is not surprising that the development of the processes is influenced by the principles of green engineering [Singh et al 2011].

Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is an alternative to the conventional organic solvent extraction. Supercritical fluids have properties of both gases and liquids, when they are raised above their critical temperature and pressure points. Profitable features of SFE are: low densities, low viscosities, diffusion coefficient, short extraction times, simple phase separation of extract and solvent, high selectivity based on adjustment of pressure and temperature [Randall et al. 1982, Herrero et al. 2006]. Since density is directly correlated with solubility, the solvent strength of a fluid can be adjusted by changing the pressure. Supercritical carbon dioxide (CO_2), is unharmful, bacteriostatic, not combustible, nonexplosive, and available in large quantities, for these reasons it can be employed in industry for extracting purposes. CO_2 is effective for the extraction of non-polar materials, for the solubilisation of polarised materials the addition of entrainers is recommended [Gopal et al. 1985, Walsh et al. 1987]. By means of supercritical CO_2, can be extracted photosynthetic pigments, in particular chlorophylls and carotenoids such as zeaxanthin, canthaxanthin, and astaxanthin as well as beta-carotene [Mendes et al. 1995, Valderrama et al. 2003]. Mendes et al. investigated supercritical fluid extraction with CO_2 on different microalgae. The solubilisation of long-chained hydrocarbon components (alkadienes and trienes) of *Botryococcus braunii* with $scCO_2$ is possible at a constant temperature of 40 °C [Mendes et al. 2003]. For pharmaceutical applications, it is important that non-toxic solvents are used in the separation process. For this reason, beta-carotene was extracted with $scCO_2$ from *Dunaliella salina* at 30 MPa and 40 °C and it improved the *cis/trans* ratio compared to extraction with acetone. Supercritical extraction with CO_2 and the cosolvent ethanol improves the extraction rate of lipids and carotenoids of *Nannochloropsis oculata* [Liau et al. 2010]. The $scCO_2$ extraction of chlorophyll from microalgae depends on the fluid density

which is a function of operating pressure and temperature. Optimum extraction condition of chlorophyll from Nannochloropsis gaditana and Synechococcus sp. were found to be 400-500 bar of pressure at 60 °C [Macias-Sánchez et al. 2005]. CO_2 is a gas at room temperature, so it is easy to remove from the product. The biomass, however, must be dried first, which entails considerable energy input [Liang et al. 2015]. Machmudah and colleagues tested different conditions to extract astaxanthin from *H. pluvialis* by scCO_2. The highest amount of total extract, astaxanthin extracted and astaxanthin content in the extract were 21.8%, 77.9% and 12.3%, respectively, at 55 MPa and 343 K [Machmudah et al. 2006]

Ionic Liquids

Ionic liquids (ILs) are used as solvents for lipid extraction, refining of carbohydrates, or extraction of high value products from algal biomass [Choi et al. 2014, Gao et al. 2016, Malihan et al. 2014, Orr et al. 2015]. They can vary considerably in their physiochemical properties such as polarity, hydrophobicity, toxicity, and thermostability [Petkovic et al. 2011, Plechova et al. 2008]. ILs have been shown to facilitate the extraction of lipids from microalgae, thanks to their ability of disrupting microalgal cell structure improving access of co-solvents to the intracellular lipids [Teixeira et al. 2012].

Reaction conditions like low temperatures are generally desirable, but many ILs exhibit high viscosities at low temperatures, so the processing of algal biomass was performed at temperature ranges of 100-120 °C. Viscosity can also be reduced by using co-solvents such as methanol. However, cosolvent addition can potentially affect lipid extraction increasing the auto-partitioning behaviour of the lipids, from the aqueous toward the IL phase, or increasing the rate of transfer between the IL and the immiscible hexane layer often used in studies for easy handling of lipids [Orr et al. 2016].

Despite their properties and advantages, only few reports have been available for utilization of ionic liquids for extraction of lipids from microalgae. One step process for lipid extraction was proposed by Salvo et al. using the ionic liquid 1-butyl-3-methylimidazolium. In this process, the microalgae cell walls were lysed with ionic liquids that subsequently formed two immiscible phases of both hydrophobic and hydrophilic layers [Di Salvo et al 2013]. The hydrophobic lipid phase due to its low density, will stratify above the hydrophilic phase, so it can be easily removed and purified. ILs has immense potential in the improvement of lipid extraction efficiency; however, more work is needed to understand the limits of its commercial viability [Kumar et al. 2017]. A novel approach was developed by extracting pigments from intact cells using a mixture of ionic liquids and organic solvents keeping the

microalgae cell walls intact, which could be followed by cell disruption and extracting the other intact components (e.g., proteins), a so-called pre-fractionation step which might open up new applications for extraction of high-value components from microalgae [Desai et al. 2016].

Switchable Solvents (SS)

Switchable solvents, which work in a phase splitting mode through changing the solvent and process conditions, are one of the alternative systems for liquid solvent extractions [Kumar et al 2017].

Switchable solvents are a subclass of ionic liquids which can be divided into two categories, switchable polarity solvents (SPS) or switchable hydrophilicity solvents (SHS). SPS are able of changing their polarity upon the addition of carbon dioxide while SHS change from a hydrophobic solvent into a hydrophilic one [Boyd et al. 2012, Jessop et al. 2011, Samorì et al. 2010]. To return ionic to non-ionic forms, switchable solvents require only CO_2 removal, possible through stripping with N_2 or heat [Singh et al. 2011]. SHS such as N,N-dimethylcyclohexylamine (DMCHA) was used to extract lipids from wet *Nannochloropsis gaditana, Tetraselmis suecica, Desmodesmus communis* biomass [Samorì et al. 2010 and 2013. Independently, DMCHA was also capable of extracting lipids from exhausted culture media of the microalgae *B. braunii*, however, only 42% of the total available lipids were recovered compared to standard chloroform methanol extraction [Boyd et al. 2012]. While SHS have shown significant potential as a low energy extraction method as distillation is not required to recover these solvents for reuse, the process can be lengthy taking up to 24 h to recover all of the available lipids [Du et al. 2013]. The advantages and disadvantages of the green solvents discussed are summarized in Table 1.3. Most of the experimental work described in this thesis concerns the employment of DMCHA and NaDESs for the refining of S. dimorphus biomass and will be exposed in the following sections.

Solvent	Advantages	Disadvantages	References
Super critical fluid technology (SCF)	Lower extraction time, Low toxicity, Mass transfer equilibrium could be favourable, Separation step is not required, Recycling the CO2 avoids greenhouse eff. ect	High equipment and operational cost.	Santana et al. 2012, Yen et al. 2015
Ionic Liquids	Less hazardous, Non-flammable and remains in liquid state for a broad range of temperatures, Possess both ions and enable to design a suitable solvent, Enable single solvent extraction, instead of biphasic system.	High cost, Studies on scaling-up are essential to explore the possibility.	Seddon 1999, Di Salvo et al. 2011
Switchable solvents	Suitable for multi-step lipid extraction, No need of replacement of solvent for next step, Easily switchable by stripping N2, Recycling can be done for several extraction	Application in lipid extractions is yet to be studied.	Jessop et al. 2007, Phan et al. 2008
Deep eutectic solvents	Fluid systems composed of two or more eutectic solvents, occurs naturally, Safe and inexpensive, highly preferred due to bio-degradability, Non-toxicity, low cost synthesis, Low volatility and wide polarity.	Showed promising results in extraction of bioactive compounds and its application is yet to be determined.	Paiva et al. 2014, Smith et al. 2014
Natural deep eutectic solvents	Composed of primary metabolites such as aminoacids, organic acids, sugars, and choline derivatives, Non-toxic, bio-degradable, stabilizes solute and low cost for synthesis, used for phenols and flavonoids extraction.	Role of these solvents on lipid extraction are yet to be studied	Choi et al. 2011, Dai et al. 2013

Table 1.3 Advantages and disadvantages of most used innovative solvents

Deep Eutectic Solvents

Deep eutectic solvents (DESs) are a new class of solvents that are liquid eutectic mixtures based on hydrogen-bonding interaction of two or three components. DESs are attractive solvents for the fractionation (or pre-treatment) of lignocellulosic and microalgal biomasses [Lu et al. 2016]. DESs are transparent liquid eutectic mixtures that generally form by simply heating two components (at least one as a solid), which act as hydrogen-bond acceptors (HBAs) and hydrogen-bond donors (HBD), respectively. Quaternary ammonium salts, such as choline chloride (ChCl) are generally employed as HBAs, whereas metal salts, urea, carboxylic acids, and polyols can be used as HBDs [Abbott et al. 2007, Francisco et al. 2013]. DESs could have tunable physicochemical properties for specific applications. A DES generally has a freezing point (Tf) or melting point (Tm) significantly lower than those of its individual components. DESs share many physical properties of ILs, including relatively broad liquid range, negligible volatility, conductivity, and low flammability [Smiglak et al. 2006]. Compared to conventional ILs, DESs provides several major benefits including simple preparation, low cost, low toxicity and high biodegradability [Radosevic et al. 2015, Zhang et al. 2012]. Lu and co-workers tested three different aqueous deep eutectic solvents with the goal of lipids extraction, i.e. aqueous choline chloride-oxalic acid (aCh-O), aqueous choline chloride-ethylene glycol (aCh-EG) and aqueous urea-acetamide (aU-A) on *Chlorella* sp. The hydrogen bonds of alpha-cellulose and hemicellulose in the cell wall were affected through hydrogen bond formation between the anions (hydroxyl, carboxyl, acylamino) in the aqueousDES and hydroxyl groups in the cell envelope, resulting in a severe modification of the structure. Results indicated that, lipid recovery rate was increased from 52.03% of untreated biomass to 80.90%, 66.92%, and 75.26% of the biomass treated by aCh-O, aCh-EG and aU-A, respectively [Lu et al. 2016].

NaDES

Natural deep eutectic solvents (NADESs) consist of primary metabolites such as amino acids, organic acids, sugars or choline derivatives. NADESs have striking advantages over DES regarding biodegradability, toxicity, solute stabilization, sustainability, and synthesis cost [Espino et al. 2016, Choi et al 2011]. The standard components of NADESs are sugars (glucose, sucrose, fructose); organic acids (lactic, malic, citric acids); urea and choline chloride, which are naturally present in all types of cells and organisms [Rengstl et al. 2014]. Enhanced efficiency of NADES (choline chloride with lactic acid) in extraction of phenols and flavonoids over other organic solvents was reported in the literature by showcasing the

efficiency of NADES constituted from natural and renewable non-toxic bioresources [Dai et al. 2013]. In 2017 was reported one of the first applications of NADESs on microalgal biomass, as described in the results and discussion section [Cicci et al. 2017]. For these reasons a further description of these solvents is reported in detail in the following chapters.

- *Microalgae primary composition and fractionation methods*

The biochemical composition of microalgae dry matter may be divided into proteins, lipids and carbohydrates, with traces of metals and pigments. These materials have not the same level of accessibility and it leads to the difficulty of DSP of microalgae cellular metabolites. PUFA's and phospholipids represent much of the lipid fraction and their presence in the cell's membrane makes them relatively inaccessible [Sharma et al. 2012]. Triacylglycerides (TAGs) are storage neutral lipids formed under certain environmental or stress conditions, they are more accessible once the cell's membrane is disrupted, and the separation can involve mild techniques [Breuer et al. 2012]. Cell wall carbohydrates may account for as much as 54% of the cell wall structure [Cheng et al. 2011]. Pigments are typically present in the cell's membrane and extractable with the aid of harsh solvents [Thompson 1996]. Microalgae have a very favourable composition with only 4–10% ash matter, therefore at least 90% of the algae biomass could be processed for marketable products [Laurens et al. 2012]. Microalgal macromolecular composition expressed as a function of its dry matter is reported in the Table 1.4.

Strain	Proteins	Carbohydrates	Lipids	Nucleic acid
Scenedesmus obliquus	50-56	10-17	12-14	3-6
Scenedesmus quadricauda	47	-	1.9	-
Scenedesmus dimorphus	8-18	21-52	16-40	-
Chlamydomonas rheinhardii	48	17	21	-
Chlorella vulgaris	51-58	12-17	14-22	4-5
Dunaliella salina	57	32	6	-
Euglena gracilis	39-61	14-18	14-20	-
Tetraselmis maculata	52	15	3	-
Porphyridium cruentum	28-39	40-57	9-14	-
Spirulina platensis	46-63	8-14	4--9	2-5
Spirulina maxima	60-71	13-16	6-7	3-4.5
Anabaena cylindrica	43-56	25-30	4-7	-

Table 1.4 Chemical Composition of Algae Expressed on A Dry Matter Basis (%). Adapted from Becker, 1994.

Fractionation is the final step in the biorefinery process, it is expensive, but it is a convenient solution for high-value products in the field of food/health/cosmetics. It is needed for further

isolation of individual components from extracts and mixtures of hydrophilic (e.g., polar proteins, carbohydrates) and hydrophobic (e.g., non-polar proteins, neutral lipids, pigments) phases into purified components. For the hydrophilic components, charged membranes or beads with alternatives such as protein precipitation can be applied [Burgess et al. 2009, Carta et al. 2010]. Ultrafiltration/diafiltration can be applied for concentrating, buffer exchange, or selective separation of proteins from carbohydrates [Gerardo et al. 2014, Safi et al. 2014, Schwenzfeier et al. 2011]. For a further fractionation of the hydrophobic phase, such as specific lipids or pigments, above discussed techniques such as solvent extraction, or supercritical methods are currently applied.

Proteins Fractioning

In the chemistry of microalgae, proteins are involved in capital roles such as growth, repair and maintenance of the cell as well as serving as chemical messengers, regulators of cellular activities and defence against pathogens. Algae are generally regarded as a viable protein source, with essential aminoacidic composition meeting Food and Agricultural Organisation (FAO) and World Health Organisation (WHO) requirements and they often have the same content of other protein sources, such as soybean and egg [FAO 1991, Fleurence 1999]. The quality of proteins can vary dramatically, depending on digestibility and the availability of essential amino acids [Boisen et al. 1991]. For example, the amino acid profile of *C. vulgaris* compares favourably and even better to the standard profile for human nutrition proposed, because the cells of *C. vulgaris* can synthesise essential and non-essential amino acids [Safi et al. 2014]. The digestibility of microalgae is investigated in a few studies involving *Scenedesmus obliquus*, *Spirulina* sp. and *Chlorella* sp. Their digestibility coefficient values reach percentage of 88.0%, 77.6%, and 76.6%, respectively [Becker 2007], in comparison to protein sources such as casein and egg with a digestibility coefficient of 95.1% and 94.2%. Protein extraction process is technically the same for all microalgae and is mainly conducted by solubilisation of proteins in alkaline solution (pH 10-12) with NaOH [Safi et al. 2013, Bajguz 2000]. Further purification can follow by precipitating the solubilised proteins with trichloroacetic acid (25% TCA) or hydrochloric acid (0.1 N HCl) [Barbarino et al. 2005, Chronakis et al. 2000]. Quantification is carried out by elemental analysis, Kjeldahl, Lowry assay, Bradford assay, Bichinchoinic acid or dye binding method. Alternative methods for enriching algal proteins can be membrane technologies used in conjunction with a cell disruption technique, such as polysaccharidase hydrolysis, UAE, or PEF. Membrane separation is a process which selectively allows mass transfer of materials from one phase

to another, usually driven by pressure, concentration, chemical or electrical potential gradient [Cheryan 1998]. Membranes are classified according to their pore size (micron pore size to angstroms): microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO). These can also be distinguished according to the fabrication material (e.g. zeolite, organic and inorganic) and configuration (e.g. spiral-wound, fibres and tubular) [Strathmann et al. 2011]. One of the most recent challenges for membrane filtration is the integration into biorefineries, since many of the downstream processes can be realised using available membrane methods. Proteins can be further separated from polysaccharides and traces of pigments using membrane filtration techniques (dead end or tangential flow filtration). With tangential flow filtration, the different components can be fractionated by filter sizes ranging from 1 to 1,000 kDa under mild conditions [Gerardo et al. 2014]. Dead end filtration is the standard method for removal of solid particles from the solution, however for the capture of specific proteins is necessary the coating the membrane with a specific ligand (e.g., charged, hydrophobic, hydrophilic) [Demmer et al. 2005]. With these so-called membrane absorbers a further fractionation of proteins and/or carbohydrates is feasible by selective binding in aqueous buffer systems [Weaver et al. 2013]. In the field of fractionation, chromatographic separation of different products is a technology mainly used for high-value products in the food/health/cosmetics market such as soluble proteins [Schwenzfeier et al. 2013]. Preliminary work was carried out with Tetraselmis species for fractionating a protein mixture using ionic exchange chromatography [Schwenzfeier et al. 2011,2013]. The final product obtained had 64% proteins and 24% carbohydrates with 100% solubility at pH above 5.5 and excellent emulsifying properties, useful in food applications requiring protein additions [Schwenzfeier et al. 2011].

Proteins such as phycoerythrin from *P. pruentum* can be purified with size exclusion chromatography or ionic exchange chromatography to achieve high purities and might be an interesting protein for the clinical research based on its pharmacological (immunomodulatory, anticancer, and antioxidant) properties [Cuellar Bermudez et al. 2015, Bermejo et al. 2006].

Proteins are easily denaturated by high temperature, acid/basic treatments and salts. For their recovery or purification mild operation conditions are advisable, in this way their functionality cannot be compromised. The challenge in separating the proteins maintaining them in their native form can be achieved with the aid of aqueous micellar systems [Tani et al. 1998].

Protein purification is possible in aqueous micellar two-phase system (AMTPS). The most used micellar solution is based on non-ionic surfactants (e.g. polyoxyethylene alkyl ether), it becomes turbid and then separates in two isotropic phases if heated above the cloud point. The aqueous phase is a surfactant depleted phase and it is rich in hydrophilic proteins, the surfactant rich phase extracts membrane proteins. This phase separation is reversible and it is reached with increasing temperature that cause the decreasing in the solubility of micelles [Gu et al. 1992]. Below the cloud point, micelles are solubilized by hydrogen bonds between water and polyoxyethylene, when the temperature increases, the hydrogen bond network collapse, resulting in an intermicellar aggregation. Alkylglucosides and sugar esters can be utilized in AMTPS for protein extraction [Liu et al. 1995, Garavito et al. 1980] with the aid of a water-soluble polymer (e.g. polyethylene glycol PEG), in a so-called polymer induced phase separation. In this case the phase separation temperature is increased with an increase in the PEG concentration [Schürholz et al. 1989, Zhao et al. 1996]. Further purification steps, such as column chromatography and electrophoresis are required after separation with AMPTS [Tani et al. 1998].

Ionic liquids (ILs) were proposed as alternative phase-forming components of Aqueous Biphasic Systems (ABSs). IL-based ABSs are formed by mixing water-miscible ILs with salts, carbohydrates, amino acids, and polymers [Gutowski et al. 2003, Freire et al. 2012]. Partitioning of proteins in these systems depends on: type and concentration of phase-forming components, pH, temperature, ionic strength and on hydrophobicity, isoelectric point, molecular weight and conformation of the polypeptide. Most used salt is imidazolium coupled with halogens or [BF_4] anions. Some studies reported the extraction of model proteins, such as Bovine Serum Albumin (BSA), in the guanidinium-based IL-rich phase without losing the native structure and maintaining protein stability [Ding et al. 2014]. Protein extraction using IL-based ABS involves two main steps: forward extraction, i.e., extraction of the protein from the initial matrix into IL-rich phase, and recovery of the protein.

In conventional ABS, proteins can be recovered by modification of system parameters, such as pH, change in salt concentration, or addition of other salts. The main goal is to achieve a high recovery of a protein with a high purity level without affecting the functionality of the protein.

The separation of proteins can be achieved by ultrafiltration and/or nanofiltration, precipitation, and chromatographic techniques, such as size exclusion chromatography and

by the use of affinity tags (HisTags) able to help in recovering the protein from the IL-rich phase by Immobilized Metal Affinity Chromatography (IMAC) [Desai et al. 2016].

Lipids fractioning

Lipids are heterogeneous group of compounds and are mainly composed of glycolipids, waxes, hydrocarbons, phospholipids, and small amounts of free fatty acids [Lee 2008, Hu et al. 2008]. Chlorophyceae, show typical biochemical composition: 30–50% proteins, 20–40% carbohydrate and 8–15% of lipids under favourable environmental conditions [Hu 2004]. During stress conditions, lipids content (mainly composed of triacyglycerols TAGs) can reach~58-60%% [Mata et al. 2010]. Unlike other lipids, triacylglycerols do not perform structural role but instead they accumulate as dense storage lipid droplets in the cytoplasm. The extraction process of total lipids is generally conducted by the method of Bligh and Dyer using a mixture of chloroform and methanol, or by hexane, or petroleum ether [Zheng et al. 2011, Lee 2001, Lee et al. 2010]. Quantification of total lipids is conducted gravimetrically after evaporating the extracting solvent, in addition column chromatography is applied in order to separate different lipid constituents [Olmstead et al. 2013].

Ideal membrane processing of microalgae metabolites would enable the extraction of protein-rich and carbohydrate-rich fractions, leaving the bulk of the lipids with the remaining biomass. Proteins and carbohydrates being separated via membrane technology based on size and charge exclusion. The bulk of the lipids would stay trapped in the biomass this would generate a significant shift in the biochemical composition. Potential applications of lipid-rich biomass would suit direct transesterification into biodiesel or thermo-chemical conversions such as hydrothermal liquefaction [Takisawa et al. 2013, Dong et al. 2013, Zhu et al. 2013, Jazrawi et al. 2013].

Recently the purification of TAGs via membrane filtration has been reported with encouraging results. TAGs may be extracted via UF membranes in the region of 1–100 kDa. Recent developments have shown the recovery of hexane from soybean edible oil is feasible using commercially available membranes in Organic solvent nano filtration (OSN) With OSN, oil recovery yield was reported to be 70–80% from oil-solvent mixtures ranging 10–35% w/w [Ribeiro et al. 2006, Darvishmanesh et al. 2011]. OSN could potentially allow the recovery of the lipid extracts at the same time as recovering the organic solvent, minimising costs of evaporation and solvent volume requirement.

Pigments fractioning

Pigments have an important role in the photosynthetic process, they have biological interesting abilities such as antioxidant, anticancer, antiinflammatory, antiobesity and neuroprotective activity. It is possible to recognise three classes of pigments in microalgae: phycobilins, chlorophylls and carotenoids.

Phycobilins (phycobiliproteins) are accessory pigments for collecting light. They have applications in molecular biology as fluorescent markers, immunoassays and dyes for microscopy [Spolaore et al. 2006]. These proteins are soluble in water and represent large portion of the total proteins. Four phycobilin classes are produced by microalgae and cyanobacteria: allophycocyanin (green-blue), phycocyanin (blue), phycoerythrin (purple) and phycoerythrocyanin (orange). Researchers have made many efforts to purify PBPs with maximum purity and yield. The purification of phycoerythrin is reported in literature through the techniques of: expended-bed adsorption chromatography, acetone precipitation, gel filtration and ion-exchange chromatography. Several researchers purified and characterized phycocyanin by using ammonium sulphate precipitation coupled with ion-exchange and hydrophobic interaction chromatography, respectively. [Román et al. 2002, Tripathi et al. 2007, Soni et al. 2008]. Zhang et al. purified phycocyanin and allophycocyanin using ion-exchange chromatography with a pH gradient to elute proteins [Zhang et al. 1999]. Aqueous two-phase separation was used to separate phycocyanin and allophycocyanin [Patil et al. 2007]. Recently, a protocol was developed, which facilitates the purification of phycobiliproteins from marine isolate *Lyngbya* sp. A09DM by the non-ionic surfactant Triton X-100 mediated ammonium sulphate precipitation [Rastogi et al. 2015]. Microwave assisted extraction was proved as a good method in the pre-purification of PBPs [Juin et al. 2015]. Furthermore, UF was used to isolate phycoerythrin protein from *Grateloupia turuturu* following cell homogenisation, which was reported to retain 100% of the protein without denaturation [Denis et al. 2009].

Chlorophylls are fat soluble pigments that convert light energy into chemical energy. Most microalgae have chlorophyll a, chlorophyll b and c are present in some division such as Dinophyta [Barsanti et al. 2014]. Cells can accumulate from 0.5% to 1% of pigment per dry weight. In order to quantify the amount of chlorophylls in a particular specie, the intracellular chlorophylls must first be extracted. The traditional method that has been employed is organic solvent extraction above mentioned [Mulders et al. 2014].

Thin layer chromatography (TLC) and high-pressure liquid chromatography (HPLC), are used to fractionate and to quantify chlorophyll and its derivative products.

Organic adsorbents such as sucrose and cellulose were found to be the most efficient stationary phases for use in two-dimensional thin layer chromatography [Wright et al. 2005]. Riley and Wilson and Co and Schanderl utilised silica gel as their stationary phase and managed to completely separate all of the plant pigments except for some minor components [Co et al. 1967, Riley et al. 1965]. However, Jeffrey found that use of silica gel promoted the formation of degradation products and resulted in multiple chlorophyll zones [Jeffrey 1968]. HPLC is superior to TLC because it requires even less sample for analysis, is faster and features automatic detection system [Mantoura et al. 1983]. In addition to these, HPLC is more precise and has a higher degree of sensitivity [Shoaf 1978]. Reverse phase HPLC is preferred to normal phase as the latter does not separate polar compounds and its polar stationary phase promotes pigment degradation [Wright et al. 1984]. There are different types of detectors that may be used to measure the concentrations of separated pigments as they exit the column. The most commonly used detectors rely on fluorescence and absorbance analyses. Jeffrey et al. states that fluorescence detection is more sensitive and more selective than absorbance detectors when used to analyze chlorophylls amongst carotenoids [Wright et al. 2005].

The use of ABSs was tested for pigment extraction and purification. Three ABSs – polyethylene glycol (PEG) 400-potassium citrate, Iolilyte 221PG-potassium citrate and PEG400-Cholinium dihydrogen phosphate (Ch DHp) were selected to evaluate the partitioning of pigments and proteins from N. oleoabundans extract. Iolilyte 221PG-citrate consists of a top phase rich in IL (Iolilyte 221PG) and a bottom phase rich in citrate. PEG400-citrate and PEG400-Ch DHp ABSs both consist of a top phase rich in polymer (PEG400) and a bottom phase rich in salts. *Neochloris oleoabundans* pigments (carotenoids and chlorophylls) are mostly hydrophobic molecules and therefore tend to partition to the least-hydrated phase (top phase) [Suarez Ruiz et al. 2018].

Carotenoids are fat soluble pigments that absorb in spectrum region in which chlorophylls are not efficient and serve as photoprotectors of photosynthetic systems, preventing the excessive absorption of light radiation and the production of reactive oxygen species (ROS). Algae can produce beta-carotene, lycopene, astaxanthin, zeaxanthin and lutein. Astaxanthin has been studied for its antioxidant activity and it is used for cosmetic and medical application, but it is gaining popularity as human dietary supplement [Machmudah et al.

2006, Zhao et al. 2006]. In addition, it has the ability to be esterified, thanks to the presence of the hydroxyl group and ketone group on each ring and is more polar than other carotenoids. The green algae *Haematococcus pluvialis*, is one of the most important biological sources of astaxanthin. Other microalgae species capable to accumulate secondary carotenoids are: *Botryococcus braunii, Chlamydomonas nivalis, Chlorella* sp, **Scenedesmus** sp., [Lemoine and Schoefs, 2010]. In contrast to the listed species, astaxanthin content in H. pluvialis represents 90% of total carotenoids. Beta -carotene is used as colorant in the food industry or as food supplement because it is the precursor of retinol. Spirulina platensis and *Dunaliella salina* are good producers of this antioxidant, reaching levels above the 14% dry weight [Spolaore et al. 2006].

The carotenoids extraction by adsorption was reported in the process of palm oil production [Ong et al. 1980, Mamuro et al. 1986]. Two types of solvent are commonly used in adsorption method: ethanol and isopropanol are used as initial solvents to build an initial layer on the surface area of the adsorbent in order to allow solute contact with liquid phase. The second solvent is n-hexane, which is preferable for eluting the carotene adsorbed on the hydrophobic surface of the adsorbent. A process of separating carotene from crude palm oil by adsorption chromatography with a synthetic polymer adsorbent was developed by [Goh et al. 1988]. However, this chromatographic process is still not commercially proven.

Carbohydrates fractioning

Photosynthetic microorganisms, such as eukaryotic microalgae and cyanobacteria capture solar energy and convert it into chemical energy through numbers of complex reactions called photosynthesis. The carbon dioxide is used as substrate for carbohydrates formation, inside the chloroplast but also in the cytosol (in the case of prokaryotes carbohydrates are synthesized in the cytosol). Production of carbohydrates serves two main purposes for algae; they act as structural components in the cell walls, and as storage components inside the cell. Carbohydrates, as storage compounds, provide the energy needed for the metabolic processes of the organisms and allow, if needed, temporary survival in dark conditions [Geider et al. 2002, Beardall et al. 2004, Kromkamp 1987] and allow microalgae to adjust their growth to the changing environmental conditions. Carbohydrates are a wide category encompassing sugars (monosaccharides) and their polymers (di-, oligo-, and polysaccharides). The reserve carbohydrates are species dependent; cyanobacteria synthesize glycogen (α-1,4 linked glucan), red algae synthesize floridean starch (hybrid of

starch and glycogen) and green algae synthesize amylopectin-like polysaccharides (starch) [Sekharam et al. 1989]. Fractionation of microalgae with the scope of carbohydrates recovery is not common, because the whole biomass serves as attractive feedstock for the production of bioethanol. According to Hu, Chlorophyta content of carbohydrates is 20-40%, but in stress condition (e.g. nitrogen depletion) microalgae increase their content in oil and saccharides.

For bioethanol production, microalgae and cyanobacteria biomass can be employed in traditional process consisting in enzymatic hydrolysis and yeast fermentation. Microalgal can accumulate carbohydrates used for the production of bioethanol, such as starch, glycogen, and cellulose. Photosynthetic organisms could be forced to produce hydrogen, acids, and alcohols (e.g. ethanol) in dark conditions (dark fermentation), however this pathway is not efficient for the production of bioethanol [de Farias Silva et al. 2016].

After upstream processes and pre-treatment for the destruction of cell wall, biomass hydrolysis is achieved through acid/base or enzyme addition. The obtaining of ethanol is possible after the fermentation step that involves the presence of yeasts (e.g. Saccharomyces sp.) or bacteria (e.g. Zymomonas genus).

Several eukaryotic microalgae and cyanobacteria produce and excrete large amounts of exopolysaccharides in their extracellular environment. these exopolysaccharides have more complex structures if compared with cell wall polysaccharides or intracellular starch. They often include up to 9-12 different monosaccharides and several non-sugars substituents. Heteropolymers produced by microalgae are mainly composed of xylose, galactose and glucose even if other monosaccharides such as mannose, fucose, rhamnose, ribose, arabinose, fructose, galacturonic acid, glucuronic acid, iduronic acid, nosturonic acid or methylated sugars are present in their structures.

Exopolysaccharides are rarely considered as high-value molecules, but more as by-products of other productions such as pigments or lipids. For this reason, the downstream processes for extraction of polysaccharides from microalgae are often not really adapted for the treatment of these low soluble polymers. Microalgae may excrete large quantities of polysaccharides to cover their cells, presumably in order to protect them from fluctuations in environmental conditions and/or predators ranging from about 0.5 g L^{-1} up to 20 g L^{-1} [Markou et al. 2013]. Exopolysaccharides from microalgae can remain associated to the cell surface (cell-bound polymers) and/or liberated into the surrounding environment as released polysaccharides [Arad and Levy-Ontman, 2010; De Philippis et al., 1998].

Exopolysaccharides biological activities described in literature are anti-inflammatory, immunomodulatory, anti-tumor, antiviral, antiparasitic, antioxidant, hypoglycaemic, and hypocholesterolemic activities, but they have also rheological and functional properties in the field of cosmetic industry, food and feed [Arad et al. 2010; De Philippis et al. 1998 Raposo et al. 2013]. A general strategy is proposed to selectively extract and purify EPS from microalgae cultures. EPS solubilised in culture media are extracted using centrifugation or microfiltration to remove microalgae [Li et al. 2011, Ye et al., 2005]. Thereafter, the soluble fraction (supernatant or filtrate) is precipitated using methanol, ethanol or isopropanol (2-3 volumes of alcohol for one volume of supernatant/filtrate) at temperatures between -20°C and 20°C [De Brouwer et al. 2004, Gloaguen et al., 2004]. This method allows the selective concentration of EPS, the possibility to recycle the alcohols by distillation and the treatment of highly viscous solutions. Tangential ultrafiltration processes were proposed as an alternative to the conventional extraction. In 2011, Li's group has developed a pilot-scale extraction and purification process of biological active EPS from culture media of diverse Cyanobacteria and microalgae such as *Chaetoceros muelleri*, *Chlorella pyrenoidosa*, *Spirulina platensis*, *Haematococcus pluvialis*, *Nostoc commune* and *Nostoc sphaeroide* [Li et al. 2011].

The technology is based on a microfiltration process (polypropylene membrane) to remove microalgae and a tangential-flow ultrafiltration system with a polyethersulfone membrane (cut-off 5000 Da) to concentrate the EPS (20 to 40 times). Patel and co-workers published that diafiltration with a 300 kDa polyethersulfone membrane was a most efficient technique to isolate EPS from *Porphyridium* cell free broths compared to solvent extraction [Patel et al. 2013]. A work on the upgrading of exhausted culture media resulting from the cultivation of the cyanobacterium *Arthrospira platensis* (Spirulina) and the extraction and purification protocol for the exopolysaccharides excreted by the cyanobacterium was recently published by our research group [Sed et al. 2017].

Chapter 2. Green Chemistry and innovative solvents in Biorefinery

2.1 Traditional solvents in chemical industry

Organic solvents play a fundamental role in different scientific and technological applications and they are the focus of most the operations in the chemical industry. Today, almost all manufacturing and processing industries depend on the extensive use of solvents or water. The solvents are used as process aids, cleaning agents, and dispersants. An even larger amount of water is used and contaminated in related processes. They contaminate our air, land, and water, even if lots of precaution are adopted. Solvents are difficult to contain and recycle and their incorrect disposal can cause environmental or human health hazards. Halogenated solvents were broadly used in the past century, thanks to their excellent ability to solubilise organic components. In 1990s almost 15 billion kilograms of organic and halogenated solvents were produced worldwide each year [Walsh 1996]. The dry-cleaning industry has been dependent on halogenated solvents, mostly on perchloroethylene (tetrachloroethylene). This solvent is highly toxic, affecting plant workers and consumers who use dry cleaners [EPA 1993]. Perchloroethylene is probably carcinogenic to humans, is a central nervous system depressant and can enter the body through respiratory or dermal exposure. Tetrachloroethylene dissolves fats from the skin, potentially resulting in skin irritations [IARC 1997, NIOSH 2009]. Tetrachloroethylene is a common soil contaminant, with a density of 1.622 g/cm^3 can cause dense nonaqueous phase liquid (DNAPLs) if sufficient quantities are released. Part of the amount of the produced tetrachloroethylene is released into the atmosphere, its degradation products observed include phosgene, trichloroacetyl chloride, hydrogen chloride, carbon dioxide, and carbon monoxide. Currently chlorinated solvents are under regulation and their use is exclusively allowed for those activities in which there are not available alternatives.

Other solvents frequently used are aromatic and aliphatic hydrocarbons, thanks to their low solubility in water they are easily separable from aqueous fractions. These solvents have elevated volatilities considered as an advantage in the industrial applications (their recovery is achieved by simple evaporation), on the other hand it contributes to increase the risk of fire, explosion and inhalation. Their big disadvantages are the high oral toxicities and the carcinogenicity (e.g. benzene, Polycyclic aromatic hydrocarbons PHAs) [Badger 1948].

Their presence in the atmosphere can contribute to the global overheating, the reduction of the ozone and the photochemical smog [www.epa.gov].

In the food industry solvents with non-favourable characteristics are often used. Hexane was classified by the U.S. Environmental Protection Agency (EPA) as a hazardous air pollutant. The same agency has attested by that more than 20 million kg of hexane were released to the atmosphere due to its use in the extraction of vegetable oils. Many are the rules that the legislations are adopting to decrease or stop the consumption of organic solvents, especially if they cause irreversible damages to the human health or to the environment. Unfortunately, they are still used in a vast range of activity contributing to the generation of enormous quantity of waste. The development of more ecological alternatives is becoming important and the substitution of toxic and dangerous solvents with new classes of organic solvents with "greener" features is one of the goals that the scientific community is pursuing.

2.2 Green Chemistry

Pursuing a sustainable production of biochemicals, food, feed and biofuels from biomasses it was necessary to integrate into the biorefinery a set of principles aimed at reducing the use and production of hazardous substances. In 1998, Paul Anastas and John Warner developed a list of twelve practical principles providing an early conception of what would make a greener chemical, process, or product. The principles gave birth to the philosophy of Green Chemistry.

It offers a protocol in the development of a biorefinery and plays an important role in the production of raw materials from biomasses [Anastas et al. 2000]. The design, development and implementation of chemical products and processes can be framed in a perspective of sustainability, using the following principles:

1) Prevention

"It is better to prevent waste than to treat or clean up waste after it has been created."

A measure of waste is the environmental factor or "E-Factor", developed by Sheldon is the ratio of the mass of the total waste generated by a reaction per unit mass of product produced [Sheldon 1994]. It better reflects a total chemical process rather than a chemical reaction alone.

2) Atom economy

"Synthetic methods should be designed to maximize incorporation of all materials used in the process into the final product."

The efficiency of a reaction can be calculated with the "percent atom economy" index [Trost 1991]: the formula weight of the desired product(s) divided by the sum of the formula weights of all the reactants. It is important to achieve maximum percent yields, but also design syntheses that maximize the incorporation of the atoms of the reactants into the desired product.

3) Less Hazardous Chemical Synthesis

"Wherever practicable, synthetic methods should be designed to use and generate substances that possess little or no toxicity to human health and the environment."

This concept can be assimilated to the concept of *best available technology* (BAT), introduced in 1984 with Directive 84/360/EEC and applied to air pollution emissions from large industrial installations. It means the most effective and advanced stage in the development of activities and their methods of operation which indicates the practical suitability of particular techniques for providing the basis for emission limit values, to prevent or to reduce emissions and the impact on the environment. For example, in the manufacture of polystyrene foam sheet packing material, chlorofluorocarbons which contribute to O_3 depletion and global warming, have now been replaced by CO_2 as the blooming agent [Tomasko et al. 2009].

4) Designing Safer Chemicals

"Chemical products should be designed to preserve efficacy of function while reducing toxicity."

Green chemistry's goal is to prevent adverse consequences of the design of chemicals by making design choices that minimize hazard. Hazard reduction must have equal status with the other physicochemical attributes associated with chemical structure and function. Efforts need to be made in the designing phase to minimize or eliminate hazard. Development of a framework to design safer chemicals will improve the possibility of reducing the generation of hazardous chemicals A framework can be drawn up to address chemists and scientists toward the design of safer chemicals. First of all, it is important to understand the mechanism of action of a hazardous chemical. It is possible through Quantitative Structure–Activity Relationships (QSAR), that are quantitative regression models that allow an estimation of

the potential activity of an untested chemical by comparing its structure with a series of chemicals with similar structural properties [Perkins et al. 2003].
Second, it is important to study the toxic-kinetics and toxic-dynamics of a substance and to understand the adsorption, distribution and metabolism and excretion of the xenobiotic in several model organisms. At the end, the quantity of chemical available for biological action (bioavailability) needs to be decreased, which leads to decreased toxicity.

5) Safer Solvents and Auxiliaries

"The use of auxiliary substances (e.g., solvents, separation agents, etc.) should be made unnecessary wherever possible and, innocuous when used."

The development of Green Chemistry redefines the role of a solvent: An ideal desirable green solvent should be natural, nontoxic, cheap, and readily available. This principle focuses on creating products in such a way that they use less hazardous solvents (such as water). In 2013 Sanofi developed the solvent selection guide, in order to help chemists in the selection sustainable solvents that will be accepted in all production sites. Solvents are divided into four classes: "recommended", "substitution advisable", "substitution requested" and "banned". This ranking is derived from Safety, Health, Environmental, Quality, and Industrial constraints. Each solvent has its own ID card that indicates the overall ranking, health, safety and environmental hazard bands, physical properties, cost, and substitution advice. as well as its ICH limit (limit imposed by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use) [Prat et al. 2013]. The guide is regularly updated and in alignment with the European regulations and Global Harmonized System (GHS). A recent update was elaborated by the CHEM21 consortium, providing a new method for the ranking score calculation (Figure 2.1) [Prat et al. 2015]. For example, Pfitzer resulted the winner of Presidential Green Chemistry Challenge in 2002, introducing a new greener three-step synthesis for the production of sertraline. Eliminating titanium chloride, toluene, tetrahydrofuran, hexane and chloromethane and using ethanol as solvent, resulted in a reduction of solvents from 60,000 to 6,000 gal/ton. The substitution of palladium on carbon catalyst with the palladium on calcium carbonate leads to higher yields.

Family	Solvent	BP (°C)	FP (°C)	Worst H3xx[a]	H4xx	Safety score	Health score	Env. score	Ranking by default	Ranking after discussion[b]
Water	Water	100	na	None	None	1	1	1	Recommended	Recommended
Alcohols	MeOH	65	11	H301	None	4	7	5	Problematic	Recommended
	EtOH	78	13	H319	None	4	3	3	Recommended	Recommended
	i-PrOH	82	12	H319	None	4	3	3	Recommended	Recommended
	n-BuOH	118	29	H318	None	3	4	3	Recommended	Recommended
	t-BuOH[c]	82	11	H319	None	4	3	3	Recommended	Recommended
	Benzyl alcohol	206	101	H302	None	1	2	7	Problematic	Problematic
	Ethylene glycol	198	116	H302	None	1	2	5	Recommended	Recommended
Ketones	Acetone	56	−18	H319	None	5	3	5	Problematic	Recommended
	MEK	80	−6	H319	None	5	3	3	Recommended	Recommended
	MIBK	117	13	H319	None	4	2	3	Recommended	Recommended
	Cyclohexanone	156	43	H332	None	3	2	5	Recommended	Problematic
Esters	Methyl acetate	57	−10	H302	None	5	3	5	Problematic	Problematic
	Ethyl acetate	77	−4	H319	None	5	3	3	Recommended	Recommended
	i-PrOAc	89	2	H319	None	4	2	3	Recommended	Recommended
	n-BuOAc	126	22	H336	None	4	2	3	Recommended	Recommended
Ethers	Diethyl ether	34	−45	H302	None	10	3	7	Hazardous	HH
	Diisopropyl ether	69	−28	H336	None	9	3	5	Hazardous	Hazardous
	MTBE	55	−28	H315	None	8	3	5	Hazardous	Hazardous
	THF	66	−14	H351	None	6	7	5	Problematic	Problematic
	Me-THF	80	−11	H318	None	6	5	3	Problematic	Problematic
	1,4-Dioxane	101	12	H351	None	7	6	3	Problematic	Hazardous
	Anisole	154	52	None	None	4	1	5	Problematic	Recommended
	DME	85	−6	H360	None	7	10	3	Hazardous	Hazardous
Hydrocarbons	Pentane	36	−40	H304	H411	8	3	7	Hazardous	Hazardous
	Hexane	69	−22	H361	H411	8	7	7	Hazardous	Hazardous
	Heptane	98	−4	H304	H410	6	2	7	Problematic	Problematic
	Cyclohexane	81	−17	H304	H410	6	3	7	Problematic	Problematic
	Me-cyclohexane	101	−4	H304	H411	6	2	7	Problematic	Problematic
	Benzene	80	−11	H350	None	6	10	3	Hazardous	HH
	Toluene	111	4	H351	None	5	6	3	Problematic	Problematic
	Xylenes	140	27	H312	None	4	2	5	Problematic	Problematic
Halogenated	DCM	40	na	H351	None	1	7	7	Hazardous	Hazardous
	Chloroform	61	na	H351	None	2	7	5	Problematic	HH
	CCl_4	77	na	H351	H420	2	7	10	Hazardous	HH
	DCE	84	13	H350	None	4	10	3	Hazardous	HH
	Chlorobenzene	132	29	H332	H411	3	2	7	Problematic	Problematic
Aprotic polar	Acetonitrile	82	2	H319	None	4	3	3	Recommended	Problematic
	DMF	153	58	H360	None	3	9	5	Hazardous	Hazardous
	DMAc	166	70	H360	None	1	9	5	Hazardous	Hazardous
	NMP	202	96	H360	None	1	9	7	Hazardous	Hazardous
	DMPU	246	121	H361	None	1	6	7	Problematic	Problematic
	DMSO[c]	189	95	None	None	1	1	5	Recommended	Problematic
	Sulfolane[c]	287	177	H360	None	1	9	7	Hazardous	Hazardous
	HMPA	>200	144	H350	None	1	9	7	Hazardous	HH
	Nitromethane	101	35	H302	None	10	2	3	Hazardous	HH
Miscellaneous	Methoxy-ethanol	125	42	H360	None	3	9	3	Hazardous	Hazardous
	Carbon disulfide	46	−30	H361	H412	9	7	7	Hazardous	HH
Acids	Formic acid	101	49	H314	None	3	7	3	Problematic	Problematic
	Acetic acid	118	39	H314	None	3	7	3	Problematic	Problematic
	Ac_2O	139	49	H314	None	3	7	3	Problematic	Problematic
Amines	Pyridine	115	23	H302	None	4	2	3	Recommended	Hazardous
	TEA	89	−6	H314	None	6	7	3	Problematic	Hazardous

Figure 2.1 CHEM21 solvent guide of "classical" solvents

6) Design for Energy Efficiency

"Energy requirements should be recognized for their environmental and economic impacts and should be minimized. Synthetic methods should be conducted at ambient temperature and pressure."

We use energy for transportation purposes and to provide electricity to our homes and businesses. Traditional methods for generating energy have been found to contribute to global environmental problems such as Global Warming and the energy used can also be a significant cost. This principle focuses on creating products and materials in a highly efficient manner and reducing associated pollution and cost**.** In a laboratory, the amount of energy and its associated cost is modest and may seem insignificant. But on the much larger industrial scale, energy/money savings are multiplied and energy efficiency becomes even

more important. To remove volatile solvent conveniently we use a rotary evaporator, which involves the combined use of a heat source, vacuum pump, rotating motor, and chiller. The heat, vacuum, and rotation vaporize the solvent and the chiller condenses the solvent vapours into a flask for removal. in a laboratory scale, these devices consume a small amount of energy, it is not the same for industrial plants, where their use waste thousands of kW/h per month. If we can develop chemical reactions that avoid solvent removal and/or simplify work-up, we can save energy and money.

One efficient manner to carry out sustainable synthesis is the biocatalysis. Enzymes are derived from renewable resources and are biodegradable, they work in mild conditions (ambient temperature and pressure in water), they are highly specific for the substrate and lead to higher quality products. In the energetic field, the traditional chemical process for the production of biofuels uses sodium methoxide for conversion of plant oil triglycerides to fatty acid methyl esters (FAMEs), which subsequently results in a product contaminated with high alkali salt content requiring costly purification. The use of lipase as a biocatalyst for esterification was researched for its efficiency at mild reaction conditions and high-purity product yields and was proposed to eliminate the need for purification. However, for biofuel production, economical implementation of such an enzymatic process requires efficient recovery and reuse of lipase due to the required scale of production, therefore necessitating an immobilised enzyme [Zhang et al. 2012]. Piedmont Biofuels announced in 2012 the successful scale-up of a continuous enzymatic transesterification of free fatty acids (FFAs) via immobilized *Candida antarctica* lipase B for biodiesel production; the enzymatic reaction eliminated the need for caustic stripping of the chemical intermediate due to its high product selectivity [Di Cosimo et al. 2013].

7) Use of Renewable Feedstocks

"A raw material or feedstock should be renewable rather than depleting whenever technically and economically practicable."

As already mentioned in the precedent chapter, in the past 10 years, significant advances have been made in the development of fuels, chemicals and materials from renewable feedstocks. These for example, have included biodiesel from plant oils and algae, bioethanol and butanol from sugars and lignocellulose, plastics, foams and thermosets from lignin and plant oils. To accomplish the seventh principle, collaborations between several disciplines involving biotechnology, agronomy, toxicology, physics, engineering are needed to reach

the goal of chemicals and materials derived from renewable feedstock with minimal impact on human health and the environment.

8) Reduce Derivatives

"Unnecessary derivatization (use of blocking groups, protection/deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste."

One of the solutions is the use of enzymes. Enzymes are specific, they can react with only one site of the molecule leaving the rest, hence protecting groups are often not required. Penicillin acylases (PA) are widely used to produce semi-synthetic β-lactam antibiotics and chiral compounds. Broad substrate specificity and high regio-, chemo- and stereoselectivity of the enzyme are used for the production of chiral compounds, as well as for the protection of hydroxy and amino groups in peptide and fine organic synthesis. In the first industrial synthesis of penicillin G the protection with its silyl ester was required before the next steps. Involving PAs in the synthesis has many advantages from a green perspective one of which is that the silyl protecting group is not required and it occurs in water at just above room temperature.

9) Catalysis

"Catalytic reagents (as selective as possible) are superior to stoichiometric reagents."

A catalyst is *a substance that changes the velocity of a reaction without itself being changed in the process*. It lowers the activation energy of the reaction and it is not consumed. It can be used in small amounts and be recycled generating any waste. In 2009 Sheldon and co-workers developed of a green two-step, three-enzyme process for the synthesis of a key intermediate in the manufacture of atorvastatin (cholesterol reducer). In the old process alkaline condition (pH 10) and temperature around 80 °C were required to reach yield of ~85% [Matsuda et al. 1999]. The new process involves the biocatalytic reduction of ethyl-4-chloroacetoacetate using a ketoreductase in combination with glucose and a NADP-dependent glucose dehydrogenase for cofactor regeneration, obtaining the ethyl-4-chloro-3-hydroxybutyrate. In the second step, a halohydrin dehalogenase (HHDH) is employed to catalyse the replacement of the chloro substituent with cyano by reaction with HCN at neutral pH and ambient temperature. The overall process has an E factor (kg waste per kg product) of 5.8 when process water is not included, and 18 if included [Ma et al. 2010].

10) Design for Degradation

"Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment."

The biggest challenge is making chemicals that are stable during usage, but don't persist in the environment. The breakdown products must be non-toxic and not persistent. The break down can be due to photodegradation, hydrolysis or biological species, often with enzymes (biodegradation). In laundry industry branched alkylbenezene sulfonate (branched detergents) have been phased out in most developed countries because they are too persistent, they do not biodegrade. Biodegradable soaps, or detergents, break down more easily in the environment. Sodium dodecylbenzenesulfonate is a common detergent and it is a linear alkylbenzene sulfonate. It is useful as a detergent because it has a polar headgroup (sulfonate) and a non-polar alkyl group, making it a surfactant. It degrades quickly in the environment under aerobic conditions, or when oxygen is present, because microbes are able to use to the linear alkyl chain as energy, via a process called β-oxidation. Once the long chain is degraded, the rest of the molecule can be degraded as well [Bautista-Toledo et al. 2014].

The commodity plastics, polyethylene (PE), polypropylene (PP), polystyrene (PS) and polyvinylchloride (PVC) have revolutionised the packaging industry. Once these materials are discarded they persist in the environment without being degraded. Biodegradable polymers have offered scientists a possible solution to waste-disposal problems associated with traditional petroleum-derived plastics. Polyhydroxyalkanoates (PHAs) are synthesized by microbial fermentation and can be degraded to water and carbon dioxide under environmental conditions by a variety of bacteria, have much potential for applications of degradable plastics [Holmes 1988]. Disadvantages compared with conventional plastics are brittleness and narrow processability window [Barham et al. 1986]. For this reason, various copolymers of polyhydroxybutyrate (poly3-hydroxybutirate-co-3-hydroxyvalerate PHBV) have been biosynthesized [Doi et al. 1995].

11) Real-time analysis for Pollution Prevention

"Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances."

Monitoring a chemical reaction as it is occurring can help prevent release of hazardous and polluting substances due to accidents or unexpected reactions. With real time monitoring,

warning signs can be spotted, and the reaction can be stopped or managed before such an event occurs.

12) Inherently Safer Chemistry for Accident Prevention

"Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires."

Working with chemicals always carries a degree of risk. However, if hazards are managed well, the risk can be minimised. This principle clearly links with numbers of the other principles that discuss hazardous products or reagents. In fact, it is practically impossible to achieve the goals of the twelfth principle without the implementation of at least one of the others. Where possible, exposure to hazards should be eliminated from processes, and should be designed to minimise the risks where elimination is not possible. This principle focuses on safety for the worker and the surrounding community where an industry resides. The most widely known examples where safe chemicals were not used and the result was disaster is that of Bhopal, India in 1984. A chemical plant had an accidental release of methyl isocyanate that resulted in thousands of lives lost and many more injuries. The disaster indicated a need for enforceable international standards for environmental safety, preventative strategies to avoid similar accidents and industrial disaster preparedness [Broughton 2005].

2.2.1 Supercritical fluids (SCFs)

The properties of a fluid in the supercritical region are generally described as a combination of those of a liquid and a gas, with the exception of compressibility and heat capacity (higher near the critical point than they are in liquid or gas). The majority of studies on SCFs have focused on four fluids: CO_2 the most widely studied, ethane, ethylene and water. CO_2 has several advantages over the others, including being non-toxic, non-flammable and available in high purity. Its critical point (73.8 bar, 31.1 °C) are also relatively accessible. These characteristics make this green solvent the most widely used. They are non-carcinogenic, nontoxic, non-mutagenic, non-flammable and thermodynamically stable.

Another major benefit refers to the possibility of adjusting the thermophysical properties of SCFs, such as diffusivity, viscosity, density or dielectric constant, by simply varying the operating pressure and/or temperature. Moreover, SCFs have excellent heat transfer properties, and have been studied as environmentally benign heat transfer fluids. They were

proposed as sustainable alternative to the fluids used today in air conditioning and refrigeration systems (chlorofluorocarbons, ammonia, sulfur dioxide, propane) and which are toxic or potent greenhouse gases.

The use of the SCFs as solvents instead of organic compounds can offer several advantages such as improved reaction rates, selectivity control and process safety.

Due to their good solvent properties, SCFs are also used in extraction, precipitation and crystallization techniques. Applications of high-pressure technologies involve extraction of valuable products (nutraceuticals, food additives, antioxidants etc.) from natural materials, Extraction by $scCO_2$ of hop constituents to make beer and extraction of caffeine from coffee beans and tea leaves for decaffeinated beverages are already performed on an industrial scale [Capuzzo et al. 2013]. Several industrial plants are in operation also for extraction of spices for food industry and natural substances for use in cosmetics [Sovilj et al. 2011]. The main disadvantage of SCF extraction refers to the high investment costs and therefore the high price of the products compared to those obtained by conventional methods. Nevertheless, organic solvent residues in products used for food and feed are subject to restrictions and SCFs allow the possibility of fractionation of special components from total extract, for these reasons the use of these gases for extractions is encouraged.

High pressure technologies for polymer processing

In the field of polymeric foams, $scCO_2$ was used as blowing agent, to replace traditional compounds such as chlorofluorocarbons, hydrochlorofluorocarbons and volatile organic solvents [Tomasko et al. 2009]. To obtain polymer or composite foams the substrate is saturated with $scCO_2$, followed by rapid depressurization at constant temperature (pressure quench). It offers also the possibility to control pore size and distribution varying the process parameters (P, T and depressurization rate). SCFs can be used for the drying (removing liquids from solids without altering the structure of the material), for the cleaning in microelectronics, (the contaminants dissolve in the SCF and then are removed together with the gas by expansion) or in low temperature pasteurization in the food or pharmaceutical sectors [Sawan et al. 1998, Darani et al. 2009]. The use of SCFs in chemical and biochemical reactions arises from a desire to replace volatile organic solvents due to the lower environmental impact and the lower energy consumption. Use of SCFs as reaction media has a high potential also in pharmaceutical industry for the production of chiral intermediates and final products.

Reported reactions in SCFs are ammonolysis of esters to amides, asymmetric and catalytic hydrogenations, synthesis of single enantiomer instead of racemic mixture, membranes breaking, for the extraction of intracellular components (e.g. enzymes) from microbial cells [Wang et al. 2000, Seki et al. 2008]. The effect of SCFs on stability and activity of biocatalysts for the performance of different biochemical reactions was reported in several studies. Supercritical gases may influence enzyme activity or selectivity by the pressure effect (acting on denaturation or solubility of reactants) [Matsuda 2013, Knez 2009, Hobbs et al. 2007].

At high pressures solute solvent interactions increase, resulting in higher solvent capacity [Habulin et. al 2001]. However, $scCO_2$ has also disadvantages, as sometimes lower catalytic activities due to formation of carbonic acid may occur [Habulin et al. 2007]. The possibility of carrying out integral green biocatalytic processes by combining ILs and SCFs (forming two-phases systems) with enzymes represents an interesting research area for the ability to perform both biotransformation and product extraction at the same time [Sureshkumar et al. 2010].

In recent years, SCFs were studied either as heat transfer media in refrigeration systems and power cycles, as alternative solution to carbon storage, as material processing media in the domain of fuel cells, or as reactants for the synthesis of biodiesel and of other biobased fuels. [Tan et. al 2011, Wen et al. 2009].

Biodiesel production is possible by the use of cooking oil and animal fats and their conversion to biodiesel through the supercritical transesterification (SCTE). SCTE takes place at high temperatures and pressure, when at least one of the reactants (usually methanol) is in supercritical state. The process does not require catalyst, can give nearly complete conversion in a short processing time, and can tolerate feedstocks with a high concentration of free fatty acids and water. Moreover, the quality of biodiesel produced by SCTE appears to be high, as the quality of the byproduct glycerol [Anitescu et al. 2012].

There are still concerns regarding the wide application of SCTE, for the high energy necessary to reach the supercritical conditions of the reactants, the specific requirements for reactors and other high pressure equipments and the safety issue of the operation. Generation of hydrogen and other compounds that may be used as fuel is one of the most studied applications of supercritical water SCW. Recently, hydrogen production from wet biomass and organic compounds in sub- and supercritical water has gained significant attention [Guo et al. 2014, Rönnlund et al. 2011].

The process can take place at lower temperatures (400-600 °C) in the presence of catalysts, or at higher temperatures (600-800 °C) without catalyst [Wen et al. 2009].
The advantages of SCW gasification over conventional biomass gasification (such as steam reforming) refers to the possibility of processing feedstocks with high water content and the generation of pressurized gases, which simplifies their transportation and further use. However, there are numbers of issues that need to be address in order to allow the technology to become commercially available.

2.2.2. Ionic Liquids

Ionic liquids (ILs) are innovative fluids composed by ions liquids at room temperature (RTILs) or with melting points below 100 °C. The story of these liquids began in 1914, with the preparation of ethylammonium nitrate by the addition of nitric acid to ethylamine and the distillation of water, resulting in a "molten salt" at room temperature [Walden 1914] In the past three decades they have received wide attention for their features, such as non or low volatility, thermal stability and large liquid ranges and the number of scientific papers published on ILs has increased exponentially [Lei et al. 2017].
The general composition of ionic liquids is based on an organic cation and an inorganic polyatomic anion, for this reason the potential number of ILs is huge. In the figure is represented the chemical structure of cations commonly employed in the synthesis of molten salts. Most common anions are: tetrafluoroborate, hexafluorophosphate, trifluoromethanesulfonate and aluminum tetrachloride.

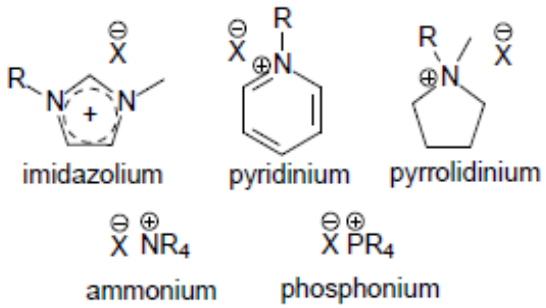

Figure 2.2 Examples of cations commonly used for the synthesis of ILs

Many new cations have recently been proposed (Figure 2.2). Ionic liquids have ionic interactions (mutual electrostatic attraction or repulsion of charged particles), which makes them very miscible with polar substances. At the same time the presence of the alkyl chain on the cation determines their solubility in less polar fluids. By changing the anionic species or the type of cation (for example by varying the length or the branching of alkyl groups), it

is possible to change physical-chemical properties such as melting point, hydrophobicity, viscosity, density and solubility. The possibility to design ILs with the necessary properties for a specific application makes them "designer solvents". ILs usually have an extremely low vapour pressure (e.g. [BMIM][PF_6] at 298.15 K as 10^{-11}Pa) that can be assumed as negligible [Kabo et al. 2004]. This property characterizes ILs as green solvents since they do not evaporate into the atmosphere, they are not explosive and can be used avoiding hazardous exposure and air pollution problems.

The main factors that influence the melting point are the charge distribution on the ions, hydrogen bonding ability, the symmetry of the ions and the van der Waals interactions. Quantitative Structure Property Relationships (QSPR) can be used as predictors for ILs melting points. Katritzky et al. Demonstrated that most important parameter for the melting point prediction are: molecular shape and symmetry of cations, electrostatic intermolecular interactions and the number of conformational and rotational degrees of freedom in the solid and liquid phases [Katritzky et al. 2002].

The majority of RTILs are denser than water with values ranging between 1 and 1.6 gcm^{-3}. The density decreases with increase in the length of the alkyl chain [Marsh et al. 2004]. The high viscosity of ILs is one of their disadvantages, being two or three orders of magnitude greater of those for organic solvents it affects mass transfer and increases the power requirement for mixing in liquid-liquid systems. Viscosity values at 20 °C are influenced by the nature of cations and anions and should be in the range of 50-500 mPa s. The increase of length of one or both alkyl chains on the cation does not necessarily result in a monotonous increase in the viscosity. [Bonhote et al. 1996, Sun et al. 1998]. The decomposition temperature varies between 100˚C and 360˚C. Protic ILs with a bis (trifluoromethane) sulfonamide anion with alkylammonium cations, the imidazolium cation, and a variety of heterocyclic cations are known as the most stable with decomposition temperatures of >200˚C [Nazari et al. 2013].

Generally, ILs have high conductivities (≈10 $mScm^{-1}$); the ionic conductivity, which depends on the available charge carriers and their mobility (which depends on viscosity), varies with the molecular weight, and size of the ion. Ion conductivity decreases by increasing the size of the cation (less mobility); consequently, the conductivity of PILs with longer alkyl chains decreases [Greaves et al. 2007]. ILs also have a significantly large electrochemical window, some values are over 6 V. This allow the electrodeposition of metal and semiconductors at room temperature.

In the past the synthesis of ILs was mainly concentrated on obtaining unique chemical-physical properties (ILs of 1st generation), such as the absence of volatility and thermal stability, or on a targeted behavior (ILs of 2nd generation), now one of the main objectives is the achievement of desirable biological characteristics (3rd generation ILs). At the same time the number of studies on the toxicity and biodegradability of ionic liquids is increased, even if this type of research still represents a small part of the whole literature [Hough et al. 2007].

ILs are engaged in fields such as chemistry, materials science, chemical engineering and environmental science. As a result of their low volatility and non-flammability, in the last decades there has been a great increase in their study due to the need for environmentally friendly green solvents. Nowadays, they are not just used as solvents, but also as catalysts or catalyst activators, electrolytes for batteries, photochemistry and electrosynthesis and even as advanced heat transfer fluids and lubricants. [Steinrueck et al 2015, Van Valkenburg et al. 2005, Barhdadi et Al. 2003, Lewandowski et al. 2009]. From a biorefinery point of view (RTILs) are emerging as attractive and green solvents for lignocellulosic biomass pre-treatment. The unique solvating properties of RTILs foster the disruption of the 3D network structure of lignin, cellulose, and hemicellulose, which allows high yields of fermentable sugars to be produced in subsequent enzymatic hydrolysis. [Mora-Pale et al. 2011].

These solvents are referred to fitting in the context of ‘‘Green Chemistry’’: due to their negligibly low vapour-pressure and reduced gaseous emissions when used instead of a conventional organic solvent. It does not automatically relate to a greener process, and many other points of view have to be taken into account before such a statement can be made.
Although the scientific community is now focused on sustainability of ionic liquids, this theme remains something unexplored. The economic-environmental impact, the cumulative demand for energy (which includes the supply of raw materials, the recycling and disposal of chemicals, the energy required for heating, agitation, cooling), and the eco-toxicological behaviour, are all extremely important parameters to be considered for a complete analysis of the sustainability of ILs, especially in order to increase the possibilities of their application on a large scale. [Kralisch et al. 2005, Zhang et al. 2008].

2.2.3 Solvents with switchable behaviour (Switchable Solvents)

Distillation is a common strategy for the separation of the solvent from the products, but it is expensive from the economic point of view because of the high energy required and from the safety and environmental point of view because it requires the use of volatile organic solvents. Furthermore, chemical processes are made up by many phases that require for each step a specific solvent. Indeed, after each phase of the process the solvent must be removed and replaced with a new one, more suitable for the next step, increasing the costs and the environmental impact of the process. These drawbacks can be avoided by using non-volatile solvents, requiring a method for removing these solvents from the products. The "switchable solvents" represent a valid response to these procedures, because their properties can be regulated by allowing the same solvent to be used for various consecutive reactions or separation stages. The switchable solvents can be considered reversible ionic liquids and constitute a new class of solvents with very interesting applications. The concept of "switchable compounds" was proposed for the first time by Jessop et al. in 2005 [Jessop et al. 2005]. The idea is based on the possibility of commuting the form, and therefore the physical-chemical properties of these solvents through the application of an external triggering agent. For example, switchable polarity solvents (SPSs) are liquids that can be reversibly modified by a marked non-polar shape to a very polar shape. While the switchable hydrophilicity solvents (SHSs) are normally very hydrophobic liquids (with a very small miscibility in water), which pass thanks to a suitable primer to a hydrophilic form completely miscible in water.

Switchable Polarity Solvents (SPSs)

The SPSs, as already mentioned, are solvents that have the ability to pass reversibly from a low polarity, to a high polarity form through the addition / removal of CO_2 at atmospheric pressure, which acts as a chemical "trigger" for the transformation (Figure 2.3). The removal of CO_2 occurs by stripping with N_2 and with a slight heating (about 60 °C). The reverse reaction occurs easily, not only because the CO_2 is a gas, but also because the thermodynamics of the reaction is fairly balanced (ΔH = -136 kJ/mol, ΔS = -425 J/mol K, ΔG = -8.6 kJ/mol): the forward reaction is favoured enthalpically, while in the other direction it is favoured entropically. The reaction is exoergonic, so it will be necessary to ensure that the temperature of non-polar SPS does not increase too much, both for thermodynamic reasons (the reaction is favoured by low temperatures), and for reasons related to the mass transfer (the solubility of CO_2 is reduced, although its diffusion coefficient increases).

The polarity difference resulting from the transformation is wide, as a result many products are soluble in only one of the solvent forms.

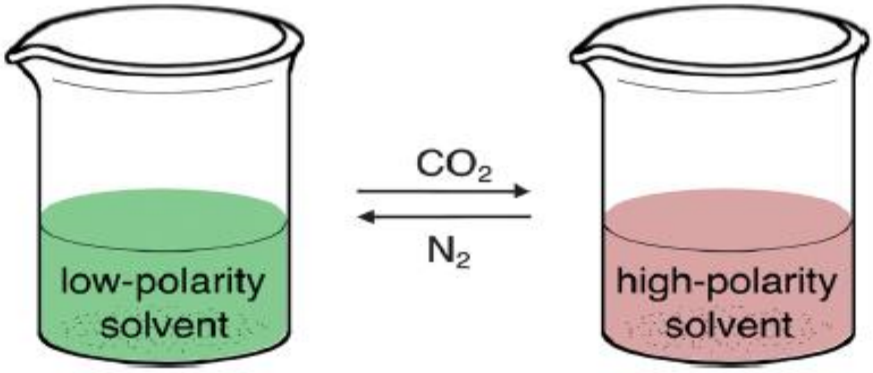

Figure 2.3 Scheme of Switchable Polarity Solvents behavior

The first SPSs proposed by Jessop et al. are based on an alcohol (or water) and an amidine / guanidine (Figure 2.4a). In the forward reaction, the CO_2 reacts with the solvent mixture to form salts of the alkylcarbonate anion.

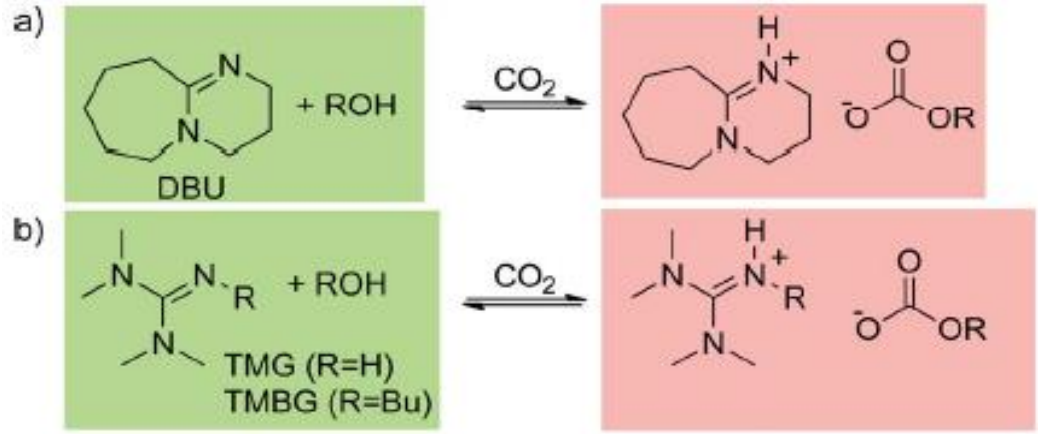

Figure 2.4 Examples of SPSs. a) mixture of amidine and alcohol, b) mixture of guanidine and alcohol

For example, an equimolar mixture of DBU (1,8-diazabicyclo- [5.4.0] -undec-7-ene) and 1-hexanol acts as a slightly polar solvent, similar to chloroform, allowing to dissolve non-polar compounds such as hydrocarbons, whereas DBU hexylcarbonate liquid, resulting after treatment with CO_2, is a polar liquid very similar to dimethylformamide, therefore immiscible with hydrocarbons (Figure 2.5).

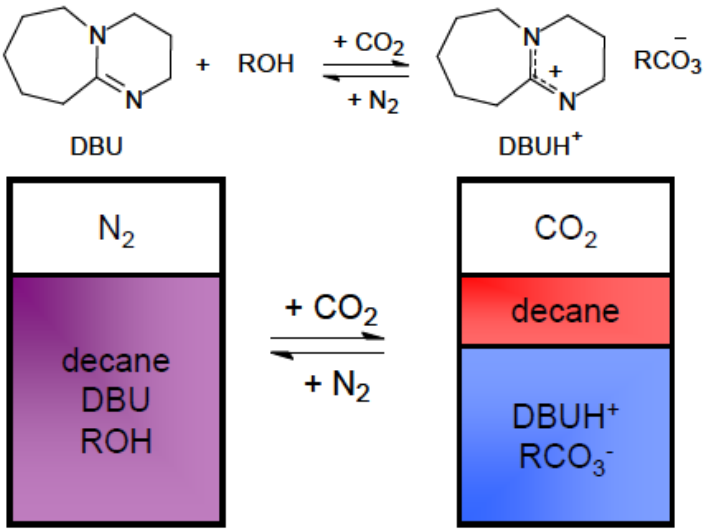

Figure 2.5 SPS based on an equimolar mixture DBU / alcohol, and variation of its polarity after addition of CO_2

Although these alkycarbonate SPSs have immediately shown great potential, they have some limitations. First of all, they have a high sensitivity to water because the reaction base /CO_2/ water to give solid [BH][HCO_3] is thermodynamically favourite respect to the reaction base/CO_2/ROH to give [BH][RCO_3] in a liquid state (where $BH^+$is the protonated base). For this reason, to avoid the formation of bicarbonate salt, the base and alcohol must be dried before use. Secondly, bases (amidine / guanidine) cannot be used as inert solvents for some reactive solutes such as alkyl halides and strong acids. The problem of water sensitivity can be easily solved by exchanging the alkylcarbonate anion with the carbamate anion; in fact Yamada et al. have shown that an SPS consisting of a primary amidine / amine mixture (Figure 2.6) is less sensitive to water than an amidine/alcohol mixture, since the primary amines form carbamate salts which are thermodynamically more stable than bicarbonate and alkylcarbonate salts [Yamada et al. 2007, Yamada et al. 2009].

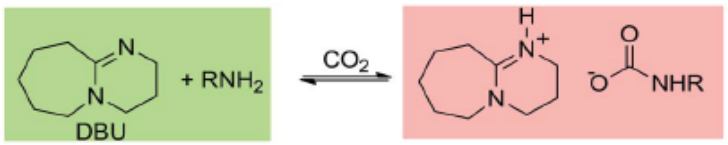

Figure 2.6 SPS based on a primary amine/amidine

The last developed SPS systems are those consisting of a single organic component, which are the most economical, due to the absence of the expensive amidine / guanidine base. Among the first to be proposed, there are the secondary amines, because they have a double

behaviour at the same time: both of a nucleophilic molecule and of a proton donor molecule (Figure 2.7).

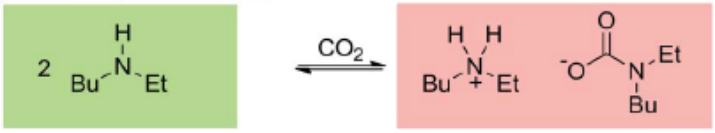

Figure 2.7 Example of a single component SPS

During the "switching" process, polarity is not the only property of the liquid that can change; for example, it was shown by Li et al. that ionic liquids containing primary amino groups can show a change of basicity in addition to that of polarity [Li et al. 2008].

The selection of the type of SPS for a specific reaction or separation depends on the desired polarity range required for the solubility / insolubility of the raw materials or products. Depending on the selected SPS system, is possible to cover a wide range of polarity. Figure 2.8 shows the range of polarities and the relative displacement of the reported SPS systems, in comparison with the common organic solvents.

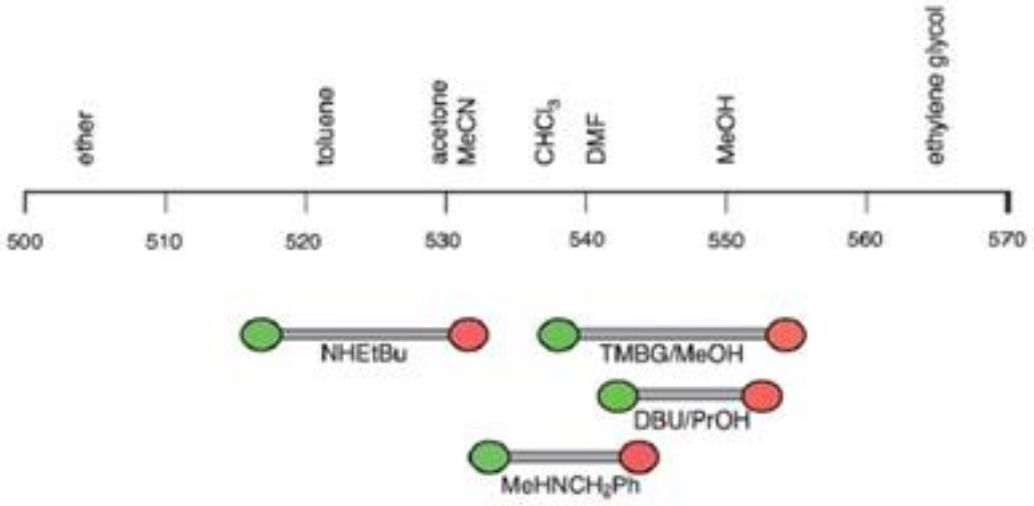

Figure 2.8. Comparison of the polarity range of different SPSs (the low-polarity form is shown in green, the high-polarity one in red). The scale represents the λmax of the solvatochromic dye "Nile Red" which has been dissolved in the solvent

As shown in the figure, the amidine/guanidine-based SPS exhibit polarity greater or equal to that of chloroform in their non-polar form; SPSs which form carbamates cover polarity ranges shifted to the left (lower values), because they are made up of primary and secondary amines generally less polar than alcohols.

SPS as reaction media

SPS are suitable as reaction media (for organic synthesis) because they facilitate the subsequent separation of products. The reactions can be carried out in one form of the solvent and when it is switched into the other form the product or the catalyst will precipitate as insoluble. For example, styrene polymerization can be carried out in a low-polarity solvent (a mixture of DBU/1-propanol), followed by precipitation of the product caused by the "switching" of the solvent in its highly polar form [DBUH][$C_3H_7CO_3$]; afterwards, the solvent can still be modified by returning to its non-polar form and then reused (Figure 2.9). As already mentioned, the SPS can be used as solvents to facilitate the separation of the catalyst from a polymeric product. The Darensbourg's group copolymerizes CO_2 with an epoxide using a chromium based homogeneous catalyst; in the original method, after polymerization, the polymer containing the catalyst was dissolved in dichloromethane, treated with hydrochloric acid to split the catalyst from the polymer chain and precipitated by addition of methanol. With the advent of SPS a more efficient method has been developed: the raw product is first dissolved in N-ethyl-butylamine and then treated with CO_2. The transition to the ionic form of the solvent provides not only the acidity (carbamic acid) required to split the catalyst from the polymer chain, but also triggers the precipitation of the polymeric product thus fractionating it from the metal. The SPS solution containing the catalyst is then distilled, leaving the catalyst still active and ready to be reused [Darensbourg et al. 2017].

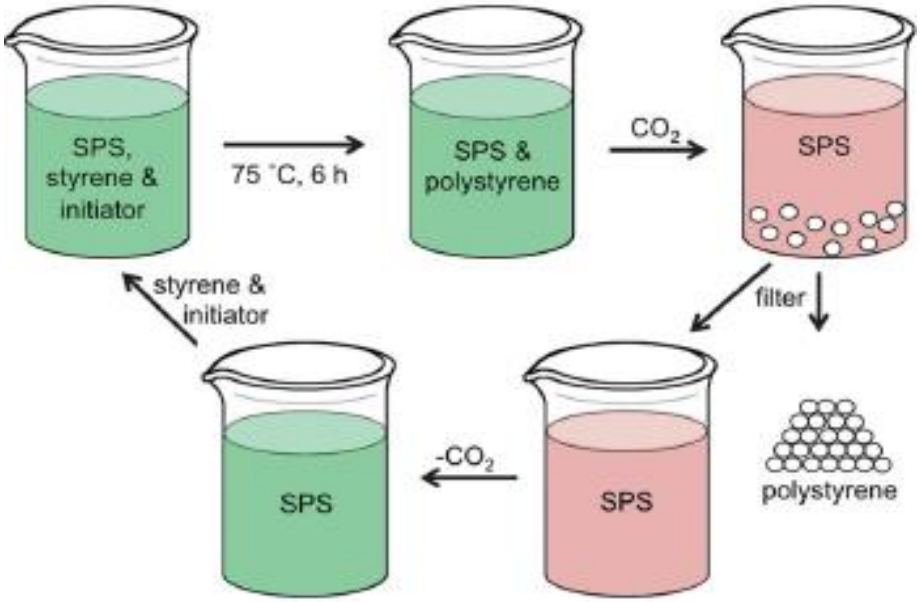

Figure 2.9 Use of SPS as a solvent for styrene polymerization. The low and high polarity shape are shown in green and pink respectively

SPS as a means of extraction

The SPSs are mainly used for extraction thanks to their switchable behaviour and the possibility of product recovery when the solvent is commuted (Figure 2.10).

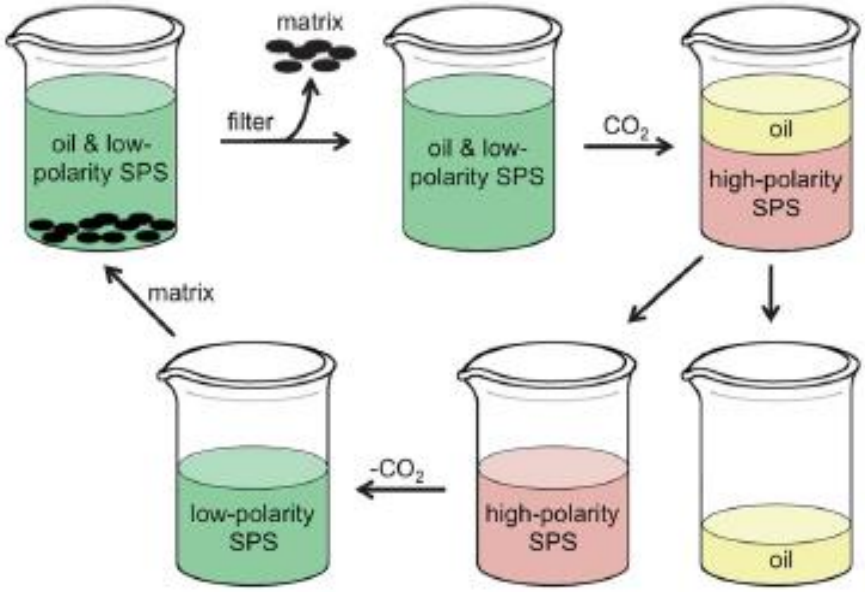

Figure 2.10 extraction of an organic non-polar product (such as oil) from a solid matrix using an SPS

For example, both methylbenzylamine and the DBU-alcohol system have been used as solvents to extract soybean oil from the seeds; while the SPS constituted by DBU-1 octanol was used by Samorì et al. for the extraction of lipids and fatty acids from exhausted microalgal culture media [Phan et al. 2009, Samorì et al. 2010].

Switchable Hydrophilicity Solvents (SHSs)

The use of switchable hydrophilicity solvents is proposed as an alternative to the traditional organic solvents, because they offer the possibility of product recovery avoiding the step of distillation. To understand the usefulness of a SHS, it is necessary to consider the general problem of the separation of a product from a solvent; after an extraction or a reaction, a solution of the product is obtained in the solvent. The traditional approach to product recovery is to carry out a distillation (undesirable for the reasons explained above), or a precipitation, by the addition of an "anti-solvent" (which involves not only a greater waste of solvent, but also the problem of separation of the different solvents employed, before their recycling). However, if the extraction is carried out with a SHS solvent in its original hydrophobic form, then this can be removed from the product by the simple addition of water and CO_2 and subsequently recovered from the water without any distillation, through the

removal of the same CO_2. At the end of the process, both the solvent and the aqueous phase can be readily recycled in the next cycle of extraction. The SHSs are solvents able to change their chemical behaviour; at first, they form a hydrophobic phase poorly miscible with water, but they can commute to a hydrophilic form widely miscible in water thanks to the application of an external triggering agent. CO_2 is an excellent triggering agent as it is abundant, economical and relatively safe to use and manage (Figure 2.11). Currently identified solvents that exhibit this behaviour, by addition and removal of CO_2, are amidines and secondary and tertiary amines (solvents with poor miscibility in water and with a basic pH).

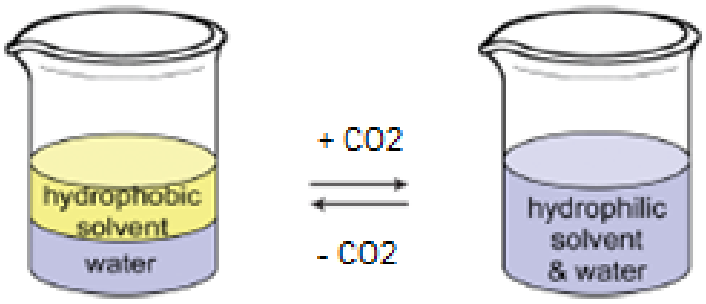

Figure 2.11 Scheme of Switchable Hydrophilicity Solvents behaviour

The change in hydrophilicity is due to an acid-base reaction, in particular to the protonation of the amino group by carbonic acid. By blowing CO_2 into a system consisting of SHS and water we have the formation of carbonic acid which dissociates in its two forms of carbonate and bicarbonate, the result is a bicarbonate salt of the protonised amine which is much more soluble in water (Figure 2.12).

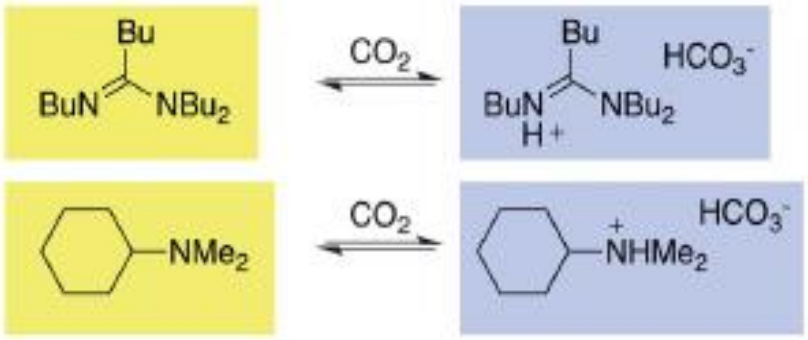

Figure 2.12 reaction mechanism of two tertiary amine based SHSs. The hydrophobic form is shown in yellow, the hydrophilic form in violet

The resulting solution is generally at a pH close to 8, which indicates that the salt present in the system is a bicarbonate rather than a carbonate, as can be easily understood from Figure 2.13 showing the equilibrium of carbon dioxide in water.

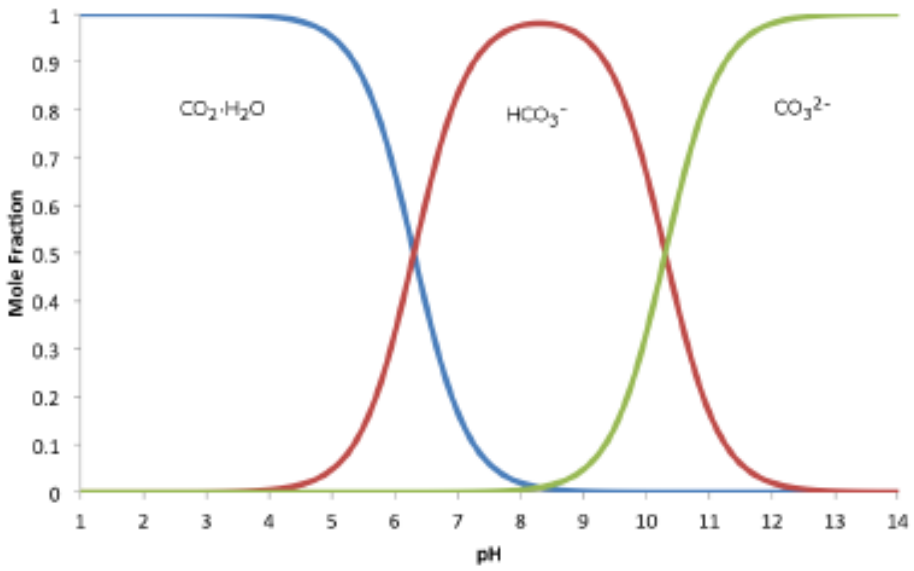

Figure 2.13 Carbon dioxide – Carbonic acid equilibrium

The following reactions affects the process:

$H_2CO_3 + NR_3 \leftrightarrow HNR_3 + HCO_3^-$ for tertiary amines

$2R_2NH + CO_2 \leftrightarrow R_2NH_2 + R_2NCO_2^-$ for secondary amines

This process is reversible: by removing CO_2 we obtain the initial two-phase system. CO_2 stripping is easily achieved by blowing air, nitrogen or argon and/or heating the system sufficiently (60-70 °C). Although the polarity change is not a characteristic required for a SHS, it occurs during the transformation, which surprisingly is much larger than that observed with any SPS solvent. In the figure 2.14 is reported the change of polarity of a mixture of N, N, N'-tributylpentanammidine and water in a 1:1 ratio comparing it with several SPSs.

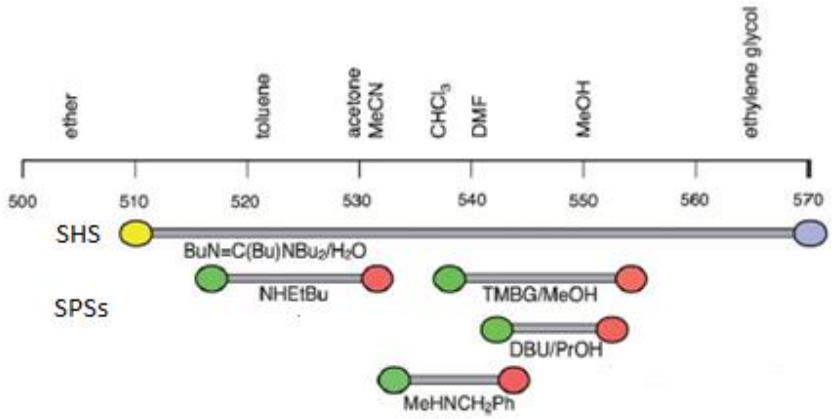

Figure 2.14 comparison of the polarity range of different SPS and the SHS (the hydrophobic form is shown in yellow, the hydrophilic one in violet)

The usefulness of SHSs has been demonstrated in many separation processes such as recovery of vegetable oil from soybeans, lipids from algae, polystyrene from expanded polystyrene waste and phenolic compounds from lignin (Figure 2.15). However, some of these compounds are particularly harmful to human health and flammable, they are characterized by higher boiling points than traditional volatile organic solvents and therefore a low percentage of their vapors is released into the atmosphere [Phan et al. 2009, Boyd et al. 2012, Fu et al. 2014, Jessop et al. 2011].

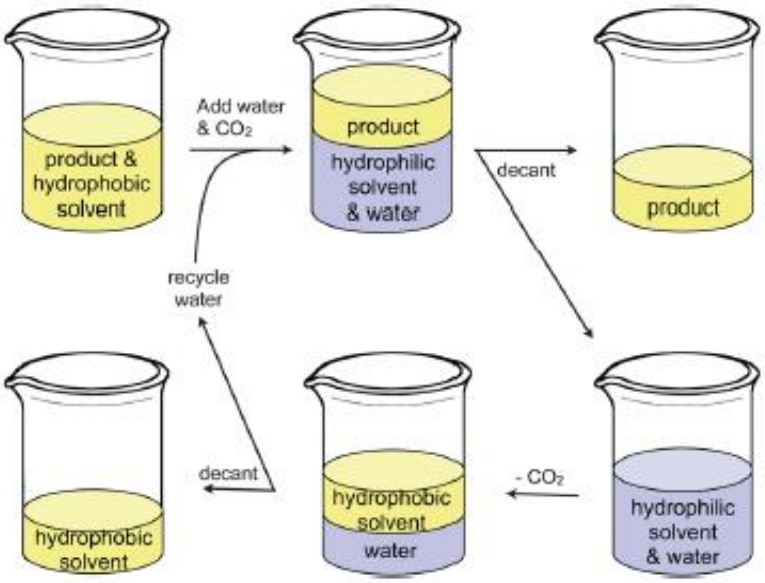

Figure 2.15 process by which a SHS can be separated from a product and recovered without the distillation operation

Identification and design of a SHS

As already mentioned in the previous paragraph the compounds that can show the commutable behaviour belong to the classes of amines, guanidines and amidines. This condition is necessary but not sufficient to define a SHS, therefore is mandatory to identify the parameters that make these solvents switchable. Several amines were tested, and it has been observed that the amines showing SHS behaviour are characterized by having an octanol-water partition coefficient ($\log K_{ow}$) between 1.2 and 2.5. Amines with a $\log K_{ow}$ coefficient lower than 1.2 are hydrophilic and form a homogeneous phase in contact with water. Amines with a $\log K_{ow}$ coefficient greater than 2.5 instead form biphasic mixtures even after the contact with CO_2. A second parameter is the pK_{aH}, which measures the strength of conjugated acid and which will generally be greater 9.5. The amine must have a sufficiently high basicity to be able to be protonated by the carbonic acid and to allow the change of hydrophilicity of the system but not extremely high, since the reaction of formation of the bicarbonate salt would be in fact irreversible. In Figure 2.16 and in Table 2.1 we can see the tested amines as a function of the $\log K_{ow}$ and pK_{aH} [Vanderveen et al. 2014].

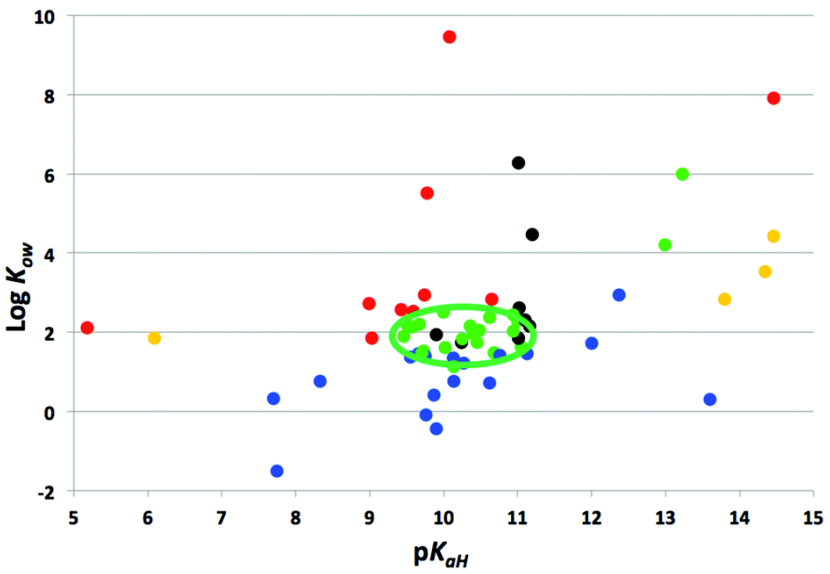

Figure 2.16 Components tested to verify SHS behaviour at room temperature as a function of pKah and logKow. The dots are coloured according to their behaviour: miscible in water (blue); biphasic (red); precipitation after CO_2 addition (black); SHS (green)

Behaviour	Compound	Ratio of compound to water (v : v)	Log K_{ow}	pK_{aH}
Monophasic	Triethanolamine	1 : 1	−1.51	7.85
Monophasic	*N*,*N*,*N*′,*N*′-Tetramethylethylenediamine	1 : 1	0.21	9.2
Monophasic	*N*,*N*,*N*′,*N*′-Tetramethylguanidine	2 : 1	0.30	13.6
Monophasic	*N*-Ethylmorpholine	1 : 1	0.30	7.70
Monophasic	1,8-Diazabicycloundec-7-ene	2 : 1	1.73	12
Monophasic	*N*-Hexyl-*N*′,*N*′-dimethylacetamidine	2 : 1	2.94	12
Irreversible	*N*″-Hexyl-*N*,*N*,*N*′,*N*′-tetramethylguanidine	2 : 1	2.82	13.6
Irreversible	*N*″-Butyl-*N*,*N*,*N*′,*N*′-tetraethylguanidine	2 : 1	3.52	13.6
Irreversible	*N*″-Hexyl-*N*,*N*,*N*′,*N*′-tetraethylguanidine	2 : 1	4.43	13.6
Switchable	Triethylamine	1 : 1	1.47	10.68
Switchable	*N*,*N*-Dimethylbutylamine	1 : 1	1.60	10.02
Switchable	*N*-Ethylpiperidine	1 : 1	1.75	10.45
Switchable	*N*-Methyldipropylamine	1 : 1	1.96	10.4
Switchable	*N*,*N*-Dimethylcyclohexylamine	1 : 1	2.04	10.48
Switchable	*N*-Butylpyrrolidine	1 : 1	2.15	10.36
Switchable	*N*,*N*-Diethylbutylamine	1 : 1	2.37	10.51
Switchable	*N*,*N*-Dimethylhexylamine	1 : 1	2.51	10.18
Switchable	*N*,*N*,*N*′-Tripropylbutanamidine	2 : 1	4.20	12
Switchable	*N*,*N*,*N*′-Tributylpentanamidine	2 : 1	5.99	12
Biphasic	*N*,*N*-Dimethylaniline	1 : 1	2.11	5.06
Biphasic	*N*,*N*-Diisopropylethylamine	1 : 1	2.28	11.0
Biphasic	Tripropylamine	1 : 1	2.83	10.70
Biphasic	*N*″-Hexyl-*N*,*N*,*N*′,*N*′-tetrabutylguanidine	2 : 1	7.91	13.6
Biphasic	Trioctylamine	1 : 1	9.45	10.9

Table 2.1 Behaviour of tested tertiary amines as function of amine : water ratio, Log K_{ow} and pK_{aH}

In the identification of optimal switchable hydrophilicity compounds, a design strategy was studied taking into account the volatility of the SHS candidate and the easiness of the switch and reswitch operation (reactivity). The choice of a SHS with very low volatility is dictated by the minimization of the vapours released into the atmosphere. Reducing volatility is possible by increasing the molecular weight in the design of the molecule, for example by increasing the length of the alkyl chains. However, a large increase in the length of the chains can cause an excessive rise in the logK_{ow} which leads to insufficient water solubility of the bicarbonate salt. Proposing molecules with medium alkyl chains, containing hydrophilic functional groups, can promote both the decrease of volatility and the logK_{ow} interval necessary for the switchable behaviour. The addition of hydrophilic functional groups on the alkyl chains linked to the central nitrogen of the amine group also influences the basicity of the amine itself, decreasing the alkalinity. The additional functional groups are: alcohols, esters, ketones and aromatic rings. A design strategy was also implemented in the identification of secondary amines from SHS behaviour. Secondary amines react more readily with CO_2 than tertiary amines, with the formation of ammonium carbamate. Systems consisting of secondary amines pass from a biphasic solution to a monophasic one after less than 10 minutes of bubbling with CO_2, unlike the phase changing of SHS consisting of tertiary amines, which lasts from 20 to 120 minutes [Vanderveen et al. 2014].

Although the increase in the reaction rate of a secondary amine is an attractive feature, the formation of the carbamate salt causes high energy costs for the regeneration phase. The strategy resides in designing secondary amines adding functional groups with a high steric bulk. The carbamates formed are unstable and are rapidly hydrolysed to bicarbonate. Carbamates are in fact formed as a kinetic product before being converted to bicarbonates, thus favouring a rapid absorption of CO_2 without great energy requirements for its subsequent removal.

Evaluation of the risks of SHS

For a potential industrial application, it is necessary to study the impact that SHS have on human and animal health and on the environment, preferably comparing them with that of traditional solvents. To evaluate these effects several factors are taking into account: lethal doses, boiling point temperature, flash point, eutrophication potential (EP) and the effect on the skin, comparing them with those of toluene and hexane. Here, to assess the toxicity of SHS, the Lethal Dose 50 (LD50) was used, which represents the amount of substance that, administrated to a sample of rats or guinea pigs, is able to kill 50% of the population. However, LD50 represents a measure of acute toxicity and solvents as agents of chronic toxicity have high LD50 such that they may appear safer than others. An example is the hexane (LD50 25g/kg) which is known as a chronic neurotoxin [Sendur et al. 2009, Ritchie et al. 2001].

Inhalation toxicity levels can be assessed with LC50 representing the concentration in the environment of a lethal substance for the 50% of the sample population. The triethylamine and the dipropylamine have respectively an LC50 of 4.1 g/m^3 and 4.4 g/m^3 [Lewis et al 1996], while the corresponding values for toluene and hexane are 30.1 g/m^3 and 169 g/m^3. Clearly low-volatility SHSs are difficult to inhale due to their low vapor pressure, which makes them less harmful than volatile solvents. For example, the vapor pressure of propyl-3-secbutylamine propanoate at 25 °C is about 13 Pa, while the vapor pressure of triethylamine, toluene and hexane are respectively 9670 Pa, 3804 Pa and 20240 Pa. It is clear that SHSs are less dangerous in terms of toxicity and therefore safer. The boiling point gives us an indication of solvent volatility as well as the flashpoint which also provides us further information on the potential flammability. It can be seen from Table that all SHS are safer in terms of flammability and volatility than hexane and toluene.

The effects on the skin is an additional factor to consider. Different SHS were classified and divided into classes, defined by the "Globally Harmonized System (GHS)", based on their level of corrosion. A corrosive substance belonging to class 1A shows effects after 3 minutes of exposure and less than one hour of observation. A corrosive substance of class 1B shows effects after one hour of exposure and less than 14 days of observation. Unfortunately, for some SHS the data related to their effect on the skin are not available [GHS 2011].

The persistence of a solvent in the environment is another problem to be taken into consideration. It is difficult to predict the degradation tendency of a substance as it can degrade in many ways. However, some behaviours are closely related to the chemical structure of the compound [Davis et a. 1981]. Quaternary chains, ramifications, heterocycles and tertiary amines tend to reduce degradability. Atoms of oxygen, in particular ester groups, alkyl chains not substituted with four or more carbon atoms, unsubstituted phenyl groups are structures which lead to an increase in degradability. SHS based on secondary amines are considered to be more biodegradable than those based on tertiary amines, tertiary amines are in fact poorly biodegradable. However, not all tertiary amines persist in the environment. The N, N-dimethylcyclohexylamine, however, is considered to be biodegradable in a watery environment despite its decomposition is not fast [IUCLID, 2000]. An ideal SHS based on a secondary amine should contain a branched group to destabilize the carbamate product, but branching contributes negatively to the biodegradability. Trialkylamines are more persistent than conventional solvents, whereas secondary and tertiary amines with a second functional group have a comparable or superior biodegradability compared to conventional solvents [Davis et al. 1981, Pasteris et al. 2002]. In Table below are therefore reported all the information related to the safety and environmental impact of some SHSs.

Substance	LD_{50} (oral, rat) (mg kg^{-1})	Boiling point (°C)	Flash point (°C)	Eutrophication potential[a]	Skin effects
N,*N*-Dimethylcyclohexylamine	348	159	43	*0.17*	Corrosive (1B)
N-Ethylpiperidine	280	128	17	*0.17*	Corrosive (1B)
N-Butylpyrrolidine	51	156	35	*0.17*	Irritant
N,*N*-Dimethylhexylamine	*500*	148	34	*0.17*	Corrosive (1B)
N,*N*-Dimethylbutylamine	188	95	−5	*0.18*	Corrosive (1A)
N,*N*-Diethylbutylamine	*300*	136	24	*0.17*	Corrosive (1B)
N-Methyldipropylamine	*267*	117	−3	*0.18*	Corrosive (1B)
Triethylamine	460	89	−9	*0.18*	Corrosive (1A)
N,*N*-Diisopropylaminoethanol	940	190	64	*0.15*	Corrosive (1B)
N,*N*-Dibutylaminoethanol	1076	230	95	*0.15*	Corrosive (1B)
N,*N*-Dimethylphenethylamine	*300*	210	71	*0.16*	Irritant
Dipropylamine	460	108	17	*0.18*	Corrosive (1A)
Di-*sec*-butylamine	*300*	135	21	*0.17*	Corrosive (1A)
N-Propyl-*sec*-butylamine	*300*	124	*15*	*0.18*	Corrosive (1B)
N,*N*-Dimethylbenzylamine	265	183	53	*0.16*	Corrosive (1B)
Toluene	636–6400	110	4	*0.13*	Irritant
Hexane	28710	69	−22	*0.15*	Irritant

Table 2.2 Properties of SHS related to safety, health and environmental impact [Vanderveen et al. 2014]

There are other considerations that can be decisive for choosing the most suitable SHS system to be used:

- some SHSs, such as for example the aminoacetals, the amino acids and the amidines can undergo hydrolysis, degrading over time (they cannot be re-used for several cycles).

- most SHSs work in a 1: 1 volume ratio with water, but some require more water to show "switchable" behaviour (2: 1, or even 5: 1). By increasing the amount of water, however, it is also necessary to increase the energy to heat the system during the phase of CO_2 removal.

- some SHSs transform faster than others; in particular, the secondary amines pass from a hydrophobic form to a hydrophilic one more rapidly than tertiary amines (as explained in the previous paragraphs). Because of these uncertainties it is not possible to determine unequivocally what is the most benign SHS system, therefore, depending on the type of application, it is necessary to evaluate the SHS with the best properties.

SHS as solvents for the extraction of biocompounds from biological matrices

The production of biofuels from microalgae is not yet considered economically feasible, due to the high energy costs associated with the extraction process. Extraction with organic solvents and supercritical extraction are the main alternatives of lipid extraction.

mainly proposed solvents are methanol / chloroform, hexane / isopropanol, hexane / ethanol, dichloromethane / ethanol. These blends have a synergistic effect. The alcohols have the ability to break the hydrogen bonds and the electrostatic forces between lipids and proteins

within the membranes, opening pores across the cell wall and making it accessible to the non-polar solvent, which is able to interact with the neutral lipids. Extractions with pure solvents such as ethanol, butanol or hexane have also been proposed.

Extraction with supercritical fluids is a valid alternative to the classic solvent extraction systems. It offers multiple advantages: it is free of toxicity, CO_2 is inert, not flammable and recyclable. At room temperature and atmospheric pressure CO_2 is a gas, which makes it easily separable from the solute once the extraction process is complete. The main disadvantage of CO_2 is its non-polar nature, for this reason, in some cases, small percentages of polar modifiers must be added, such as methanol and acetonitrile, to increase its solubilizing power.

In the figures below are shown process diagram. Figure 2.17 shows the scheme associated to extraction with organic solvent, in particular hexane, while Figure 2.18 represents an extraction made with supercritical CO_2.

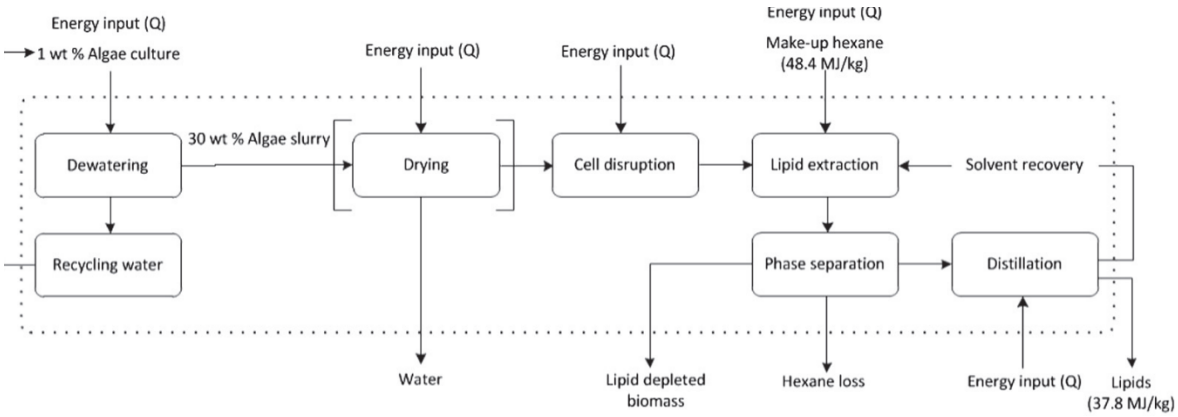

Figure 2.17 Process diagram for lipid extraction with organic solvent (hexane) [Du et al. 2015]

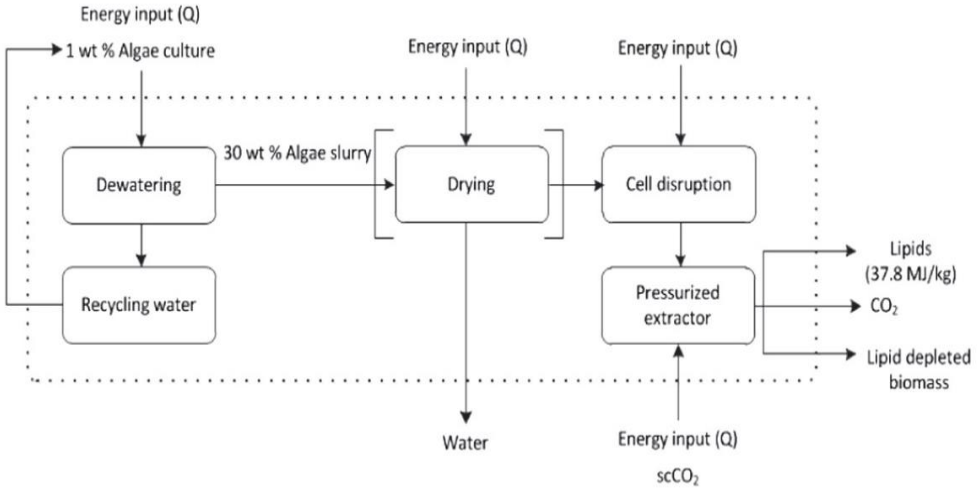

Figure 2.18 Process diagram for lipid extraction with $scCO_2$ [Du et al. 2015]

The higher energy costs are associated with two phases of the process:

• Preliminary drying of the biomass: Because of the immiscibility of the traditional organic solvents used to dissolve the lipids, a preliminary drying step of the biomass is necessary, which also acts in the breaking of the cell wall. The water contained in the wet algal sample, and around the algal cells, acts as a sort of barrier reducing the extraction capacity of the solvent. In terms of extraction yield it is preferable to carry out the extractions on the dried algal biomass. If the extraction is carried out directly on wet algal samples, the energy costs related to the drying of the biomass would be avoided, but it would be necessary to carry out a mechanical, chemical, or biological pre-treatment of cell wall destruction to improve extraction efficiency of the solvent.

• Separation of the solvent from the extracted lipid phase: An evaporation or distillation step is required since the recovery of the solvent is necessary.

The switchable hydrophilicity solvents are considered a possible alternative respect to the organic solvents, they act positively on both the critical aspects of the process. A possible process diagram is shown in Figure 2.19.

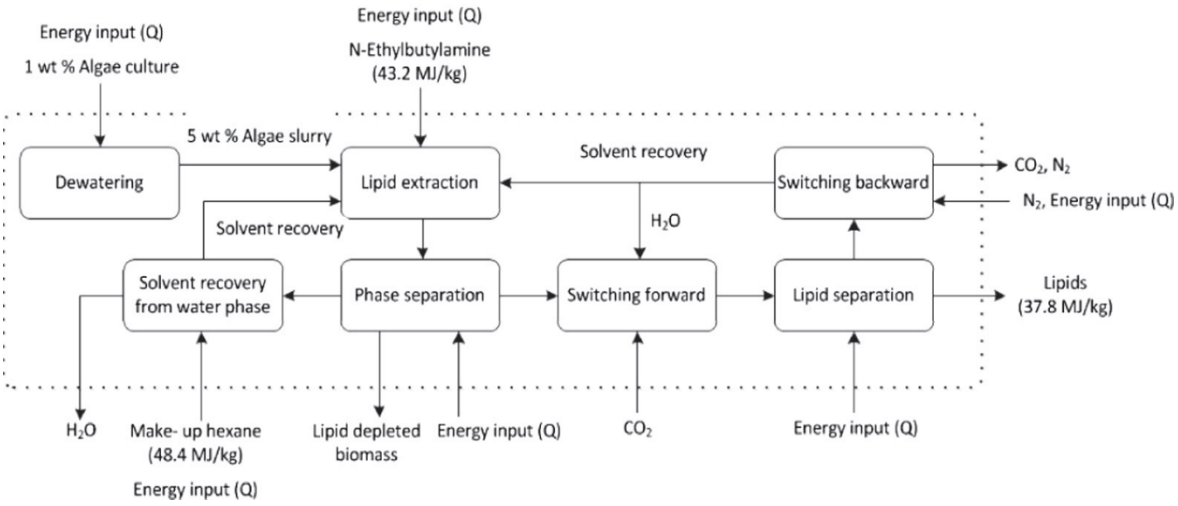

Figure 2.19 Process diagram for lipid extraction with Switchable Solvents [Du et al. 2015]

An estimate of the energy costs in the three types of extraction was made by Ying Du and co-workers, demonstrating that the extraction with SHS requires lower energy costs and therefore could represent the best choice. Figure 2.20 shows energy request for each of the extraction methods.

From the figure we can see how in dry extractions the main energy costs are inherent to the drying of the biomass while in wet extraction a greater energy use is required in the breaking

of the cell wall. In the process that involves SHS it can be seen that the main energy use is linked to solvent recovery. Part of the solvent remains in the aqueous phase and therefore a further recovery or treatment step is required which cannot be released into the environment. [Du et al. 2015].

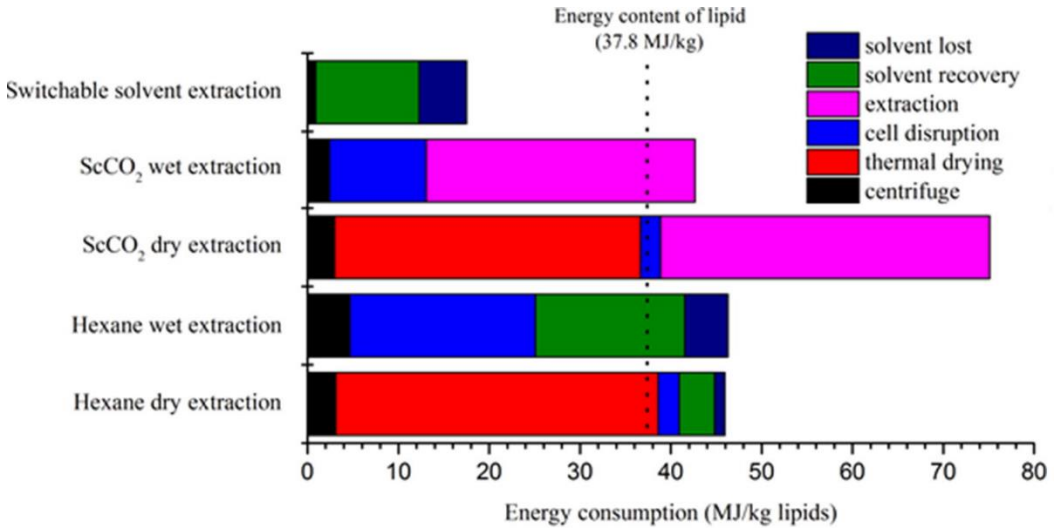

Figure 2.20 Energy request for lipid production with different extraction methods

2.2.4 Deep Eutectic Solvents (DESs)

Deep eutectic solvents, or DES, are also compounds with a ionic character which often exhibit low toxicity and high biodegradability. The concept of DES has been described for the first time by Abbot et al. in 2003. DESs are made up of a mixture of compounds organic (an acid and a Lewis or Brønsted base) which presents melting temperature significantly lower than that of the pure compounds (Figure 2.21) [Clouthier et al., 2012].

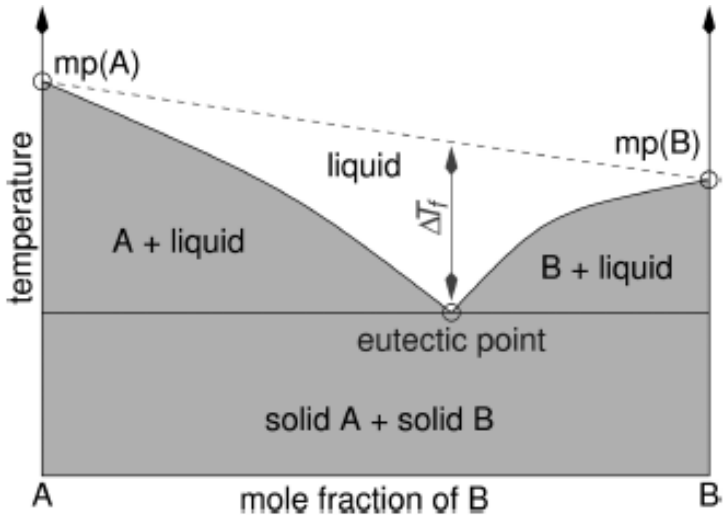

Figure 2.21 Generic phase diagram of an eutectic system

They can be easily synthesized by mixing the components in standard conditions. DESs are actually ionic liquids and possess physical properties comparable to ILs, but constitute a new class of solvents because their chemical properties are quite different from the commonly used ILs [Smith et al., 2014]. They are formed in a simple way and a great number of DESs has been studied, they are all united by some characteristics:

• low conductivity

• high viscosity

• low vapor pressure

• high thermal stability

Like ILs, DESs are generally less volatile than the classic organic solvents. Their low vapor pressure makes them not flammable and reduces the risk of inhalation. Their cost and their environmental friendliness make them theoretically more attractive than ionic liquids, whose biodegradability is often questioned. Since the discovery of DES in 2003 there has been a rapid development with over 300 publications between 2009 and 2013 [Tang et al. 2013]. As regards their technological use the knowledge of DESs is however still in its infancy. The lack of information and classifications of the their properties means that their use is not yet completely consolidated in many applications, for example in the electrodeposition of the metals, in the extraction of metal oxides, in the adsorption of gases, but in the pharmaceutical field or in the purification of biodiesel, they are already employed [Smith et al. 2014]. It has also been shown that some DESs can substitute analogous ILs, having similar properties but lower cost and less toxicity, as well as great simplicity of preparation [Zaijun et al. 2011].

Structure and behaviour of DES

Solvent synthesis

DESs are composed of large asymmetric ions with low reticular energy and consequently low melting points. They are generally composed of a supramolecular complex of ammonium quaternary salt or a hydrogen bonding acceptor (HBA) and a metal salt or a hydrogen bonding donor (HBD). Their structure resembles liquid crystals. Deep eutectic solvents can generally be described from the following formula: Cat^+X^-zY, in which Cat^+ is any cation (ammonium, phosphonium, sulfonium, etc.) and X is a Lewis base (usually a halide). Y is instead a Lewis or Brønsted acid forming an anionic complex, in ratio Y / X =

z, with X^-. Most of the studies focused on quaternary ammonium cations or imidazolium with a special emphasis on choline chloride (ChCl). Choline chloride is a salt similar to vitamin B, biodegradable and non-toxic. ChCl has a relatively low cost compared to other salts used. In the European Union it is classified as a provitamin (i.e. a substance capable of turning into a vitamin through the metabolic processes of an organism) and is produced on a large scale (Mt / year) as a supplement for feed. Typical compounds used to form DES are listed in Figure 2.22.

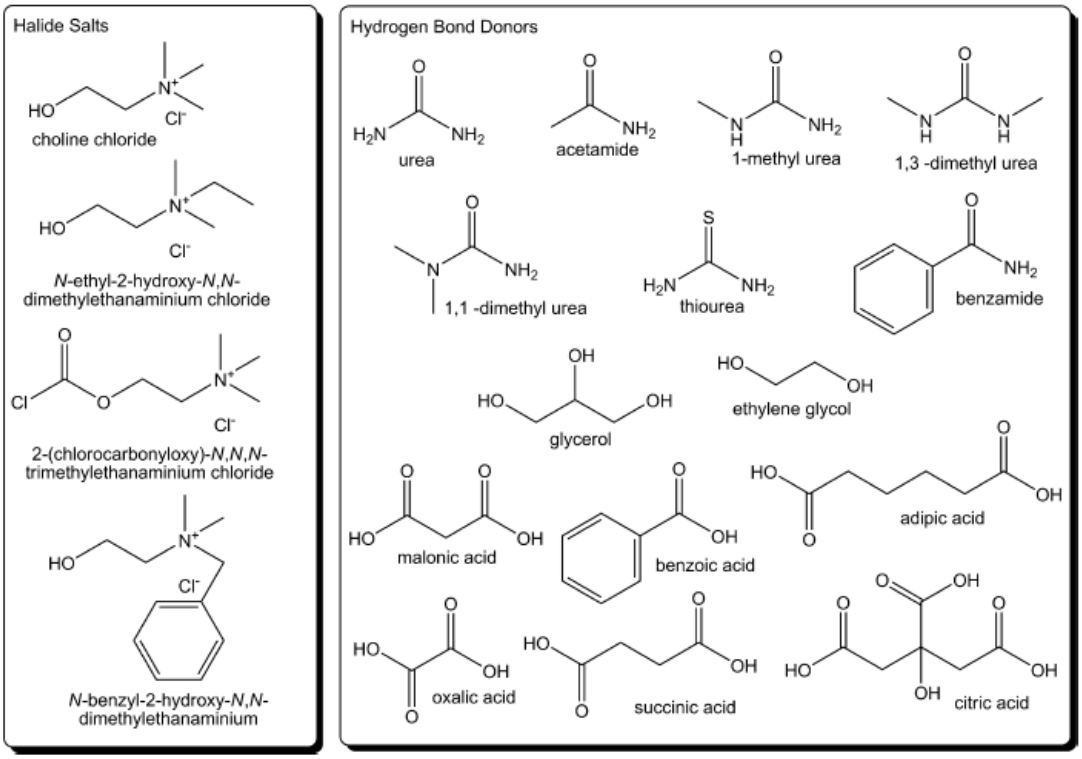

Figure 2.22 Principal organic molecules used as DES components [Smith et al. 2013].

As previously stated, the deep eutectic solvents and conventional ILs have similar physical properties. In particular, they are both designer solvents that can be customized to achieve the desired chemical behaviour. They have also a low vapor pressure, a large field of existence of the liquid phase and are not flammable. Furthermore, they are less toxic than ILs, it makes less problematic the transport of large quantity, allowing the use on industrial scale. However, DESs are often less inert chemically. The production of DESs takes place through the mixing of two compounds at a moderate temperature, generally involving a lower production costs compared to conventional Ils, allowing large-scale productions. While the toxicological properties of individual components forming the DES are very well known, there is very little information that concern the eutectic mixtures. The fact that individual components have very different behaviours than the mixture, demonstrates that

even at the toxicological level things can change. Hayyan et al. studied the toxicity and cytotoxicity of choline chloride with various HBDs such as glycerine, ethylene glycol and urea. They found that the cytotoxicity of DES is much higher than that of the individual components. They also demonstrated that toxicity and cytotoxicity vary with the structure of the mixture. Further studies will be necessary before the statement of non-toxicity and biodegradability of DESs. [Hayyan et al. 2013 a-b].

2.2.5 Natural Deep Eutectic Solvents (NaDESs)

"Natural Deep Eutectic Solvents" (NaDESs) are mixtures formed by natural primary metabolites present in all organisms, such as sugars, polyalcohol, amino acids, organic acids and choline derivatives, which have lower melting points than the individual components that constitute them. In particular, these primary metabolites, due to the formation of intermolecular hydrogen bonds, change their state from solid to liquid when mixed in a certain ratio, thus forming eutectic systems. Combining different natural metabolites, more than 100 combinations of NaDESs were found [Choi et al. 2011].

Five main groups can be distinguished: ionic liquids constituted by an acid and a base, NaDESs based on sugars with neutral compounds, NaDESs based on sugars with bases, NaDESs based on sugars with acids and NaDESs based on sugars with amino acids. Furthermore, different types of mixtures of sugars or organic acids can form liquids, such as the fructose-glucose-sucrose mixture or maleic-acidic acid; even other combinations of more than two components can form clear liquids, such as glucose-sorbitol-malic acid or choline-proline-malic chloride. NaDESs can extend the range of "green" solvents because they have different advantages compared to synthetic ionic liquids: low cost, biodegradability, non-toxicity, simple methods of separation. They also show excellent chemical-physical properties such as solvents: negligible volatility, liquid state even below 0 ° C, adjustable viscosity, high polarity range and high solubilizing capacity for a wide variety of compounds.

NaDESs in nature

NaDESs have also been observed in nature, in plant secretions and in conditions of extreme drought or cold. Hence it has been hypothesized that they perform important physiological functions as liquid medium alternative to water and lipids in cells and living organisms. For example, the NMR spectrum of flower nectar shows that it is composed primarily of sugars

that individually are solids at room temperature; however, the composition of the sugar mixture in this secretion makes it a liquid, the NaDES fructose-glucose-sucrose. The same is true for the components found in honey (composed of glucose and fructose). The components of NaDES have also been observed in plants of the *Selaginela* species (native of the Chihuahua desert, in Central America), in microorganisms such as lichens, and in barley seeds, which can survive for long periods without water [Dai et al. 2013].

Various studies have shown that the level of primary metabolites, with a lack of water, increases with respect to normal growth conditions even for normal plants: for example, the Arabidopsis species shows an increase in sugar levels (sucrose), amino acids (proline, alanine, arginine), organic acids (succinic acid, fumaric acid, malic acid) and amines (choline) in drought conditions. Cold resistance could also be related to the presence of NaDESs, as shown by cryoprotectants based on sugars, polyalcohols, proline (frequent ingredients) commonly used for plants. In addition, NaDESs show a high solubilizing ability for macromolecules such as gluten, starch and DNA, thus explaining the biosynthesis and storage of these compounds, poorly soluble in water, in organisms. NaDESs are able to solubilize also various poorly water-soluble secondary plant metabolites, such as flavonoids.

Structure of NaDES

NMR spectroscopy applied to NaDES in search of the causes that lead to the formation of eutectic, showed the existence of hydrogen bonds between the different components of NaDES. First of all, Abbott et al. observed, through the HOESY spectroscopy, a cross-correlation between the fluoride ion of choline fluoride and the protons of urea [Abbott et al. 2004]. The HOESY spectrum of NaDES 1,2 propanediol-choline-water chloride (PCH) detected a signal corresponding to the proton of the methyl group of 1,2 propanediol, which interacts with both the carbon of the methyl group and the methylene carbon associated with choline chloride nitrogen; while the NOESY spectrum showed a strong interaction between the protons of the choline chloride hydroxyl groups, of the 1,2 propanediol and of the water, thus demonstrating the presence of hydrogen bonds.

To evaluate the stability of a NaDES, the same mixture was prepared but in different molar ratios: in the case of the choline glucose-chloride mixture a 2:5 molar ratio makes the NaDES stable, while with molar ratios 2:1, 1:1, 1:4 a solid precipitate gradually appears, although it is still possible to prepare a transparent liquid. These observations have led to the conclusion that chloride ion of choline chloride can form two hydrogen bonds with two hydroxyl groups of sugars; the same behaviour is also observed in a mixture of choline chloride and a

carboxylic acid. It can be said that the formation of hydrogen bonds leads to the complexation of the starting solid components, producing a liquid with a supramolecular structure. NaDESs have structures that are very similar to that of liquid crystals, in which all the molecules are arranged in a matrix through intermolecular hydrogen bonds between the components.

The number of acceptors or donors groups of the hydrogen bond significantly influences the formation and stability of NaDESs: for example, in the case of organic acids, succinic acid does not form a liquid with choline salts, unlike it happens with malic acid, citric acid, tartaric acid; in the case of organic acid-sugar combinations, the acids having more carboxylic groups, such as citric acid, can form stable liquids with a greater variety of sugars, respect to those with few carboxyl groups, such as malic acid. Considering the structure of these acids, it can be stated that the presence of more hydroxyl/carboxylic groups in the acid allows the formation of more hydrogen bonds, thus leading to an increase in the stability of the liquids.

The spatial arrangement of hydroxyl groups also has a high influence on the formation and stability of hydrogen bonds. For example, the liquid formed by galactose and choline chloride is not stable and precipitates, while that formed from choline chloride and glucose (which has the same number of hydroxyl groups as galactose) is a stable liquid. This phenomenon has also been observed with other sugars and polyalcohol, such as sorbitol which can form a stable liquid with choline chloride, the same cannot be said for mannitol.

NaDES diluted with water

The physical properties and the solubilizing capacity of NaDES can be changed by adding small amounts of water. Considering that an interesting application of NaDES is their potential use as solvents, the addition of small amounts of water (generally between 5-10% by weight) reduces the temperature at which the liquid is formed, thus making the NaDES exploitable as a means of extraction/reaction at room temperature or even lower [Widegren et al. 2005, Kodderman et al. 2006, Najdanovic-Visak et al. 2003]. However, an excessive dilution of NaDES involves the loss of existing hydrogen bonds, and consequently, the disappearance of the particular NaDES structure [Gutiérrezet al. 2009]. The table shows several NaDESs diluted with water, listing for each of them, in addition to some properties (density, viscosity, polarity, etc.), the molar ratio of the water compatible with the stability of liquid NaDES at room temperature.

The addition of water to the NaDES also entails other effects such as the reduction of the preparation time and temperature, and the reduction of their viscosity. The main advantage of diluted NaDES is that their physical properties, such as the solubilizing capacity, can be regulated by varying the water content (Table 2.3).

name	composition (mole ratio)	water (wt %)	water activity (40 °C)	density(40 °C) g/cm^3	viscosity (40 °C) mm^2/s	T_d[a] / °C	T_g[b] / °C	E_{NR}[c] (kca/ mol)
MCH	malic acid:choline chloride:water(1:1:2)	11.6%	0.195	1.2303	445.9	201	-71.32	44.81
GlyCH	glycerol:choline chloride:water(2:1:1)	5.26%	0.126	1.1742	51.3	187	-101.6	49.55
FCH	fructose: choline chloride: water (2:5:5)	7.84%	0.151	1.2078	280.8	160	-84.58	49.81
XCH	xylose: choline chloride: water (1:2:2)	7.74%	0.141	1.2095	308.3	178	-81.8	49.81
SCH	sucrose: choline chloride: water (1:4:4)	7.40%	0.182	1.2269	581	>200	-82.96	49.72
FGSH	fructose:glucose:sucrose : water (1:1:1:11)	22.0%	0.662	1.3657	720	138	-50.77	48.21
GCH	glucose: choline chloride: water (2:5:5)	7.84%	0.162	1.2069	397.4	170	-83.86	49.72
PCH	1,2-propanediol: choline chloride: water (1:1:1)	7.70%	0.242	1.0833	33	162	-109.55	50.07
LGH	lactic acid:glucose: water (5:1:3)	7.89%	0.496	1.2497	37	135	-77.06	44.81
SoCH	sorbitol: choline chloride: water (2:5:6)	9.23%	0.12	1.1854	138.4	>200	-89.62	49.98
XoCH	xylitol:choline chloride: water (1:2:3)	11.2%	0.116	1.17841	86.1	>200	-93.33	49.72
H_2O	water	100%	1	0.992	≈1	-	-	48.21
MeOH	methanol	-	-	0.791	-	-	-	51.89

[a] decomposition temperature; [b] glass transition temperature; [c] $E_{NR} = hcN_A/\lambda_{max} = 28591/\lambda_{max}$.

Table 2.3 Physical-chemical characteristics of some NaDES [Dai et al. 2013].

Structure of NaDES diluted with water

The results of the research conducted by Y. Dai et al. have confirmed the existence of hydrogen bonds between the hydroxyl groups of 1,2-propanediol and chloride ion of choline chloride and between all the hydroxyl groups of 1,2-propanediol, choline chloride and water [Dai et al. 2013]. This example therefore suggests that water participates in the supramolecular structure of NaDES. However, increasing the amount of water in the NaDES, the relative number of protons detected on the hydroxyl groups (relative to the protons on the methyl group) decreases during dilution, implying the breaking of hydrogen bonds between 1,2-propanediol and choline chloride. The analysis of the spectra obtained by increasing the degree of dilution of NaDES, showed that the complex super-molecular interactions of PCH are preserved up to a dilution of 50% (by weight); a further increase in

the quantity of water inside the NaDES involves the hydration of each component and, therefore, the formation of a solution of the free forms of individual components in water [Gutiérrez et al. 2010].

Properties of NaDES diluted with water

Some important physical properties of NaDES diluted with water have been reported. NaDESs have a high decomposition temperature (Td), between 170-200 °C, with the exception of LGH and FGSH (based on sugars) which decompose at a lower temperature, respectively at 135 °C and 138 °C [Dai et al. 2013].

All NaDESs have glass transition points (Tg) lower than -50 °C, but do not have melting points, which confirms that these are supermolecular complexes, with a stable liquid state over a wide temperature range. From these two considerations it is possible to conclude that NaDESs can be used as solvents in a range of at least between -20 / 100 °C, without incurring thermal decomposition or phase change problems. The density of NaDES is higher than that of water and decreases linearly as the degree of dilution increases.

The high viscosity of NaDESs, due to the presence of an extensive network of hydrogen bonds between the components, represents a major obstacle for their application as a means of extraction. However, through the dilution with water the interactions between the components weaken (as explained in the previous paragraph) and consequently the viscosity of the NaDESs is reduced. The viscosity is, therefore, strongly influenced by the water content present in the system: for example, if 5% and 10% (v/v) of water are added in the GCH, the viscosity is reduced respectively to 132 mm^2/s and to 39.74 mm^2/s. When the total water content in the GCH is equal to 25% (v/v), it is possible to obtain a high viscosity decrease, up to about 7.2 mm^2/s (approaching the water value), while preserving the supermolecular structure typical of NADES. Clearly, the viscosity of a NaDES is also influenced by the temperature, it decreases as the latter increases.

Polarity is another important property of NaDESs, since it affects their solubilizing capacity. NaDESs based on organic acids (such as MCH, LGH) are the most polar (44.81 kcal/mole) followed by those based on amino acids and pure sugars (such as PMH, FGSH) that have a polarity similar to the water (48.21 kcal/mole). NaDESs based on sugars and/or polyalcohol (such as PCH, GCH) are the least polar, with values close to those of methanol (51.89 kcal/mole). On the polarity scale of figure 2. 23 (referring to the λmax of the solvatochromic dye Nile Red dissolved in the solvent), the polarity values of some NaDESs are reported, in comparison with the polarities of the SHS/SPS systems.

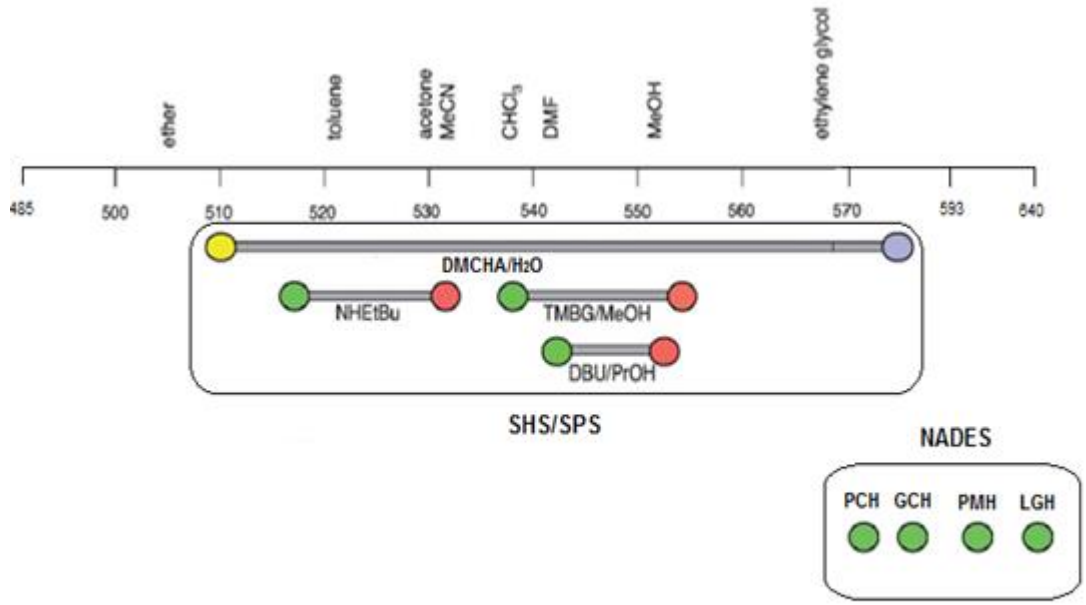

Figure 2.23 comparison of the polarities of four NaDES in their native form respect to SHS and SPS solvents.

Like viscosity, even polarity is significantly influenced by the addition of water. Table 2.4 shows how the polarity of PCH and LGH varies with water content changes. In particular, it can be observed how the polarity of PCH remains constant when the water content is between 0% and 25%, increases considerably with 50% of dilution and finally reaches a polarity similar to that of water with a degree of dilution of 75%. On the contrary, as the degree of dilution increases, the polarity of LGH is reduced because it has a polarity higher than that of water: up to 10% of dilution there is no change in polarity, from 15% to 50% the polarity decreases and finally at 75% the polarity stabilizes at a value similar to that of water.

	E_{NR} (kcal/mol)	
% of added water (v/v)	**PCH**	**LGH**
0	50.07	44.81
5	50.16	44.81
10	49.90	44.81
15	49.64	44.81
25	49.13	44.88
50	48.38	47.97
75	48.38	48.13

Table 2.4 Polarity behaviour of PCH and LGH as function of their water content

NaDES polarity tests were performed using the "Nile Red" solvatochromic dye. The value of λmax, determined with a UV / Vis spectrophotometer, is inserted in the following formula to determine the E_{NR} polarity scale:

$$E_{NR}\ (\text{kcal/mole}) = h\ c\ N_A/\lambda_{max} = 28591/\lambda_{max}$$

Where:

h = Planck's constant (J·s)

c = light speed (m/s)

N_A = Avogadro's number (mol^{-1})

λ_{max} = wavelenght of the absorbance peak (nm)

Table 2.5 shows the electrical conductivity of NaDES diluted with water. Large differences in conductivity between NaDES can be observed: GlyCH (glycerol: choline chloride: water 2:1:1) has the highest conductivity value (13.75 mS / cm), whereas FGSH (fructose: glucose: sucrose: water 1:1:1:11) has the lowest conductivity value (0.001 mS / cm) similar to water (0.002 mS / cm) and methanol (0.004 mS / cm). In this perspective, NaDES composed of sugars can be considered practically neutral. The conductivity decreases in the following order: base-polyalcohol > base-organic acid ≈ base-sugar > organic-amino acid > organic-sugar acid > sugar-sugar.

Composition	NaDES	Conductivity
Fructose:glucose:sucrose:water (1:1:1:11)	FGSH	0.0012
Lactic acid:glucose:water (5:1:3)	LGH	0.114
Proline:malic acid:water (1:1:3)	PMH	1.06
Fructose:choline chloride:water (2:5:5)	FCH	3.1
Sorbitol:choline chloride:water (2:5:6)	SoCH	6.77
Glucose:choline chloride:water (2:5:5)	GCH	6.81
1,2 propanediol:choline chloride:water (1:1:1)	PCH	12.09
Water		0.0021
Methanol		0.0042

Table 2.5 Formulation and conductivity of different NaDES compared with water and methanol

The water content has effects on the conductivity of the NaDES: initially the conductivity increases as the amount of water increases, and then decreases after reaching a peak value of about 10-100 times superior to that of a pure NaDES (Figure 2.24). SuCH and GCH

assume the highest value of conductivity when the water content is 60% by weight, while the LGH reaches its value at 80% of water. Thus, in the same way of viscosity and polarity, conductivity can be modified by changing the water content of NaDES.

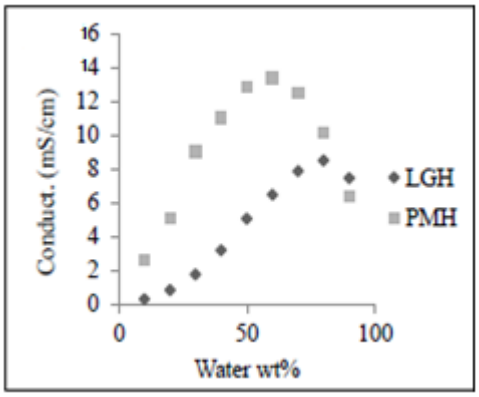

Figure 2.24 Conductivity of LGH and PMH as function of their water content

NADES diluted with water: tunable natural solvents

The most interesting application of NaDES is their use as a means of extraction. Moreover, the possibility of adjusting the properties of NaDES, quantitatively, by adding water makes them suitable to the extraction of different compounds. In particular, Dai's group studied the ability of some NaDES diluted with water (LGH, GCH, PCH) to solubilize some natural, poorly water-soluble products such as rutin, quercetin, cinnamic acid, cartamin and taxol [Dai et al. 2013]. Research results show that the solubility of many of the compounds tested is higher in PCH, which is reasonable, considering that PCH is the least polar NaDES. It is interesting to note that the solubility of these compounds in NaDES has increased from 18 to 460000 times compared to that in water.

The solubility in NaDES of some macromolecules present in cells, such as DNA, gluten and starch, has also been studied: starch is more soluble in GCH, forming solutions that are clear at room temperature. In contrast, gluten and DNA are more soluble in LGH, according to their polarity values. The solubility of the DNA and gluten in the LGH increases respectively by 34 and 101 compared to that in water. The next step was to analyse the influence of water content on the solubilizing capacity of the above NaDES.

The dilution with water of PCH involves an increase in its polarity and, consequently, a decrease in its ability to solubilize non-polar compounds. For example, the solubility of quercetin (a widely used flavonoid in plants) in PCH decreases slightly at first, from 5% to 10% (v/v) of water, and then drastically decreases when a higher quantity of water is added at 10%. On the contrary, the trend of the solubility of cartamine (a natural dye extracted from

safflower flowers, with antioxidant properties) in PCH, according to the percentage of water present in it, presents a maximum at 5%, probably due to the medium polarity of cartamine.

The optimal water content depends on the compound that is to be solubilized and therefore it represents an important factor for the optimization of a NaDES. In fact, as said above, quercetin presents the greatest solubility in pure PCH while the cartamine in GCH has a water content of 10% (v/v). This is due to the different polarity of the two compounds: the non-polar compounds have the highest solubility in pure NaDES, while the dilution with 5% -10% of water can increase the solubility of medium polarity compounds.

The optimal water content does not depend only on the polarity of the solutes but is also related to the viscosity of the NaDES. For example, cartamine has the highest solubility in GCH with 10% (v/v) of water, while it shows the maximum solubility in PCH with 5% (v/v) of water. Despite the polarity of the GCH is higher than that of the PCH, to achieve the maximum solubility of the cartamine, it is therefore necessary to add more water in the GCH than in the PCH. This phenomenon is due to the higher viscosity of the GCH, which forms a strong structure like a liquid crystal, where there is no space to dissolve the various solutes; however, by increasing the water content, it is possible to weaken its highly bound structure and thus obtain a greater solubilizing capacity.

Possible uses of NaDESs

The NaDESs fully represent the principles of green chemistry, for this reason their use in many sectors is quite tempting. In the last years several applications have been suggested for these solvents [Dai et al. 2013, Paiva et al. 2014].

- Biocatalysis: despite many NaDESs contain degrading agents such as citric acid and urea, some enzymes such as the antarctic white lipase B (CALB), show high activity and stability in choline chloride-based NaDESs [Durand et al. 2013]. These enzymes have been used in the transesterification of ethylvalerate with 1-butanol. Using NaDES such as ChCl / Urea as cosolvent, CALB shows a lot of stability greater than the use of an aqueous solution [Gorke et al. 2010]. NaDESs based on glycerol could be a valid alternative in the biocatalytic biodiesel production. Lipases are widely used in the enzymatic production of biodiesel and, in 2013, Zhao et al. have studied the use of a NaDES ChCl / Glycerol in this process. CALB maintain a high biocatalytic activity in the aforementioned NaDES, allowing to obtain a high yield in enzymatic trans-esterification of triglycerides with ethanol in this type of solvent. It can be

assumed that their use in biocatalysis will increase considerably especially in the pharmaceutical, nutraceutical and cosmetic field [Zhao et al. 2013].

- Extraction: the extraction capacity of a compound depends on its own solubilizing properties. NaDES, like all DESs, have the ability to accept and yield protons and electrons. They are therefore able to create hydrogen bonds which increase their solubilizing attitude [Zhang et al.,2012]. Choi's group studied the extraction of phenolic compounds from safflower using various NaDES: lactic acid - glucose, glucose - ChCl, fructose - glucose -sucrose. They found that NaDESs warrant a high level of extraction towards the phenolic compounds, related to the interactions of the hydrogen bonds between the molecules and the solvent [Dai et al. 2013]. Optimizing parameters of the NaDES such as viscosity, polarity and temperature as described previously, Dai et al. found higher yields in extraction with NaDES compared to those obtained with conventional solvents such as water or ethanol. Furthermore Morrison et al. in 2009 have shown that capacity extraction of NaDES (ChCl-Urea, ChCl-maloic acid) to poorly soluble compounds such as benzoic acid, griseofulvin, danazol, itraconazole (all bioactive molecules used in pharmaceuticals) is from 5 to 22,000 times greater than water [Morrison et al. 2009]. Since NaDESs are environmentally friendly and safer alternatives than many other solvents, their good properties such as being liquid at room temperature and having adjustable characteristics, promote their use in the extraction of natural products for various applications.
- CO_2 capture: one of the biggest drawbacks in the processes of chemical capture of CO_2 with the use of aqueous amine solutions is the inevitable humidification of the gaseous stream. Given their low vapor pressure and their high affinity towards carbon dioxide, ionic liquids can be employed as substitutes in the transaction. However, their costs limit the economy of the process. Just like ILs, NaDESs have a negligible vapor pressure but they are cheaper. Plus, their biodegradability makes their disposal easier and cheaper. NaDESs based on amines could be excellent candidates for capture of atmospheric CO_2, leading to cheaper processes than those currently in use.
- Biomedical applications: because of their versatility, non-toxicity and biodegradability, some NaDESs have already found employment in the biomedical sector. It is claimed that NaDES can dissolve drugs increasing their solubility, permeation and absorption, thus warranting the TheDES (Therapeutic DES) acronym. Tuntarawongsa et al. studied the preparation of NaDES as therapeutic

compounds that solubilize ibuprofen. The reported amount of soluble ibuprofen is greater respect the concentration that can be solubilized in water. [Tuntarawongsa et al. 2012]. The combination of NaDES and bioactive molecules such as menthol, ibuprofen or mandelic acid is definitely an interesting alternative for the production of drugs conveyor systems or other biomedical applications.

2.2.6 Hydrophobic NADES

Eutectic Solvents from natural resources that have been proposed in the literature are almost hydrophilic or very hydrophilic. NADES with a certain hydrophobic character were missing, until their development in 2015 by Marrucho's group. These eutectic mixtures were based on DL-Menthol and natural occurring acids such as acetic acid, lauric acid, pyruvic acid and L-lactic acid. These NADES showed a low viscosity, with values lower than hydrophilic NADES. For example DL-menthol : acetic acid mixture at 40 ° C showed a viscosity of 4.6 mPa·s, lower than the one of PCH at the same temperature (35.8 mPa·s). The hydrophobicity of this solvent compared to the classic NADES based on Choline chloride is also confirmed by the amount of water contained in the matrix of this NaDESs, which varies from a minimum of 0.4% to a maximum of 1,567% by weight according to the authors. These solvents were employed for the extraction of four different biomolecules such as caffeine, tryptophan, vanillin and isophtalic acid from aqueous solutions [Ribeiro et al. 2015]. Unfortunately, only one of these mixtures (DL-menthol : Lauric acid) proved to be stable in contact with water, as stated by the authors in a subsequent article [Florindo et al. 2017]. In the same work, they presented new solvents based on menthol and saturated fatty acids, such as octanoic acid and decanoic acid, which have proved to be stable in contact with aqueous phases. These NADES are able to remove pesticides from aqueous environments, but their extraction capability decreases over cycles (from 75% to 33% at fourth cycle. In 2018 the same research group proposed innovative binary mixtures based only on saturated fatty acids (dodecanoic, decanoic, nonanoic and octanoic acid). Here, the dodecanoic acid acts as an acceptor of electrons (HBA) and the others as donors (HBD). Researchers once again focused on the extraction of pollutants from the aqueous solutions obtaining extraction efficiencies ranging from 76% to 88% for the Bisphenol A [Florindo et al. 2018].

Contrary to hydrophilic NaDESs, these solvents cannot change their polarity, since a change in the molar ratios of the components causes the failure in the formation of the eutectic. These hydrophobic and low viscosity NaDES could carve out an important role in various

sectors due to the low cost of their components and their complete eco-compatibility. At the moment these mixtures have been used only for the removal of harmful components from the aqueous environments, therefore their potentiality in extracting interesting components from biomass is not yet known. The extraction efficiency of hydrophilic and hydrophobic NaDESs on microalgal biomolecules will be discussed in Chapters 4 and 5.

Aim of the Thesis

The aim of this thesis is to biorefine microalgal biomass through innovative "green" solvents and methods that allow greater efficiency and recovery of commercial biomolecules. For this reason, in Chapter 3 Switchable Hydrophilicity Solvents, solvents with the ability to switch their behaviour from hydrophilic to hydrophobic, have been tested using the "Circular Extraction" method on a synthetic model matrix and on the microalgal strain *Scenedesmus dimorphus*. In Chapter 4 the tunable hydrophilic Natural Deep Eutectic Solvents, whose polarity and viscosity can be modified according to their formulation, were tested on the biomass. In Chapter 5 the idea of solvent commutability and the bio-friendly characteristics of the natural solvents were merged to create the first switchable NaDES, whose performance in the extraction of model and microalgae compounds proved to be very promising if coupled with the circular extraction protocol.

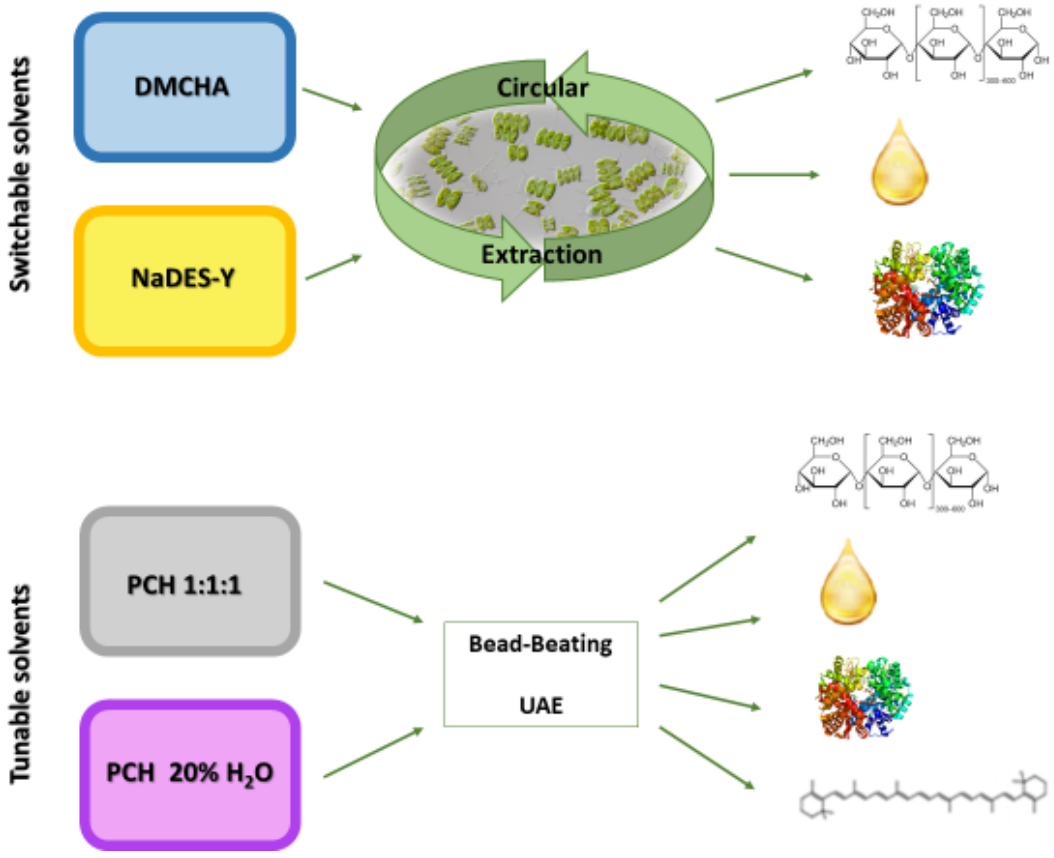

Figure 2.25 Graphical abstract of the Thesis

Chapter 3. Circular Extraction: an innovative use of switchable solvents for the biomass biorefinery

3.1 DMCHA Circular Extraction application on *S. dimorphus* and model matrix

The environmental impact of biomass processing can best be minimized by utilizing many components of the biomass and by getting maximum utility out of any solvent used. A Switchable-Hydrophilicity Solvent, the N,N-dimethylcyclohexylamine, has been used for a complete extraction of hydrophobic and hydrophilic molecules in the solvent's opposite-hydrophilicity states, thus simplifying microalgal biomass extraction and increasing the solvent usefulness. The N,N-dimethylcyclohexylamine, is a tertiary amine which at room temperature is a colourless liquid with a characteristic odour whose molecular structure is reported in Figure 3.1.

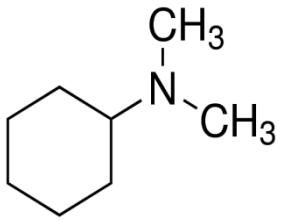

Figure 3.1. N,N-Dimethylcyclohexylamine structure

It is one of those tertiary amines that falls within the category of solvents with reversible behaviour characterized by a pKa_H equal to 10.48 and by a $logK_{ow}$ equal to 2.04. At room temperature it presents a very low solubility in water, about 10 g / L, but in presence of water and carbon dioxide bubbling it change its nature and from a purely hydrophobic solvent we obtain a hydrophilic solvent consisting of bicarbonate salt of the amine. As demonstrated by literature, it is a solvent with considerable extraction capacity. In particular in the field of extraction of components from algal matrices, it has been seen its ability to extract biomolecules from moist samples without the pre-treatment step which leads to the breaking of the cell wall. Its behaviour as SHS also allows the recovery of the solvent through change of hydrophilicity. It has a boiling point between 162-165 ° C and an ignition temperature of 43 ° C. However, it is a toxic compound due to inhalation and skin contact, it is corrosive and can cause eye damage and skin burns. It is harmful both for the environment and for humans, with an LD50 of 348 mg kg^{-1}. It is highly toxic to aquatic organisms and poorly

biodegradable in water, for this reason, the aqueous residues must be treated appropriately. It can react violently with oxidising materials and is dangerous if exposed to heat as it is flammable. Despite present interesting features at the process level, for its use it is necessary to adopt security measures.

In the decade of the fastest expansion of renewable energy sources ever, microalgae and cyanobacteria, which are among the fastest-growing photosynthetic biomasses on Earth, are still confined to the specialty food market. [Draaisma et al. 2013, Spolaore et al. 2006].
Downstream processing of microalgae can either proceed through thermal treatment of the whole microalgal suspension (through which bioliquids or intermediates are produced but the original biomass components are mostly destroyed) or by extracting the valuable compounds (lipids, carbohydrates, pigments, etc.) [Chiaramonti et al. 2017, Molina Grima et al. 2003]. While the former approach may permit producing a drop in feedstock in biofuel production, many useful molecules that can be deployed in high added value markets are destroyed [Kim et al. 2013]. This not only represents waste from an environmental perspective but also from a financial perspective. Besides increasing the efficiency of raw biomass production, and thus lowering its production cost, the evaluation and optimization of biorefining costs are just as important [Moroni et al. 2014, Cicci et al. 2014]. Compared to the level of optimization in oil refineries, biorefining is still a clumsy set of operations [Di Paola et al. 2015].
Switchable hydrophilicity solvents (SHSs) are a new class of solvents that are able to change their nature, from hydrophobic to hydrophilic and vice versa [Jessop et al. 2010, Vanderveen et al. 2014]. This ability may be used to improve existing liquid–solid and liquid–liquid extraction unit operations [Holland et al. 2012]. Their use has the power to green biorefinery operations because biomass processing can be performed from the wet state, thus resulting in a significant simplification of the required pre-treatment steps and reduction of the relevant energy requirement. In the case of lipid extraction from microalgae in (concentrated) aqueous suspensions, the high affinity of the switchable solvent system (SSS) towards non-polar compounds is exploited to extract oil from algae. Afterwards, switching the SHS to its hydrophilic form by contact with CO_2 makes it possible to recover oil from the SSS after the induced solvent–lipid phase separation. The SSS cycle is completed by transforming the SSS to the non-polar form again, by removing the CO_2 by stripping with N_2, with- or without applied heating [Du et al. 2015]. In microalgae processing, these solvents have been applied essentially in the extraction of lipids from freeze-dried microalgae samples and from wet, undisrupted biomass. After the lipid extraction, solvent

recovery is normally accomplished by switching the solvent hydrophilicity with CO_2, which induces phase splitting [Boyd et al. 2012, Samorì et al. 2013]. As mentioned above, switchable hydrophilicity solvents have only been used in their hydrophobic form to extract hydrophobic solutes. However, the treated biomass might also contain hydrophilic solutes, as well as debris that might not be soluble in either hydrophobic or hydrophilic solvents. The hydrophilic form of the SHS, obtained after switching, may thus be suitable for extraction of hydrophilic material from raw biomass or separation of the hydrophilic material from the solid residue, obtained after biomass has undergone extraction by the SHS in the hydrophobic form. To the best of our knowledge, this dual use of SHSs has never been proposed before and it is original both in the concept and in the experimental approach; it increases the utility of the extraction process and of the solvent itself. Indeed, during the regeneration stage, the solvent duplicates as a second solvent and performs a second, complementary biomass extraction task, which usual solvents cannot perform. Thus, any environmental impacts associated with the solvent are not only amortized over several cycles due to the repeated recycling of the solvent but are now amortized over two extractions per cycle. The SHS N,N-dimethylcyclohexylamine has been tested on a model matrix and on the oleaginous microalga *Scenedesmus dimorphus* through two different systems, where the first contact of the biomass with the solvent was alternately in a hydrophobic or hydrophilic state, followed by a second contact in the opposite state.

3.1.1 Materials and Methods

Scenedesmus dimorphus (UTEX 1237) was obtained from the Culture Collection of Algae at the University of Texas at Austin, USA. The strain on agar was inoculated into 3NB culture medium that in a litre contains: $CaCl_2$ 0.17 mmol, $NaNO_3$ 8.82 mmol, $MgSO_4 \cdot 7H_2O$ 0.3 mmol, K_2HPO_4 0.43 mmol, KH_2PO_4 1.29 mmol, NaCl 0.43 mmol, $Na_2EDTA \cdot 2H_2O$ 2 mmol, $FeCl_3 \cdot 6H_2O$ 0.36 mmol, $MnCl_2 \cdot 4H_2O$ 0.21 mmol, $ZnCl_2$ 0.037 mmol, $CoCl_2 \cdot 6H_2O$ 0.0084 mmol and $Na_2MoO_4 \cdot 2H_2O$ 0.017 mmol. Cultures were grown in 400 mL cylindrical glass tubes, with a diameter of 6 cm, fed with filtered and humidified air; flow rate was $130*10^3$ Nm^3/h. Photoperiod of light provided by cold white fluorescent lamps (400-700 nm, 865 K, 32 W, 80 mmol photons m^2 s^{-1}) 16 h followed by a period of darkness equal to 8 h. The temperature was maintained constant at 28 ±1 °C. The biomass was harvested every 8-10 days when it reached the stationary phase of growth. The *S. dimorphus* cell density was inferred from the absorbance measured at a wavelength of 690 nm (A690). In the stationary

phase, when cells were harvested, A690 was 5.5, corresponding to a wet weight of 8 g L^{-1}, and a dry weight of 3.1 g L^{-1}. A 50 mL aliquot of culture suspension was centrifuged; settled cells were separated from the supernatant and washed 3 times by resuspending them in 50 mL of distilled water, repeating centrifugation and discarding the supernatant. 30 mL of DMCHA were added to the biomass sample in an Erlenmeyer's flask, obtaining a liquid to solid weight ratio equal to 166 : 1 so as to assess the solvent's capability to exhaust the algal matrix without solvent saturation concerns. The suspension was then agitated for 24 hours in an orbital shaker. Subsequently, the suspension was centrifuged to separate the undissolved biomass residue from the extract. The extract was supplemented with the same volume of distilled H_2O and blown with CO_2 to cause the switch of DMCHA to its hydrophilic form according to the reaction:

$$NR_3 + H_2O + CO_2 \rightleftarrows NR_3H^+ + HCO_3^-$$

During the switching process, two further phases appear: the hydrophilic phase and the oily phase originated from the switched solvent. Upon completion of the solvent switchover, the original hydrophobic phase disappears and only the hydrophilic solvent and the extracted oily phase remain. In our bench-scale set-up, the hydrophobic-to-hydrophilic switch takes 20 minutes (CO_2 being sparged by a microsparger), while the hydrophilic-to-hydrophobic switch takes 30 minutes (stripping being promoted by heating). Both switching times could be easily reduced in an industrial setting by recycling CO_2, by improving mixing, by sparging N_2 and by suitable equipment design choices. The hydrophilic solvent and the oily phase (quantified gravimetrically) were separately removed from the vessel. The undissolved microalgal biomass, that is, the solid residue from the extraction carried out by "native" DMCHA, was then mixed with the hydrophilic solvent, that is, the liquid obtained by the switching chemical reaction reported above, under the same conditions reported before. The undissolved residue was recovered from the suspension after centrifugation and the supernatant was heated at 70 °C for 30 minutes, under agitation, to reverse the above reaction and cause DMCHA to switch back to its hydrophobic form. 83% of the DMCHA was recovered after one complete 2-extraction stage run performed in our bench scale experiments. The incomplete recovery is due to the fact that, to avoid unnecessary complications at this stage, no recycle streams have been set up. Industrial-scale use of DMCHA would result in much higher recovery, for a number of reasons briefly indicated in the following. DMCHA has a very limited volatility (normal boiling point being 162 °C) and

the vapour DMCHA content of the released CO_2 stream is negligible. DMCHA entrainments in the released CO_2 stream are possible but, in an industrial setting, CO_2 released during the hydrophilic-to-hydrophobic switch would be recycled to be fed into the hydrophobic-to-hydrophilic switch reactor, thus virtually cancelling any DMCHA losses with the gas phase. DMCHA being slightly water soluble, some solvent also remains in the water phase after the split. By recycling the water phase obtained during the hydrophilic-to hydrophobic solvent switch, DMCHA loss could be minimised, along with the entailed water treatment needs. 70 °C may seem a somewhat high temperature both from a green perspective and from the point of view of the preservation of temperature-labile solute integrity. It should be noted, thus, that CO_2 could be stripped at lower temperature under vacuum or by insufflation with N_2, thus avoiding the need for heating altogether. However, doing so would result in a less valuable (from the point of recyclability) N_2/CO_2 gas mixture, since CO_2 absorption in the subsequent hydrophobic-to-hydrophilic switch process would likely be slowed down by the diffusional hindrance due to the lower CO_2 partial pressure. During the hydrophilic-to-hydrophobic switch, hydrophobic DMCHA separates, leaving a hydrophilic phase constituted by water and water-loving solutes behind. These latter might be recovered as a precipitate, be found enclosed in micelles, or be dissolved as true solutes; a staged recovery from each state according to existing methods could therefore be adopted. Conceptually, the extraction procedure may follow these two orders: (1) Hydrophobic first, then hydrophilic after forward switch; (2) Hydrophilic first, by forward-switching pure DMCHA before using it for extraction, then hydrophobic after backward switch.

We call here the first sequence “forward-mode” (Figure 3.2), starting with DMCHA in its native form (hydrophobic), then followed by the hydrophilic extraction (after forward switch).

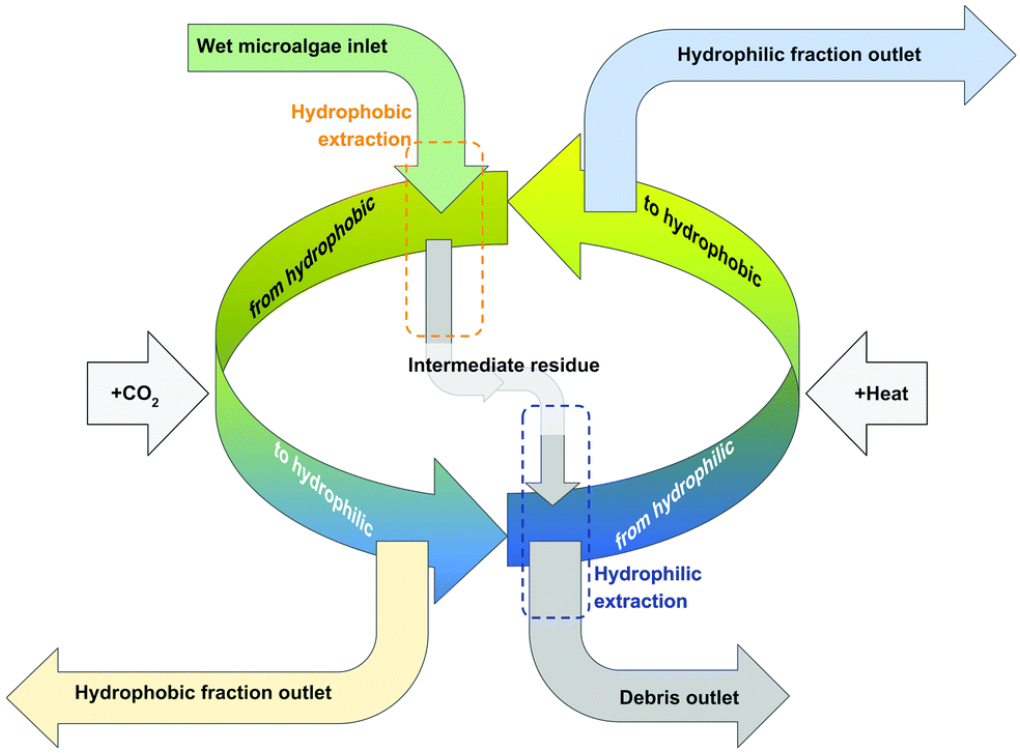

Figure 3.2 Sequential two-stage extraction in the forward mode (hydrophobic first)

The microalgal biomass was preliminarily compositionally assessed and it has been found to contain 37.9% ± 1.8% proteins, 40.4% ± 2.1% carbohydrates and 12.1% ± 1.9% neutral lipids. Proteins and carbohydrates were quantified colourimetrically with spectrophotometry; total proteins were quantified by the Lowry assay [Lowry et al. 1951], total carbohydrates were quantified using the Dubois assay [Dubois et al. 1956]. Since pure DMCHA is hydrophobic, and the results published so far have only concerned the extraction potential of pure (hydrophobic) DMCHA, we will start by discussing the forward mode. In this sequence, the biomass is first brought into contact with pure DMCHA. Afterward we will discuss the backward-mode approach and compare the two modes in terms of their biomass fractionation performance. All extractions, undergone by the microalgal biomass in each stage of the dual-stage extraction scheme, were evaluated by submitting the intermediate debris and the final debris to total protein and total carbohydrate assays. With the aim to solve the mass balance for each fraction, the difference with respect to the initial value in the raw biomass was calculated.

The dual-extraction with the two sub-extractions in reversed order will be denoted the "backward-mode" (Figure 3.3).

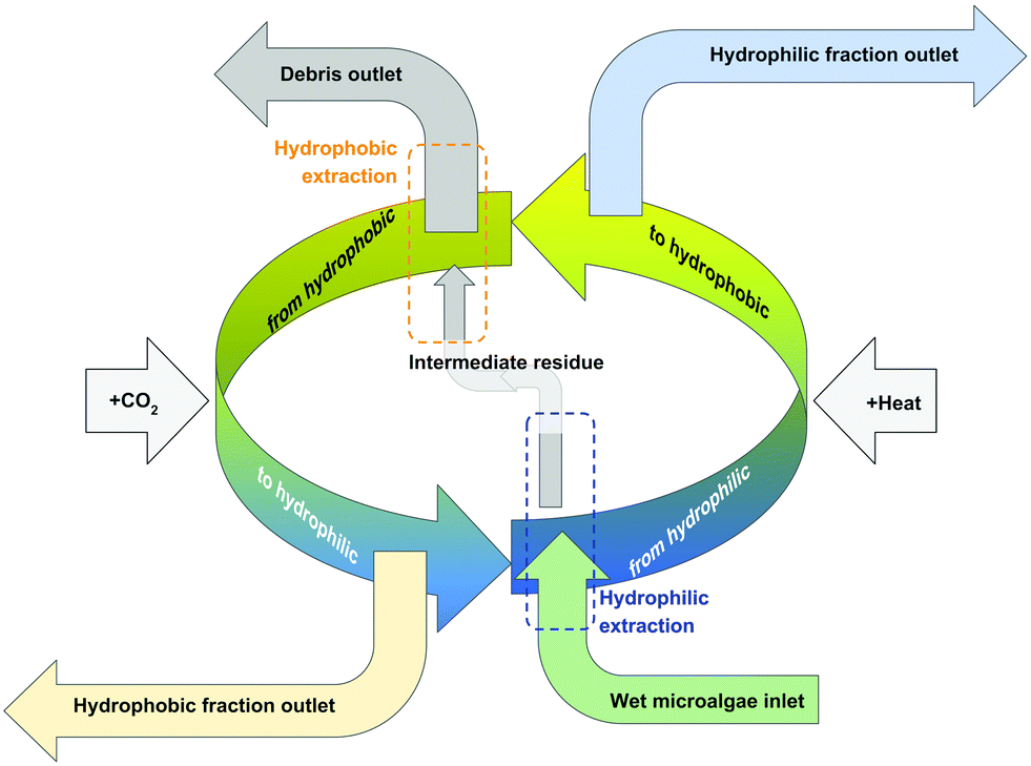

Figure 3.3 Sequential two-stage extraction in the backward mode (hydrophilic first).

Contemporarily, the composition of a model matrix was chosen to simulate the measured composition of *S. dimorphus* and the water content of "wet" microalgal biomass separated by centrifugation of the cultured suspension (20 minutes at 2600 g).

Model matrix was prepared by blending starch (0.38 g), glucose (0.091 g), casein (0.177 g, adopted as a widely available hydrophobic protein), albumin (0.158 g, adopted as a widely available hydrophilic protein), sunflower oil (0.09 g adopted as a widely available triacylglycerides pool), soy lecithin (0.04 g adopted as a widely available phospholipids pool to represent cellular membranes) and water (0.75 g adopted to represent intrinsic and extrinsic water Subsequently, the extraction of the model matrix was carried out with SHS, in its native hydrophobic state. The model matrix (1.58 g) was thoroughly mixed with 30 mL of DMCHA and agitated for 24 h in the presence of glass beads. Then agitation was interrupted and any insoluble material was separated and stored for the second-stage extraction. 30 mL of water were added and CO2 was injected in the biphasic system thus causing the phase split that expels oily fractions. The now-hydrophilic solvent was then collected and used to further treat the insoluble material that had been stored at the end of the first extraction stage, again by prolonged thorough mixing. Finally, any still insoluble material was removed and the liquid was switched back to its initial state stripping the CO2 by heating. By following the two extraction stages described here, the complete "forward-

mode", dual-stage circular extraction was complete, and the extraction capability of the SHS was assessed by characterising both the supernatant streams and the solid residuals.
In a separate experiment, the extraction order was reversed, thus following what was named the "backward-mode" circular extraction. In this case the model matrix was first treated with the hydrophilic form of the amine, producing an aqueous liquid phase and an intermediate solid residue that was stored. After heating (stripping of CO_2 from the liquid phase), the supernatant (the hydrophobic SHS), was decanted from the aqueous phase that contained the extracted hydrophilic components from the model biomass mixture. The recovered DMCHA was then used to extract hydrophobic components from the intermediate solid residue, thus obtaining a second extract and a final insoluble residue. All streams were compositionally assessed as before, thus completely characterising the backward-mode dual-stage extraction in the synthetic matrix. Results are reported in Figures 3.6 and 3.7.

3.1.2 Results and discussion

The obtained results for the Forward-mode extraction with DMCHA are reported in Table 3.1.

System	Metabolites		
Forward-mode dual extractions	***Proteins***	***Carbohydrates***	***Lipids (Neutral)***
Stage I extraction - Hydrophobic	7.0% ± 1.1%	42.0% ± 3.6%	96.1% ± 1.9%
Stage II extraction - Hydrophilic	34.1% ± 2.1%	9.4% ± 1.4%	0%
Total extraction	41.1% ± 2%	51.4% ± 3.3%	96.1% ± 1.9%

Table 3.1 Fractional extraction efficiencies in the forward-mode

The reported results should be interpreted as follows: during the forward-mode extraction, 42% of the carbohydrates contained in the cells were dissolved with DMCHA, which has a hydrophobic character. Switched DMCHA, which has a hydrophilic character, managed to extract a further 9% of the initial cell carbohydrates that had remained in the cell debris after the first stage of the forward-mode extraction. The total carbohydrate extraction reached therefore 51%. It should be noted that the total extraction is split between two streams;

therefore, their recovery should be performed through both the hydrophobic-to-hydrophilic switching and the hydrophilic-to-hydrophobic switching.

The backward-mode extraction has different extraction yields, as indicated by the figures reported in Table 3.2, but the same concept holds.

System	Metabolites		
Backward-mode dual extractions	***Proteins***	***Carbohydrates***	***Lipids (Neutral)***
Stage I extraction - Hydrophilic	49.9% ± 4.9%	46.6% ± 5.7%	0%
Stage II extraction - Hydrophobic	3.3% ± 0.07%	3.7% ± 0.07%	92.6 % ± 1.7%
Total extraction	52.0% ± 1.4%	50.3% ± 6.0%	92.6 % ± 1.7%

Table 3.2 Fractional extraction efficiencies in the backward-mode

Based on the results of the forward-mode dual-stage extraction, separate consideration may be made for proteins, carbohydrates and lipids, and their distribution in the two subsequently extract streams obtained. If we consider the total extraction obtained from both stages, it can be observed that neutral lipids are extracted almost quantitatively, while (roughly) only 40% of the proteins and 50% of carbohydrates can be extracted. From the point of view of fraction distribution between extracts, neutral lipids are exclusively obtained from the hydrophobic extract. The protein and carbohydrate extraction task, on the other hand, is split between the two subsequently staged extractions in an opposite way: while 80% of the extractable carbohydrates are picked up during the hydrophobic extraction stage, only 20% of the extractable proteins are dissolved during hydrophobic extraction; the remaining 80% are dissolved during the hydrophilic extraction stage. Reversing the extraction order, that is, adopting the backward-mode dual-extraction, impacts both total extraction efficiency and fractional (stage-related) extraction efficiencies. About 50% instead of 40% proteins can be extracted, while an unchanged fraction (50%) of carbohydrates can be extracted. The Figures 3.4 and 3.5 shows the percentage of extraction efficiency in both extraction modes in order to compare them allowing a better understanding of the data contained in the Tables.

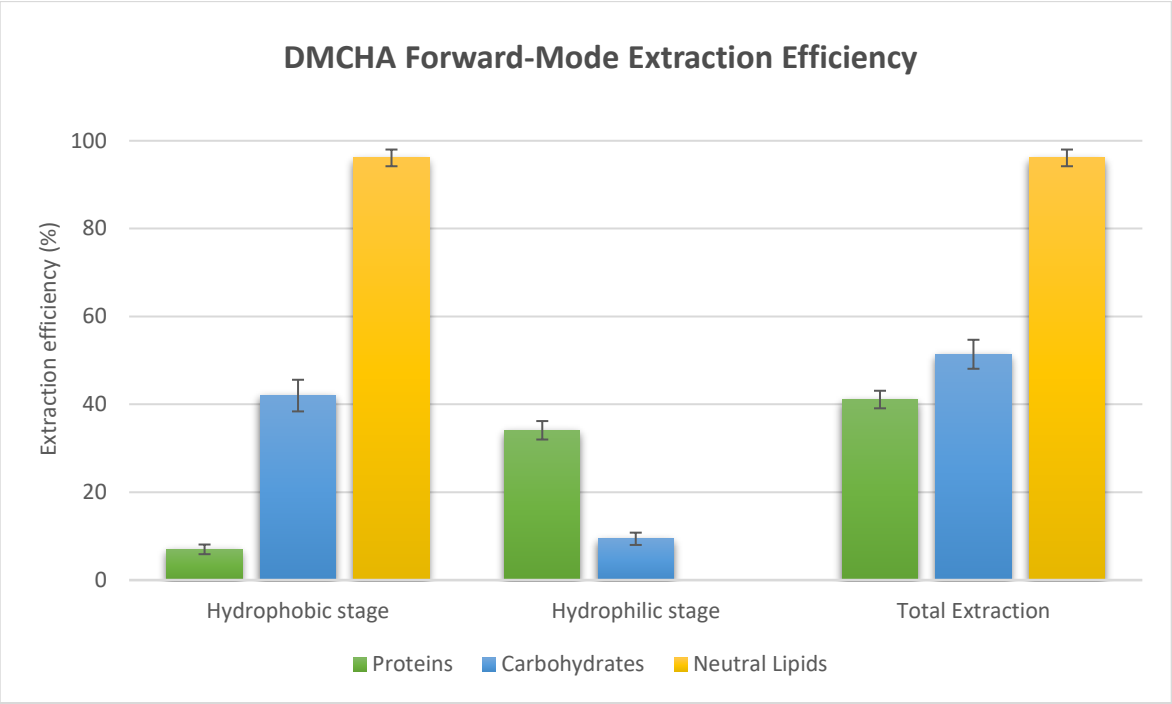

Figure 3.4 DMCHA forward-mode extraction efficiency % for proteins, carbohydrates and neutral lipids

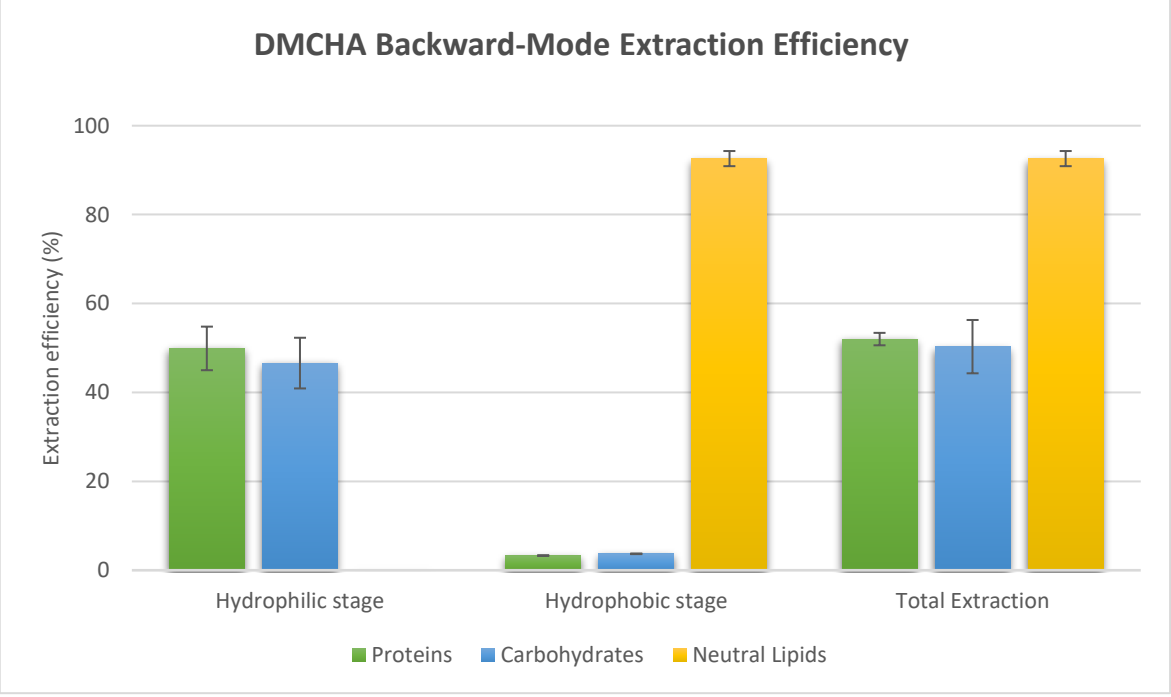

Figure 3.5 DMCHA backward-mode extraction efficiency % for proteins, carbohydrates and neutral lipids.

Therefore, it appears that the microalgal wall is successfully broken down by switched DMCHA too, thus allowing the insides of the cell to be accessed by the solvent. The hydrophilic character of the solvent might play a role in enhancing extractability during the backward-mode extraction in multiple ways, including ensuring a more thorough local

contact of the cell matrix with the solvent itself, or modifying the structure of the fractions (e.g. protein denaturation). When looking at the partial extraction, the difference appears even more striking. When carrying out backward extractions, less than 2% of the extractable proteins and less than 4% of the extractable carbohydrates are left behind after the hydrophilic extraction stage for the hydrophobic stage to pick up. Neutral lipid recovery appears to be essentially concentrated in the hydrophobic stage, and the neutral lipid extent of extraction appears to be independent of stage ordering. This means that, for the process developer, starting biomass processing with the hydrophilic stage provides both a superior protein and carbohydrate recovery and the absence of neutral lipids in the extracted hydrophilic fraction.

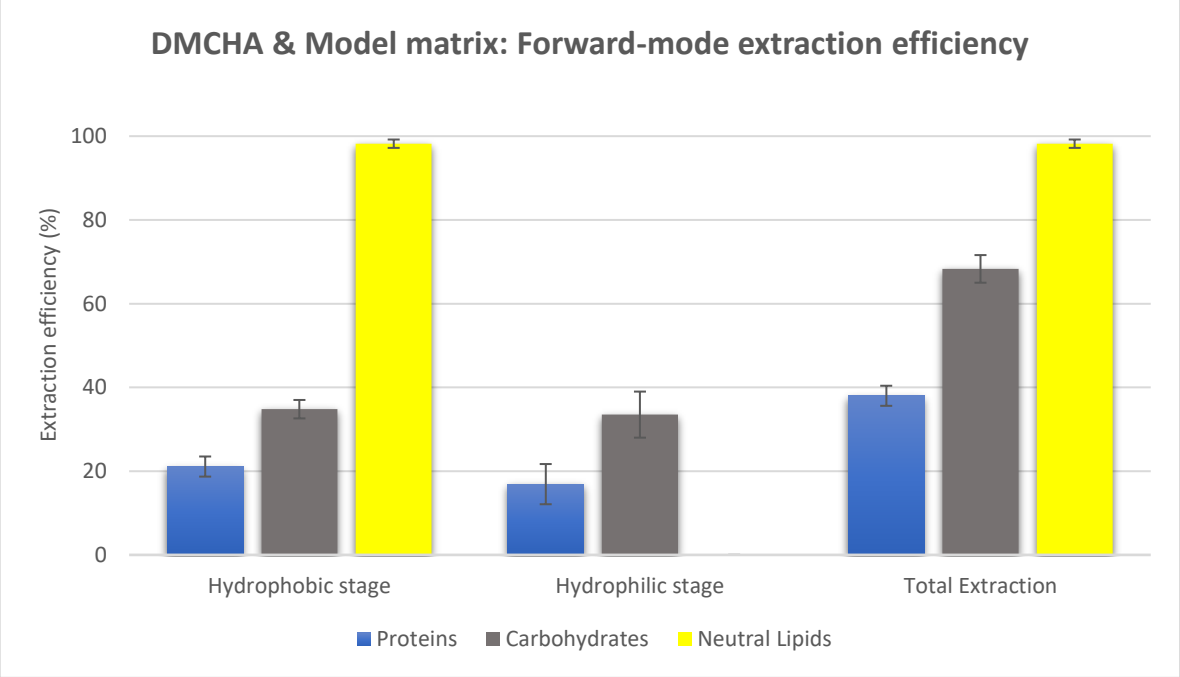

Figure 3.6 DMCHA Fractional extraction efficiencies on model system extracted in Forward-mode

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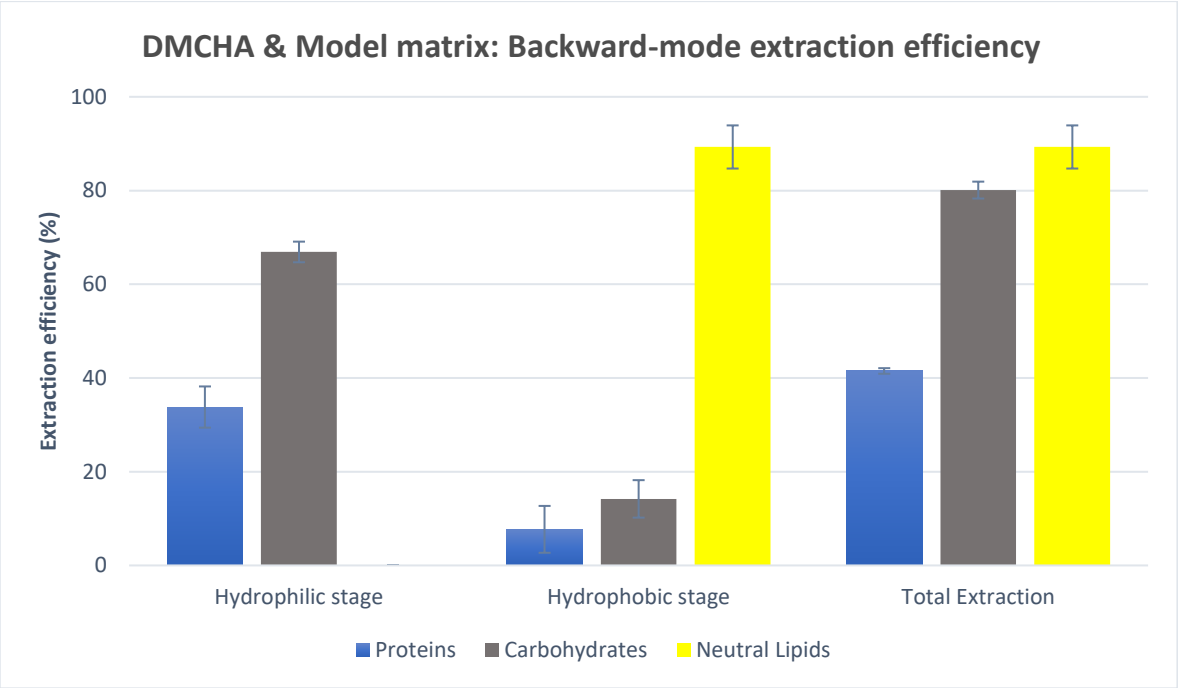

Figure 3.7 DMCHA Fractional extraction efficiencies on model system extracted in Backward-mode.

The reported results should be interpreted as follows: during the forward-mode extraction (Figure 3.6), 34.8% of the carbohydrates contained by the model matrix were dissolved by DMCHA, which has a hydrophobic character. Switched DMCHA, which has a hydrophilic character, managed to extract a further 33.5% of the initial carbohydrates that had remained in the matrix residue after the first stage of the forward-mode extraction. The total carbohydrates extraction reached therefore 68.3%. It should be noted that the total extraction is split between two streams; therefore, their recovery should be performed through both the hydrophobic-to-hydrophilic switching and the hydrophilic-to-hydrophobic switching. The backward-mode extraction (Figure 3.7) has different extraction yields, but the same concept holds.

Synthetic matrix extractions results (Figure 3.6 and 3.7) show that, as far as total extraction is concerned, separate consideration may be made for proteins, carbohydrates and neutral lipids, and their distribution in the two subsequent extract streams obtained.

Neutral lipid extraction is almost quantitative whatever orientation (forward or backward) of the operation is adopted and protein extraction is nearly quantitative, with a slight preference for forward rather than backward orientation (98% vs. 89%). In the case of carbohydrates, a more marked difference between forward and backward orientation is observed (68.3% vs. 80.1%), with an advantage of the backward mode over the forward mode. From the point of view of fraction distribution between extracts, neutral lipids are

exclusively obtained from the hydrophobic extract and are absent in the hydrophilic extract, whatever the orientation of the operation.

Protein extraction, on the other hand, appears to be split between the two subsequently staged extractions in the opposite way. The extraction occurring in the first place picks up a large fraction of the proteins (21.1% if it is hydrophobic, 33.8% if it is hydrophilic), leaving a much smaller fraction to be accomplished by the extraction performed subsequently (16.9% if it is the hydrophilic follow-up of a forward-mode extraction, 7.7% if it is the hydrophobic follow-up of a backward-mode extraction). Carbohydrates extraction shows a behaviour that is similar, albeit less marked, to that of proteins. The extraction occurring first picks up a comparatively larger fraction of the carbohydrates originally contained in the matrix (34,8% if it is hydrophobic, 66,9% if it is hydrophilic), leaving a somewhat smaller fraction for the extraction coming later (33,5% if it is the hydrophilic follow-up of a forward-mode extraction, 14.2% if it is the hydrophobic follow-up of a backward-mode extraction). In combination with a cell wall rupturing technique, therefore, the anticipated extraction potential toward biologic fractions recorded during synthetic matrix extraction experiments was well confirmed on microalgal matrix for lipids and proteins, while a lower extraction was recorded for carbohydrates, most likely due to residual diffusional hindrances and inter-fraction cross-link effects which are the fundamental (non-compositional) difference between the synthetic and the natural matrix.

3.1.3 Conclusions

From an application point of view, it appears therefore that using both the hydrophilic and hydrophobic forms of a switchable-hydrophilicity solvent can be beneficial for separating microalgal biomass into its primary fractions, i.e. proteins, carbohydrates and lipids. Furthermore, it appears that the hydrophilic form of the DMCHA-based solvent is equally able to cause the disruption of the microalgal biomass and permit the leaching of its fraction content. Whatever the order followed in performing the dual-mode extraction, the hydrophilic stage is maximally able to pick up proteins and carbohydrates, but does not pick up neutral lipids; conversely, the hydrophobic stage picks up neutral lipids and only part of the contained proteins and carbohydrates. Starting the microalgal biomass processing with the hydrophilic stage of a prospective “circular” dual-stage extraction provides superior protein and carbohydrate recovery, with little neutral lipids in the extracted hydrophilic fraction.

3.2 Switching characterisation of DBAE an DIPAE as less toxic SHSs

Since N,N-dimethylcyclohexylamine is a toxic solvent, with possible harmful effects on the operator and the environment, following the advice of Professor Philip G. Jessop, our research group decided to investigate the extractive ability of two different SHSs: dibutylaminoethanol and diisopropylaminoethanol. The above explained experimental set-up was maintained, as the idea of solvent employment for the circular extraction.

N, N-dibutylaminoethanol, DBAE, at room temperature is a colourless liquid, whose structure formula can be seen in Figure 3.8 below. It is part of the category of tertiary amines with commutable behaviour characterized by a pKah = 9.67 and a $\log K_{ow}$ = 2.20. It is characterized by a boiling temperature of 230 ° C and a flash point of 95 ° C. The substance is corrosive, its vapours are irritants for the respiratory tract and it is harmful both for the environment and for humans (LD50 equal to 1070 mg kg^{-1}).

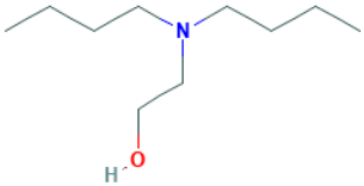

Figure 3.8 N,N-dibutylaminoethnol structure formula

N, N-diisopropylaminoethanol, DIPAE, is a colourless switchable tertiary amine, liquid at room temperature, whose structure formula can be seen in Figure 3.9 below. It is included in the category of tertiary amines with invertible behaviour characterized by a pKah = 10.14 and by $\log K_{ow}$ = 1.16. It is characterized by a boiling temperature of 190 ° C and a flash point of 64 ° C. The substance is corrosive, toxic if ingested or inhaled (LD50 of 940 mg kg^{-1}). The vapours can be irritants to the eyes and the respiratory tract.

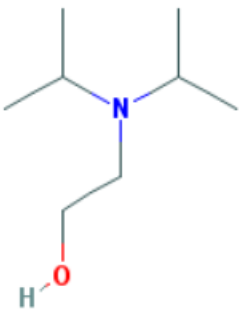

Figure 3.9 N,N-diisopropylaminoethanol structure formula

In particular, we can see the comparison between the three SHS used in this work in the Table 3.3 below.

	DMCHA	DBAE	DIPAE
pK_{aH}	10.48	9.67	10.14
$LogK_{ow}$	2.04	2.20	1.16
Boiling point ° C	163	230	190
Flash Point ° C	43	95	64
LD50 (mg / kg)	348	1070	940
Solubility in water	10 g / L	n.d.	n.d.
Vapour pressure (at 20 °C)	3.6 mm Hg	<2 mm Hg	<1 mm Hg

Table 3.3 Comparison of physical-chemical characteristics of DMCHA, DBAE and DIPAE

3.2.1 Switchable behaviour of DBAE and DIPAE

Several temperatures were tested to find the optimal one for switching improvement of DBAE. The complete switch was obtained keeping the system temperature constant at 10 ° C as we can see in further results, it was also necessary to stir the solution with a magnetic stirrer because of the viscosity of the solvent (clearly higher than that of the DMCHA). Table 3.4 shows the temperatures at which the switch was tested with the respective required times for the complete conversion.

Temperature	**20°C**	**15°C**	**8 ± 2°C**
Time (min)	>180	70	50

Table 3.4 Time trend of DBAE switch in function of temperature

It was not possible to complete the operation in acceptable time at room temperature. The CO_2 absorption reaction is strongly exothermic and it has been necessary to remove the generated heat and complete the operation at lower temperatures. To complete the operation in times comparable to those of the DMCHA, it is necessary to refrigerate the system up to a temperature of about 10 ° C to favour the process thermodynamics.

The condition of switch back was then identified and therefore the possibility of recovering the hydrophobic solvent. The solvent was placed inside a glass cylinder in which air was insufflated, the cylinder was placed inside a thermocryostat. In this case reswitch operation

is much simpler than that of the DMCHA, it is conducted at a temperature of 40 °C and the time required to complete the recovery of the solvent was 30 minutes.

Several temperatures were tested to find the optimal one for DIPAE switching improvement. The complete switch was obtained at room temperature. In this case, unlike the DBAE, lowering the temperature only slowed the process kinetics. Moreover, the system resulted much more viscous, making the phase of absorption of CO_2 more complicated. The reswitch condition was then identified and therefore the possibility of recovering the hydrophobic solvent. The solvent was placed inside a glass cylinder in which air was blown, the cylinder was placed inside a temperature controlled thermocryostat. The reswitch was performed at 70 °C and the time required to complete the operation was 15 min.

3.2.2 Comparison of switch and reswitch conditions for the three SHSs

The switch operation is compared in terms of time necessary to completely change the solvent and in terms of temperatures at which the process is carried on. For all three solvents CO2 is insufflated thanks to the use of a microsparger to allow a greater gas-liquid contact surface and therefore favour the diffusion of CO_2 in water. The CO_2 flow rate is in all three cases 30 NL/h. In Table 3.5 below are reported the main parameters.

Solvent	Temperature °C	Time (min)
DMCHA	20	35-40
DBAE	10	50-60
DIPAE	20	35-40

Table 3.5 Parameters of switch operation for DMCHA, DBAE and DIPAE

The time required to complete the switch operation is essential as it is directly proportional to the amount of CO_2 required. Comparing the data, it is clear that DBAE is the solvent that requires more CO_2 to complete the switch operation, while DIPAE and DMCHA do not differ significantly. The switch operation of the DBAE also requires lower temperatures and therefore additional costs to refrigerate the system. The reaction is in fact strongly exothermic and therefore it is necessary to remove the heat generated and favour the absorption of CO_2. The DBAE also possesses a higher viscosity than the other two solvents, which in combination with the lowering of the temperature requires the use of a magnetic stirrer to promote absorption. In conclusion, while the DIPAE has similar conditions to those of the DMCHA, the switch operation of the DBAE is instead much more onerous in terms of costs for the refrigeration, for the agitation of the solution and for the amount of CO_2

required. In the Figure 3.10 we can see the trend of the position of the interface between the two phases and therefore the percentage of conversion from the hydrophobic to the hydrophilic form over time. As it can be seen from the graph the trend of the DIPAE curve is very in line with that of the DMCHA while that of the DBAE is different.

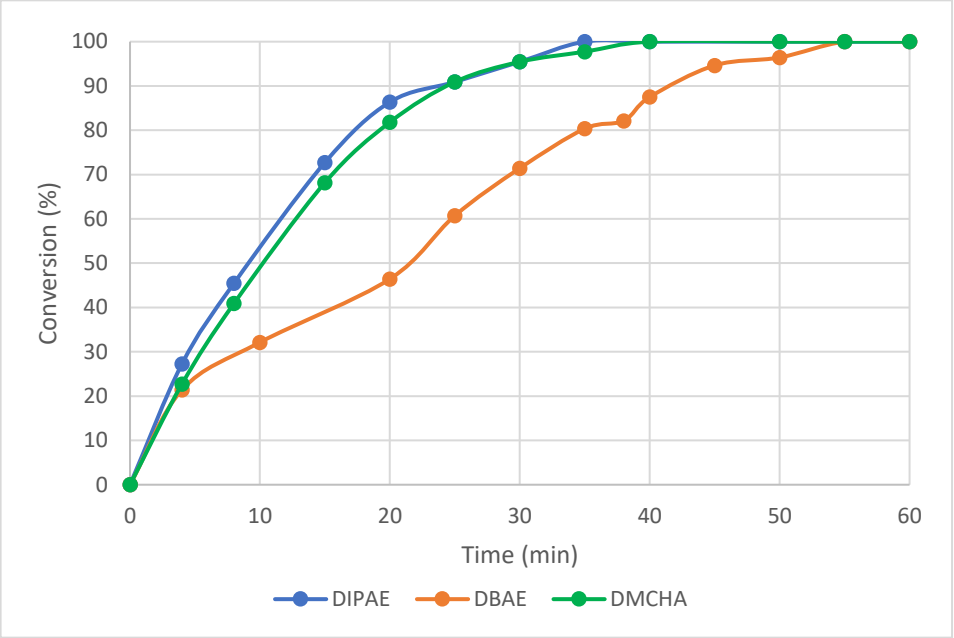

Figure 3.10 Trend of the interface position between the two phases hydrophilic and hydrophobic as a function of the switching time

The reswitch and solvent recovery conditions are reported in Table 3.6. It can be seen how the conditions of the DBAE recovery are much less burdensome. The time required is comparable to that of DMCHA but the temperature is halved. The percentage of solvent recovery is higher due above all to the very low solubility of the compound in water. The DIPAE also in this case shows characteristics very similar to those of DMCHA, its more hydrophilic character and therefore its greater solubility in water, however, lead to very low recovery efficiency of the solvent and equal to 60%.

Solvent	Temperature °C	Time (min)	Recovery (%)
DMCHA	70	30-40	80
DBAE	40	30	90
DIPAE	70	20	60

Table 3.6 Parameters of reswitch operation and recovery percentage of the native form of the solvent

3.2.3 Extraction performances of DBAE and DPAE compared with DMCHA

The three studied solvents were also compared in terms of switch and reswitch conditions and extraction ability. Each replica was quantified in terms of proteins, carbohydrates and lipids extracted from the tested solvent. Because of the inability to test the extract, as the amines interfere with Lowry and Dubois assays, the solid residue of the biomass was assayed for each extraction stage. The concentration of extracted products was obtained by subtraction from the initial composition, according to the mass balance reported below.

$$C^{ext} = C^{o} - C^{res}$$

$$p^{ext} = p^{o} - p^{res}$$

The extraction capacity of the solvents was evaluated in terms of extraction efficiencies by evaluating the individual extractions separately. The extractions made with the three solvents in their hydrophobic forms resulted in the efficiencies reported in Table 3.7, which are also compared in the Figure 3.11 below.

Solvent	E% Proteins	E% Carbohydrates	E% Neutral Lipids
Hydrophobic DMCHA	7.0% ± 1.1%	42.0% ± 3.6%	96.1% ± 1.9%
Hydrophobic DBAE	3.6% ± 0.7%	22.3% ± 2.5%	47.1 ± 2.7%
Hydrophobic DIPAE	21.1 ± 3.6%	41.4 ± 0.8 %	15.1 ± 1.4%

Table 3.7 Extraction efficiencies % of hydrophobic DMCHA, DBAE and DIPAE

As we can see the percentage of carbohydrates extracted by DMCHA and DIPAE is around 40% and similar for the two solvents. Only the DBAE shows a slightly lower extraction percentage, this could be due to the high viscosity of the solvent which interferes with the mass transfer.

The percentage of extracted lipids is very different in the three cases. The extraction efficiency of DIPAE is extremely low, around 15%, while that of DBAE reaches ~50%. In both cases we deviate a lot from DMCHA, that manages to extract almost all the neutral lipids. This can be caused by the viscosity of the DBAE and the results of DIPAE find an explanation in its nature. DIPAE presents a logarithm of water octanol distribution equal to 1.16, very low compared to that of DMCHA which is equal to 2.02, this indicates that it is a solvent with a much less hydrophobic character respect to DMCHA and therefore not very

similar to the lipophilic fractions. This also explains the significantly higher extraction of proteins by DIPAE, which reaches a percentage of 20% compared to the percentage extracted by the other two solvents.

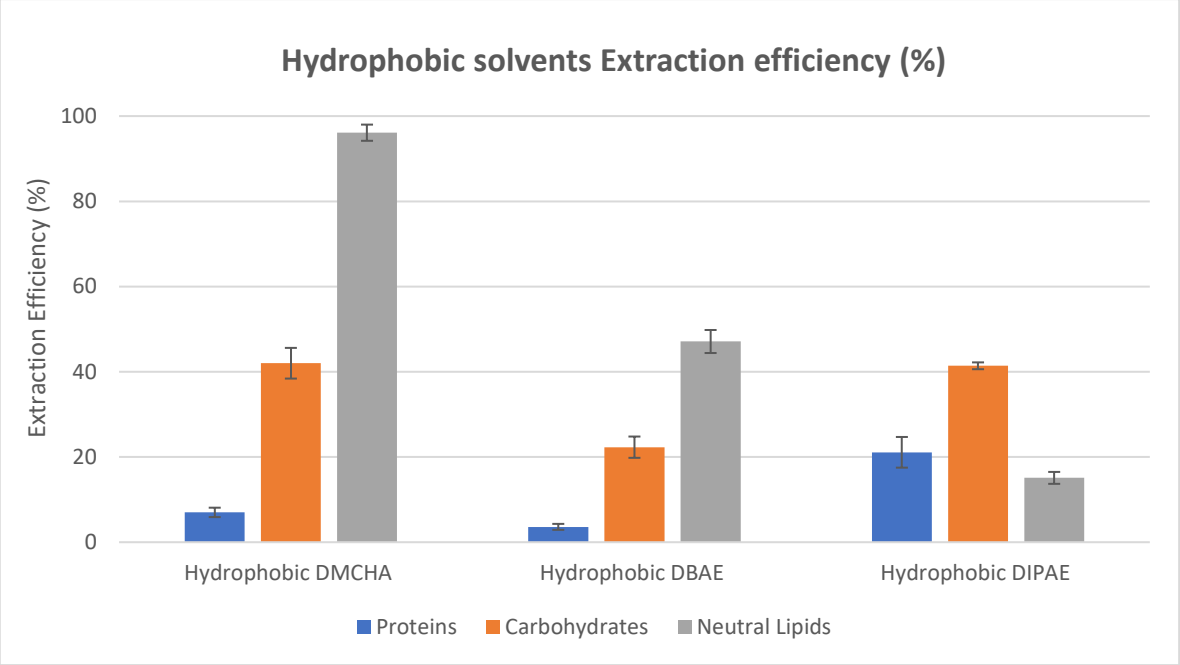

Figure 3.11 Extraction efficiencies % of hydrophobic DMCHA, DBAE and DIPAE

The comparison on the results of the hydrophilic stage of extraction (carried out by using pre-switched SHS) is carried out exclusively between DIPAE and DMCHA as switched DBAE is unstable and returns progressively to the hydrophobic form by losing CO_2 over the extraction time frame. Keeping the solvent under suitable thermal and pressure conditions, could allow its use in the extraction process. The results are shown in Table 3.8 and can be seen in the Figure 3.12.

Solvent	E% Proteins	E% Carbohydrates	E% Neutral Lipids
Hydrophilic DMCHA	34.2% ± 2.1%	9.4% ± 1.4%	0%
Hydrophilic DIPAE	8.7% ± 4.0%	13.35% ± 1.4 %	0%

Table 3.8 Extraction efficiencies % of hydrophilic DMCHA and DIPAE

In this case the percentage of carbohydrates extracted from DIPAE is slightly higher than that of DMCHA. The same cannot be said for proteins. In fact, DIPAE extracts a high percentage of proteins already in the first stage of extraction. The solvent put in contact is a solvent already charged and the driving force is considerably reduced.

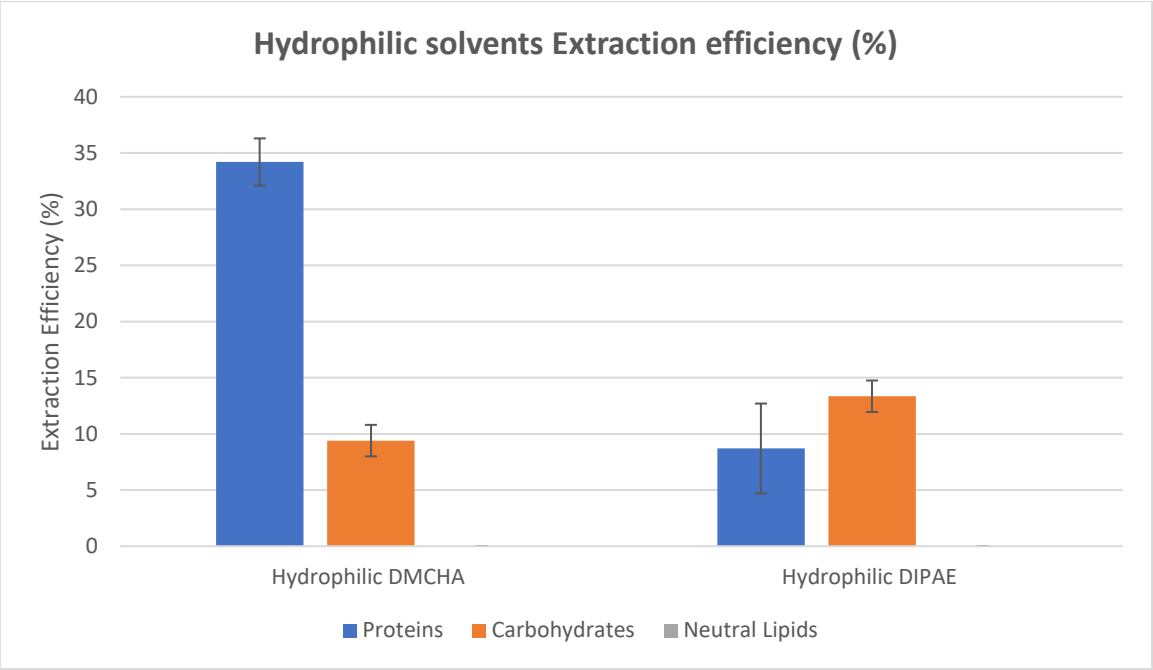

Figure 3.12 Extraction efficiencies % of hydrophilic DMCHA and DIPAE

Finally, to complete the complete extraction data of the two extraction stages are reported, which are nothing but the sum of the previous stages (Table 3.9 and Figure 3.13).

Solvent	E% Proteins	E% Carbohydrates	E% Neutral lipids
DMCHA total extraction	41.2% ± 1.9%	51.4% ± 3.2%	96.1% ± 1.9%
DBAE total extraction	3.6% ±0.7%	22.3% ± 2.5%	47.1±2.7%
DIPAE total extraction	29.8 ± 5.1%	54.7± 0.6%	15.1 ±1.4%

Table 3.9 Total Extraction efficiencies % of DMCHA, DBAE and DIPAE in forward-mode

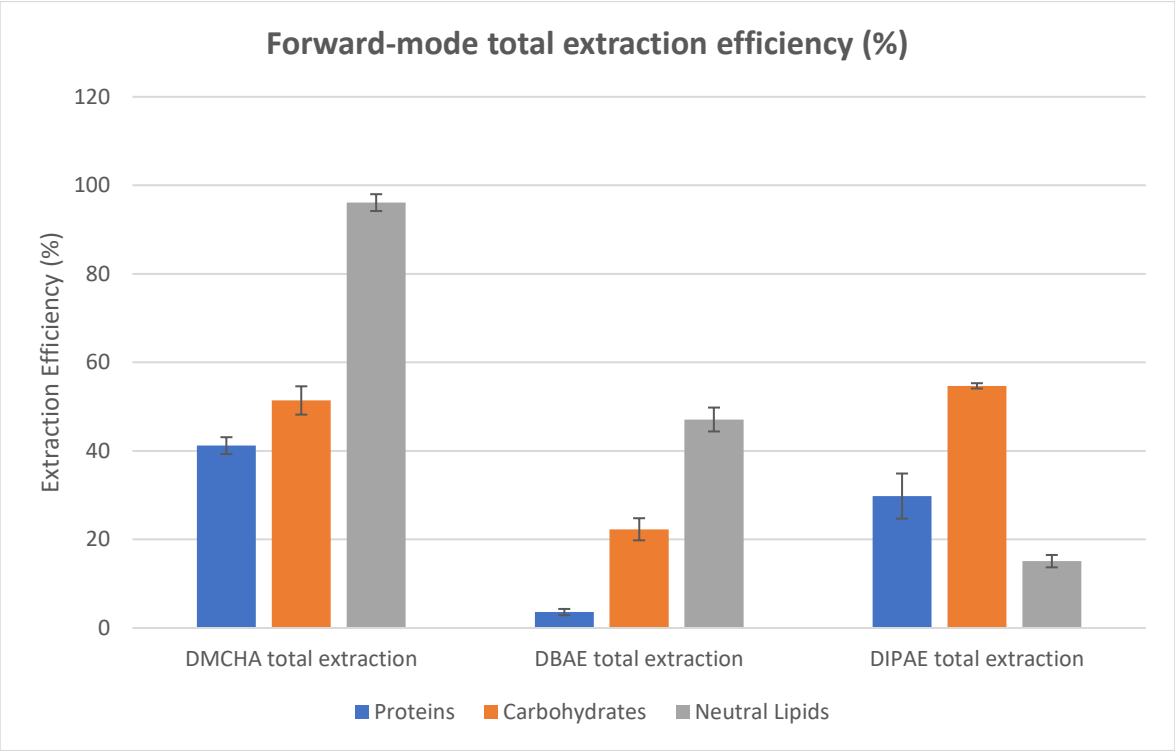

Figure 3.13 Total Extraction efficiencies % of DMCHA, DBAE and DIPAE in forward-mode

DMCHA is able to solubilize a wide range of biomolecules in both its hydrophilic and hydrophobic forms. Furthermore, the realisation of a double extraction has led to an increase in the percentage of extraction of proteins and carbohydrates. The efficiency is greater, then, if the biomass is put first in contact with the hydrophilic solvent and then with the hydrophobic one, thus demonstrating that both forms are capable of breaking the cell wall. The percentage of extracted lipids is independent of the extraction order and comparable to that of traditional solvents.

DBAE, inserted in the experimental plan because of its lower toxicity, has lower extraction efficiencies than DMCHA due to the higher viscosity of the solvent combined with the impossibility of carrying out a hydrophilic extraction. The hydrophilic form of the solvent is in fact unstable at room temperature due to the spontaneous stripping of CO_2. Recovery of the lipid fraction is more onerous as it requires constant refrigeration of the system. The switch operation, in order to be completed, is carried out at the temperature of 10 ° C under constant mechanical stirring. Thanks to the ease of CO_2 stripping and the very low solubility of DBAE in water, the solvent recovery operation is simpler and equal to 90%.

DIPAE like DBAE has lower level of toxicity than DMCHA. Regarding switch and reswitch operating conditions, DIPAE has similarities with DMCHA but extraction efficiencies are much lower. The percentage of extracted lipids is about 15% compared to 96% of DMCHA,

mainly due to its minor hydrophobicity and therefore it is less analogous to the lipid fraction. Moreover, being more soluble in water, the percentage recovered after the reswitch operation is lower than the other two solvent, approximately 60%.

3.3 Kinetic model for the extraction of carbohydrate and protein through hydrophilic and hydrophobic form of DMCHA

Fundamental aspects for the evaluation of the efficiency of a liquid-solid extraction process are essentially based on experimental kinetic tests of biomass depletion. The extraction rate is the most important parameter for the process dimensioning of an extractor. In this part of the work, through kinetic tests, the fundamental parameters of the liquid-solid extraction of carbohydrates and proteins from biomass have been estimated with DMCHA in its two forms, hydrophilic and hydrophobic. Neutral lipids were not estimated, because in previous experiments the plateau of extraction efficiency was reached in the first 6 hours of biomass-solvent contact. Two mathematical models have been chosen, both are based on the fundamental kinetics laws and mass transfer. In particular:

1) a first stationary mass transfer model in which it is assumed that the main mechanism controlling the rate of extraction of the biomass components is the mass transfer from solid to the liquid phase bulk. The model can be represented by an equation like:

$$\frac{dC}{dt} = \mathrm{k}_L a(C_{eq} - C)$$

It describes the change in concentration of the component in the extract over time and in which $k_L a$ is the volumetric mass transfer coefficient. This equation must be integrated with the initial condition that at time t = 0 the concentration in the extract is 0.

Therefore, we obtain:

$$C(t) = C_{eq}[1 - exp(-k_L at)]$$

Or in terms of yield, dividing by the initial mass of component present in the solid:

$$Y(t) = Y_{eq}[1 - exp(-k_L at)]$$

Where Y and Y_{eq} are the yields of the component in question in the liquid bulk and at the steady-state respect to the mass of initially present component.

2) a second kinetic model proposed by Harouna-Oumarou (2006) that considers the extraction process of proteins, lipids and carbohydrates from plants, a second-order extraction process that starts from the assumption that there are essentially two phenomena during the extraction: initially there is an intense dissolution in which there is the maximum extraction rate and then a second slower step which essentially corresponds to the diffusion in the liquid bulk. The dissolution of carbohydrates, proteins and lipids contained in the biomass can be described by the equation:

$$\frac{dC}{dt} = k(C_s - C)^2$$

Where k is the second order kinetic constant of extraction (l * $g^{-1}h^{-1}$), Cs is the concentration at saturation and C would be the concentration of carbohydrates or proteins at any time t. This equation must be integrated with the initial condition that at time t = 0 the concentration C in the extract is equal to 0. We obtain:

$$C = \frac{C_s^2 * k * t}{1 + C_s * k * t}$$

With the previously described method, were evaluated the extraction capacities of the hydrophilic virgin solvent (i.e. pre-switched, pure solvent) along the 6, 24, 48 and 65 hours and along the 6, 15, 24, 48, 65, 72, 96 hours for the hydrophobic virgin solvent. In the figure is reported the biomass suspension in distilled water after 6, 15 and 24 h of hydrophobic extraction. The figure 3.14 reported biomass resuspended in water after 6, 15 and 24 h of hydrophobic DMCHA treatment.

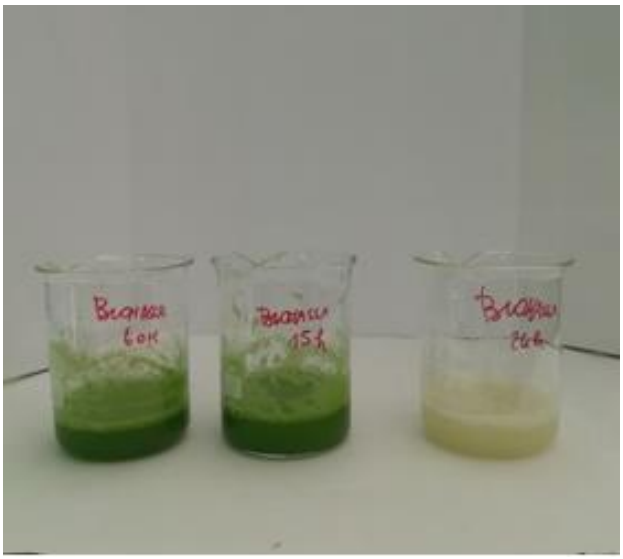

Figure 3.14 Variation in the colour of the biomass after extraction at 6, 15 and 24 hours

Two runs of parallel extractions were performed, first with virgin DMCHA in its hydrophobic form and second with DMCHA in virgin hydrophilic form. At the end of each extraction the residue was tested, calculating the percentage of carbohydrates and proteins extracted from the solvent. Each extraction was made by contacting 0.2 g of dry biomass and 30 ml of solvent for the hydrophobic form and 60 ml for the hydrophilic form. The data obtained were then interpolated with the Origin data analysis program. In the following paragraphs the results obtained for both solvent forms are reported in detail.

3.3.1 Kinetic model results with virgin hydrophilic DMCHA

The Figures 3.15 and 3.16 represent the experimental kinetic data obtained from carbohydrate and protein extraction tests on microalgal biomass in contact with virgin hydrophilic DMCHA. In the figures the experimental data are represented by small squares while the experimental model used is represented by a continuous line. The Tables 3.10 and 3.11 show the data about the concentrations in the extract obtained for proteins and carbohydrates as a function of time.

Time (h)	Carb's conc in the extract [g/l]	Carb's extraction efficiency %
0	0	0
6	0.483	30.2
24	1.013	46.6
48	1.058	65.7
65	1.133	70.3

Table 3.10 Concentration of carbohydrates in extract and extraction efficiency as a function of the contact time of DMCHA in its virgin hydrophilic form

Time (h)	Prot's conc in the extract [g/l]	Prot's extraction efficiency %
0	0	0
6	0.550	42.7
24	0.694	49.9
48	0.875	68.5
65	0.883	69.1

Table 3.11 Concentration of protein in extract and extraction efficiency as a function of the contact time of DMCHA in its virgin hydrophilic form.

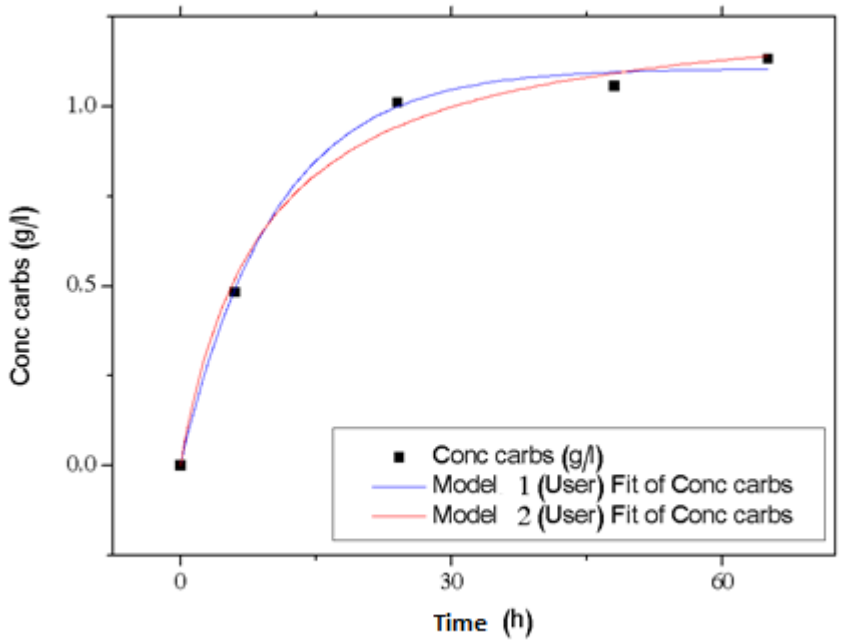

Figure 3.15 Comparison between kinetic model 1 and 2 for carbohydrate extraction with hydrophilic solvent

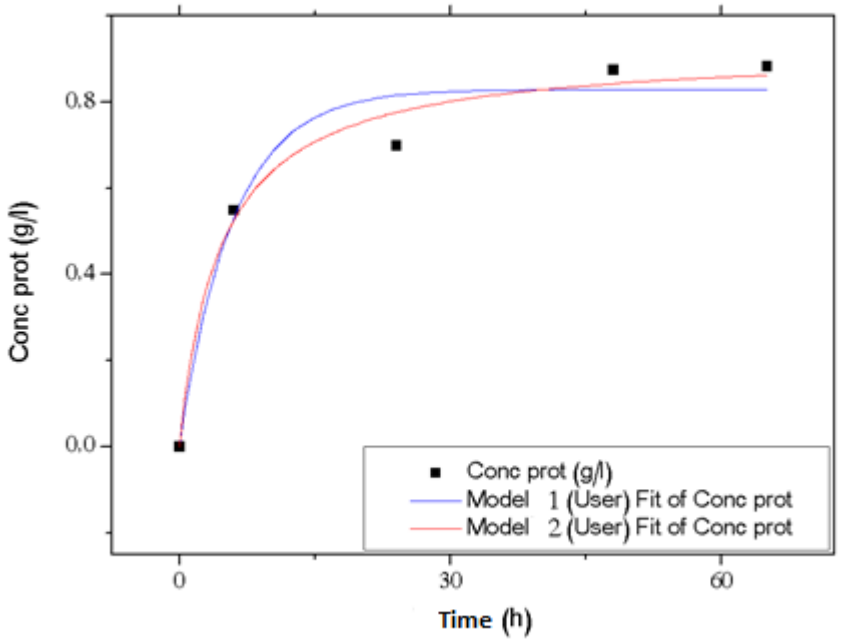

Figure 3.16 Comparison between kinetic model 1 and 2 for protein extraction with hydrophilic solvent

Through a non-linear interpolation of the data carried out by the Origin data analysis program it was possible to identify the characteristic parameters k_La and k, the mass transfer coefficients and saturation concentration (Figure 3.15 and 3.16). The transfer coefficients

give us an index of characteristic time of the process that has been calculated and reported together with the other results in the Tables 3.12 and 3.13.

Carbohydrates	Model 1	Model 2
r^2	0.99	0.99
k_La (s^{-1})	0.097	-
C_{eq} (g/l)	1.11	-
k ($l*g^{-1}h^{-1}$)	-	0.08
C_s (g/l)	-	1.30
Characteristical time (h)	10.51	9.1

Table 3.12 Parameters obtained for carbohydrate extraction by virgin hydrophilic DMCHA

Proteins	Model 1	Model 2
r^2	0.95	0.98
k_La (s^{-1})	0.17	-
C_{eq} (g/l)	0.83	-
k ($l*g^{-1}h^{-1}$)	-	0.23
C_s (g/l)	-	0.93
Characteristical time (h)	6	5

Table 3.13 Parameters obtained for protein extraction by virgin hydrophilic DMCHA

3.3.1.1 Comparison between the developed models for the hydrophilic DMCHA

Comparing the results obtained for both models, no marked differences were found in both kinetic of extraction for carbohydrates and proteins. In fact, both classes of studied compounds have similar polarity characteristics and therefore the fact that the trends are similar is an indication of the reliability of the obtained models. Regarding the choice of the model that could best represent the extraction of these compounds through the hydrophobic solvent, both models have very high correlation coefficients (r^2> 0.9). Data analysis was performed through the Akaike's test. The Akaike Information Criterion (AIC), is a method for the evaluation and comparison of statistical models developed by the Japanese mathematician Hirotsugu Akaike in 1971 and presented to the mathematical community in 1974. It provides a measure of the quality of the estimation for a statistical model taking into account both the data fitness respect to the model and its complexity. It is based on the concept of information entropy and offers a relative measure of lost information when a

model is used to describe the reality. Models with the lower AIC are better for representing a phenomenon. The model that presented a lower and therefore more reliable value of AIC, is the first model or the model that assumes that the mass transfer is essentially controlled by the diffusion in the bulk of the liquid phase. This seems to be an acceptable conclusion explicable through the analysis of the microalgal structure interfacing with the solvent. The biomass after the centrifugation operation and after the separation of the solid from the aqueous phase in which it was suspended has a certain degree of humidity. The solvent first is in contact with the cell wall of the cell, which is essentially composed of cellulose. The fundamental unit that constitutes cellulose is the cellobiose which is composed of two glucose molecules. Therefore, the OH^- groups are solvated by water. For this reason, the first resistance that the solvent should override is that of the water boundary layer. The high agitation and the fact that the solvent is very basic, however, makes sure that the cellulose easily undergoes basic hydrolysis and therefore this resistance is easily to overcome. Once the cell wall is attacked and hydrolysed in some places, the cell membrane becomes accessible. The cell membrane has a double nature, it is a lipid bi-layer of phospholipids with polar heads and non-polar tails. The hydrophilic DMCHA, due to its nature, can easily overcome both the humidity layer and the polar heads of the cell membrane, confirming that the resistance is essentially diffusive.

3.3.2 Kinetic model results with virgin hydrophobic DMCHA

The Figures 3.17 and 3.18 represent the experimental kinetic data obtained from carbohydrate and protein extraction tests on microalgal biomass in contact with virgin hydrophobic DMCHA. The Table 3.14 and 3.15 shows the data about the concentrations of carbohydrates and proteins obtained in the extract as a function of time.

Time (h)	Carb's conc in the extract [g/l]	Carb's extraction efficiency %
0	0	0
6	0.73	15.9
15	1.94	39.1
24	2.01	43.5
48	2.05	44.2
65	2.01	43.3
72	1.96	42.2
96	2.01	43.5

Table 3.14 Carbohydrate concentration in the extract and extraction efficiency as a function of the contact time of DMCHA in its virgin hydrophobic form.

Time (h)	Prot's conc in the extract [g/l]	Prot's extraction efficiency %
0	0	0
6	0.80	15.6
15	1.40	24.7
24	1.77	34.4
48	2.30	44.8
65	2.27	44.2
72	2.33	45.5
96	2.30	44.8

Table 3.15 Protein concentration in the extract and extraction efficiency as a function of the contact time of DMCHA in its virgin hydrophobic form.

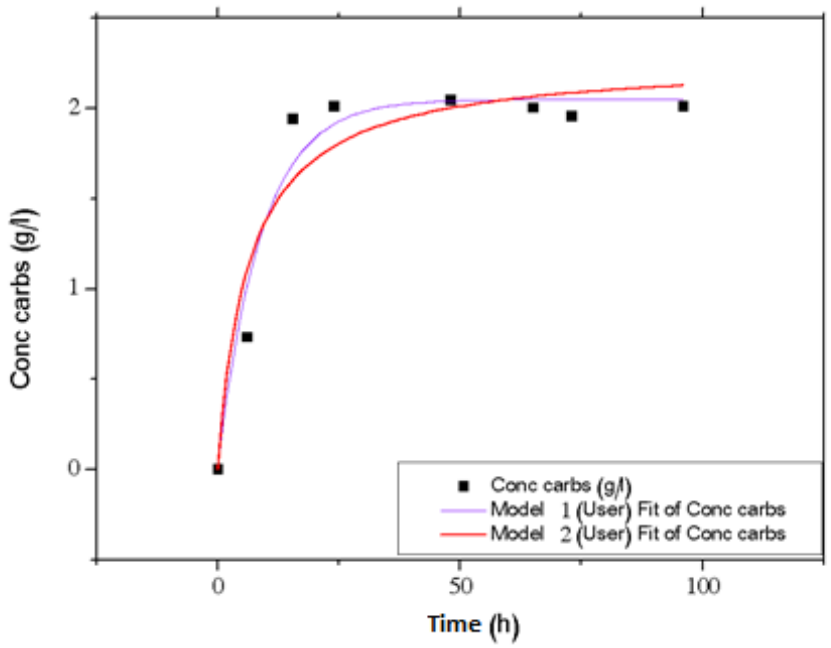

Figure 3.17 Comparison between kinetic model 1 and 2 for carbohydrate extraction with hydrophobic DMCHA

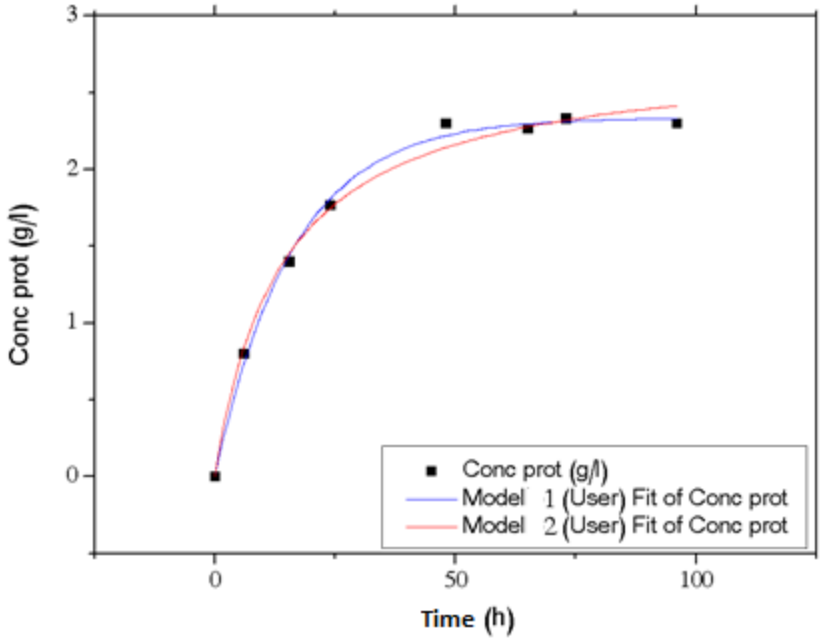

Figure 3.18 Comparison between kinetic model 1 and 2 for protein extraction with hydrophobic DMCHA

Through a non-linear interpolation of the data, carried out by the Origin data analysis program it was possible to identify the characteristic parameters k_La and k, mass transfer coefficients and the concentration at saturation. The mass transfer coefficients and the parameter $1/k_La$ give us index of the characteristic time of the process that has been calculated and reported together with the other results in the Tables 3.16 and 3.17.

Carbohydrates	Model 1	Model 2
r^2	0.956	0.909
k_La (s^{-1})	0.112	-
C_{eq} (g/l)	2.05	-
k ($l*g^{-1}h^{-1}$)	-	0.0686
C_s (g/l)	-	2.269
Characteristical time (h)	9	6.4

Table 3.16 Parameters obtained for carbohydrate extraction by hydrophobic DMCHA

Proteins	Model 1	Model 2
r^2	0.995	0.990
k_La (s^{-1})	0.061	-
C_{eq} (g/l)	2.33	-
k ($l*g^{-1}h^{-1}$)	-	0.025
C_s (g/l)	-	2.766
Characteristical time (h)	16.5	14.5

Table 3.17 Parameters obtained for protein extraction by hydrophobic DMCHA

3.3.2.1 Comparison between the developed models for the hydrophobic DMCHA

Comparing the results obtained for both models, no marked differences were found for carbohydrates and proteins extraction. Both classes of studied compounds have similar polarity characteristics and therefore the fact that the trends are similar is an indication of the reliability of the obtained models.

Regarding the choice of the model that could best represent the extraction of these compounds through the hydrophobic solvent, both models have very high correlation coefficients (r^2> 0.9). Data analysis was performed through the AIC. The model that presented a lower (and therefore more reliable) value of AIC is the second model or the two-step model. For hydrophobic DMCHA this could be explained by referring to the comparison already made in the paragraph concerning the kinetics of the hydrophilic solvent. The DMCHA, assisted by a constant agitation, but above all being a strong base can easily overcome the layer of water and to attack the cell wall in a very fast way. When it crosses the cell wall, unlike the hydrophilic form that does not meet resistance, the hydrophobic DMCHA standing in front of polar heads finds something opposite that is totally different from its nature. For this reason, we can say that the hydrophobic solvent meets an obstacle due to the fact that it is interfacing with something that has a completely different nature. However, being a strong base, it can saponify the phospholipids by altering the membrane structure and the extraction process occurs. The extraction speed slows-down and the resistance can be related to the extraction of the residual components.

Chapter 4. Extraction of microalgal metabolites with a Choline Chloride – based Natural Deep Eutectic Solvent

Circular extraction has provided excellent results, as the SHS solvents are suitable both for the biomass pre-treatment, being able to break microalgae walls, the extraction of biomolecules and the subsequent fractionation of neutral lipids. Thus, whilst they are safer and more convenient compared to traditional organic solvents when applied in the process industry, due to their low vapor tensions and no need for distillation, their toxicity for humans and environment pushes researchers to pursue the goal of even more "green" solvents.

In a typical chemical process, the solvents are widely used for the dissolution of the reagents, to favour the kinetics and the thermodynamics of a chemical reaction, for the extraction of products, for the separation of mixtures. However most of the currently used organic solvents are characterized by different properties harmful to human health and the environment.

Among the principles of Green Chemistry are that solvents should be innocuous to Man and to the Environment (safer solvents) and that the substances used in a chemical process should be chosen to minimize the potential for chemical accidents (intrinsically safe processes).

Biorefining, the biomass Era counterpart of oil refining is likely going to be extraction-based, and thus heavily solvent-dependent, much as the Oil Era was based on distillation and hence heat-dependent.

The overall goal of green chemistry combined with a biorefinery approach is the production of genuinely green and sustainable chemical products. Any new chemical process being developed should aim at using sustainable feedstocks. In the field of sustainable energy, microalgae can warrant renewable biofuels such as third-generation biodiesel obtained by the non-polar lipids, but the maximum utilization of the feedstock mass in final products is required. Lipids are not the only important fraction from the biomass, microalgae and cyanobacteria are important sources of carbohydrates, proteins, polyunsaturated fatty acid (PUFA) with high nutritional values, carotenoids such as β-carotene and astaxanthin, phycobiliproteins, which are used as natural dyes and antioxidant compounds for pharmaceutical and cosmetic applications. Biorefineries of the future will incorporate the production of fuels, energy and value-added chemicals, via the processing of biomass, into a single site (Kamm et al., 2006). The most popular extraction method for recovering oil and high value products from microalgae is solvent extraction because it guarantees reproducible results and it is a relatively inexpensive technique; its drawbacks are the high flammability

and/or toxicity of the organic solvents, the high cost of solvent recovery and the request of large volume of solvent. Organic solvents can lead to contamination in the form of solvent residues being present in the final product. These are the reasons why most organic solvents may have limited application in food processing. Downstream processing for the potential commercial production of microalgae products not only must consider economic costs, but should also consider minimizing environmental impacts, in order to attain sustainable production processes. (Mercer and Armenta, 2011). The green technology facilitates the minimum use of non-hazardous media, new environmentally acceptable solubilization techniques by controlling physical properties of media such as temperature and pressure and developing new green solvents. This liquid was found to have interesting solvent properties that are comparable to ambient temperature ionic liquids and a wide variety of solutes were found to exhibit high solubility. DES are obtained by mixing solid compounds forming a eutectic mixture with a melting point much lower than either of the individual components due to the formation of hydrogen bonds. Natural products are a plentiful and ideal source of ILs and DES due to their enormous chemical diversity, biodegradability and pharmaceutically acceptable toxicity profile. Different mixtures of various abundant cellular constituents (primary metabolites) such as sugars, sugar alcohols, amino acids, organic acids, and choline derivatives were tested and many combinations of these compounds were found to be liquids. The exploration of different combinations of these common metabolites abundantly present in all types of cells and organisms provided over 100 combinations of NaDESs. These eutectic solvents present densities higher than that of the water, the viscosity is determined by water content and temperature. The polarity range varies from 44.81 kcal mol^{-1} (higher than water) to 50.07 kcal mol^{-1} (comparable to methanol), but the polarity depends on the amount of water present in the solvent. (Dai et al., 2013).

In this part of the work, the PCH (1,2-propanediol, choline chloride, water 1:1:1) NaDES was used to treat microalgal biomass and carry out the extraction of cellular components, such as lipids, proteins, carbohydrates and photosynthetic pigments (chlorophylls and carotenoids) from the biomass itself.

Three sets of experiments were carried out based on different contact time between biomass and PCH: 24 and 72 hours, with and without pre-treatment with ultrasound. Biomass was shaken together with the PCH solvent in the presence of glass beads to promote the extraction efficiency. The analysis of the extract composition was carried out spectrophotometrically for pigments (chlorophylls and carotenoids), with biochemical assays for proteins and carbohydrates and gravimetrically for the determination of lipids.

The results showed the ability of PCH, coupled with the mechanical destruction of cell walls, to solubilize a wide range of polar biomolecules at room temperature.

4.1 Materials and Methods

1,2-propanediol-choline chloride-water (PCH) was prepared following the molar ratio of 1:1:1. Components were mixed in a pyrex bottle with a magnetic bar and a cap and heated in a water bath at 50 °C in agitation for 45 minutes, until the solution became clear and transparent. Choline chloride (>99 %), 1,2-propanediol (≥ 95 %), dichloromethane (>99.8 %) and methanol (>99.9 %) were purchased from Sigma-Aldrich. *S. dimorphus* (UTEX 1237) was cultivated in cylindrical glass tubes in batch mode under artificial light, in sterile 3NB synthetic medium as reported in the paragraph 3.1.1. Physical properties of the PCH were investigated. Viscosity was measured with the rotational viscometer Haake instruments Rotovisco RV 12, conductivity with the Delta Ohm probe HD 8706-R1 and pH with pH-meter of Hanna Instruments HI 8418. Carbohydrates and proteins were quantified colourimetrically with an UV-Vis Mapada spectrophotometer; total carbohydrates were quantified through the Dubois assay (Dubois et al. 1956) and total proteins were quantified with Lowry assay (Lowry et al. 1951). Chlorophylls and carotenoids were determined spectrophotometrically adopting Lichtenthaler equations (Lichtenthaler, 1987). Cell destruction was obtained in the presence of PCH with bead-beating method with glass beads of 2.5 mm diameter and it lasted for 24 h. It was compared with the Ultrasound Assisted Extraction (UAE) coupled with the solvent. Ultrasound pulsed cycle was effectuated in a refrigerated vessel and lasted for 40 minutes with a frequency of 20 kHz and a range of 70%. The amount of extracted chlorophylls and carotenoids was quantified spectrophotometrically in the solvent, extracted lipids were evaluated gravimetrically, proteins and carbohydrates were quantified through the difference between the wet on the residual biomass and the extracted were the difference in content from starting biomass and residual one. A graphic sequence of the employed method is reported in the Figure 4.1

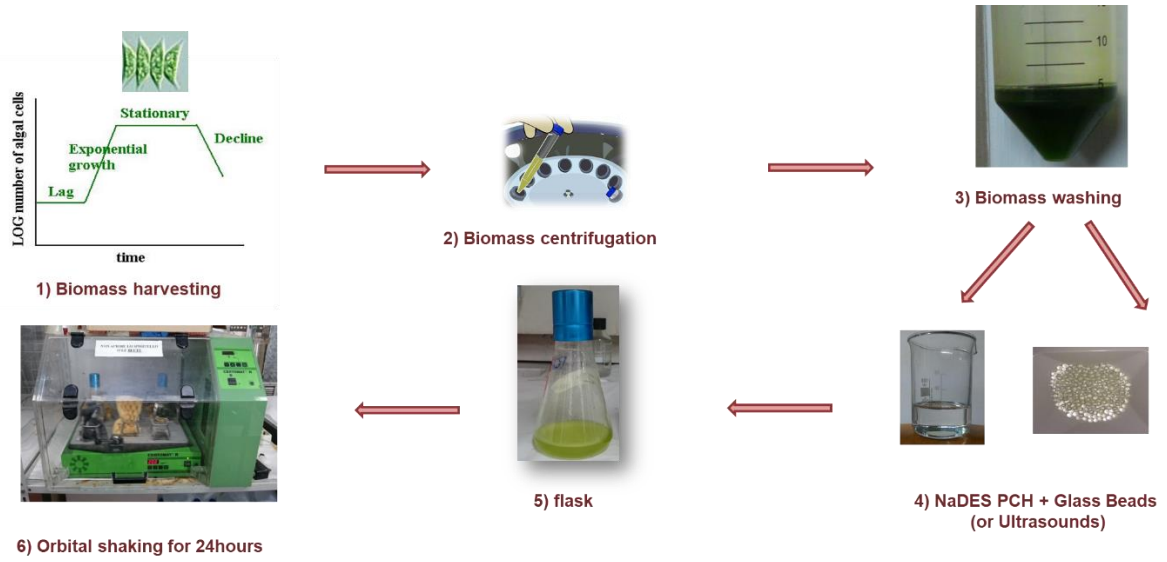

Figure 4.1 Methods employed: biomass harvesting, centrifugation and washing, mechanical pre-treatment and solvent extraction for 24 hours.

4.2 Results and discussion

4.2.1 Physical-chemical properties of PCH as function of its water content

The effect of the water contained in the solvent on properties of conductivity, viscosity and polarity, was studied. It increases with increasing of water amount formulation, reaching a peak when H_2O is 60 wt%, then it decreases. (Figure 4.2.)

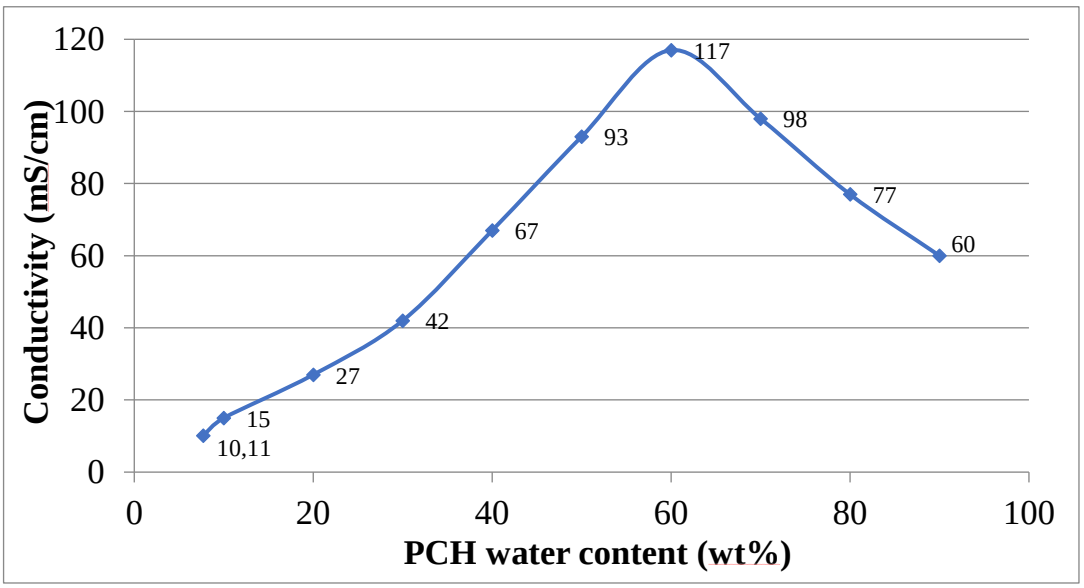

Figure 4.2. Variation of conductivity in accordance with the water amount in PCH

The huge viscosity of NaDES, due to the presence of a large net of hydrogen bonds established among its components, is a big obstacle in extraction protocols. When PCH is diluted with water, the interactions among the components are weakened and viscosity decreases as shown in Figure 4.3. Reached the degree of dilution of 50 %, the viscosity value is close to the one of water and the solvent still maintains its intermolecular structure.

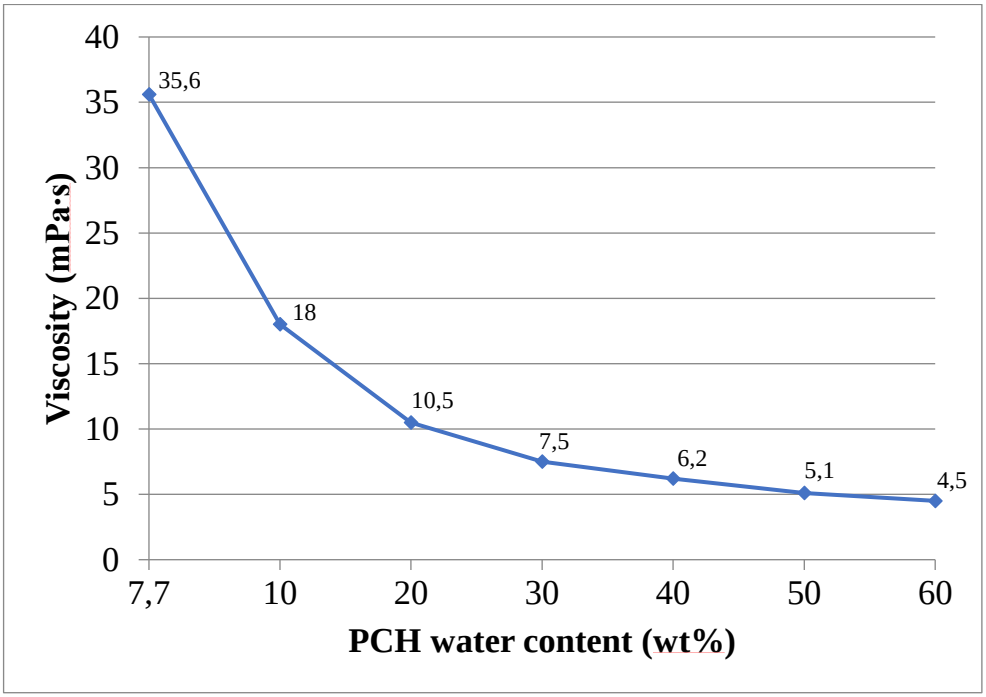

Figure 4.3. Variation of viscosity in accordance with the water amount in PCH

To understand polarity variation under the influence of water amount in PCH, miscibility tests were performed with an organic solvent. PCH has a polarity close to that of methanol; for this reason, it could have its same miscibility behaviour. Butyl alcohol is a solvent miscible with methanol and with PCH, they form an only phase, but it is immiscible with water (and in theory with PCH with high water content). When the water content in the NaDES is 25 wt% the system PCH – butyl alcohol forms two separated liquid phases, because PCH reaches a polarity value close to the one of water (Figure 4.4).

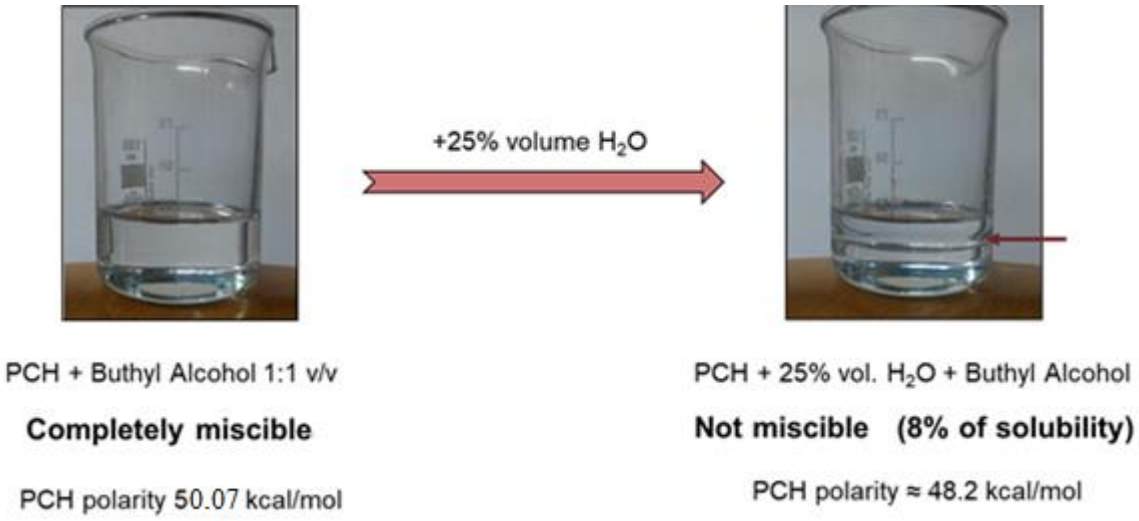

Figure 4.4 miscibility behaviour of PCH as function of its water content.

The investigated properties of PCH are summarized in Table 4.1.

PCH (1:1:1)	
pH	5
Conductivity at 25 °C (mS/cm)	10.1
Viscosity at 40 °C (mPa•s)	35.6
Density at 40 °C (g/cm^3)	1.1

Table 4.1. Physical-chemical properties of PCH

4.2.2 Extraction of biomolecules with PCH from wet samples of *S. dimorphus* biomass

Firstly, the action of PCH on microalgae cell walls, without any intervening mechanical rupturing method, was investigated. 0.6 g of wet biomass was inserted in a flask with 30 mL of PCH at room temperature and was shaken at 250 rpm for 24 hours in an orbital shaker. After the sample centrifugation, the PCH recovered was completely transparent and no peaks resulted from spectrophotometer. Presuming a solvent diffusional problem, due to its huge viscosity, a new extraction lasting 72 hours was performed with the same results. PCH alone is not able to destroy and penetrate microalgae cell walls (Figure 4.5).

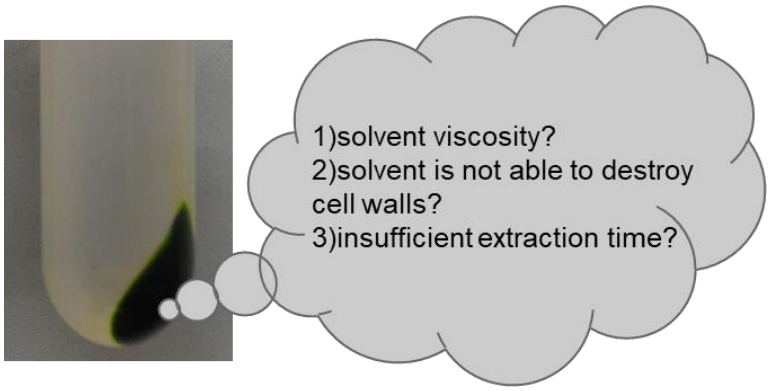

Figure 4.5 PCH and cells centrifugation after 72 hours of cell-solvent contact

For this reason, mechanical destruction was applied on the biomass and two protocols were set up. Bead-beating extraction with PCH is a simple and economic way to destroy cell walls and can be easily accomplished by means of glass beads. Before the incubation in the orbital shaker, the solution containing 0.6 g of microalgae, 2.5 g of glass beads and 50 mL of NaDES, was vortexed for 15 minutes at 1500 rpm. Then it was put in a 250-mL flask with 50 mL of PCH. The beads-assisted extraction lasted for 24 hours at room temperature in an orbital shaker set to 250 rpm. The solution was recovered and ultracentrifuged at 17000 rpm at 20 °C for 30 minutes, 2.5 mL of solvent with the accompanying extract were spectrophotometrically analysed to quantify the extracted photosynthetic pigments, while the residual was put in a (previously weighed) flask with 100 mL of dichloromethane and methanol for 24 hours. In this way, extracted lipids were quantified gravimetrically. The cell pellet was washed twice to remove salts and its content of proteins and carbohydrates was quantified, the extracted were calculated through the difference with the starting content (Figure 4.6).

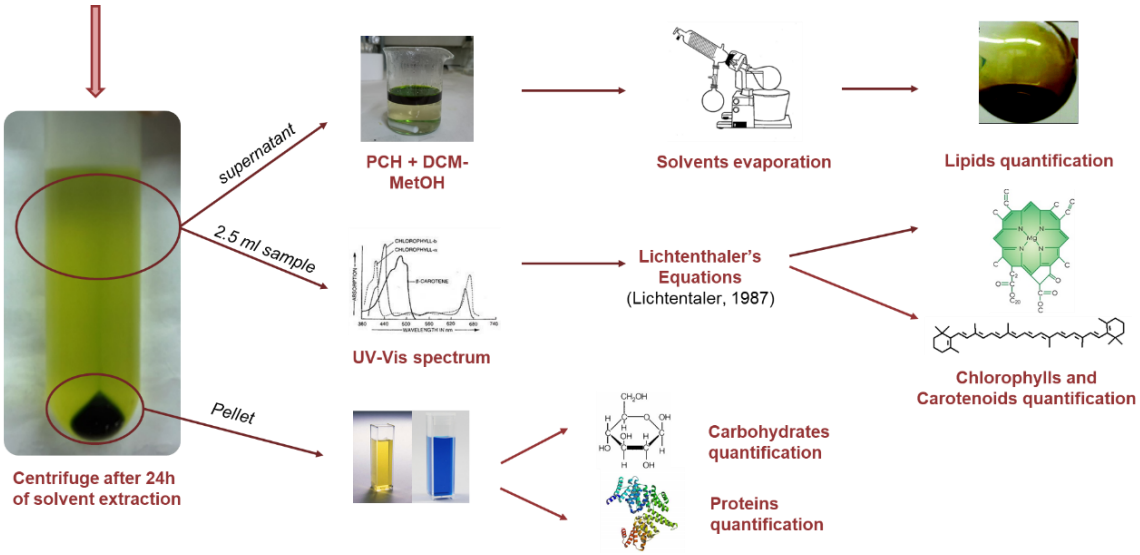

Figure 4.6 Methods employed for solvent and biomass recovery and extraction evaluation

A pellet sample was seen at optical microscope showing that a very small quantity of cells was effectively lysed (Figure 4.7a). To improve the efficiency in the cell walls breaking, the next step was UAE in presence of PCH. 0.6 g of wet biomass in 100 mL of NaDES was treated in a jacketed vessel, refrigerated with water and ethylene glycol at 0 °C, for 40 minutes. Every ten minutes a sample of 1 mL was seen at microscope to supervise the effective break of cells. Figure 4.7b shown the lysed biomass after 40 minutes of UAE.

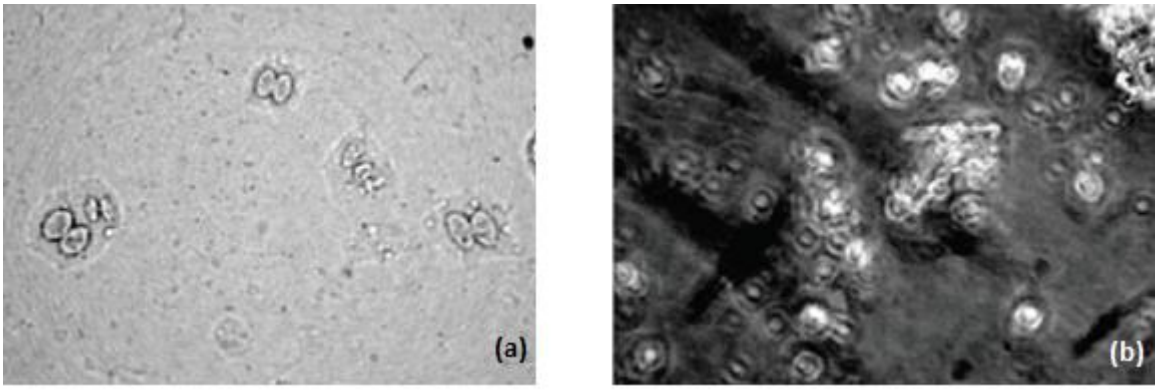

Figure 4.7a Living cells after 24 PCH treatment
Figure 4.7b Lysed cells after 40 minutes of UAE in PCH

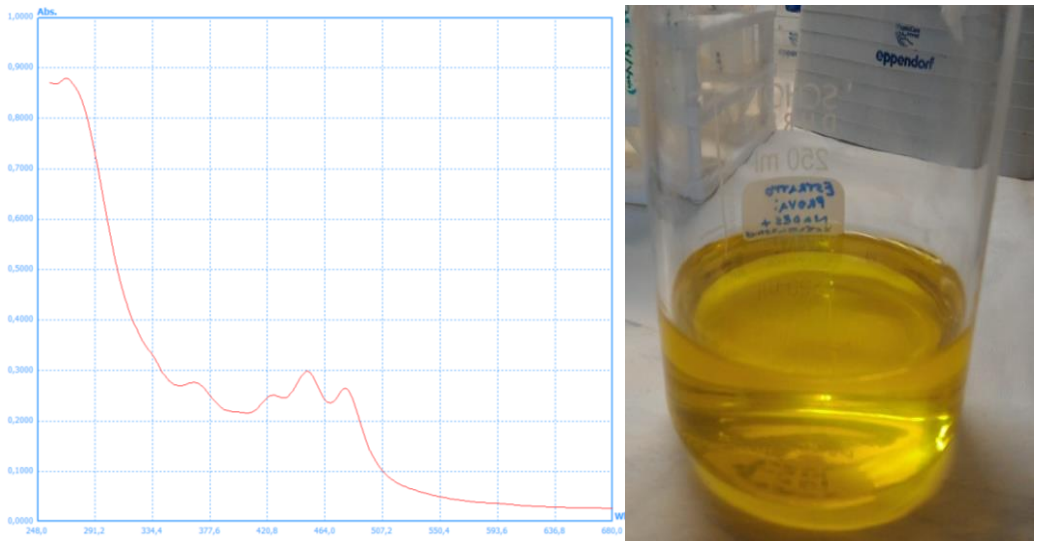

Figure 4.8 Absorbance spectrum of PCH after UAE and PCH after 24 hours of contact with biomass

After the ultrasound cycle the suspension was transferred in a flask and shaken for 24 hours. Biomolecules extraction was evaluated as explained before. In the figure 4.8 is showed the absorbance spectrum of the PCH (diluted 1:1) after UAE pre-treatment and 24 h of contact with biomass, the peak in the region of 480 nm is related to the chlorophyll and the two peaks at 450-430 to the extracted carotenoids. Figure 4.9 reports the percent of extracted molecules, comparing the two methods of cell destruction. The comparison between the two protocols shown a better extraction efficiency for the UAE. This was predictable thanks to the strong lytic action of ultrasounds on cell walls, but it implicates a higher energy waste. In both cases, PCH showed low affinity for nonpolar compounds as neutral lipids, and a satisfactory extractive ability for photosynthetic pigments, carbohydrates and proteins.

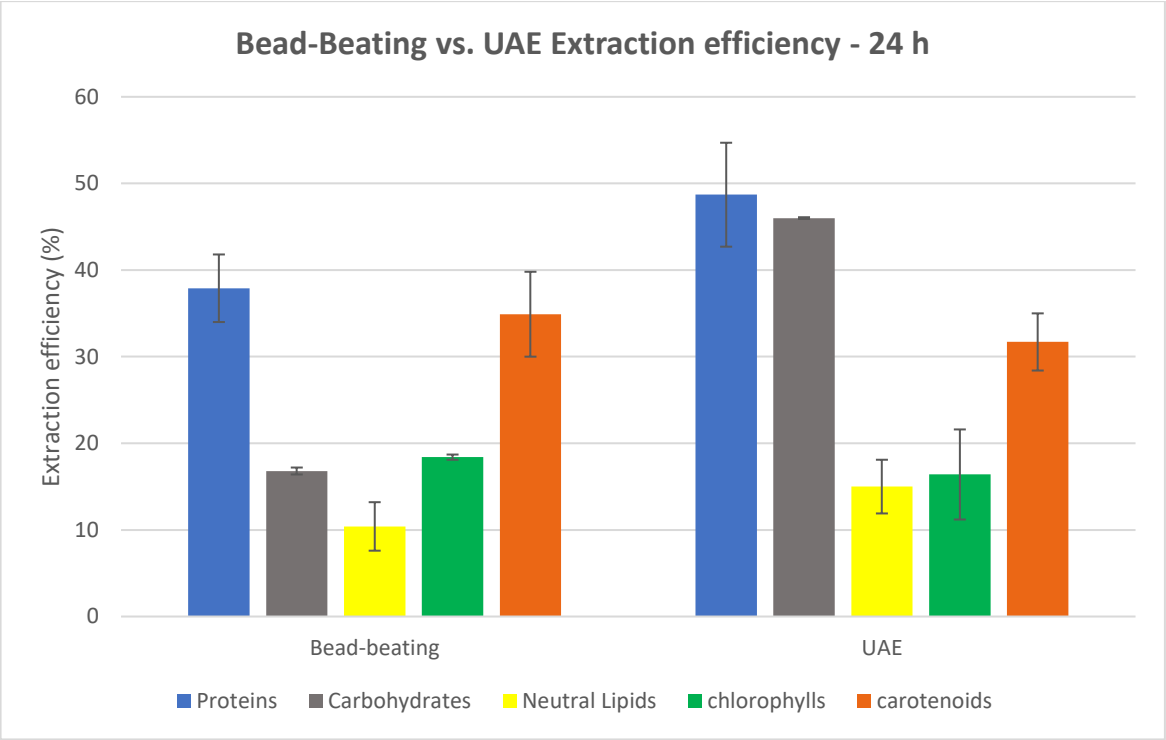

Figure 4.9 S. dimorphus biomolecules extraction efficiency % with Bead-beating mechanical disruption and UAE coupled with PCH.

Biomass exhaustion is one of green chemistry's goal, so the extraction of the greatest part of interesting biomolecules from microalgae is part of the objectives of this thesis. What is interesting to understand is whether the solvent extraction kinetics has reached a plateau in 24 hours or the solvent is still potentially able to extract components. Another set of extractions, which lasted 72 hours, was completed under the same experimental conditions described above, but this time with the only aid of bead beating mechanical treatment. The results of extraction efficiency obtained are shown in the Figure 4.10 and compared with the results obtained in the previous experiment.

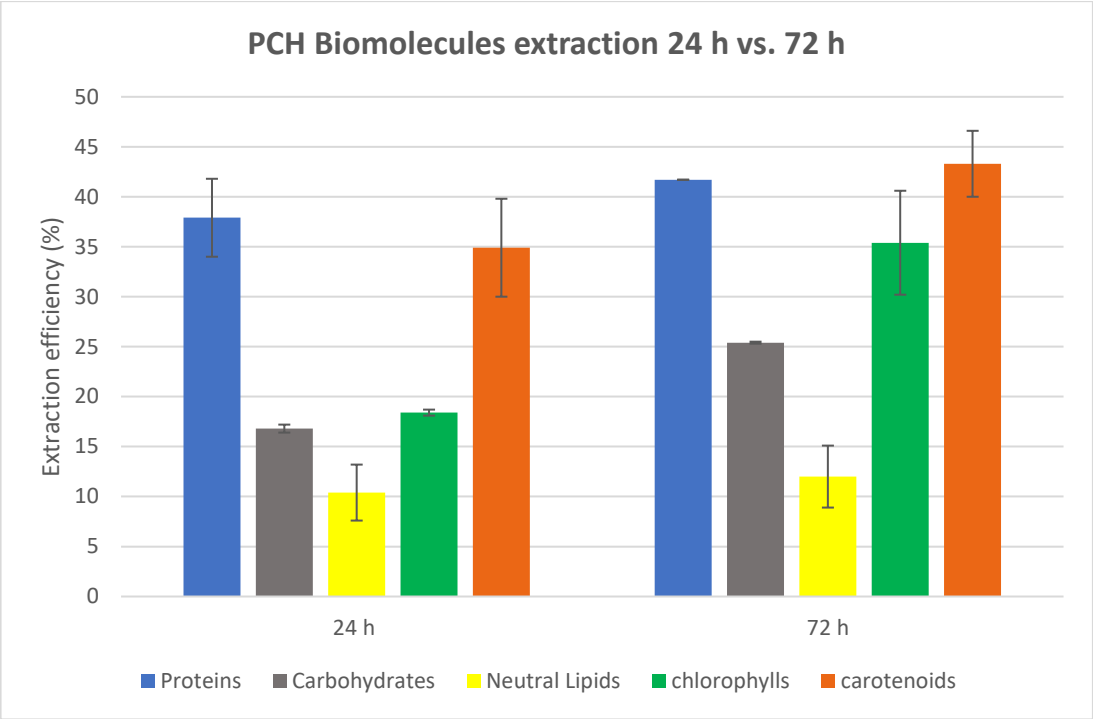

Figure 4.10 PCH Extraction efficiency (%) of proteins, carbohydrates, lipids, chlorophylls and carotenoids after 24 and 72 hours of cells-solvent contact

The chart above show that the concentration of carbohydrates, chlorophylls and carotenoids in the extract increases as the time of extraction is extended from 24 to 72 hours, whereas the extraction efficiency of lipids and proteins remains constant. Proteins and lipids seem to have reached their saturation value in the solvent in only 24 hours and even the ultrasound treatment does not improve their extraction.

When mixed in certain ratios, NaDES change their state from solid to liquid, forming a supramolecular structure, constituted a large network of hydrogen bonds. The viscosity of NaDES in its original equimolar formulation is around the value of 36 mPa*s at the temperature of 40 ° C. These high values do not allow NaDES to be largely employed as extraction solvents, due to the resistance to the mass transfer. Ternary systems, which includes water as one of the components, are particularly interesting because adding water allows tuning physical properties of the liquid, like viscosity, density and polarity. The NaDES in question maintains its characteristics of solvent up to a 50% (wt) of water, after this value the intermolecular structure of hydrogen bonds is weaker and propanediol and choline chloride become single components dissolved in water. Studying the behaviour of the viscosity of the solvent in function of its water content we have seen that for PCH formulated with 20% (wt) water the value goes down to 10.5 mPa*s, the conductivity instead is affected by an increase reaching the value of 27 mS / cm. In this further section, our study

examines how well PCH with a different water ratio (20 wt%) performs in the extraction of cellular components from algal biomass compared to PCH 1: 1: 1 molar ratio (about 7.7% wt).

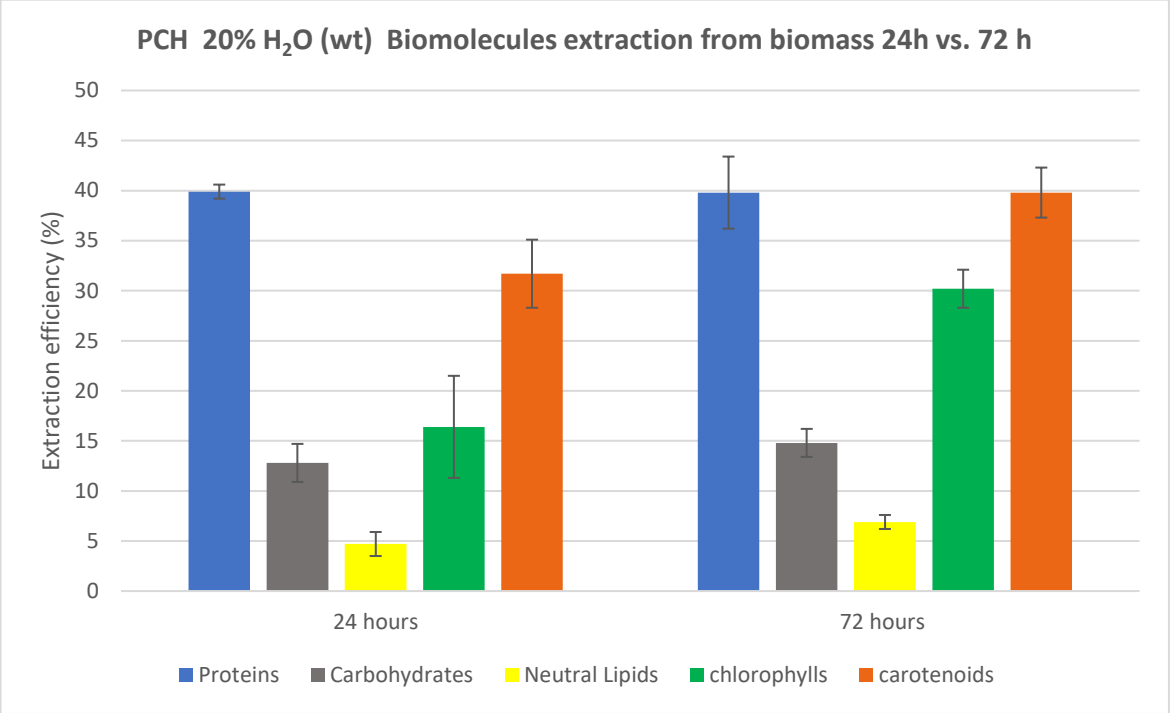

Figure 4.11 PCH diluted with 20% of H_2O extraction efficiency (%) of proteins, carbohydrates, lipids, chlorophylls and carotenoids after 24 and 72 hours of cells-solvent contact

The figure 4.11 shows that the concentration of carbohydrates, chlorophylls and carotenoids increases as the time of extraction is extended from 24h to 72h, for diluted PCH solvent. The extraction efficiencies related to PCH (1:1:1 molar ratio) are always slightly higher. Therefore, viscosity was not an issue in the extraction of molecules for this type of NaDES. The amount of proteins extracted after 24 hours was close to saturation for both solvents. The graphs also show that there was little difference amongst the extraction efficiencies of proteins between the two types of PCH solvents used.

NaDESs could represent good candidates for solvent-based extractions in the food industry, pharmaceutical industry and most processes where biorefinery is involved. For instance, a previous study showed that NaDES PCH alone is not able to attack the cellular walls of algal biomass through osmotic lysis [Cicci et al. 2017].

4.3 Conclusions

When mixed in certain ratios, primary metabolites can form Natural Deep Eutectic Solvents. Most attractive NaDESs have water as a key constituent because, by varying the water content, some major physical-chemical properties of the solvent can be tuned. Here, the PCH NaDES properties and solubility toward microalgal biomass was investigated. The (water-free) PCH has shown to possess a high viscosity, that can be reduced significantly by tuning the PCH:Water composition. NaDESs are liquid solvents and maintain their supermolecular structure at room temperature. PCH covers a fairly large range of polarity, that can be modulated, again, by changing the H_2O content in the DES. For this reason, NaDESs come close to the concept of "switchable solvents" generally attached to tertiary amines and, even though their change of polarity is not as large as that of the mentioned amines case, NaDESs fully implement the nature of "tunable" solvent that switchable solvents do not feature because they only possess two states. Thanks to these features, natural eutectic solvents are suitable to solubilize a wide selection of molecules, which are scarcely soluble, or completely insoluble, in water, such as DNA, pigments, proteins and polysaccharides, as it was shown here with microalgal biomass. One of its most attractive qualities is its sustainability and biodegradability, conferred by their natural and nontoxic ingredients. NaDES ingredients are cheap, the mixtures are easy to prepare with a low energetic waste and solvents have a high temperature of breakdown. These features encourage their utilization in the food, cosmetics, chemical and pharmaceutical industry. Combining PCH with a mechanical destruction method (UAE/Bead-beating) a protocol with potential application in biorefinery for principal classes of biomolecules can be devised.

In this study we also tested the extraction capacity of PCH against its own diluted version, containing 20% wt of water. Two sets of extraction were conducted, one lasting 24 hours and the other 72 hours.

The results showed that PCH, in either form, was able to extract a great amount of components from the biomass with the help of bead-beating, as device of mechanical destruction. The higher extraction efficiencies are related to proteins, carotenoids and carbohydrates.

The most interesting fact was that PCH proved particularly selective towards proteins and carotenoids, reaching concentration values close to saturation after 24 hours of extraction. Even after diluting PCH, its ability to extract proteins remained unaffected, showing

efficiencies above 40%. This is particularly relevant, as NaDES' viscosity is generally one of the major impediments to their applications to chemical processes, and this case proves to be an exception. Moreover, water is cheaper than choline chloride and 1,2 propanediol so, proportionately, the process using diluted PCH is even more efficient when looking at the overall costs. To date, the limited change to nonpolar behaviour limits the PCH potential in lipids extraction. Although PCH investigation is still in its infancy a deeper study on PCH molecular structure could widen its polarity limits and lead to an improvement of its solubility toward nonpolar substances and enable a more complete extraction for the whole biomass.

Chapter 5. A novel switchable-hydrophilicity, natural deep eutectic solvent (NaDES)-based system for the bio-safe biorefinery

Switchable hydrophilicity solvents (SHS) are a new class of solvents that are able to change their nature from hydrophobic to hydrophilic and vice-versa [Jessop et al. 2011]. So far, SHS systems have been created by biphasic systems composed of a hydrophobic liquid organic base and an aqueous layer. Upon addition of CO_2, the liquid base becomes protonated and the resulting bicarbonate salt is fully miscible with water, converting the entire mixture into a single phase. Common SHS functional groups include alkylated amidines or secondary and tertiary amines that act as liquid bases to deprotonate carbonic acid or hydrated CO_2 [Vanderveen et al. 2014]. Switchable hydrophilicity solvents have been used in their hydrophobic form to extract hydrophobic solutes, such as oil from soybean flakes and microalgae, astaxanthin from microalgae, phenols from lignin-derived bio-oils and herbicides from water samples [Jessop et al. 2010, Samorì et al. 2013, Huang et al. 2018, Fu et al. 2014, Lasarte-Aragonés et al. 2015]. In all of these studies the focus is limited to the extraction of only one compound or fraction from the biomass (or the liquid phase), the switching serving the purpose of separating that compound or fraction from the solvent. The SHS in the switched hydrophilic state is not used and must therefore be brought back to the initial hydrophobic condition prior to further use, so that the hydrophilic state of the SHS is necessary for the overall process but useless as far as extraction is concerned. Solvents that exhibit this behaviour are toxic and require complex synthesis procedures. Furthermore, the main problem in the use of these solvents are solvent losses in the process. The water used in the switch procedure and the aqueous phase that were in contact with the solvent are both residues of the process and must be treated appropriately. The solvent toxicity also makes them unsuitable for all those applications where biocompatibility is essential as the food, feed and pharmaceutical industry. It could be interesting to find a natural, biocompatible and easily obtainable solvent with SHS behaviour. Recently, Chen et al. described a switchable hydrophilicity system where the solvent is a fatty acid and the hydrophilic phase to make it switch is based on a dilute aqueous solution of a water-soluble amine [Chen et al. 2017]. The switching mechanism, in this case, is different from that described above, in that the amine is able to create a complex with the fatty acid, so that the oily phase is entirely dissolved into the watery phase and the whole system becomes hydrophilic. Addition of CO_2 reprotonates the carboxylate anion, causing the hydrophobic carboxylic acid to phase separate from the

aqueous phase. In this Chapter, a switchable-hydrophilicity solvent system, consisting of a fatty acids-based natural deep eutectic solvent, complemented by a bio-friendly dilute amine solution, has been introduced. The potential of the most benign switchable solvent system has been characterised in microalgae biorefining according to the recently proposed "Circular Extraction" scheme.

In this part of the study we identified a NaDES, (for us NaDES-Y), which could be commuted in certain conditions. NaDES-Y is a hydrophobic solvent that in contact with water forms two distinct phases. Mixed with a 5% (w/w) aqueous solution of a water-soluble primary amine, it commutes and becomes miscible with water. Injecting CO_2, or adding HCl, the reverse reaction takes place, making the hydrophobic NaDES available again. By removing the absorbed CO_2, heating the solution to 60 ° C, the NaDES is again complexed. Figure shows the switchable behaviour of NaDES-Y.

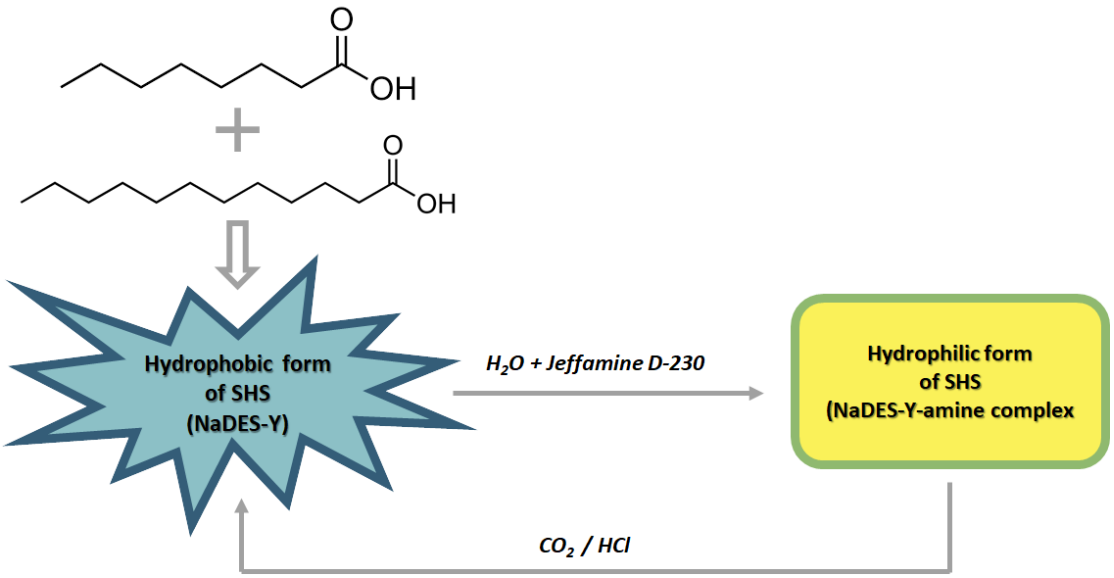

Figure 5.1. NaDES-Y switching system, from hydrophobic form to hydrophilic and vice-versa.

5.1 Materials and Methods

5.1.1 Nades-Y properties

NaDES-Y is an eutectic mixture based on two ingredients: octanoic and dodecanoic acid, mixed in the ratio 1:3. Octanoic acid acts as HBD, dodecanoic acid acts as HBA. This NaDES is highly hydrophobic and exhibits a solidification temperature of 9 °C (compared to 16 °C and 43.8 °C of the individual acids), so that it is normally in the liquid state at ambient temperature. This solvent presents the thermo-physical characteristics at 25 °C reported in Table 5.1 [Florindo et al. 2018]:

Property	Value
Density [g/cm^3]	0.905
Dynamic Viscosity [mPa*s]	8
pH	2
Melting Temperature [°C]	9.1
Water Content (saturation) [wt%]	1.35

Table 5.1 thermo-physical characteristic of NaDES-Y at room temperature

Dynamic viscosity values obtained are lower than those of the hydrophilic NaDES-Y present in the literature and can be explained considering the density values of the used carboxylic acids. The obtained viscosities are very low when compared with those of other low viscosity hydrophobic NaDES such as those based on DL-menthol or on quaternary ammonium salts, whose values typically vary between 11-50 mPa*s and 173-783 mPa*s respectively [Florindo et al. 2017].

5.1.2 Optimization of the direct and inverse switching of NaDES-Y

NaDES-Y switching operation (from hydrophobic to hydrophilic form)

From a detailed study of the literature, it has been concluded that the switch of NaDES-Y could be possible changing the pH of the aqueous solution which the eutectic mixture is in contact with. Three bases have been tested in a NaDES-Y / H_2O biphasic system, in quantities sufficient to reach a pH of at least three units higher than the pK_a of the carboxylic acid mixture.

The characteristics of the hydrophilic NaDES-Y once a single phase was obtained are shown in the Table 5.2.

	Na_2CO_3	NH_3 solution	Jeffamine D-230
pH	8.5	8.9	8.65
Toxicity	LD50 4000 mg/kg	LD50 350 mg/kg	LD50 2885 mg/kg
NaDES-Y : H_2O	-	1:10	1:13
Visual aspect	Turbid (presence of precipitate)	Transparent	Transparent (light-bluish shadow)

Table 5.2 pH of hydrophilic acqueous solution, LD50 of the bases, Nades:solution required for the switch, visual aspect of the hydrophilic NaDES.

Sodium carbonate was discarded due to the formation of precipitate which is not acceptable from the process point of view.

Ammonia was preliminarily tested to confirm that the switchability of NaDES-Y was a pH related issue. The reaction was fast and the required quantities were small if related to the NaDES volume. It was discarded for considerations regarding its toxicity (LD50 equal to 350 mg / kg) and amount of acid required in the subsequent reswitch phase. Being a strong base, a high amount of acid is needed to reduce the pH until the desired value.

Finally, the complexation of NaDES-Y with Jeffamine D-230 was a very fast and exothermic reaction that required moderate agitation. Jeffamine has an oral LD50 value equal to 2885 mg / kg, higher than that of DMCHA and therefore more sustainable and less dangerous. Jeffamine is a weak base and this facilitates the subsequent reswitch operation. All these considerations led us to discard the two options previously described, concentrating the follow-up of the experimentation only on this primary amine.

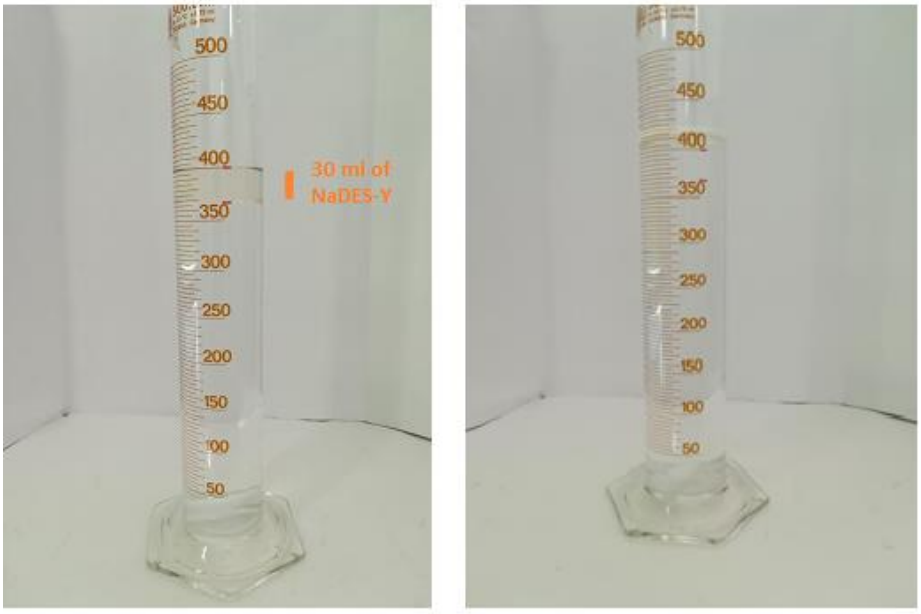

Figure 5.2 Hydrophobic NaDES-Y in contact with water and Hydrophilic NaDES-Y after Jeffamine addition and mixing

NaDES-Y reswitch operation (from hydrophilic to hydrophobic form)

In the first part of the experiment, 30 mL of NaDES-Y were mixed with the 5% (wt) amine solution, in the volume ratio 1:13, and thoroughly mixed, thus obtaining a single, hydrophilic phase and demonstrating that the NaDES-Y can be switched *in situ* (Figure 5.2).

The mixture, initially biphasic, was stirred until a homogeneous aqueous solution was formed (NaDES was complexed by the amine). In the second part of the experiment, Once the aqueous solution was a single homogeneous phase, CO_2 was bubbled into the mixture. CO_2 was infused at a flow rate of 15 NL / h keeping the system refrigerate at a constant temperature of 15 °C, to promote the absorption of carbon dioxide. After 30 minutes, a change in turbidity of the aqueous solution was observed, which turned from transparent to cloudy. Observing the solution, we could see internal flows, probably the hydrophobic phase (hydrophobic NaDES) rose after a period of rest (overnight), because it was possible to observe a lighter phase above the aqueous phase. In the reswitch, CO_2 acted breaking the complex formed between the Jeffamine and the mixture of carboxylic acids reprotonating the carboxylate anions of the NaDES-Y and causing a phase splitting between the NaDES-Y and the amine solution. The CO_2 had indeed encouraged the breakdown of the complex making the recovery of NaDES possible. The solution was then heated to 60 °C for 20 min to allow the CO_2 stripping. At the end of the operation, the hydrophobic DES passed again in aqueous solution. By the CO_2 removal, the amine in the aqueous solution was in fact again available to complex the NaDES. The various stages are shown in the Figure 5.3.

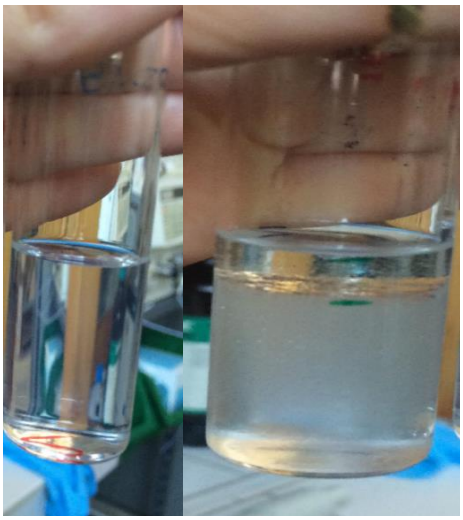

Figure 5.3 Hydrophilic NaDES sample, switched hydrophobic NaDES after CO_2 bubbling

It should be noted that the reversal of the system to the initial split-phase state can be obtained not only by injecting CO_2 but also by acidification with strong acids, such as HCl, although the use of the latter would likely increase the overall environmental impact of the method and would not be reversible, e.g. upon flushing with a gas stream such as air.

The required ratio HCl: NaDES-Y: H_2O is 1: 1: 12.

We have ascertained that the hydrophobic phase that separates from this phase splitting is still the original NaDES-Y by checking that its solidification temperature is unchanged, such as the density, thus demonstrating that the NaDES-Y can also be switched-back *in situ* (Table 5.3).

Density [g/cm^3][l]	Melting temperature [°C]
0.902	**8.7**

Table 5.3 Density and Melting temperature values of hydrophobic NaDES-Y obtained after back-switching

In order to test the suitability of this method for lipid solubilization and release, sunflower oil was used as a model system for triacylglycerols [Jessop et al. 2012]. 1 mL of NaDES-Y and 5 mL of sunflower oil were mixed. A single phase was obtained as results in the Figure 5.4 (b). Upon adding the aqueous amine solution and mixing, a phase split was produced between a hydrophilic phase consisting of the water and ammonium carboxylate salts of the hydrophilic NaDES-Y, and a hydrophobic phase made by sunflower oil (c). In Figure (e) it is possible to see how the NaDES stratifies above the aqueous solution after the CO_2 bubbling for 30 minutes (the orange arrow indicates the ml of regenerate hydrophobic

NaDES). the CO_2 stripping, also in this case, allows the aqueous solution to be able to re-complex the NaDES.

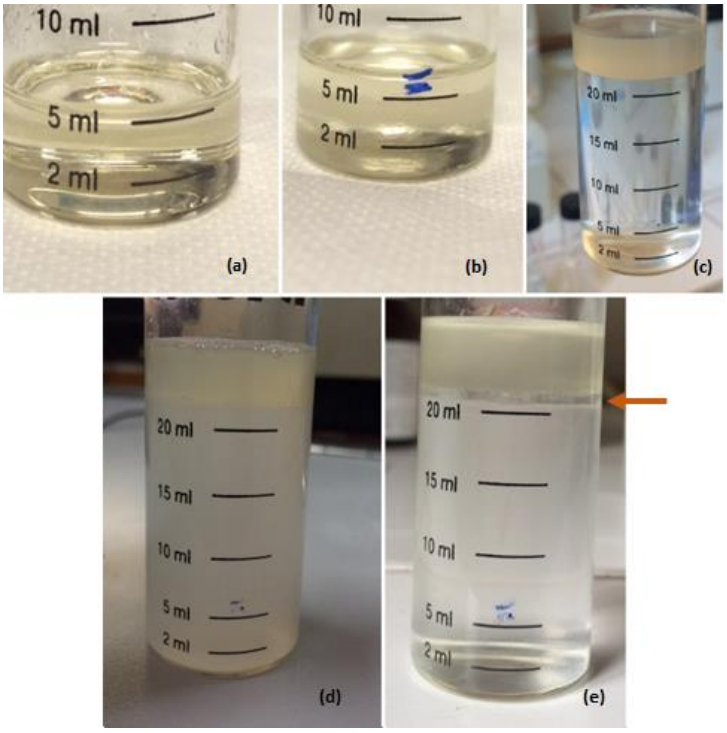

Figure 5.4 5 ml of sunflower oil (a), mixed with 1 ml of Nades-Y (b) in contact with the solution of Jeffamine 5% Nades-Y is switched in its hydrophilic form (c), after the bubbling with CO_2 (d) and overnight rest is possible to see 1 ml of switched back Nades-Y (e)

In Chapter 3 was discussed the possibility of increasing the extraction of microalgal biomass components by exploiting both the native form of the SHS and the hydrophilic form obtained after the switching process, thereby increasing the overall utility of both the algae and the solvent. Was also showed the power of this approach in contributing to the biomass fractionation into the main classes of biologically-relevant substances, and the entailed opportunity for optimising this fractionation by adopting the “forward-mode” (carrying out the extraction first by using the hydrophobic form of the SHS, and then the hydrophilic form) or the “backward-mode” (carrying out the extraction first by using the hydrophilic form of the SHS, and then switching back to the hydrophobic form for solvent recovery and then extraction of hydrophobic components) that can be adopted for the overall solid-liquid extraction unit operation (Figure 5.5). It should be noted that the figure refers only to conceptual facts and does not care about their time sequence. Square and circle mean "hydrophilic step" and "hydrophobic step". Arrows tell the reader what goes in and out, without caring about time. The curved arrows going from the circle to the square and vice-versa indicate that the hydrophobic phase becomes hydrophilic and vice-versa.

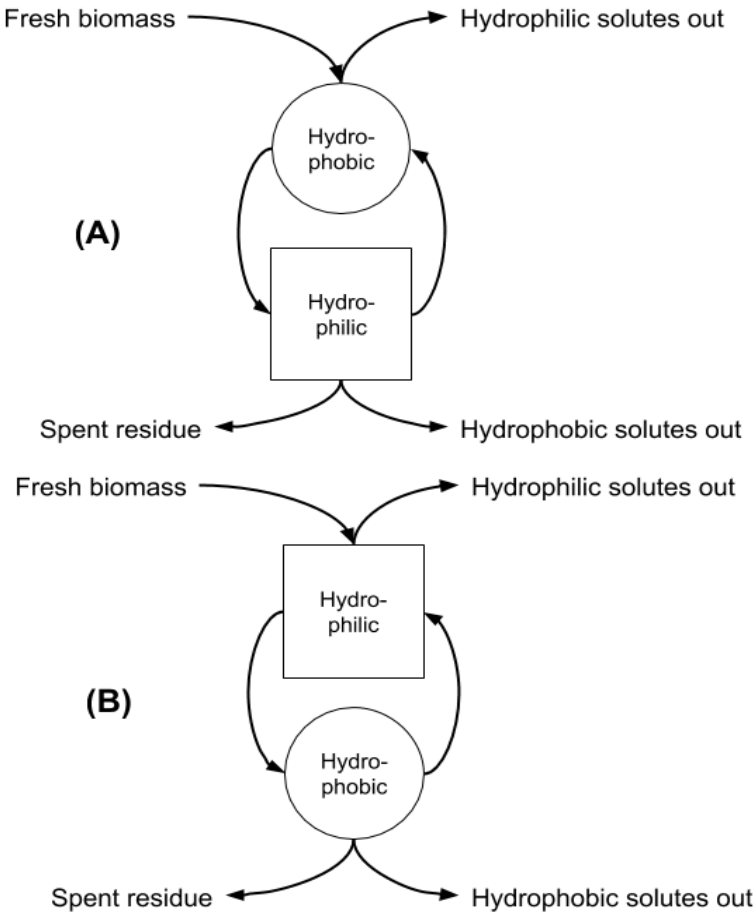

Figure 5.5 Sequential two-stage extraction in forward (hydrophobic first, A) and backward (hydrophilic first, B) modes.

5.1.3 Model matrix formulation

A model matrix was prepared with the same ingredients and the same concentrations as the one shown in the paragraph 3.1.1. The composition of this matrix was chosen to simulate the measured composition of *S. dimorphus* and the water content of "wet" microalgal biomass separated by centrifugation of the cultured suspension (20 minutes at 2600 g). Subsequently, the extraction of the model matrix was carried out with NaDES-Y, in its native hydrophobic state. The model matrix (1.58 g) was thoroughly mixed with 30 mL of NaDES-Y and agitated for 24 h in the presence of glass beads. Then, the agitation was interrupted and any insoluble material was separated and stored for the second-stage extraction. The aqueous amine solution was added to the homogeneous NaDES-Y thus causing the phase split that expels oily fractions. The now-hydrophilic solvent was then collected and used to further treat the insoluble material that had been stored at the end of the first extraction stage, again by prolonged thorough mixing. Finally, any still insoluble material was removed and the liquid was switched back to its initial state. By following the two extraction stages

described here, the complete “forward-mode”, dual-stage circular extraction was complete, and the extraction capability of the SHS was assessed by characterising both the supernatant streams and the solid residuals.

In a separate experiment, the extraction order was reversed, thus following what was named the “backward-mode” circular extraction in our previous work [Cicci et al. 2018]. In this case the model matrix was first treated with NaDES-Y that had been already mixed with the amine solution, thus becoming hydrophilic, producing an aqueous liquid phase and an intermediate solid residue that was stored. After addition of CO_2 to the liquid phase, triggering a phase split, the supernatant (the hydrophobic NaDES-Y), was decanted from the aqueous phase that contained the extracted hydrophilic components from the model biomass mixture. The recovered NaDES-Y was then used to extract hydrophobic components from the intermediate solid residue, thus obtaining a second extract and a final insoluble residue. All streams were compositionally assessed as before, thus completely characterising the backward-mode dual-stage extraction in the synthetic matrix.

5.2 Results and Discussion

The reported results should be interpreted as follows: during the forward-mode extraction (Figure 5.6), 41% of the carbohydrates contained by the model matrix were dissolved by NaDES-Y, which has a hydrophobic character. Switched NaDES-Y, which has a hydrophilic character, managed to extract a further 26% of the initial carbohydrates that had remained in the matrix residue after the first stage of the forward-mode extraction. The total carbohydrates extraction reached therefore 67%. It should be noted that the total extraction is split between two streams; therefore, their recovery should be performed through both the hydrophobic-to-hydrophilic switching and the hydrophilic-to-hydrophobic switching.

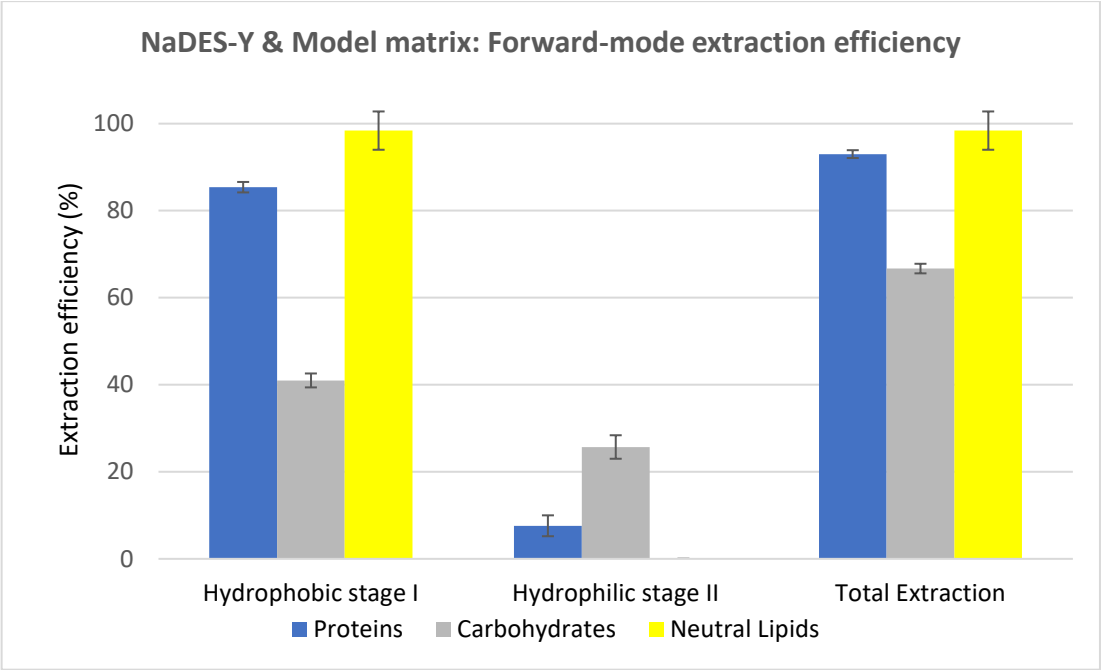

Figure 5.6 Fractional extraction efficiencies on model system extracted in forward-mode.

The backward-mode extraction (Figure 5.7) has different extraction yields, but the same concept holds.

Synthetic matrix extractions results (Figure 5.6 and 5.7) show that, as far as total extraction is concerned, separate consideration may be made for proteins, carbohydrates and neutral lipids, and their distribution in the two subsequent extract streams obtained.

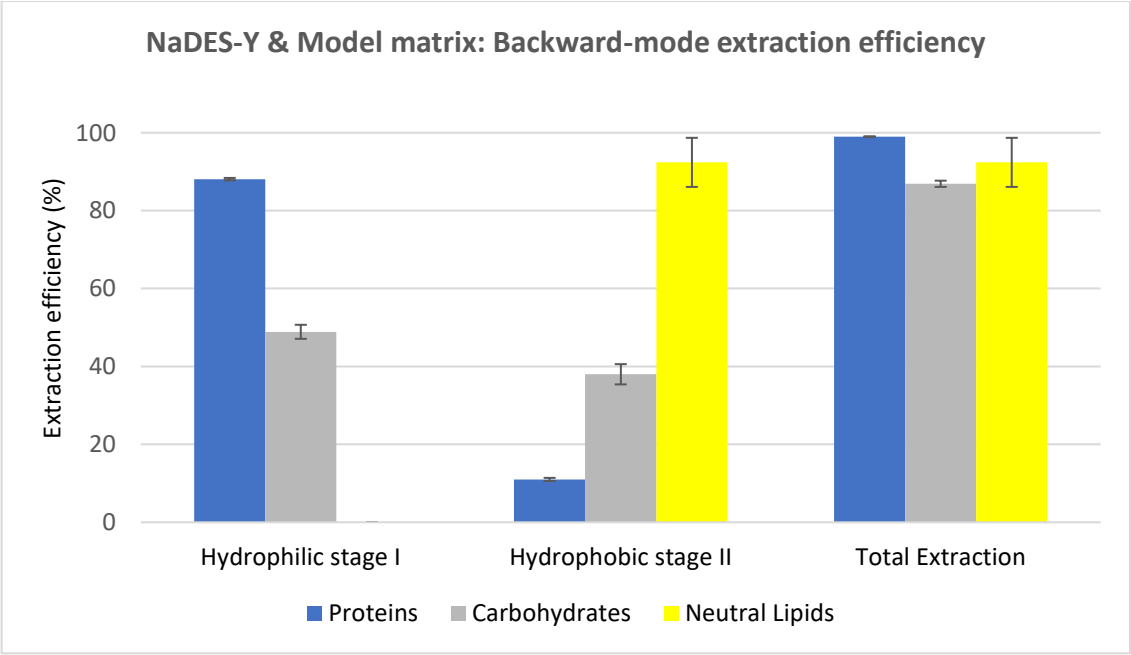

Figure 5.7 Fractional extraction efficiencies on model system extracted in backward-mode.

Neutral lipid extraction is almost quantitative whatever orientation (forward or backward) of the operation is adopted and protein extraction is nearly quantitative, with a slight preference for backward rather than forward orientation (99% vs. 93). In the case of carbohydrates, a more marked difference between forward and backward orientation is observed (66% vs. 86%), again with an advantage of the backward mode over the forward mode. From the point of view of fraction distribution between extracts, neutral lipids are exclusively obtained from the hydrophobic extract and are absent in the hydrophilic extract, whatever the orientation of the operation.

Protein extraction, on the other hand, appears to be split between the two subsequently staged extractions in the opposite way. The extraction occurring in the first place picks up a large fraction of the proteins (85% if it is hydrophobic, 88% if it is hydrophilic), leaving a much smaller fraction to be accomplished by the extraction performed subsequently (8% if it is the hydrophilic follow-up of a forward-mode extraction, 11% if it is the hydrophobic follow-up of a backward-mode extraction). Carbohydrates extraction shows a behaviour that is similar, albeit less marked, to that of proteins. The extraction occurring first picks up a comparatively larger fraction of the proteins originally contained in the matrix (41% if it is hydrophobic, 49% if it is hydrophilic), leaving a somewhat smaller fraction for the extraction coming later (26% if it is the hydrophilic follow-up of a forward-mode extraction, 38% if it is the hydrophobic follow-up of a backward-mode extraction).

While the result observed for neutral lipids was expected, explanation for the behaviour of the proteins is less intrinsically clear. Proteins are equally distributed between hydrophilic- and hydrophobic-character in the synthetic matrix. However, well above the available amount of protein matching the type of solvent (hydrophilic vs. hydrophobic) is extracted in the extraction stage coming first. This might be due to the formation of micellar systems created by the phospholipids that were added to represent cell membranes and eased the extraction, pretty much as it occurs in micelle-assisted protein recovery techniques [Liu et al. 2008]; indeed, the rough solubility of casein (representing hydrophobic proteins) in native (hydrophobic) NaDES-Y is 0.24 g/L, while the calculated casein concentration in our hydrophobic extract (first stage of the forward-mode extraction of the synthetic matrix) is 4.60 g/L; the rough solubility of albumin (representing hydrophilic proteins) in native NaDES-Y is 0.22 g/L, while the calculated albumin concentration in our hydrophobic extract is 4.00 g/L. Analogously, the rough solubility of casein in the hydrophilic form of NaDES-Y is 0.06 g/L, while the calculated casein concentration in our hydrophilic extract (from the first stage of the backward-mode extraction of the synthetic matrix) is 4.35 g/L; the rough

solubility of albumin in hydrophobic-to-hydrophilic-switched NaDES-Y is 0.52 g/L, while the calculated albumin concentration in our hydrophilic extract is 4.45 g/L. Carbohydrates are not only more extensively extracted in backward-mode in the term of the total amount, but they are also dissolved more in each partial step of it (both the first and the second step show a +10% increase).

Prior to the fourth part of the work, microalgal biomass of *Scenedesmus dimorphus* (UTEX 1237) was cultivated in our laboratory and then compositionally assessed. Carbohydrates and proteins were quantified colourimetrically with spectrophotometry; total carbohydrates were quantified by the Dubois assay and total proteins were quantified by the Lowry assay [Dubois et al. 1956; Lowry et al. 1951].

All of the above extraction procedures, both in forward mode and in backwards mode, were also performed on the microalgal biomass rather than the synthetic matrix. First, however, we investigated the capability of the solvent to break the cell wall by agitating a microalgal cells-in-solvent suspension for 24 h. The very poor yield in a subsequent forward-mode extraction indicated that the mildly acidic pH (~2) of the solvent was unable to cause cell rupture by itself, and that an additional cell disruption method would be necessary. Further extractions were performed in the presence of glass beads, which greatly improved the extraction of lipids, and moderately improved the extraction of proteins and carbohydrates.

A further provision to boost the extraction of the lagging fractions was the application of microwaves (90 s at 300 W in a household oven). However, when carrying out the "forward-mode" extraction, we realised that the combined effect of microwaving (which implies heating dipoles such as water) in the presence of an acidic pH (imparted by the fatty acids) caused an extensive degradation of chlorophyll (to pheophytin), a well-known undesired outcome of misperformed sterilisation processes of vegetables in the food industry. The solution, here, came from the very solution adopted in vegetables sterilisation, that is inducing a mild alkalinity in the food mass that must be sterilised. However, in Circular Extraction, it is not necessary to actually perform any alkalinisation, because it is sufficient to adopt the "backward-mode" instead of the forward mode extraction. Indeed, achieved the desired outcome of higher yields without extensive degradation of chlorophyll.

The quantitative results are reported in Figures 5.8, 5.9, 5.10 (microalgal biomass, various extraction implementations in forward- and backward-mode).

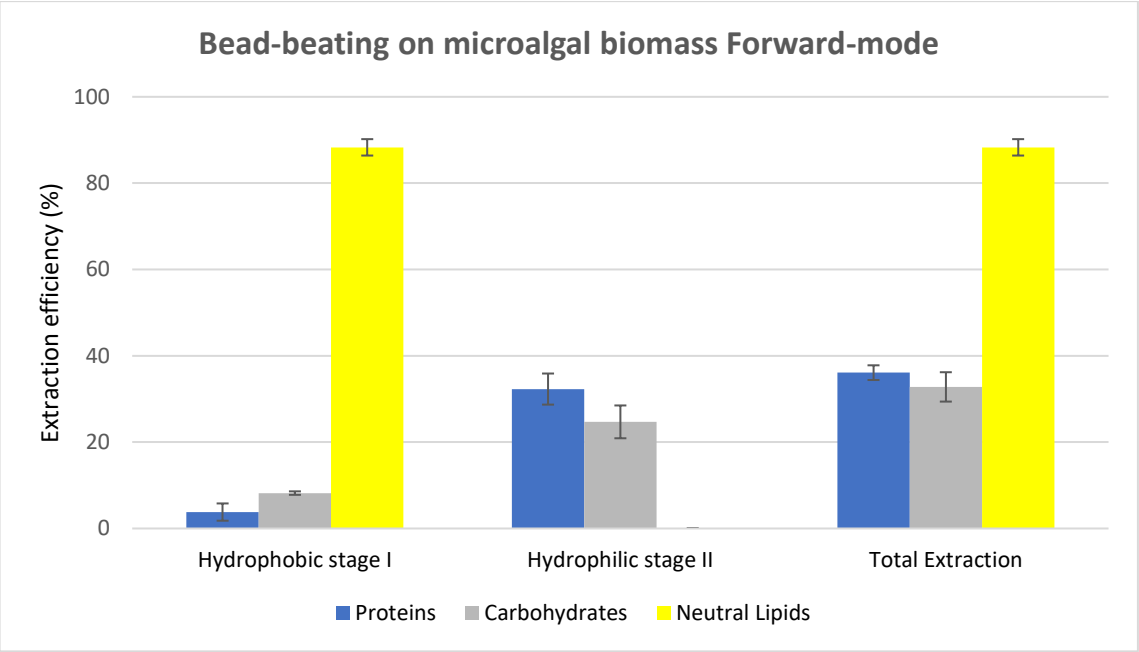

Figure 5.8 Fractional extraction efficiencies on bead beating-assisted microalgal suspension extraction in forward-mode.

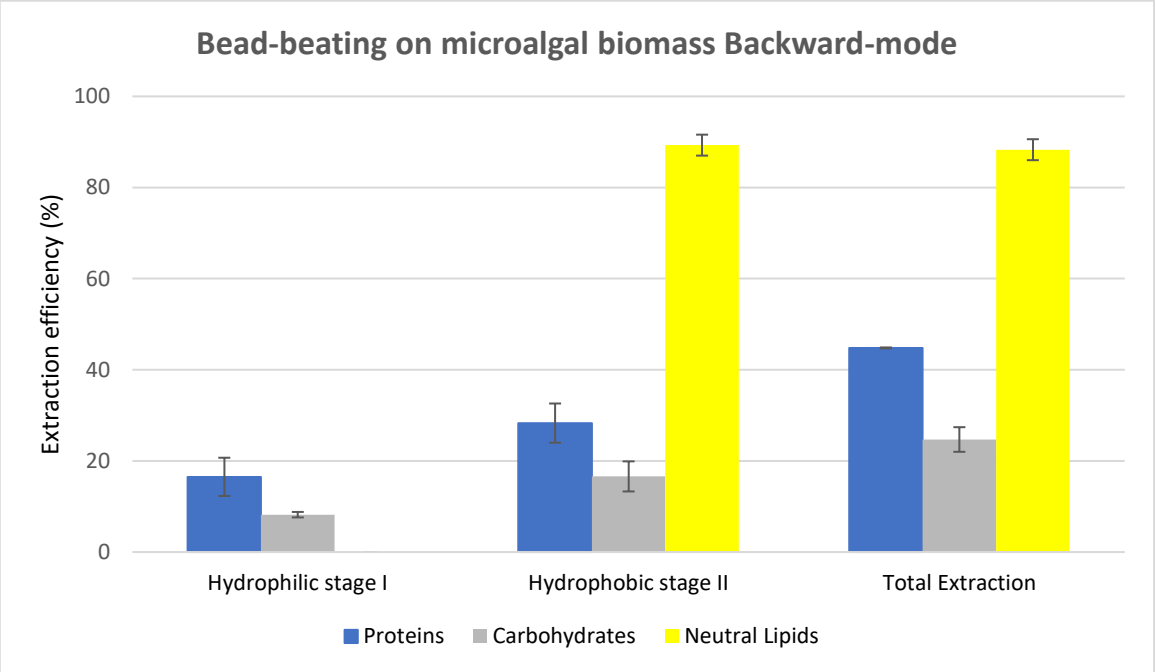

Figure 5.9 Fractional extraction efficiencies on bead beating-assisted microalgal suspension extraction in backward-mode.

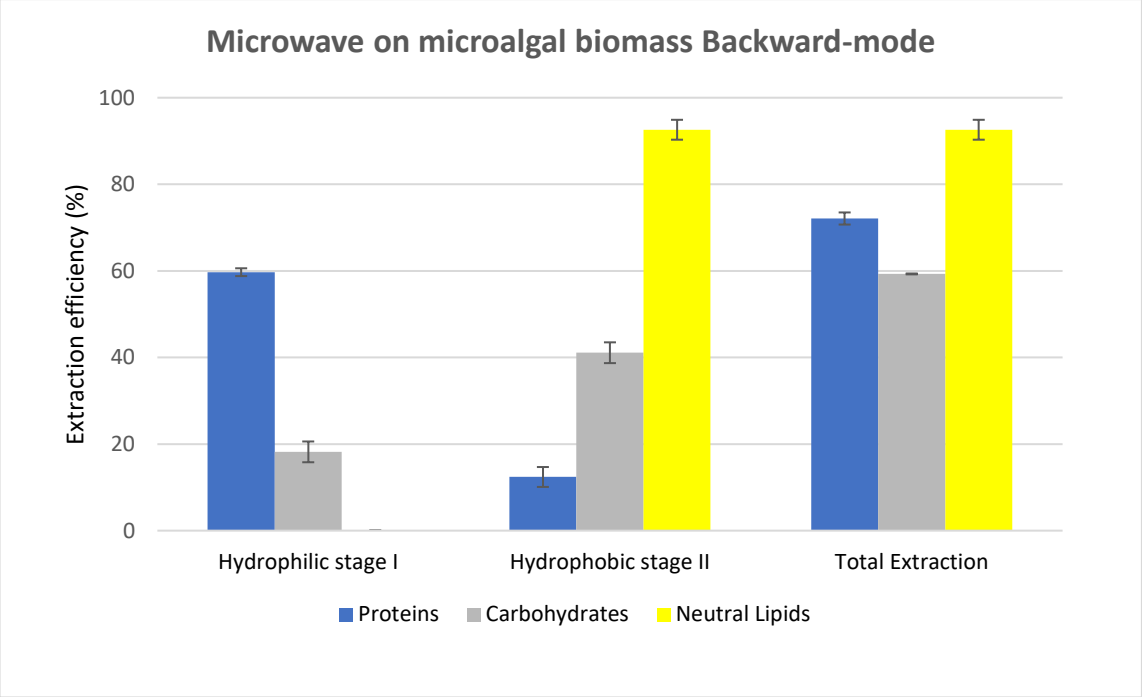

Figure 5.10 Fractional extraction efficiencies on microwave-assisted microalgal suspension extraction in backward-mode.

The results of the work show that the method of cell disruption has a strong effect on the yield of extract. As mentioned above, barely agitating a microalgal suspension in either hydrophobic or pre-switched NaDES-Y does not succeed in ensuring a significant extraction yield most likely due to the unbroken cell wall hindrance and was not investigated further (the extraction efficiency resulted to be 2.0% ± 2.6% for proteins, 5.1% ± 4.4% for carbohydrates, with only traces of neutral lipids). When bead beating was added, a significant improvement to lipid yield was recorded during the hydrophobic step of the forward-mode extraction (88%) (Figure 5.8), while protein and carbohydrate extraction was essentially unchanged. However, during the subsequent hydrophilic stage of the forward-mode extraction, a further 32% and 25% of the microalgal proteins and carbohydrates, respectively, were picked up from the microalgal matrix, thus leading to an overall extraction of 36% of the original proteins and 33% of the original carbohydrates. When reversing the extraction (backward-mode) (Figure 5.9), 17% and 8% of the original proteins and carbohydrates, respectively, were dissolved during the hydrophilic stage; after hydrophilic-to-hydrophobic switching the solvent, a further 28% (proteins) and 17% (carbohydrates) extraction was possible, thus attaining an overall extraction ratio of 45% and 25%, respectively, of the original proteins and carbohydrates of the microalgal matrix. Lipids were extracted exclusively in the hydrophobic stage, and total extraction did not vary appreciably between the forward-mode and the backward-mode conduite of the dual-stage extraction. It is therefore apparent that beads beating ensures a fair yield in lipids, while proteins and

carbohydrate extraction is promoted to a lesser degree. The second cell disruption technique tested, microwave-assisted extraction, could only be applied to the hydrophilic phase and, although it could also have been limited to the hydrophilic stage of the forward-mode extraction, in this study it was limited to (hydrophilic) first stage of the backward-mode extraction. Results (Figure 5.10, "Microwave-assisted microalgal suspension extraction") show a clear improvement in extractability of proteins during the first (hydrophilic) stage of the extraction (54% vs 17% in the first stage of the bead-beaten extraction), while protein extraction in the second stage (hydrophobic, un-microwaved) became less efficient (12% vs 28% in the first stage of the bead-beaten extraction). Overall protein extraction ratio, however, jumped from 45% to 72%. Carbohydrates resembled the proteins behaviour in a mirrored way: their extractability during the first (hydrophilic) stage of the extraction was only slightly promoted by the microwave treatment (18% vs 8% in the first stage of the bead-beaten extraction), while in the second stage (hydrophobic, un-microwaved) their extraction was boosted (41% vs 17% in the second stage of the bead-beaten extraction). Overall carbohydrate extraction, therefore, was significantly promoted, from 25% to 59%.

In combination with a cell wall rupturing technique, therefore, the anticipated extraction potential toward biologic fractions recorded during synthetic matrix extraction experiments was well confirmed on microalgal matrix for lipids, while a 20%-60% lower extraction was recorded for proteins and carbohydrates, most likely due to residual diffusional hindrances and inter-fraction cross-link effects which are the fundamental (non-compositional) difference between the synthetic and the natural matrix.

Compared to the case study reported in the original 'Circular Extraction' article, in the present work extraction ratios are broadly comparable in the cases where NaDES-Y extraction is assisted by some means which is capable of breaking cell walls. However, depending on the type of cell disruption technique deployed, the results may be inferior, comparable, or superior to those obtained with DMCHA. Thus, bead beating-assisted extraction comes close to DMCHA extraction for proteins (36% vs 41%) and lipids (88% vs 96%) but is less efficient with carbohydrates (33% vs 51%) in forward-mode extraction. In backward-mode extraction NaDES-Y, again, comes close to DMCHA efficiency for proteins (45% vs 52%) and lipids (89% vs 93%), while carbohydrate extracting power is half that of DMCHA (25% vs 50%). If microwaved extraction is used the extraction with NaDES-Y improves significantly, as noted before, and thus NaDES-Y ranks as the best extracting medium for proteins (72% vs 52%) and carbohydrates (59.3% vs 51.4%) and comes very close to DMCHA efficiency for lipids (93% vs 96%).

From a biorefinery application perspective, adopting the bead beaten-assisted forward-mode extraction may warrant extracting lipids (88%) during the hydrophobic stage while proteins and carbohydrates extraction task is covered by the hydrophilic stage. On the other hand, by adopting the microwave-assisted extraction backward-mode extraction, during the hydrophilic stage NaDES-Y is able to extract more proteins than DMCHA (54% vs 50%), with less contamination by co-extracted carbohydrates (18% instead of 47%), while the subsequent hydrophobic stage can extract more carbohydrates than DMCHA-based would (41% vs 4%) in the same stage. While it may be observed that lipids would be co-extracted, this would not actually cause any recovery problem, given that the subsequent hydrophobic-to-hydrophilic form switch bring their separation.

5.3 Conclusions

In the present chapter, we demonstrate that NaDES-Y: can be made to switch, thus forming a hydrophilic phase; can be made to switch back, with appropriate means, thus returning to the initial hydrophobic state. It can be also used to solubilise triacylglycerides, and that these ones can be recovered upon switching the SHS to its hydrophilic form and it can be used to extract the biological fractions (proteins, carbohydrates and lipids) making up biomass. This latter part of the work was carried out according to the "Circular Extraction" paradigm in two subsequent steps and in both the "forward-mode" and "backward-mode": a synthetic matrix representing the microalgal biomass, made of selected proteins, lipids, carbohydrates, and water, was extracted by NaDES-Y and the extraction streams were characterised; a sample of the adopted, wet microalgal biomass was extracted by NaDES-Y and the extraction streams were characterised.

NaDES systems are often touted as 'designer solvents' because they offer the possibility of regulating their hydrophilicity by adding water. However, the range of hydrophilicity variation is modest and can be used, at most, to optimise the extraction of certain solutes. Moreover, only hydrophilic NaDESs (and hydrophobic – but not natural – DES) have been found to be amenable to such a tuning scheme. Hydrophilicity cannot be reversed the way SHS allow. In this work, we have devised a way to go back and forth from the hydrophobic to the hydrophilic behaviour and vice versa by reversibly modifying a hydrophobic NaDES with a lean amine in water solution, so that its hydrophobic components are complexed and the resulting solution is hydrophilic. This finding introduces therefore the first switchable

NaDES-based solvent system described in the open literature, although the exact nature of the hydrophilic phase (and, in particular, whether the supramolecular structure is retained, or is destroyed and then reconstituted upon hydrophilic-to-hydrophobic switch) remains to be clarified. The modest amine content of the water solution and the very low toxicity of the adopted amine also duplicate this as a practically bio-safe SHS (at the working concentration of Jeffamine in the hydrophilic phase, the lethal dose is >50 g/kg). Together, the two findings mark a significant step toward the identification of a destination-neutral biorefining tool for the obtainment of biologic fractions from biomasses with a wide range of potential market applications. From an application point of view, this solvent is not able to fracture cell walls, and thus requires a complementary step to achieve this (such as bead beating or microwaving). However, NaDES-Y assisted with a complementary cell-breaking step features an extraction performance which is nearly equal to that of DMCHA for all the examined biological fractions (with beads beating) and may be even higher than that of DMCHA for proteins and carbohydrates (in microwave-assisted extractions). NaDES-Y, with the combined freedom of choice of forward-mode or backward-mode circular extraction and of the (required) cell wall fracturing provision, may supply a powerful tool in the hands of the biochemical process engineer.

Conclusions

In recent years a new class of solvents has emerged, the Switchable Hydrophilicity Solvents (SHSs), which can finally represent a sustainable and economic alternative to conventional volatile solvents used in the chemical industry.

The first SHS studied in this work was the DMCHA and its ability to perform metabolites extraction from microalgal biomass was tested. In order to achieve the best leaching of the microbial cell the "Circular Extraction" was proposed, showing promising extraction efficiencies in both the forward mode and the backward-mode. Chapter 3 introduces and describes a totally new way of using switchable hydrophilicity solvents that at the same time pushes compliance with the principles of Green Chemistry to a new level and introduces new biorefinery strategies. The innovative method disclosed therein makes minimal use of solvents and minimal use of additional chemical compounds by enhancing the utility of a single solvent that, by changing its hydrophilicity, duplicates as two complementary solvents. At the same time, by increasing the range of products recovered and separated, the method reduces waste to a minimum. The operations comprised in the novel method are carried out at atmospheric pressure and moderate temperature and are energy efficient. Their use has the power to revolution biorefinery operations because the splitting of microalgae components from the wet state of integral cells can be performed from the wet state, thus resulting in a significant simplification of the required pretreatment steps, reduction of the relevant energy requirement and efficiency enhancement in the obtainment of the basic biological fractions (proteins, carbohydrates and lipids) from renewable feedstocks. The described method is not limited to the solvent and the biomass resource it was tested upon, but is general in its novelty, has profound implications in the innovation of extraction systems design, and is quite a substantial step toward intrinsic safety in biomass processing.

It may be used to innovate existing liquid-solid extraction unit operations. Continuous solids extraction is normally performed by countercurrent stage-wise contactors (such as the Bollman, Kennedy, Bonotto and Rotocell extractors). The solvent trickles through multiple beds of the extracted biomass or is thoroughly mixed with subsequent confined amounts of biomass, until it reaches saturation, the leanest solids always being contacted with the leanest solvent and the strongest solid always being contacted with the strongest solvent according to the countercurrent extraction principle.

In the present case, two sets of stages would be used and there are two “lean” and two “strong” solvents (the hydrophobic ones and the hydrophilic ones), the leanest hydrophilic solvent being obtained by switching the strongest hydrophobic solvent, and the leanest hydrophobic solvent being obtained by back-switching the strongest hydrophilic solvent. Separate devices would be required for switching the solvents back and forth and collect extracted solutes which, in a laboratory setting, often tend to stick to the container wall, or settle to its bottom.

Then, we focused on tests of biomass depletion in order to obtain kinetic models on both solvent forms, for the extraction of proteins and carbohydrates. Two models have been identified in the literature that could describe the liquid-solid extraction process of DMCHA on the biomass. Data interpolation allowed us to identify the most appropriate model for the two solvent forms and to calculate the characteristic parameters such as mass transfer coefficient and saturation concentration. While the solvent in its hydrophilic phase has limitations only on components diffusion in the liquid bulk, for the hydrophobic form there is an additional resistance due to the different nature of the solvent respect to the water in the cell boundary layer. It can be said that by applying “Circular Extraction” and starting from the hydrophilic solvent it would be possible to extract biomolecules from humid biomass without considering wall diffusion limitations.

Considering the toxicity of the DMCHA, DBAE and DIPAE were tested on the microalgal biomass. DBAE at room temperature it is not stable in its hydrophilic form and tends to commute back to its native form, permitting a better recovery of the original solvent. DIPAE is less hydrophobic than DMCHA therefore its recovery can reach lower percentage, but none of the two alcohols is comparable with the DMCHA in terms of biomolecules extraction efficiencies.

Following the fifth principle of Green Chemistry, a hydrophilic Natural Deep Eutectic Solvent, the PCH, was tested for biorefining. It is a tuneable solvent, its polarity and viscosity can be modulated with the addition of water in its formulation. NaDES alone was inappropriate for extracting microalgal components but coupled with mechanical pretreatments such as bead-beating and UAE it reached acceptable extraction efficiencies.

In the fourth part of the work, a very recent innovative solvent was identified in the literature, NaDES-Y, a hydrophobic Natural Deep Eutectic Solvent. Its chemical nature fully respects the principles of Green Chemistry and REACH regulation. It is interesting for its very low solidification temperature value (only 9 °C) which allows it to be used in its liquid form in

ambient conditions and for its extremely low viscosity (≈7 mPa s) when compared with other NaDESs present in the literature. The first commutable NaDES system was developed switching its hydrophobic form into a hydrophilic one by simply altering its pH in contact with an aqueous solution of a weak amine.

The results on commutability allow us to confirm that the switching reaction is reversible and the recovery of the hydrophobic solvent can be obtained through the addition of an acidifying agent such as carbon dioxide or another inorganic acid. The use of NaDES-Y coupled with pH-modifying agents such as Jeffamine D-230 and CO_2 would contribute to making the ecosystem sustainable and could be said to be in accordance with the principles of Green Chemistry.

The next phase concerned microalgal biorefining through the protocol of Circular Extraction, using reversible NaDES-Y. The main purpose of the work was to identify a solvent that could be competitive with the other solvents tested up to now in the laboratory, e.g. the DMCHA. As for the tertiary amine, the concept of circular extraction was applied, first on a synthetic matrix simulating microalgae biomass and then directly on *Scenedesmus dimorphus* samples. The results obtained from the synthetic matrix were very promising. The solvent in the two forms was able to solubilize high amounts of proteins, carbohydrates and lipids. The percentages obtained have led us to use the solvent on a more complex system such as the algal matrix.

Results obtained on microalgal biomass test highlighted the fact that there are a whole series of mechanisms and complexities that a model matrix cannot explain. The percentages of extraction using only the bead-beating method are slightly lower than the DMCHA in the case of direct extraction but starting from hydrophobic form of NaDES-Y the percentages are quite comparable. In the case of reverse extraction, starting from the hydrophilic solvent led to lower percentage of recovery. By using a biomass pretreatment, such as microwaves, NaDES-Y proves to be competitive, with comparable extraction efficiency respect to the DMCHA, being able to overcome diffusional resistances that slowed down the process.

The kinetic models obtained on DMCHA, both hydrophilic and hydrophobic, have confirmed that it manages to break cell walls, being a very basic solvent and therefore very aggressive. NaDES-Y, in its hydrophobic form is slightly aggressive and its acidity leads to an excessive degradation of chlorophyll. This led us to consider that from the application point of view, it would be convenient to use the "Circular extraction" to effectively separate the fractions of the biomass but it would be better to use the backward-mode.

The model matrix gave us an idea of the potential of the NaDES-Y in the extraction of biomolecules, which were partly confirmed by the biomass tests. It seems necessary for to perform a biomass pretreatment operation, such as microwaving that can facilitate the solvent in the extraction and at the same time does not degrade the biocomponents. The extraction with DMCHA is obviously a more consolidated process without pretreatment requirement, but the very low toxicity and the food grade formulation of the NaDES-Y make it a much more engaging adversary.

The reversibility of NaDES-Y represents a great achievement, considering its switchable formulation that took place in our laboratory. It is at the beginning of its potential development, the first switchable NaDES was the result of efforts in scientific research and in my humble opinion the employment of solvents with the double characteristic of being food grade and switchable in downstream biorefinery processes, is not utopia but it could be implemented in the very near future.

Scientific Production

Papers published in international scientific journals

Sed G., Cicci A., Jessop, P. G. Bravi M. “A novel switchable-hydrophilicity, natural deep eutectic solvent (NaDES)-based system for the bio-safe biorefinery.” *RSC Advances* 8 (2018): 37092-37097

Cicci, A., **Sed, G.,** Jessop, P. G., & Bravi, M. “Circular extraction: an innovative use of switchable solvents for the biomass biorefinery”. *Green Chemistry*, *2018*, *20*(17), 3908-3911.

Sed, G., Cicci, A., and Bravi, M. "Growth of Microalgae in Spectrum-Neutral, Volume-Distributed Light Restriction as the Baseline of Wastewater Exploitation." *Chemical Engineering Transactions* 64 (2018): 667-672.

Mazzelli, A., Cicci, A., **Sed, G.,** & Bravi, M. “Development of a Semi-Theoretical Light Radiation and Photosynthetic Growth Model for the Optimal Exploitation of Wastewaters by Microalgae.” *Chemical Engineering Transactions* 64 (2018): 685-690.

Sed G., Cicci A., Bravi M. “Extraction and Purification of Exopolysaccharides from Exhausted Arthrospira platensis (Spirulina) Culture Systems.” *Chemical Engineering Transactions* 57 (2017): 211-216.

Cicci, A., **Sed, G**., and Bravi, M. "Potential of Choline Chloride–Based Natural Deep Eutectic Solvents (NaDES) in the Extraction of Microalgal Metabolites." *Chemical Engineering Transactions* 57 (2017): 61-66.

Cicci, A., **Sed, G.,** Tirillò, J., Stoller, M., & Bravi, M. “Production and Characterization of Silver Nanoparticles in Cultures of the Cyanobacterium A. Platensis (Spirulina)”. *Chemical Engineering Transactions* 57 (2017): 1405-1410.

References

Abbott, Andrew P., et al. "Deep eutectic solvents formed between choline chloride and carboxylic acids: versatile alternatives to ionic liquids." *Journal of the American Chemical Society* 126.29 (2004): 9142-9147.

Abbott, Andrew P., et al. "Eutectic-based ionic liquids with metal-containing anions and cations." *Chemistry–A European Journal* 13.22 (2007): 6495-6501.

Abomohra, Abd El-Fatah, Mostafa El-Sheekh, and Dieter Hanelt. "Pilot cultivation of the chlorophyte microalga Scenedesmus obliquus as a promising feedstock for biofuel." *biomass and bioenergy* 64 (2014): 237-244.

Adenan, Nurul Salma, Fatimah Md Yusoff, and Mohamed Shariff. "Effect of salinity and temperature on the growth of diatoms and green algae." *Journal of Fisheries and Aquatic Science* 8.2 (2013): 397-404.

Alvarez-Guerra, Enrique, and Angel Irabien. "Separation of proteins by ionic liquid-based three-phase partitioning." *Ionic Liquids in Separation Technology*. 2014. 207-234.

Anastas, Paul T., and John Charles Warner. *Green chemistry: theory and practice*. Vol. 30. Oxford: Oxford university press, 2000.

Anitescu, George, and Thomas J. Bruno. "Fluid properties needed in supercritical transesterification of triglyceride feedstocks to biodiesel fuels for efficient and clean combustion–A review." *The Journal of Supercritical Fluids* 63 (2012): 133-149.

Arad, Shoshana Malis, and Oshrat Levy-Ontman. "Red microalgal cell-wall polysaccharides: biotechnological aspects." *Current opinion in biotechnology* 21.3 (2010): 358-364.

Badger, G. M. "The carcinogenic hydrocarbons: chemical constitution and carcinogenic activity." *British journal of cancer* 2.4 (1948): 309.

Bajguz, Andrzej. "Effect of brassinosteroids on nucleic acids and protein content in cultured cells of Chlorella vulgaris." *Plant Physiology and Biochemistry* 38.3 (2000): 209-215.

Bakuei, Najmeh, et al. "Optimal cultivation of Scenedesmus sp. microalgae in a bubble column photobioreactor." (2015).

Barbarino, Elisabete, and Sergio O. Lourenço. "An evaluation of methods for extraction and quantification of protein from marine macro-and microalgae." *Journal of Applied Phycology* 17.5 (2005): 447-460.

Barham, P. J., and A. Keller. "The relationship between microstructure and mode of fracture in polyhydroxybutyrate." *Journal of Polymer Science Part B: Polymer Physics* 24.1 (1986): 69-77.

Barhdadi, Rachid, et al. "Room-temperature ionic liquids as new solvents for organic electrosynthesis. The first examples of direct or nickel-catalysed electroreductive coupling involving organic halides." *Chemical Communications* 12 (2003): 1434-1435.

Barreiro, Diego López, et al. "Hydrothermal liquefaction (HTL) of microalgae for biofuel production: state of the art review and future prospects." *Biomass and Bioenergy* 53 (2013): 113-127.

Barsanti, Laura, and Paolo Gualtieri. *Algae: anatomy, biochemistry, and biotechnology*. CRC press, 2014.

Bautista-Toledo, María Isidora, et al. "Removal of the surfactant sodium dodecylbenzenesulfonate from water by processes based on adsorption/bioadsorption and biodegradation." *Journal of colloid and interface science* 418 (2014): 113-119.

Beardall, John, and John A. Raven. "The potential effects of global climate change on microalgal photosynthesis, growth and ecology." *Phycologia* 43.1 (2004): 26-40.

Becker, E. W. "Micro-algae as a source of protein." *Biotechnology advances* 25.2 (2007): 207-210.

Becker, E. Wolfgang. *Microalgae: biotechnology and microbiology*. Vol. 10. Cambridge University Press, 1994.

Ben-Amotz, Ami, and Mordhay Avron. "The role of glycerol in the osmotic regulation of the halophilic alga Dunaliella parva." *Plant Physiology* 51.5 (1973): 875-878.

Benemann, John. "Microalgae for biofuels and animal feeds." *Energies* 6.11 (2013): 5869-5886.

Bermejo, R., et al. "Expanded bed adsorption chromatography for recovery of phycocyanins from the microalga Spirulina platensis." *Chromatographia* 63.1-2 (2006): 59-66.

Bionergy I. *IEA Bioenergy Task 42 on Biorefinery*. http://www.biorefinery.nl/fileadmin/biorefinery/docs/Brochure_Totaal_definitief_HR_opt.pdf (Accessed 12 September 2018).

Bitog, J. P., et al. "Application of computational fluid dynamics for modeling and designing photobioreactors for microalgae production: a review." *Computers and Electronics in Agriculture* 76.2 (2011): 131-147.

Blecher, L., and J. Schwedes. "Energy distribution and particle trajectories in a grinding chamber of a stirred ball mill." *Comminution 1994*. 1996. 617-627.

Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Physiol Pharmacol* 1959;**37**:911.

Boisen, S., and B. O. Eggum. "Critical evaluation of in vitro methods for estimating digestibility in simple-stomach animals." *Nutrition research reviews* 4.1 (1991): 141-162.

Bonhote, Pierre, et al. "Hydrophobic, highly conductive ambient-temperature molten salts." *Inorganic chemistry* 35.5 (1996): 1168-1178.

Borines, Myra G., Mark P. McHenry, and Rizalinda L. de Leon. "Integrated macroalgae production for sustainable bioethanol, aquaculture and agriculture in Pacific island nations." *Biofuels, Bioproducts and Biorefining* 5.6 (2011): 599-608.

Borowitzka, Michael A. "Commercial production of microalgae: ponds, tanks, and fermenters." *Progress in industrial microbiology*. Vol. 35. Elsevier, 1999. 313-321.

Borowitzka, Michael A. "Microalgae as sources of pharmaceuticals and other biologically active compounds." *Journal of Applied Phycology* 7.1 (1995): 3-15.

Boyd, Alaina R., et al. "Switchable hydrophilicity solvents for lipid extraction from microalgae for biofuel production." *Bioresource technology* 118 (2012): 628-632.

Breuer, Guido, et al. "The impact of nitrogen starvation on the dynamics of triacylglycerol accumulation in nine microalgae strains." *Bioresource Technology* 124 (2012): 217-226.

Broughton, Edward. "The Bhopal disaster and its aftermath: a review." *Environmental Health* 4.1 (2005): 6.

Burgess, Richard R., and Murray P. Deutscher, eds. *Guide to protein purification*. Vol. 463. Academic Press, 2009.

Butler, Thomas O., et al. "Media screening for obtaining Haematococcus pluvialis red motile macrozooids rich in astaxanthin and fatty acids." *Biology* 7.1 (2017): 2.

Campbell, Peter K., et al. *Greenhouse gas sequestration by algae: energy and greenhouse gas life cycle studies*. CSIRO Energy Transformed Flagship, 2009.

Capuzzo, Andrea, Massimo E. Maffei, and Andrea Occhipinti. "Supercritical fluid extraction of plant flavors and fragrances." *Molecules* 18.6 (2013): 7194-7238.

Carlson AS, van Beilen JB, Möller R, Clayton D. *Micro- and macro-algae: utility for industrial applications. Tall Gables, The Sydings, Speen, Newbury*, Berks: CPL Press; 2007. p. 86.

Carroll, Andrew, and Chris Somerville. "Cellulosic biofuels." *Annual review of plant biology* 60 (2009): 165-182.

Carta, Giorgio, and Alois Jungbauer. *Protein chromatography: process development and scale-up*. John Wiley & Sons, 2010.

Cartens, M., et al. "Eicosapentaenoic acid (20: 5n-3) from the marine microalga Phaeodactylum tricornutum." *Journal of the American Oil Chemists' Society* 73.8 (1996): 1025-1031.

Carvalho, Ana P., and F. Xavier Malcata. "Transfer of carbon dioxide within cultures of microalgae: plain bubbling versus hollow-fiber modules." *Biotechnology progress* 17.2 (2001): 265-272.

Chen, Lin, et al. "Improved aqueous extraction of microalgal lipid by combined enzymatic and thermal lysis from wet biomass of Nannochloropsis oceanica." *Bioresource technology* 214 (2016): 138-143.

Chen, Qianqian, et al. "A fatty acid solvent of switchable miscibility." *Journal of colloid and interface science* 504 (2017): 645-651.

Cheng, Yu-Shen, et al. "The impact of cell wall carbohydrate composition on the chitosan flocculation of Chlorella." *Process Biochemistry* 46.10 (2011): 1927-1933.
Cherubini, Francesco, et al. "Toward a common classification approach for biorefinery systems." *Biofuels, Bioproducts and Biorefining* 3.5 (2009): 534-546.

Cheryan, Munir. *Ultrafiltration and microfiltration handbook*. CRC press, 1998.

Chiaramonti, David, et al. "Review and experimental study on pyrolysis and hydrothermal liquefaction of microalgae for biofuel production." *Applied Energy* 185 (2017): 963-972.

Chinnasamy, Senthil, et al. "Biomass production potential of a wastewater alga Chlorella vulgaris ARC 1 under elevated levels of CO2 and temperature." *International journal of molecular sciences* 10.2 (2009): 518-532.

Chisti, Yusuf, and Murray Moo-Young. "Disruption of microbial cells for intracellular products." *Enzyme and Microbial Technology* 8.4 (1986): 194-204.

Chisti, Yusuf. "Biodiesel from microalgae." *Biotechnology advances* 25.3 (2007): 294-306.
Choi, Sun-A., et al. "Effects of ionic liquid mixtures on lipid extraction from Chlorella vulgaris." *Renewable Energy* 65 (2014): 169-174.

Choi, Young Hae, et al. "Are natural deep eutectic solvents the missing link in understanding cellular metabolism and physiology?" *Plant physiology* 156.4 (2011): 1701-1705.

Chronakis, Ioannis S., et al. "The behaviour of protein preparations from blue-green algae (Spirulina platensis strain Pacifica) at the air/water interface." *Colloids and Surfaces A: Physicochemical and Engineering Aspects* 173.1-3 (2000): 181-192.

Cicci, A., M. Stoller, and M. Bravi. "Microalgal biomass production by using ultra-and nanofiltration membrane fractions of olive mill wastewater." *Water research* 47.13 (2013): 4710-4718.

Cicci A. and M. Bravi, *Chem Eng Trans.*, 2014, **38**, 181-186.

Cicci, Agnese, Giorgia Sed, and Marco Bravi. "Potential of Choline Chloride–Based Natural Deep Eutectic Solvents (NaDES) in the Extraction of Microalgal Metabolites." *Chemical Engineering Transactions* 57 (2017).

Cicci A., G. Sed, P.G. Jessop, M. Bravi, 2018, Green Chemistry. DOI: 10.1039/C8GC01731

Clark, H. J., Deswarte, E.I.F., & Farmer, J.T., 2009, The integration of green chemistry into future biorefineries. *Biofuels, Bioproducts and Biorefining*, *3*(1), 72-90.

Co, Denise YC Lynn, and S. H. Schanderl. "Separation of chlorophylls and related plant pigments by two-dimensional thin-layer chromatography." *Journal of Chromatography A* 26 (1967): 442-448.

Control of Exposure to Perchloroethylene in Commercial Dry cleaning Archived September 1, 2009, at the Wayback Machine. Hazard Controls: Publication 97-157. National Institute for Occupational Safety and Health.

Cuellar-Bermudez, Sara P., et al. "Extraction and purification of high-value metabolites from microalgae: essential lipids, astaxanthin and phycobiliproteins." *Microbial biotechnology* 8.2 (2015): 190-209.

Dai, Yuntao, et al. "Natural deep eutectic solvents as new potential media for green technology." *Analytica chimica acta* 766 (2013): 61-68.

Darani, Kianoush Khosravi, and Mohammad Reza Mozafari. "Supercritical fluids technology in bioprocess industries: a review." *Journal of Biochemical Technology* 2.1 (2010): 144-152.

Darensbourg, Donald J. "Switchable catalytic processes involving the copolymerization of epoxides and carbon dioxide for the preparation of block polymers." *Inorganic Chemistry Frontiers* 4.3 (2017): 412-419.

Darvishmanesh, Siavash, et al. "Performance of nanofiltration membranes for solvent purification in the oil industry." *Journal of the American Oil Chemists' Society* 88.8 (2011): 1255-1261.

Dasgupta, Chitralekha Nag, et al. "Recent trends on the development of photobiological processes and photobioreactors for the improvement of hydrogen production." *International Journal of Hydrogen Energy* 35.19 (2010): 10218-10238.

Dauta, Alain, et al. "Growth rate of four freshwater algae in relation to light and temperature." *Hydrobiologia* 207.1 (1990): 221-226.

Davis, E. M., et al. "Basic microbial degradation rates and chemical byproducts of selected organic compounds." *Water Research* 15.9 (1981): 1125-1127.

De Brouwer, J. F. C., and L. J. Stal. "Does warm-water extraction of benthic diatoms yield extracellular polymeric substances or does it extract intracellular chrysolaminaran?." *European Journal of Phycology* 39.2 (2004): 129-131.

de Farias Silva, Carlos Eduardo, and Alberto Bertucco. "Bioethanol from microalgae and cyanobacteria: a review and technological outlook." *Process Biochemistry* 51.11 (2016): 1833-1842.

de Jesus Raposo, Maria Filomena, Rui Manuel Santos Costa de Morais, and Alcina Maria Miranda Bernardo de Morais. "Health applications of bioactive compounds from marine microalgae." *Life sciences* 93.15 (2013): 479-486.

de Jong E, van Ree R, Sanders JPM, Langeveld H. Biorefineries: giving value to sustainable biomass use. In: Langeveld, Hans, Johan Sanders, and Marieke Meeusen, eds. *The biobased economy: Biofuels, materials and chemicals in the post-oil era*. London: Earthscan Publishers; 2010. p. 111–30

de Jong, Ed, and Gerfried Jungmeier. "Biorefinery concepts in comparison to petrochemical refineries." *Industrial Biorefineries & White Biotechnology*. 2015. 3-33.

De Philippis, Roberto, and Massimo Vincenzini. "Exocellular polysaccharides from cyanobacteria and their possible applications." *FEMS Microbiology Reviews* 22.3 (1998): 151-175.

Demirbas, A., & Demirbas, M. F., 2011, Importance of algae oil as a source of biodiesel. *Energy conversion and management*, *52*(1), 163-170.

Demmer W, Fischer-Fruehholz S, Kocourek A, Nusbaumer D, Wuenn E (2005) Adsorption membrane comprising microporous polymer membrane with adsorbent particles embedded in pores, useful in analysis, for purification or concentration. Patent DE10344820 A1

Denis, Claire, et al. "Concentration and pre-purification with ultrafiltration of a R-phycoerythrin solution extracted from macro-algae Grateloupia turuturu: process definition and up-scaling." *Separation and Purification Technology* 69.1 (2009): 37-42.

Desai, Rupali K., et al. "Extraction of proteins with ABS." *Ionic-Liquid-Based Aqueous Biphasic Systems*. Springer, Berlin, Heidelberg, 2016. 123-134.

Desai, Rupali K., et al. "Novel astaxanthin extraction from Haematococcus pluvialis using cell permeabilising ionic liquids." *Green Chemistry* 18.5 (2016): 1261-1267.

Di Cosimo, Robert, et al. "Industrial use of immobilized enzymes." *Chemical Society Reviews* 42.15 (2013): 6437-6474.

Di Paola L., A. Cicci and M. Bravi, *Chem Eng Trans.*, 2015, **47**, 1321-1326.

Di Salvo, Roberto, et al. "Lipid extraction from microalgae using a single ionic liquid." U.S. Patent No. 8,450,111. 28 May 2013.

Ding, Xueqin, et al. "Design of functional guanidinium ionic liquid aqueous two-phase systems for the efficient purification of protein." *Analytica chimica acta* 815 (2014): 22-32.

Doi, Yoshiharu, Shiro Kitamura, and Hideki Abe. "Microbial synthesis and characterization of poly (3-hydroxybutyrate-co-3-hydroxyhexanoate)." *Macromolecules* 28.14 (1995): 4822-4828.

Dong, Tao, et al. "Two-step in situ biodiesel production from microalgae with high free fatty acid content." *Bioresource Technology* 136 (2013): 8-15.

Draaisma, Rene B., et al. "Food commodities from microalgae." *Current opinion in biotechnology* 24.2 (2013): 169-177.

Du, Ying, et al. "Secondary amines as switchable solvents for lipid extraction from non-broken microalgae." *Bioresource technology* 149 (2013): 253-260.

Du, Ying, et al. "Opportunities for switchable solvents for lipid extraction from wet algal biomass: An energy evaluation." *Algal research* 11 (2015): 271-283.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. T., & Smith, F., 1956, Colorimetric method for determination of sugars and related substances. *Analytical chemistry*, *28*(3), 350-356.

Durand, Erwann, Jérôme Lecomte, and Pierre Villeneuve. "Deep eutectic solvents: Synthesis, application, and focus on lipase-catalyzed reactions." *European journal of lipid science and technology* 115.4 (2013): 379-385.

Economic Impact Analysis of Halogenated Solvent Cleaning (National Emission Standards for Hazardous Air Pollutants, Environmental Protection Agency, Washington, DC, 1993).

Eriksen, Niels T. "The technology of microalgal culturing." *Biotechnology letters* 30.9 (2008): 1525-1536.

Espino, Magdalena, et al. "Natural designer solvents for greening analytical chemistry." *TrAC Trends in Analytical Chemistry* 76 (2016): 126-136.

FAO/WHO Expert Consultation; FAO: Rome, Italy, 1991.

FAS Biofuels Annual, EU Energy and Transport and GHG Emissions Trends to 2050, AEBIOM Statistical Report, Wageningen University Report no 1376, and the Bio-based Industries Consortium (BIC).

Fleurence, Joel. "Seaweed proteins: biochemical, nutritional aspects and potential uses." *Trends in food science & technology* 10.1 (1999): 25-28.

Florindo, C., L. C. Branco, and I. M. Marrucho. "Development of hydrophobic deep eutectic solvents for extraction of pesticides from aqueous environments." *Fluid Phase Equilibria* 448 (2017): 135-142.

Florindo, Catarina, et al. "From phase change materials to green solvents: hydrophobic low viscous fatty acid–based deep eutectic solvents." *ACS Sustainable Chemistry & Engineering* 6.3 (2018): 3888-3895.

Food and Agriculture Organization; World Health Organization. Protein Quality Evaluation—Report of Joint.

Francisco, Maria, Adriaan van den Bruinhorst, and Maaike C. Kroon. "Low-transition-temperature mixtures (LTTMs): A new generation of designer solvents." *Angewandte Chemie international edition* 52.11 (2013): 3074-3085.

Freire, Mara G., et al. "Insight into the interactions that control the phase behaviour of new aqueous biphasic systems composed of polyethylene glycol polymers and ionic liquids." *Chemistry–A European Journal* 18.6 (2012): 1831-1839.

Fu, Dongbao, et al. "Extraction of phenols from lignin microwave-pyrolysis oil using a switchable hydrophilicity solvent." *Bioresource technology* 154 (2014): 101-108.

Furuki, Takao, et al. "Rapid and selective extraction of phycocyanin from Spirulina platensis with ultrasonic cell disruption." *Journal of Applied Phycology* 15.4 (2003): 319-324.

G. Pasteris, D. Werner, K. Kaufmann and P. Hohener, Environ. Sci. technol., 2002, 36, 30-39.

Gao, Kai, Valerie Orr, and Lars Rehmann. "Butanol fermentation from microalgae-derived carbohydrates after ionic liquid extraction." *Bioresource technology* 206 (2016): 77-85.

Garavito, R. Michael, and JIJRG P. Rosenbusch. "Three-dimensional crystals of an integral membrane protein: an initial x-ray analysis." *The Journal of cell biology* 86.1 (1980): 327-329.

Geider, Richard J. "Light and temperature dependence of the carbon to chlorophyll a ratio in microalgae and cyanobacteria: implications for physiology and growth of phytoplankton." *New Phytologist* 106.1 (1987): 1-34.

Geider, Richard J., and Julie La Roche. "Redfield revisited: variability of C [ratio] N [ratio] P in marine microalgae and its biochemical basis." *European Journal of Phycology* 37.1 (2002): 1-17.

Gerardi, Michael H. *The Biology and Troubeshooting of Facultative Lagoons*. Vol. 7. John Wiley & Sons, 2015.

Gerardo ML, Oatley-Radcliffe DL, Lovitt RW (2014) Integration of membrane technology in microalgae biorefineries. J Membr Sci 464:86–99

Gerardo, Michael L., Darren L. Oatley-Radcliffe, and Robert W. Lovitt. "Integration of membrane technology in microalgae biorefineries." *Journal of Membrane Science* 464 (2014): 86-99.

Giménez, A. Giménez, et al. "Downstream processing and purification of eicosapentaenoic (20: 5n-3) and arachidonic acids (20: 4n-6) from the microalga Porphyridium cruentum." *Bioseparation* 7.2 (1998): 89-99.

Gloaguen, Vincent, et al. "The extracellular polysaccharide of Porphyridium sp.: an NMR study of lithium-resistant oligosaccharidic fragments." *Carbohydrate research* 339.1 (2004): 97-103.

Globally Harmonized System of Classification and Labelling of Chemicals (GHS), United Nations, New York and Geneva, 4 th edn, 2011.

Goettel, Martina, et al. "Pulsed electric field assisted extraction of intracellular valuables from microalgae." *Algal Research* 2.4 (2013): 401-408.

Goh, S.W. and S.K. Toh, 1988. Recovery of carotenoids, tocopherols, tocotrienols and sterols from esterified palm oil. GB 2218989.

Gong, Mengyue, and Amarjeet Bassi. "Carotenoids from microalgae: a review of recent developments." *Biotechnology advances* 34.8 (2016): 1396-1412.

Gong, Qitao, et al. "Effects of light and pH on cell density of Chlorella Vulgaris." *Energy Procedia* 61 (2014): 2012-2015.

González-Delgado, Ángel-Darío, and Viatcheslav Kafarov. "Microalgae based biorefinery: Issues to consider." *CT&F-Ciencia, Tecnología y Futuro* 4.4 (2011): 5-22.

Gopal, Jairam S., Gerald D. Holder, and Esin Kosal. "Solubility of solid and liquid mixtures in supercritical carbon dioxide." *Industrial & Engineering Chemistry Process Design and Development* 24.3 (1985): 697-701.

Gorke, Johnathan T., Friedrich Srienk, and Romas J. Kazlaukas. "Deep eutectic solvents for Candida antarctica lipase B-catalyzed reactions." *ACS symposium series*. Vol. 1038. Oxford University Press, 2010.

Greaves, Tamar L., and Calum J. Drummond. "Protic ionic liquids: properties and applications." *Chemical reviews* 108.1 (2008): 206-237.

Greenwell, H. C., et al. "Placing microalgae on the biofuels priority list: a review of the technological challenges." *Journal of the Royal Society Interface* (2009): rsif20090322.

Grobbelaar, J. U.. Turbulence in mass algal cultures and the role of light/dark fluctuations. *Journal of Applied Phycology* 1994 6(3), 331-335.

Grobbelaar, Johan U. "Do light/dark cycles of medium frequency enhance phytoplankton productivity?." *Journal of Applied Phycology* 1.4 (1989): 333-340.

Gu, Tiren, and Johan Sjöblom. "Surfactant structure and its relation to the Krafft point, cloud point and micellization: some empirical relationships." *Colloids and surfaces* 64.1 (1992): 39-46.

Günerken, E., et al. "Cell disruption for microalgae biorefineries." *Biotechnology advances* 33.2 (2015): 243-260.

Guo, Y., et al. "Review of catalytic supercritical water gasification for hydrogen production from biomass." *Renewable and Sustainable Energy Reviews* 14.1 (2010): 334-343.

Gutiérrez, María C., et al. "Bacteria incorporation in deep-eutectic solvents through freeze-drying." *Angewandte Chemie* 122.12 (2010): 2204-2208.

Gutiérrez, María C., et al. "Freeze-drying of aqueous solutions of deep eutectic solvents: a suitable approach to deep eutectic suspensions of self-assembled structures." *Langmuir* 25.10 (2009): 5509-5515.

Gutiérrez, Raquel, et al. "Harvesting microalgae from wastewater treatment systems with natural flocculants: effect on biomass settling and biogas production." *Algal research* 9 (2015): 204-211.

Gutowski, Keith E., et al. "Controlling the aqueous miscibility of ionic liquids: aqueous biphasic systems of water-miscible ionic liquids and water-structuring salts for recycle, metathesis, and separations." *Journal of the American Chemical Society* 125.22 (2003): 6632-6633.

H. Matsuda, T. Shibata, H. Hashimoto and M. Kitai, U.S. Patent, 5, 908,953, 1999 to Mitsubishi Chemical Corporation.

Habulin, Maja, and Z. Knez. "Pressure stability of lipases and their use in different systems." *Acta Chimica Slovenica* 48.4 (2001): 521-532.

Habulin, Maja, Mateja Primozic, and Zeljko Knez. "Supercritical fluids as solvents for enzymatic reactions." *Acta chimica slovenica* 54.4 (2007): 667-677.

Hahn, Thomas, et al. "Novel procedures for the extraction of fucoidan from brown algae." *Process biochemistry* 47.12 (2012): 1691-1698.

Hanotu, James, et al. "Microalgae recovery by microflotation for biofuel production using metallic coagulants." *Biofuels* 4.4 (2013): 363-369.

Harouna-Oumarou, H. Adamou. *Extraction solid-liquide aqueuse de l'aubier de tilleul en regimes discontinue et continue: caracterisation. cinetique, modelisation, developpement pilote*. Diss. PhD Thesis, Universite Pierre et Marie Curie, Paris, 31st Jan, 2006.

Harris, Patricia O., and Gerald J. Ramelow. "Binding of metal ions by particulate biomass derived from Chlorella vulgaris and Scenedesmus quadricauda." *Environmental science & technology* 24.2 (1990): 220-228.

Harun, Razif, et al. "Bioprocess engineering of microalgae to produce a variety of consumer products." *Renewable and Sustainable Energy Reviews* 14.3 (2010): 1037-1047.

Hayyan, Maan, et al. "Are deep eutectic solvents benign or toxic?" *Chemosphere* 90.7 (2013): 2193-2195.

Hedenskog, Gudmund, and Lars Ebbinghaus. "Reduction of the nucleic acid content of single-cell protein concentrates." *Biotechnology and bioengineering* 14.3 (1972): 447-457.

Hedenskog, Gudmund, et al. "Investigation of some methods for increasing the digestibility in vitro of microalgae." *Biotechnology and bioengineering* 11.1 (1969): 37-51.

Herrero, Miguel, Alejandro Cifuentes, and Elena Ibañez. "Sub-and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae: A review." *Food chemistry* 98.1 (2006): 136-148.

Hobbs, Helen R., and Neil R. Thomas. "Biocatalysis in supercritical fluids, in fluorous solvents, and under solvent-free conditions." *Chemical reviews* 107.6 (2007): 2786-2820.

Holland, Amy, et al. "Separation of bitumen from oil sands using a switchable hydrophilicity solvent." *Canadian Journal of Chemistry* 90.10 (2012): 805-810.

Holmes, P. A. "Biologically produced (R)-3-hydroxy-alkanoate polymers and copolymers." *Developments in crystalline polymers*. Springer, Dordrecht, 1988. 1-65.

Hough, Whitney L., et al. "The third evolution of ionic liquids: active pharmaceutical ingredients." *New Journal of Chemistry* 31.8 (2007): 1429-1436.

https://www.epa.gov/sites/production/files/2014-09/documents/pvicvi.pdf. Accessed 1 October 2019

Hu, Qiang, et al. "Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances." *The plant journal* 54.4 (2008): 621-639.

Huang, Jianke, et al. "Development of a novel multi-column airlift photobioreactor with easy scalability by means of computational fluid dynamics simulations and experiments." *Bioresource technology* 222 (2016): 399-407.

Huang, Wen-Can, et al. "Effective astaxanthin extraction from wet Haematococcus pluvialis using switchable hydrophilicity solvents." *ACS Sustainable Chemistry & Engineering* 6.2 (2018): 1560-1563.

IARC monograph. Tetrachloroethylene, Vol. 63, p. 159. Last Updated May 20, 1997. Last retrieved June 22, 2007.

IUCLID Dataset for Cyclohexyldimethylamine, European Commision-European Chemicals Bureau, 2000

Jacob-Lopes, Eduardo, et al. "Microalgal biorefineries." *Biomass Production and Uses*. InTech, 2015.

Janssen, Marcel, et al. "Enclosed outdoor photobioreactors: Light regime, photosynthetic efficiency, scale-up, and future prospects." *Biotechnology and bioengineering* 81.2 (2003): 193-210.

Jazrawi, Christopher, et al. "Pilot plant testing of continuous hydrothermal liquefaction of microalgae." *Algal Research* 2.3 (2013): 268-277.

Jeffrey, S. W. "Profiles of photosynthetic pigments in the ocean using thin-layer chromatography." *Marine Biology* 26.2 (1974): 101-110.

Jeffrey, S. W. "Quantitative thin-layer chromatography of chlorophylls and carotenoids from marine algae." *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 162.2 (1968): 271-285.

Jessop, Philip G., et al. "Green chemistry: Reversible nonpolar-to-polar solvent." *Nature* 436.7054 (2005): 1102.

Jessop, Philip G., et al. "A solvent having switchable hydrophilicity." Green Chemistry 12.5 (2010): 809-814.

Jessop, Philip G. "Searching for green solvents." *Green Chemistry* 13.6 (2011): 1391-1398.

Jessop, Philip G., et al. "Tertiary amine solvents having switchable hydrophilicity." *Green Chemistry* 13.3 (2011): 619-623.

Jessop, Philip G., Sean M. Mercer, and David J. Heldebrant. "CO_2-triggered switchable solvents, surfactants, and other materials." *Energy & Environmental Science* 5.6 (2012): 7240-7253.

John, R. P., and G. S. Anisha. "Macroalgae and their potential for biofuel." *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* 6.038 (2011): 1-15.

Jorquera, Orlando, et al. "Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors." *Bioresource technology* 101.4 (2010): 1406-1413.

Juin, Camille, et al. "Microwave-assisted extraction of phycobiliproteins from Porphyridium purpureum." *Applied biochemistry and biotechnology* 175.1 (2015): 1-15.

Junying, Z. H. U., R. O. N. G. Junfeng, and Z. O. N. G. Baoning. "Factors in mass cultivation of microalgae for biodiesel." *Chinese Journal of Catalysis* 34.1 (2013): 80-100.

Kabo, Gennady J., et al. "Thermodynamic properties of 1-butyl-3-methylimidazolium hexafluorophosphate in the condensed state." *Journal of Chemical & Engineering Data* 49.3 (2004): 453-461.

Kamm, B., and M. Kamm. "Principles of biorefineries." *Applied microbiology and biotechnology* 64.2 (2004): 137-145.

Kamm, B., and M. Kamm. "Biorefineries–multi product processes." *White Biotechnology*. Springer, Berlin, Heidelberg, 2007. 175-204.

Kamm, B., Gruber, P. R., & Kamm, M., 2007, *Biorefineries–industrial processes and products*. Wiley-VCH Verlag GmbH & Co. KGaA.

Kampa, Marilena, and Elias Castanas. "Human health effects of air pollution." *Environmental pollution* 151.2 (2008): 362-367

Katritzky, Alan R., et al. "QSPR correlation of the melting point for pyridinium bromides, potential ionic liquids." *Journal of chemical information and computer sciences* 42.1 (2002): 71-74.

Khoo, H. H., et al. "Bioenergy co-products derived from microalgae biomass via thermochemical conversion–life cycle energy balances and CO2 emissions." *Bioresource technology* 143 (2013): 298-307.

Kim, Young-Hoo, et al. "Ionic liquid-mediated extraction of lipids from algal biomass." *Bioresource technology* 109 (2012): 312-315.

Kim, Jungmin, et al. "Methods of downstream processing for the production of biodiesel from microalgae." *Biotechnology advances* 31.6 (2013): 862-876.

King D. *The future of industrial biorefineries*. http://www3.weforum.org/docs/WEF_FutureIndustrialBiorefineries_Report_2010.pdf

Knez, Željko. "Enzymatic reactions in dense gases." *The Journal of Supercritical Fluids* 47.3 (2009): 357-372.

Köddermann, Thorsten, et al. "The association of water in ionic liquids: a reliable measure of polarity." *Angewandte Chemie International Edition* 45.22 (2006): 3697-3702.

Kommareddy, Anil, and Gary Anderson. "Study of Light as a Parameter in the Growth of Algae in a Photo-Bio Reactor (PBR)." *2003 ASAE Annual Meeting*. American Society of Agricultural and Biological Engineers, 2003.

Kotnik, Tadej, et al. "Electroporation-based applications in biotechnology." *Trends in biotechnology* 33.8 (2015): 480-488.

Kralisch, Dana, et al. "Energetic, environmental and economic balances: Spice up your ionic liquid research efficiency." *Green Chemistry* 7.5 (2005): 301-309.

Kromkamp, Jacco. "Formation and functional significance of storage products in cyanobacteria." *New Zealand Journal of Marine and Freshwater Research* 21.3 (1987): 457-465.

Kumar, SP Jeevan, et al. "Sustainable green solvents and techniques for lipid extraction from microalgae: A review." *Algal Research* 21 (2017): 138-147.

Lai, YenJung Sean, et al. "Multiple synergistic benefits of selective fermentation of Scenedesmus biomass for fuel recovery via wet-biomass extraction." *Algal Research* 17 (2016): 253-260.

Lasarte-Aragonés, Guillermo, et al. "Use of switchable hydrophilicity solvents for the homogeneous liquid–liquid microextraction of triazine herbicides from environmental water samples." *Journal of separation science* 38.6 (2015): 990-995.

Laurens, Lieve ML, et al. "Algal biomass constituent analysis: method uncertainties and investigation of the underlying measuring chemistries." *Analytical chemistry* 84.4 (2012): 1879-1887.

Lee RE. Phycology. 4th ed. Cambridge, England; New York: Cambridge University Press; 2008.

Lee, Jae-Yon, et al. "Comparison of several methods for effective lipid extraction from microalgae." *Bioresource technology* 101.1 (2010): S75-S77.

Lee, S. Y., A. P. J. Middelberg, and Y. K. Lee. "Poly (3-hydroxybutyrate) production from whey using recombinant Escherichia coli." *Biotechnology Letters* 19.10 (1997): 1033-1035.

Lee, Yuan-Kun. "Microalgal mass culture systems and methods: their limitation and potential." *Journal of applied phycology* 13.4 (2001): 307-315.

Lei, Zhigang, et al. "Introduction: ionic liquids." (2017): 6633-6635.

Lemoine, Yves, and Benoît Schoefs. "Secondary ketocarotenoid astaxanthin biosynthesis in algae: a multifunctional response to stress." *Photosynthesis research* 106.1-2 (2010): 155-177.

Lewandowski, Andrzej, and Agnieszka Świderska-Mocek. "Ionic liquids as electrolytes for Li-ion batteries—an overview of electrochemical studies." *Journal of Power Sources* 194.2 (2009): 601-609.

Lewis, Richard J., and N. Sax. *Sax's dangerous properties of industrial materials*. Vol. 12. New York, 1996.

Li, Haifeng, et al. "Pilot-scale isolation of bioactive extracellular polymeric substances from cell-free media of mass microalgal cultures using tangential-flow ultrafiltration." *Process biochemistry* 46.5 (2011): 1104-1109.

Li, Wenjing, et al. "Switching the basicity of ionic liquids by CO 2." *Green Chemistry* 10.11 (2008): 1142-1145.

Liang, Yanna, et al. "Algal biorefineries." *Industrial Biorefineries & White Biotechnology*. 2015. 35-90.

Liau, Bing-Chung, et al. "Supercritical fluids extraction and anti-solvent purification of carotenoids from microalgae and associated bioactivity." *The Journal of Supercritical Fluids* 55.1 (2010): 169-175.

Lichtenthaler, H. K., 1987, Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods in enzymology*, *148*, 350-382.

Liu, C-L., Y. J. Nikas, and D. Blankschtein. "Partitioning of proteins using two-phase aqueous surfactant systems." *AIChE Journal* 41.4 (1995): 991-995.

Loorbach, Derk. "Transition management for sustainable development: a prescriptive, complexity-based governance framework." *Governance* 23.1 (2010): 161-183.

Lorenz, R. Todd, and Gerald R. Cysewski. "Commercial potential for Haematococcus microalgae as a natural source of astaxanthin." *Trends in biotechnology* 18.4 (2000): 160-167.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J., 1951, Protein measurement with the Folin phenol reagent. *J biol Chem*, *193*(1), 265-275.

Lu, Weidong, et al. "A new approach of microalgal biomass pretreatment using deep eutectic solvents for enhanced lipid recovery for biodiesel production." *Bioresource technology* 218 (2016): 123-128.

Lundquist, Tryg J., et al. "A realistic technology and engineering assessment of algae biofuel production." *Energy Biosciences Institute* (2010): 1.

Luque-Garcıa, J. L., and MD Luque De Castro. "Ultrasound: a powerful tool for leaching." *TrAC Trends in Analytical Chemistry* 22.1 (2003): 41-47.

Lutzu, Giovanni Antonio, Antonio Mario Locci, and Giacomo Cao. "Effect of medium composition on the growth of Nannochloris eucaryotum in batch photobioreactors." *Journal of Biobased Materials and Bioenergy* 6.1 (2012): 94-100.

M.D. Guiry in Guiry, M.D. & Guiry, G.M. 2018. *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org; searched on 10 September 2018.

Ma, Steven K., et al. "A green-by-design biocatalytic process for atorvastatin intermediate." *Green Chemistry* 12.1 (2010): 81-86.

Machmudah, Siti, et al. "Extraction of astaxanthin from Haematococcus p luvialis using supercritical CO2 and ethanol as entrainer." *Industrial & engineering chemistry research* 45.10 (2006): 3652-3657.

Macıas-Sánchez, M. D., et al. "Supercritical fluid extraction of carotenoids and chlorophyll a from Nannochloropsis gaditana." *Journal of Food Engineering* 66.2 (2005): 245-251.

Maeda, K., et al. "CO 2 fixation from the flue gas on coal-fired thermal power plant by microalgae." *Energy Conversion and Management* 6.36 (1995): 717-720.

Malihan, Lenny B., et al. "Blended ionic liquid systems for macroalgae pretreatment." *Renewable Energy* 66 (2014): 596-604.

Mamuro, H., Y. Kubota and H. Shiina, 1986. Carotene concentrates. Japanese Patent No. 61282357.

Mandal, Shovon, and Nirupama Mallick. "Microalga Scenedesmus obliquus as a potential source for biodiesel production." *Applied microbiology and biotechnology* 84.2 (2009): 281-291.

Mantoura, R. F. C., and C. A. Llewellyn. "The rapid determination of algal chlorophyll and carotenoid pigments and their breakdown products in natural waters by reverse-phase high-performance liquid chromatography." *Analytica Chimica Acta* 151 (1983): 297-314.

Markou, Giorgos, and Elias Nerantzis. "Microalgae for high-value compounds and biofuels production: a review with focus on cultivation under stress conditions." *Biotechnology advances* 31.8 (2013): 1532-1542.

Marsh, K. N., J. A. Boxall, and R. Lichtenthaler. "Room temperature ionic liquids and their mixtures—a review." *Fluid Phase Equilibria* 219.1 (2004): 93-98.

Mata, Teresa M., Antonio A. Martins, and Nidia S. Caetano. "Microalgae for biodiesel production and other applications: a review." *Renewable and sustainable energy reviews* 14.1 (2010): 217-232.

Matsuda, Tomoko. "Recent progress in biocatalysis using supercritical carbon dioxide." *Journal of bioscience and bioengineering* 115.3 (2013): 233-241.

Medina, A. Robles, et al. "Concentration and purification of stearidonic, eicosapentaenoic, and docosahexaenoic acids from cod liver oil and the marine microalgaIsochrysis galbana." *Journal of the American Oil Chemists' Society* 72.5 (1995): 575-583.

Mendes, Rui L., et al. "Applications of supercritical CO2 extraction to microalgae and plants." *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental AND Clean Technology* 62.1 (1995): 53-59.

Mendes, Rui L., et al. "Supercritical carbon dioxide extraction of compounds with pharmaceutical importance from microalgae." *Inorganica Chimica Acta* 356 (2003): 328-334.

Mendes-Pinto, M. M., et al. "Evaluation of different cell disruption processes on encysted cells of Haematococcus pluvialis: effects on astaxanthin recovery and implications for bio-availability." *Journal of Applied Phycology* 13.1 (2001): 19-24.

Menz, Fredric C., and Hans M. Seip. "Acid rain in Europe and the United States: an update." *Environmental Science & Policy* 7.4 (2004): 253-265.

Mercer, Paula, and Roberto E. Armenta. "Developments in oil extraction from microalgae." *European journal of lipid science and technology* 113.5 (2011): 539-547.

Metting, F. B. "Biodiversity and application of microalgae." *Journal of industrial microbiology* 17.5-6 (1996): 477-489.

Middelberg, Anton PJ. "Process-scale disruption of microorganisms." *Biotechnology advances* 13.3 (1995): 491-551.

Middelberg, Anton, Brian O'Neill, and Connor Thomas. "A simplified model for the disruption of Escherichia coli: the effect of cell septation." *Biotechnology progress* 10.1 (1994): 109-113.

Minhas, Amritpreet K., et al. "A review on the assessment of stress conditions for simultaneous production of microalgal lipids and carotenoids." *Frontiers in microbiology* 7 (2016): 546.

Moheimani, Navid Reza. "Long-term outdoor growth and lipid productivity of Tetraselmis suecica, Dunaliella tertiolecta and Chlorella sp (Chlorophyta) in bag photobioreactors." *Journal of applied phycology* 25.1 (2013): 167-176.

Molina, Emilio, et al. "Tubular photobioreactor design for algal cultures." *Journal of biotechnology* 92.2 (2001): 113-131.

Molina Grima, E., et al. "Recovery of microalgal biomass and metabolites: process options and economics." *Biotechnology advances* 20.7-8 (2003): 491-515.

Molina Grima, Emilio, G. A. Fernández, and A. Robles Medina. "Downstream processing of cell mass and products." *Handbook of microalgal culture: applied phycology and biotechnology* 2 (2013): 267-309.

Mora-Pale, Mauricio, et al. "Room temperature ionic liquids as emerging solvents for the pretreatment of lignocellulosic biomass." *Biotechnology and bioengineering* 108.6 (2011): 1229-1245.

Moroni, Monica, Agnese Cicci, and Marco Bravi. "Experimental investigation of a local recirculation photobioreactor for mass cultures of photosynthetic microorganisms." *Water research* 52 (2014): 29-39.

Morrison, Henry G., Changquan C. Sun, and Sesha Neervannan. "Characterization of thermal behavior of deep eutectic solvents and their potential as drug solubilization vehicles." *International journal of pharmaceutics* 378.1-2 (2009): 136-139.

Mulders, Kim JM, et al. "Phototrophic pigment production with microalgae: biological constraints and opportunities." *Journal of phycology* 50.2 (2014): 229-242.

Najdanovic-Visak, Vesna, et al. "Pressure, isotope, and water co-solvent effects in liquid− liquid equilibria of (ionic liquid+ alcohol) systems." *The Journal of Physical Chemistry B* 107.46 (2003): 12797-12807.

National Research Council. *Climate change science: An analysis of some key questions*. National Academies Press, 2001.

Nazari, Shidokht, et al. "Physicochemical properties of imidazo-pyridine protic ionic liquids." *Journal of Materials Chemistry A* 1.38 (2013): 11570-11579.

Nurra, C., et al. "Biorefinery concept in a microalgae pilot plant. Culturing, dynamic filtration and steam explosion fractionation." *Bioresource technology* 163 (2014): 136-142.

Olmstead, Ian LD, et al. "A quantitative analysis of microalgal lipids for optimization of biodiesel and omega-3 production." *Biotechnology and bioengineering* 110.8 (2013): 2096-2104.

Ong, A.S.H. and P.L. Boey, 1980. An improved method for extraction of carotene. British Patent No. 1562794.

Orr, Valerie CA, and Lars Rehmann. "Ionic liquids for the fractionation of microalgae biomass." *Current Opinion in Green and Sustainable Chemistry* 2 (2016): 22-27.

Orr, Valerie CA, et al. "Disruption and wet extraction of the microalgae Chlorella vulgaris using room-temperature ionic liquids." *ACS Sustainable Chemistry & Engineering* 4.2 (2015): 591-600.

Paiva, Alexandre, et al. "Natural deep eutectic solvents–solvents for the 21st century." *ACS Sustainable Chemistry & Engineering* 2.5 (2014): 1063-1071.

Park, Ji-Yeon, et al. "Acid-catalyzed hot-water extraction of lipids from Chlorella vulgaris." *Bioresource technology* 153 (2014): 408-412.

Park, Ji-Yeon, et al. "Effects of anionic surfactant on extraction of free fatty acid from Chlorella vulgaris." *Bioresource technology* 166 (2014): 620-624.

Pasquet, Virginie, et al. "Study on the microalgal pigments extraction process: Performance of microwave assisted extraction." *Process Biochemistry* 46.1 (2011): 59-67.

Patel, Anil Kumar, et al. "Separation and fractionation of exopolysaccharides from Porphyridium cruentum." *Bioresource technology* 145 (2013): 345-350.

Patil, Ganapathi, and K. S. M. S. Raghavarao. "Aqueous two-phase extraction for purification of C-phycocyanin." *Biochemical Engineering Journal* 34.2 (2007): 156-164.

Perkins, Roger, et al. "Quantitative structure-activity relationship methods: Perspectives on drug discovery and toxicology." *Environmental Toxicology and Chemistry* 22.8 (2003): 1666-1679.

Petkovic, Marija, et al. "Ionic liquids: a pathway to environmental acceptability." *Chemical Society Reviews* 40.3 (2011): 1383-1403.

Phan, Lam, et al. "Soybean oil extraction and separation using switchable or expanded solvents." *Green chemistry* 11.1 (2009): 53-59.

Plechkova, Natalia V., and Kenneth R. Seddon. "Applications of ionic liquids in the chemical industry." *Chemical Society Reviews* 37.1 (2008): 123-150.

Posten, Clemens. "Design principles of photo-bioreactors for cultivation of microalgae." *Engineering in Life Sciences* 9.3 (2009): 165-177.

Postma, P. R., et al. "Selective extraction of intracellular components from the microalga Chlorella vulgaris by combined pulsed electric field–temperature treatment." *Bioresource technology* 203 (2016): 80-88.

Prat, Denis, et al. "CHEM21 selection guide of classical-and less classical-solvents." *Green Chemistry* 18.1 (2015): 288-296.

Prat, Denis, et al. "Sanofi's solvent selection guide: a step toward more sustainable processes." *Organic Process Research & Development* 17.12 (2013): 1517-1525.

Praveenkumar, Ramasamy, et al. "Breaking dormancy: an energy-efficient means of recovering astaxanthin from microalgae." *Green Chemistry* 17.2 (2015): 1226-1234.

Presidency, E. U. "En route to the knowledge-based bio-economy." *Cologne, Cologne Summit of the German Presidency* (2007).

Pulz, Otto. "Photobioreactors: production systems for phototrophic microorganisms." *Applied microbiology and biotechnology* 57.3 (2001): 287-293.

Radošević, Kristina, et al. "Evaluation of toxicity and biodegradability of choline chloride based deep eutectic solvents." *Ecotoxicology and environmental safety* 112 (2015): 46-53.

Randall, Lenore G. "The present status of dense (supercritical) gas extraction and dense gas chromatography: impetus for DGC/MS development." *Separation science and technology* 17.1 (1982): 1-118.

Rastogi RP, Sonani RR, Madamwar D. Effects of PAR and UV Radiation on the Structural and Functional Integrity of Phycocyanin, Phycoerythrin and Allophycocyanin Isolated from the Marine Cyanobacterium Lyngbya sp. A09DM. Photochem Photobiol. 2015;91:837–844.

Rastogi, Rajesh Prasad, Datta Madamwar, and Ashok Pandey, eds. *Algal Green Chemistry: Recent Progress in Biotechnology*. Elsevier, 2017.

Rengstl, Doris, Veronika Fischer, and Werner Kunz. "Low-melting mixtures based on choline ionic liquids." *Physical Chemistry Chemical Physics* 16.41 (2014): 22815-22822.

Ribeiro, Ana Paula Badan, et al. "Solvent recovery from soybean oil/hexane miscella by polymeric membranes." *Journal of Membrane Science* 282.1-2 (2006): 328-336.

Ribeiro, Bernardo D., et al. "Menthol-based eutectic mixtures: hydrophobic low viscosity solvents." *ACS Sustainable Chemistry & Engineering* 3.10 (2015): 2469-2477.

Richmond A. (2004). Biological Principles of Mass Cultivation. In: Richmond, A. (Ed.), Handbook of Microalgal Culture.Biotechnology and Applied Phycology. Blackwell Publishing, Oxford, UK, p. 125-177.

Riley, J. P., and T. R. S. Wilson. "The use of thin-layer chromatography for the separation and identification of phytoplankton pigments." *Journal of the Marine Biological Association of the United Kingdom* 45.3 (1965): 583-591.

Ritchie, Glenn D., et al. "A review of the neurotoxicity risk of selected hydrocarbon fuels." *Journal of Toxicology and Environmental Health Part B: Critical Reviews* 4.3 (2001): 223-312.

Robles-Medina, A., et al. "Biocatalysis: towards ever greener biodiesel production." *Biotechnology advances* 27.4 (2009): 398-408.

Román, R. Bermejo, et al. "Recovery of pure B-phycoerythrin from the microalga Porphyridium cruentum." *Journal of Biotechnology* 93.1 (2002): 73-85.

Rönnlund, I., et al. "Waste to energy by industrially integrated supercritical water gasification–Effects of alkali salts in residual by-products from the pulp and paper industry." *Energy* 36.4 (2011): 2151-2163.

Ruiz, Jesús, et al. "Towards industrial products from microalgae." *Energy & Environmental Science* 9.10 (2016): 3036-3043.

Safi, Carl, et al. "Aqueous extraction of proteins from microalgae: effect of different cell disruption methods." *Algal Research* 3 (2014): 61-65.

Safi, Carl, et al. "Influence of microalgae cell wall characteristics on protein extractability and determination of nitrogen-to-protein conversion factors." *Journal of applied phycology* 25.2 (2013): 523-529.

Safi, Carl, et al. "Release of hydro-soluble microalgal proteins using mechanical and chemical treatments." *Algal Research* 3 (2014): 55-60.

Samorì, Chiara, et al. "Effective lipid extraction from algae cultures using switchable solvents." *Green chemistry* 15.2 (2013): 353-356.

Samorì, Chiara, et al. "Extraction of hydrocarbons from microalga Botryococcus braunii with switchable solvents." *Bioresource technology* 101.9 (2010): 3274-3279.

Sánchez, J. F., et al. "Biomass and lutein productivity of Scenedesmus almeriensis: influence of irradiance, dilution rate and temperature." *Applied microbiology and biotechnology* 79.5 (2008): 719-729.

Sanders, Johan, and Diederik Van der Hoeven. "Opportunities for a Bio-based Economy in the Netherlands." *Energies* 1.3 (2008): 105-119.

Sanders, Johan, et al. "Bio-refinery as the bio-inspired process to bulk chemicals." *Macromolecular bioscience* 7.2 (2007): 105-117.

Sarada, R., et al. "An efficient method for extraction of astaxanthin from green alga Haematococcus pluvialis." *Journal of agricultural and food chemistry* 54.20 (2006): 7585-7588.

Sawan, Samuel P., *Supercritical fluid cleaning: fundamentals, technology and applications*. Elsevier, 1998.

Sazdanoff, Nicholas. *Modeling and simulation of the algae to biodiesel fuel cycle*. Diss. The Ohio State University, 2006.

Schürholz, Theo, Anke Gieselmann, and Eberhard Neumann. "Miscibility gap of octyl glucoside-phosphatidylcholine micellar solutions. Partition of the nicotinic acetylcholine receptor into the surfactant-rich phase." *Biochimica et Biophysica Acta (BBA)-Biomembranes* 986.2 (1989): 225-233.

Schwenzfeier, Anja, et al. "Emulsion properties of algae soluble protein isolate from Tetraselmis sp." *Food hydrocolloids* 30.1 (2013): 258-263.

Schwenzfeier, Anja, et al. "Foam properties of algae soluble protein isolate: Effect of pH and ionic strength." *Food Hydrocolloids* 33.1 (2013): 111-117.

Schwenzfeier, Anja, Peter Alexander Wierenga, and Harry Gruppen. "Isolation and characterization of soluble protein from the green microalgae Tetraselmis sp." *Bioresource technology* 102.19 (2011): 9121-9127.

Sed, Giorgia, Agnese Cicci, and Marco Bravi. "Extraction and Purification of Exopolysaccharides from Exhausted Arthrospira platensis (Spirulina) Culture Systems." *Chemical Engineering Transactions* 57 (2017): 211-216.

Sekharam, K. Madhavi, L. V. Venkataraman, and P. V. Salimath. "Structural studies of a glucan isolated from blue-green alga Spirulina platensis." *Food chemistry* 31.2 (1989): 85-91.

Seki, Tsunetake, Jan-Dierk Grunwaldt, and Alfons Baiker. "Heterogeneous catalytic hydrogenation in supercritical fluids: potential and limitations." *Industrial & engineering chemistry research* 47.14 (2008): 4561-4585.

Sendur, Omer Faruk, et al. "Toxic neuropathy due to N-hexane: report of three cases." *Inhalation toxicology* 21.3 (2009): 210-214.

Sharma, Kalpesh K., Holger Schuhmann, and Peer M. Schenk. "High lipid induction in microalgae for biodiesel production." *Energies* 5.5 (2012): 1532-1553.

Sheldon, Roger A. "Consider the environmental quotient." *CHEMTECH;(United States)* 24.3 (1994).

Shelef, G., A. Sukenik, and M. Green. *Microalgae harvesting and processing: a literature review*. No. SERI/STR-231-2396. Technion Research and Development Foundation Ltd., Haifa (Israel), 1984.

Shoaf, W. Thomas. "Rapid method for the separation of chlorophylls a and b by high-pressure liquid chromatography." *Journal of Chromatography A* 152.1 (1978): 247-249.

Sim, T-S., A. Goh, and E. W. Becker. "Comparison of centrifugation, dissolved air flotation and drum filtration techniques for harvesting sewage-grown algae." *Biomass* 16.1 (1988): 51-62.

Singh, Anoop, Poonam Singh Nigam, and Jerry D. Murphy. "Renewable fuels from algae: an answer to debatable land-based fuels." *Bioresource technology* 102.1 (2011): 10-16.

Smiglak, Marcin, et al. "Combustible ionic liquids by design: is laboratory safety another ionic liquid myth?" *Chemical Communications* 24 (2006): 2554-2556.

Smith, Emma L., Andrew P. Abbott, and Karl S. Ryder. "Deep eutectic solvents (DESs) and their applications." *Chemical reviews* 114.21 (2014): 11060-11082.

Soman, Abhinav, and Yogendra Shastri. "Optimization of novel photobioreactor design using computational fluid dynamics." *Applied Energy* 140 (2015): 246-255.

Song, Liang, et al. "Micronutrient requirements for growth and hydrocarbon production in the oil producing green alga Botryococcus braunii (Chlorophyta)." *PLoS One* 7.7 (2012): e41459.

Soni B, Trivedi U, Madamwar D. A novel method of single step hydrophobic interaction chromatography for the purification of phycocyanin from Phormidium fragile and its characterization for antioxidant property. Bioresour Technol. 2008;99:188–194

Sovilj, Milan N., Branislava G. Nikolovski, and Momčilo Đ. Spasojević. "Critical review of supercritical fluid extraction of selected spice plant materials." *Macedonian Journal of Chemistry and Chemical Engineering* 30.2 (2011): 197-220.

Spolaore, Pauline, et al. "Commercial applications of microalgae." *Journal of bioscience and bioengineering* 101.2 (2006): 87-96.

Steinrueck, Hans-Peter, and Peter Wasserscheid. "Ionic liquids in catalysis." *Catalysis Letters* 145.1 (2015): 380-397.

Stephens, Evan, et al. "15 Microalgal production systems: Global impact of industry scale-up." (2012).

Strathmann, Heinrich, Lidietta Giorno, and Enrico Drioli. *Introduction to membrane science and technology*. Vol. 544. Weinheim: Wiley-VCH, 2011.

Suarez Ruiz, Catalina A., et al. "Selective and mild fractionation of microalgal proteins and pigments using aqueous two-phase systems." *Journal of Chemical Technology & Biotechnology* 93.9 (2018): 2774-2783.

Sun, J., M. Forsyth, and D. R. MacFarlane. "Room-temperature molten salts based on the quaternary ammonium ion." *The Journal of Physical Chemistry B* 102.44 (1998): 8858-8864.

Sureshkumar, Manthiriyappan, and Cheng-Kang Lee. "Biocatalytic reactions in hydrophobic ionic liquids." *Journal of Molecular Catalysis B: Enzymatic* 60.1-2 (2009): 1-12.

Takisawa, Kenji, et al. "Hydrolysis for direct esterification of lipids from wet microalgae." *Bioresource technology* 144 (2013): 38-43.

Tan, Kok Tat, and Keat Teong Lee. "A review on supercritical fluids (SCF) technology in sustainable biodiesel production: Potential and challenges." *Renewable and Sustainable Energy Reviews* 15.5 (2011): 2452-2456.

Tang, Baokun, and Kyung Ho Row. "Recent developments in deep eutectic solvents in chemical sciences." *Monatshefte für Chemie-Chemical Monthly* 144.10 (2013): 1427-1454.
Tani, Hirofumi, Tamio Kamidate, and Hiroto Watanabe. "Aqueous micellar two-phase systems for protein separation." *Analytical sciences* 14.5 (1998): 875-888.

Teixeira, Rodrigo E. "Energy-efficient extraction of fuel and chemical feedstocks from algae." *Green chemistry* 14.2 (2012): 419-427.

The Nova Institute. Industrial material use of biomass in Europe 2013. http://nova-institut.de/bio/data/images/1312_industrial_material_use_of_biomass_in_europe_2013_nova_institut.pdf

Thompson Jr, Guy A. "Lipids and membrane function in green algae." *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism* 1302.1 (1996): 17-45.

Tomasko, David L., et al. "Development of CO2 for polymer foam applications." *The Journal of Supercritical Fluids* 47.3 (2009): 493-499.

Tredici, Mario R. "Mass production of microalgae: photobioreactors." *Handbook of microalgal culture: Biotechnology and applied phycology* 1 (2004): 178-214.

Tredici, Mario R. "Photobiology of microalgae mass cultures: understanding the tools for the next green revolution." *Biofuels* 1.1 (2010): 143-162.

Trema Verfahrenstechnik GmbH.

http://www.trema.de/home,0,de.html?3705796042052294353219 6017124073; [accessed 05/09/2018]

Tripathi, S. N., Shivali Kapoor, and Alpana Shrivastava. "Extraction and purification of an unusual phycoerythrin in a terrestrial desiccation tolerant cyanobacterium Lyngbya arboricola." *Journal of applied phycology* 19.5 (2007): 441-447.

Trost, Barry M. "Atom economy—a challenge for organic synthesis: homogeneous catalysis leads the way." *Angewandte Chemie International Edition in English* 34.3 (1995): 259-281.

Tuntarawongsa, Sarun, and Thawatchai Phaechamud. "Polymeric eutectic drug delivery system." *Journal of Metals, Materials and Minerals* 22.2 (2012).

Ugwu, C. U., H. Aoyagi, and H. Uchiyama. "Photobioreactors for mass cultivation of algae." *Bioresource technology* 99.10 (2008): 4021-4028.

Valderrama, Jose O., Michel Perrut, and Wieslaw Majewski. "Extraction of astaxantine and phycocyanine from microalgae with supercritical carbon dioxide." *Journal of Chemical & Engineering Data* 48.4 (2003): 827-830.

Van Valkenburg, Michael E., et al. "Thermochemistry of ionic liquid heat-transfer fluids." *Thermochimica Acta* 425.1-2 (2005): 181-188.

Vandermeulen, Valerie, et al. "Industry expectations regarding the transition toward a biobased economy." *Biofuels, Bioproducts and Biorefining* 6.4 (2012): 453-464.

Vanderveen, Jesse R., Jeremy Durelle, and Philip G. Jessop. "Design and evaluation of switchable-hydrophilicity solvents." *Green Chemistry* 16.3 (2014): 1187-1197.

Vanderveen, J. R., et al. "A virtual screening approach to identifying the greenest compound for a task: application to switchable-hydrophilicity solvents." *Green Chemistry* 17.12 (2015): 5182-5188.

Vanthoor-Koopmans, Marieke, et al. "Biorefinery of microalgae for food and fuel." *Bioresource technology* 135 (2013): 142-149.

Vasumathi, K. K., M. Premalatha, and P. Subramanian. "Parameters influencing the design of photobioreactor for the growth of microalgae." *Renewable and Sustainable Energy Reviews* 16.7 (2012): 5443-5450.

Vermuë, M. H., et al. "Multi-product microalgae biorefineries: From concept towards reality." *Trends in biotechnology* (2017).

Virot, Matthieu, et al. "Microwave-integrated extraction of total fats and oils." *Journal of Chromatography A* 1196 (2008): 57-64.

Vree, Jeroen H., et al. "Comparison of four outdoor pilot-scale photobioreactors." *Biotechnology for biofuels* 8.1 (2015): 215.

Vunjak-Novakovic, Gordana, et al. "Air-lift bioreactors for algal growth on flue gas: mathematical modeling and pilot-plant studies." *Industrial & Engineering Chemistry Research* 44.16 (2005): 6154-6163.

Walden, P. I. "Ueber die Molekulargrösse und elektrische Leitfähigkeit einiger geschmolzenen Salze." *Известия Российской академии наук. Серия математическая* 8.6 (1914): 405-422.

Walsh, John M., George D. Ikonomou, and Marc D. Donohue. "Supercritical phase behavior: the entrainer effect." *Fluid Phase Equilibria* 33.3 (1987): 295-314.

Walsh, K. A. "Oxygenated products survive global slump." *Chemical week* 158.5 (1996).

Wang, Bei, Christopher Q. Lan, and Mark Horsman. "Closed photobioreactors for production of microalgal biomasses." *Biotechnology advances* 30.4 (2012): 904-912.

Wang, Shaoning, and Frank Kienzle. "The syntheses of pharmaceutical intermediates in supercritical fluids." *Industrial & engineering chemistry research* 39.12 (2000): 4487-4490.

Weaver, Justin, et al. "Anion exchange membrane adsorbers for flow-through polishing steps: Part II. Virus, host cell protein, DNA clearance, and antibody recovery." *Biotechnology and bioengineering* 110.2 (2013): 500-510.

Weissman JC, Goebel RP. *Design and analysis of microalgal open pond systems for the purpose of producing fuels*. Colorado: Golden; 1987.

Weissman, J. C., R. P. Goebel, and J. R. Benemann. "Photobioreactor design: comparison of open ponds and tubular reactors." *Bioeng. Biotech* 31 (1988): 336-344.

Weissman, Joseph Charles, and Raymond P. Goebel. *Production of liquid fuels and chemicals by microalgae. Final subcontract report*. No. SERI/STR-231-2649. Microbial Products, Inc., Fairfield, CA (USA), 1985.

Wen, Dongsheng, H. Jiang, and Kai Zhang. "Supercritical fluids technology for clean biofuel production." *Progress in Natural science* 19.3 (2009): 273-284.

Werpy, Todd, et al. *Top value added chemicals from biomass. Volume 1-Results of screening for potential candidates from sugars and synthesis gas*. No. DOE/GO-102004-1992. Department of Energy Washington DC, 2004.

Widegren, Jason A., Arno Laesecke, and Joseph W. Magee. "The effect of dissolved water on the viscosities of hydrophobic room-temperature ionic liquids." *Chemical Communications* 12 (2005): 1610-1612.

Wijffels, René H. "Potential of sponges and microalgae for marine biotechnology." *Trends in biotechnology* 26.1 (2008): 26-31.

Wiltshire, Karen H., et al. "Extraction of pigments and fatty acids from the green alga Scenedesmus obliquus (Chlorophyceae)." *Aquatic Ecology* 34.2 (2000): 119-126.

Wright, S. W., S. W. Jeffrey, and R. F. C. Mantoura, eds. *Phytoplankton pigments in oceanography: guidelines to modern methods*. Unesco Pub., 2005.

Wright, Simon W., and John D. Shearer. "Rapid extraction and high-performance liquid chromatography of chlorophylls and carotenoids from marine phytoplankton." *Journal of Chromatography A* 294 (1984): 281-295.

Wu, Chongchong, et al. "Aqueous enzymatic process for cell wall degradation and lipid extraction from Nannochloropsis sp." *Bioresource technology* 223 (2017): 312-316.

Wu, Hualian, et al. "A comparative analysis of fatty acid composition and fucoxanthin content in six Phaeodactylum tricornutum strains from diff erent origins." *Chinese journal of oceanology and limnology* 34.2 (2016): 391-398.

Wu, Zhe, et al. "The effects of light, temperature, and nutrition on growth and pigment accumulation of three Dunaliella salina strains isolated from saline soil." *Jundishapur journal of microbiology* 9.1 (2016).

Wymer, P. E. O., and Brenda Thake. "The importance of phosphorus in microalgal growth and species composition in mixed populations: experiments and simulations." *Proc. R. Soc. Lond. B* 209.1176 (1980): 333-353.

Yamada, Taisuke, et al. "Reversible, room-temperature ionic liquids. Amidinium carbamates derived from amidines and aliphatic primary amines with carbon dioxide." *Chemistry of materials* 19.5 (2007): 967-969.

Yang, Liu, Dong Xiaoyan, and Sun Yan. "New development of reverse micelles and applications in protein separation and refolding." *Chinese Journal of Chemical Engineering* 16.6 (2008): 949-955.

Ye, Shuhong, et al. "Antioxidant activities of an exopolysaccharide isolated and purified from marine Pseudomonas PF-6." *Carbohydrate Polymers* 87.1 (2012): 764-770.

Yoo, Gursong, et al. "Direct lipid extraction from wet Chlamydomonas reinhardtii biomass using osmotic shock." *Bioresource technology* 123 (2012): 717-722.

Young, G., Nippgen, F., Titterbrandt, S., & Cooney, M. J., 2010, Lipid extraction from biomass using co-solvent mixtures of ionic liquids and polar covalent molecules. *Separation and Purification Technology*, *72*(1), 118-121.

Yun, Yeoung-Sang, et al. "Carbon dioxide fixation by algal cultivation using wastewater nutrients." *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental and Clean Technology* 69.4 (1997): 451-455.

Zaijun, Li, Sun Xiulan, and Liu Junkang. "Ionic liquid as novel solvent for extraction and separation in analytical chemistry." *Ionic liquids: Applications and perspectives*. InTech, 2011.

Zbinden, Mauricio D. Antezana, et al. "Pulsed electric field (PEF) as an intensification pretreatment for greener solvent lipid extraction from microalgae." *Biotechnology and bioengineering* 110.6 (2013): 1605-1615.

Zhang, Baohua, et al. "Enzyme immobilization for biodiesel production." *Applied microbiology and biotechnology* 93.1 (2012): 61-70.

Zhang, Endong, et al. "Ammonia–nitrogen and orthophosphate removal by immobilized Scenedesmus sp. isolated from municipal wastewater for potential use in tertiary treatment." *Bioresource technology* 99.9 (2008): 3787-3793.

Zhang, Qinghua, et al. "Deep eutectic solvents: syntheses, properties and applications." *Chemical Society Reviews* 41.21 (2012): 7108-7146.

Zhang, Yi, Bhavik R. Bakshi, and E. Sahle Demessie. "Life cycle assessment of an ionic liquid versus molecular solvents and their applications." *Environmental science & technology* 42.5 (2008): 1724-1730.

Zhang, Yi-Ming, and Feng Chen. "A simple method for efficient separation and purification of c-phycocyanin and allophycocyanin from Spirulina platensis." *Biotechnology Techniques* 13.9 (1999): 601-603.

Zhao, Guo-Xi, and Jin-Xin Xiao. "Aqueous two-phase systems of the aqueous mixtures of cationic–anionic surfactants." *Journal of colloid and interface science* 177.2 (1996): 513-518.

Zhao, Hua, Cheng Zhang, and Tanisha D. Crittle. "Choline-based deep eutectic solvents for enzymatic preparation of biodiesel from soybean oil." *Journal of Molecular Catalysis B: Enzymatic* 85 (2013): 243-247.

Zhao, Liyan, et al. "Different effects of microwave and ultrasound on the stability of (all-E)-astaxanthin." *Journal of agricultural and food chemistry* 54.21 (2006): 8346-8351.

Zheng, Hongli, et al. "Disruption of Chlorella vulgaris cells for the release of biodiesel-producing lipids: a comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves." *Applied biochemistry and biotechnology* 164.7 (2011): 1215-1224.

Zhu, Yunhua, et al. "Development of hydrothermal liquefaction and upgrading technologies for lipid-extracted algae conversion to liquid fuels." *Algal Research* 2.4 (2013): 455-464.

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