



Emerging techniques for cell disruption and extraction of valuable bio-molecules of microalgae *Nannochloropsis* sp.

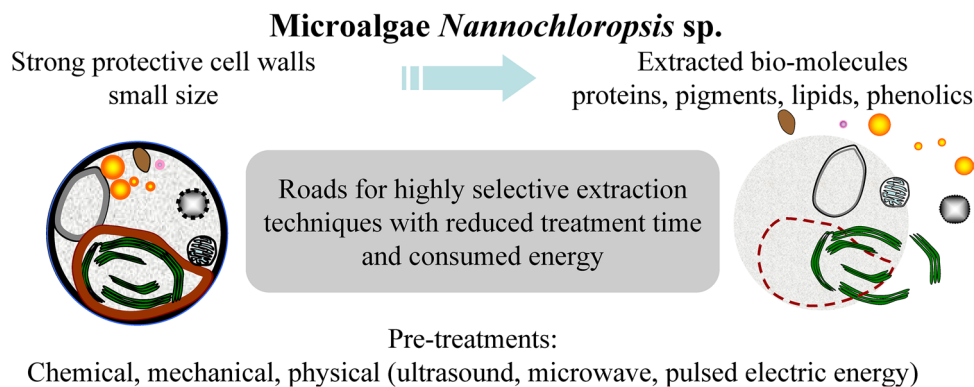
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Abstract

Microalgae of *Nannochloropsis* sp. present valuable source of bio-molecules (pigments, lipids, proteins) that have nutritional potential for the prevention and treatment of human diseases. Moreover, some species of *Nannochloropsis* are the promising sources of biofuels and excellent candidates for the replacement of classical biofuel crops. This review describes and compares the efficiency of different conventional and novel techniques that can be used for cell disruption and recovery of bio-molecules from *Nannochloropsis* sp. Classification of different extraction techniques includes chemical, enzymatic, mechanical and other physical methods. The detailed analysis of extraction efficiency assisted by pressure and temperature (subcritical and supercritical fluids, hydrothermal liquefaction), ultrasound, microwaves, and pulsed electric energy (pulsed electric fields and high voltage electrical discharges) is presented. The general discussion includes comparison between techniques, their effectiveness for cell disruption and selectivity of bio-molecules extraction from *Nannochloropsis* sp. The cost-effectiveness, benefits and limitations of different techniques are also analyzed.

Graphical abstract



Keywords Microalgae · *Nannochloropsis* sp. · Extraction · Bio-molecules · Disruption techniques

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Introduction

Microalgae are evaluated as an alternative source of ingredients for food additives, feed, cosmetics, and pharmaceutical applications, mainly due to their abundant nutritional components [1, 2]. A large number of microalga species such as *Nannochloropsis* sp., *Dunaliella* sp., *Chlorella* sp., and *Scenedesmus* sp., and the cyanobacteria *Spirulina* sp., are available and their genetic modification is possible. Microalgae can be used for industrial production of compounds of commercial relevance such as valuable lipids, proteins, carbohydrates, polyphenols, amino acids, polysaccharides, minerals, chlorophylls, and carotenoids [3, 4]. Most of these components have high potential for the prevention of human diseases and they show antimutagenic and antitumorigenic effects as well as anti-inflammatory activity [5].

Microalgae are grown on non-arable areas and they can be considered as promising sources of biofuels/biooils/bioenergy. They also are excellent candidates for the replacement of crops [6–8]. Microalgae-based hydrogen production is also regarded as a very promising pathway for producing alternative and renewable energy resources [9]. Microalgae demonstrated high productivity, fast growth that is unaffected by weather conditions, the possibility of use wastewater as a source of nutrients and many other advantages as compared with classical biofuel crops [10, 11]. Reuse of farm wastewater can be promising for both remediation of water and cost-effective microalgae production [12]. Particularly it was demonstrated that fish farm effluents can be suitable growth media for *Nannochloropsis gaditana* [13].

Novel strategies for microalgal cultivation and selection of appropriate strains with high lipid yields or optimal growth conditions gives promising perspectives for the development of algal-based biofuel industry [4]. Over the past decades, the socio-economic significance of extraction of different bio-molecules from microalgae has attracted the grown attention of academic and industrial. However, the most important bio-molecules are enclosed inside microalgae cells and their extraction (and above all, selective extraction) is not an easy task. The micron-sized microalgae cells are covered with rather thick and strong cell walls and their damage can be accompanied with degradation of bio-molecules and production of impurities in extracts [14]. The true commercial implementation of large-scale extraction equipment is also a great challenge [15].

This review concentrates on the application of different techniques for extraction of bio-molecules from the microalga *Nannochloropsis* sp. This microalga is rich in pigments and oils (e.g., palmitic, oleic and linoleic acids)

suitable for the production of the biodiesel, bio-hydrogen and high added-value compounds. The short discussion on structure, composition, and nutritional benefits of *Nannochloropsis* sp., and classification of extraction techniques, as well as different chemical, mechanical and physical methods, including thermal-pressure, ultrasound, microwaves, pulsed electric fields and high voltage electrical discharges assisted extractions are presented.

Structure, composition and nutritional benefits of microalgae and particularly *Nannochloropsis* sp.

The patterns of cellular organization in algae can be divided into prokaryotic and eukaryotic groups. In prokaryotic algae, the nuclear material, DNA, is uniformly distributed throughout the entire cell, and the membrane-bounded plastids, endoplasmic reticulum, mitochondria and Golgi apparatus are absent. Their cell walls show some chemical similarity to those of bacteria. In eukaryotic algae, the nuclear material (deoxyribo-nucleic acid, DNA), similarly to the higher plants, is isolated by nuclear membrane. The eukaryotic green microalgae *Nannochloropsis* sp. (Eustigmataceae family) present collection of six species of *Nannochloropsis* (*gaditana*, *granulate*, *limnetica*, *oceanica*, *oculata*, *salina*). *Nannochloropsis* cells reproduce asexually, dividing to yield two daughter cells.

The *Nannochloropsis* sp. have a rather small average diameter ($\approx 2.5 \mu\text{m}$) and they can be classified as picoplankton. Figure 1 shows the schematic structure (a) and example transmission electron microscopy (TEM) image (b) of *Nannochloropsis* cell. The protoplast is bounded by plasmalemma and it can be differentiated into cytoplasm, nucleus, chloroplast, one or more pyrenoids, mitochondria, and Golgi bodies. The chloroplasts (photosynthetic lamellae, disc or thylakoids) confined within membranes may have the different structures (massive, parietal or star-like structures). In many eukaryotic microalgae, the pyrenoids are present within the plastids that are the centers for enzymatic condensation of glucose into starch. For the synthesis of pigments, the chloroplasts (the double membrane-bound plastids, heterogeneous organelles) are responsible.

The genus *Nannochloropsis* has a relatively high growth rate. Commonly the microalgal growth rate is 5–10 times higher than that of plants. It can be cultivated in resources not used with conventional agriculture and can be harvested all year. The chemical composition of *Nannochloropsis* depends on strains and cultivation environments [17]. *Nannochloropsis* cells have a rather rich lipid content [between 25 and 35% of their dry weight (dw)] and they have potential for the production of high nutritional-value omega-3 fatty acids such as eicosapentaenoic acid. The light-harvesting

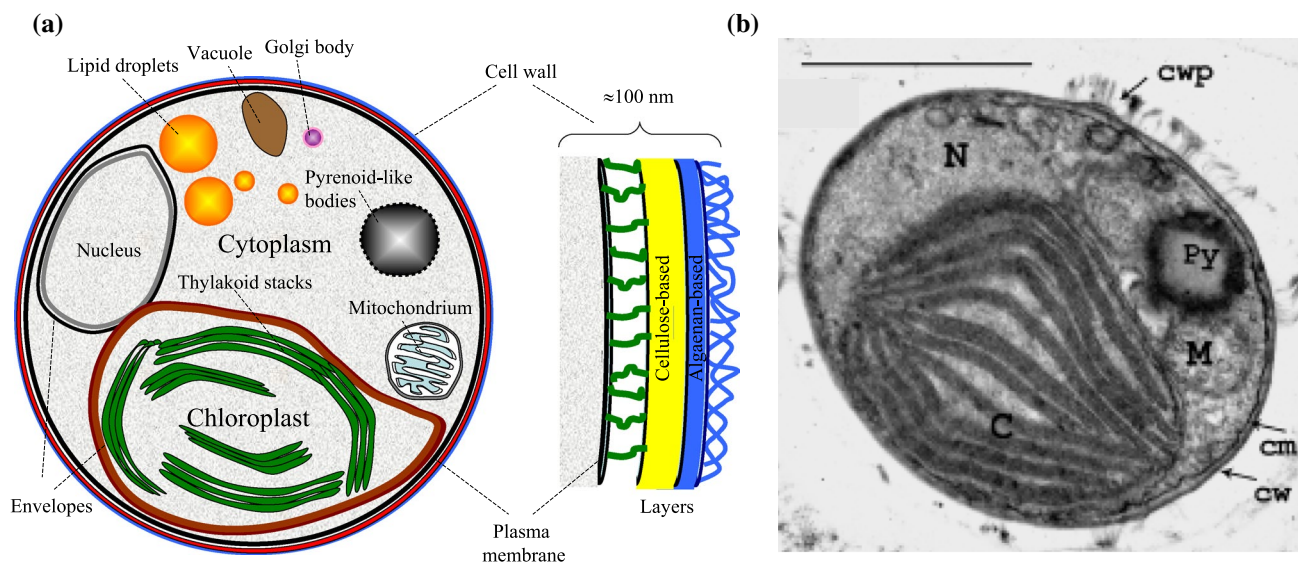


Fig. 1 **a** Schematic presentation of the structure of *Nannochloropsis gaditana* cell. The chloroplast contains a series of parallel lamellae formed by thylakoids. The cell wall contains cellulose- and algaenan-based layers. **b** Example of transmission electron microscopy (TEM) image of *Nannochloropsis* strain baik03. Here, **C** is the chloroplast,

M is the mitochondrion, **N** is the nucleus, **PY** is the pyrenoid-like bodies (the sites of carbohydrate storage), **cm** is the cell membrane, **cw** is the cell wall, and **cwp** is the cell wall papilla. Scale bar is 1 μ m. **b** with permission from [16]

pigments of *Nannochloropsis* are chlorophyll *a* and violaxanthin. The carotenoids from these microalgae can be used for nutrition supplements (food and feed), food dyes, pharmaceutical and cosmetic products. Other natural members of the carotenoid group, such as lutein (a yellow pigment) and β -carotene, still play an important role in beneficial biological activities of microalgae, referring to antioxidant, anti-inflammatory, anti-angiogenic, anti-obesity, anti-carcinogen and neuroprotective activities. The perspective of lutein production from microalga biomass was recently discussed [18]. Some microalga species also contain a high quantity of water-soluble vitamins and lipid-soluble vitamins that can be used as ingredients or food supplements. They can be considered as a source of nearly all important vitamins (A, E, C, B1, B2, B3, B6, B9, B12), carbohydrates, bio-active acids (folic, pantothenic, nicotinic), microelements (Ca, K, Na, Mg, Zn, Fe, etc.), and other bioactive chemicals.

However, *Nannochloropsis* cells have rather thick and strong cell walls (≈ 0.06 – 0.11 μ m). They consist of distinct layers with different composition and functions (Fig. 1a). They serve for protecting the cells from external pressures and form mass transfer barriers against dewatering and extraction of bio-molecules. For *Nannochloropsis gaditana* (strain CCMP 526), the cell wall has a bilayer structure with a cellulose-based inner layer (75% of the mass balance) protected by an outer hydrophobic algaenan layer [19]. The cellulose-based layer can also include several sub-layers and they are finely striated with parallel cellulose fibrils. The algaenan-based layer (algaenan is insoluble and

non-hydrolysable biopolymer) is responsible for cell wall hardness. The constituents of cell walls include carbohydrates (glucose, rhamnose, mannose, ribose, xylose, fucose, and galactose), proteins, lipids, carotenoids, tannins, and lignins. Polysaccharides of cell walls include cellulose, chitin-/chitosan-like molecules, hemicelluloses, pectins, fucans, alginates, ulvans, carrageenans, and lichenins. More details on the structure of *Nannochloropsis* sp. cell walls can be found in recent review [20]. Considerable variations in both lipid accumulation and cell wall thickness were observed for different species [21]. The comparison of cell wall structures in different microalga species was recently presented [22].

The numerous membrane barriers and cell wall envelopes in *Nannochloropsis* sp. present the serious obstacles for extraction of bio-molecules from microalgae. These walls cannot be easily digested or degraded [23]. Different extraction techniques aimed at disintegration and degradation of membrane and complex cell walls were tested in application to *Nannochloropsis* sp.

Classification of extraction techniques

Among conventional extraction techniques applied to microalga species the maceration/diffusion, Soxhlet extraction, expressing at different temperatures and pressures can be mentioned. However, the conventional techniques are not very efficient, rather expensive and require long processing time. For processing at elevated temperatures,

the risk of degradation of extracted bio-molecules cannot be excluded. In addition, the costly procedures of preliminary dewatering and drying are usually required to extract the hydrophobic constituents from microalgae [23].

Efficiency of the conventional techniques can be significantly improved with using of the supplementary treatments to disturb integrity of microalgae, cause the damage of membranes and cell walls, and intensify the mass transfer processes during extraction. Recently, the several comprehensive reviews have discussed the cell disruption for microalgae biorefineries [14, 20]. The existing techniques can be roughly divided on chemical, enzymatic, mechanical and other physical (ultrasound and microwave-assisted, pulsed electric fields, high-voltage electrical discharges) methods.

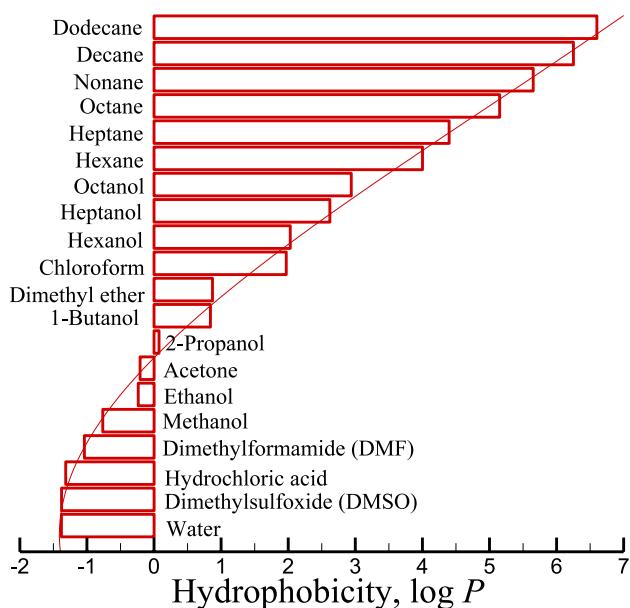
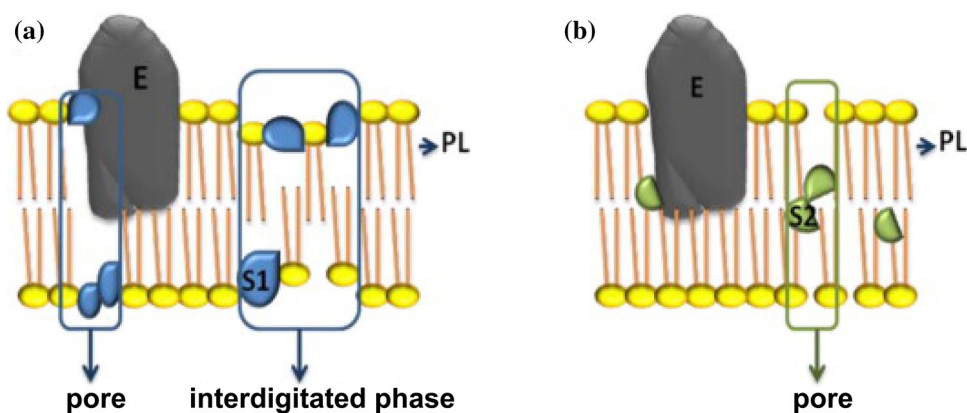


Fig. 2 Octanol/water partition coefficients for different solvents

Fig. 3 Cell membrane structure of *Nannochloropsis* sp. in the presence of hydrophilic **a** and hydrophobic **b** solvents. Here, **E** is the enzyme, **PL** is the phospholipids with polar head-groups and non-polar tail-groups, **S1** is the hydrophilic solvent, **S2** hydrophobic solvent (with permission from [25])



Chemical methods

A suitable organic solvent should preferentially solubilize the compounds of interest, and be volatile enough to ensure low energy distillation to separate the lipid from solvents [24]. The effects of organic solvents on the *Nannochloropsis* sp. growth, lipids extractability, and cell membrane integrity were investigated [25].

The solvent hydrophobicity can be characterized by the value of $\log P$ that is defined as the partition coefficient of a given solvent in a mixture of octanol and water. The solvents with $\log P \leq 0$ beyond to the relatively hydrophilic solvents whereas with $\log P > 0$ to the more hydrophobic ones. The parameters of $\log P$ for different solvents are presented in Fig. 2.

The solvents that have direct contact with microalga cells can interact with the phospholipid bilayers of cell membranes in a different manner in dependence of value of $\log P$. The mechanism of the solvents interactions within the membrane structure of *Nannochloropsis* sp. is schematically presented in Fig. 3 [25].

The relatively hydrophilic solvents strongly interact with the head groups of phospholipid molecules (Fig. 3a), whereas the hydrophobic ones can accumulate inside membranes (Fig. 3b), eventually cause the formation of pore canals or an interdigitated phases. The formation of such structures can affect the barrier properties of the membranes. The results showed that hydrophobic solvents with $\log P > 5.5$ were biocompatible while the less hydrophobic solvents with $\log P < 5.5$ were toxic to *Nannochloropsis* sp [25]. The solvents with $\log P < 5.5$ have non-negligible solubility in water and they can penetrate into the membranes that result in increasing of the membrane permeability and deactivation of dehydrogenase activity.

For extraction of the relatively hydrophobic pigments or lipids from *Nannochloropsis* biomass, the traditional organic solvents such as methanol, ethanol, acetone, chloroform, isopropanol, 1-butanol, dimethyl ether, ethyl acetate and hexane were used [26]. Typically, the hexane

is most widely used as a solvent for large-scale extraction as well as cheap and reusable. The mixtures of chloroform/methanol, n-hexane/ethanol, and dichloromethane/methanol were also tested [27]. For example, the mixture of chloroform/methanol (1/2, v/v) is frequently used to extract lipids from microalga biomass at analytical and lab-scale.

Optimisation of solvent composition allows a significant improvement of extraction efficiency. For instance, using of chloroform: methanol: water in the volume ratio of 5.7: 3: 1 allowed one-step recovery of more than 96% of the lipids from *Nannochloropsis salina* biomass paste [28].

The dry or wet routes (extraction from dried or wet biomass) can be selected for the organic solvent assisted extraction [29]. However, both the drying operation in the dry route and wet oil extraction process in the wet route consume a significant amount of energy. The energy balance can be improved by applying more efficient dryer/extraction processes.

Ionic liquid solvents (liquid organic salts) were tested for the extraction of microalga lipids [24, 30]. Ionic liquids can be used as replacements for harmful organic solvents. These solvents can dissolve the cellulose of the cell walls and disrupt the cellular structure of the microalgae. For example, the ionic liquid $[P(CH_2OH)_4]Cl$ extraction proved especially suitable for lipid extraction from *Nannochloropsis oculata*, giving even higher extraction yields from dry biomass, 14.9% and 12.8%, respectively [31]. Remarkably, that the ionic liquid can be successfully reused in many extraction circles.

Additives of surfactants, using of switchable polarity solvent (a smart solvent that can be switched reversibly between liquids that have very varied properties), acid/alkaline treatment and their combinations were tested for extraction purposes [32]. The surfactants additives (sodium dodecylsulphate, Triton X-100, Tween 20 and Polyacrylamide) have been used to promote lipid extraction from *Nannochloropsis oceanica* (slurry containing 96.0% of moisture) into the water [33]. The lipid extraction efficiency of 88.3% was achieved under optimal conditions. A switchable solvent of the class of tertiary amines (*N,N*-dimethyl cyclohexyl amine, DMCHA) has been applied for the extraction of lipids from *Nannochloropsis gaditana* [34]. The DMCHA was recovered by adding Carbon dioxide (CO_2), switching of DMCHA into a hydrogen carbonate ammonium salt and formation of a separate liquid lipid phase. The solvent system can be recycled (switched-back) by CO_2 removal. The mixed-polarity azeotropic solvents have been applied for efficient extraction of lipids from *Nannochloropsis* [35]. The superior results were obtained in testing Soxhlet extraction with hydrocarbon/alcohol azeotropic mixture compared to

the commonly used hydrocarbon solvents (i.e., hexane, cyclohexane).

Enzymatic methods

Enzymatic reactions can disintegrate the cell walls and increase their permeability [36]. The enzymatic hydrolysis was shown to be an effective and nontoxic procedure for improving of extraction efficiency of intracellular compounds [37]. In several recent works the different enzymatic methods have been applied to break cell walls and extract lipids and other valuable components from microalga *Nannochloropsis* sp [33, 38, 39]. The combined thermal and enzymatic (cellulose from *Aspergillus niger*, lipase and protease) treatments were applied to the wet biomass of *Nannochloropsis oceanica* (96.0% moisture) to enhance recovery of lipids and proteins [33]. Several surfactants (Triton X-10, Tween-20, sodium-dodecyl sulphate) were added singly or jointly into water to promote oil extraction. At the optimal conditions, extraction yields for both lipid (88.3%) and protein (62.4%) were significantly higher as compared with those of traditional hexane extraction. Optimized ternary mixtures of suitable enzymes (cellulase and two hemicellulases) allowed the recovery of up to 37.2 g of lipids per 100 g of dry biomass [38]. Scanning electron microscope (SEM) and TEM images revealed extensive cell damage and degradation of the cell walls. Combination of alkaline pretreatment and subsequent enzymatic treatment was used as cell disruption methods to increase recovery of lipids from *Nannochloropsis* sp [40]. The combination of commercialize enzymes (cellulase, protease, lysozyme, and pectinase) was used. The optimum reaction conditions were established accounting for the compromise between economic feasibility and lipid yield and 90.0% of lipid was extracted under optimal conditions. The disruption of *Nannochloropsis gaditana* was performed using either enzymatic treatment with Alcalase or high-pressure homogenization (HPH) [41]. Enzymatic treatment resulted in a smaller release of proteins (35.0%) in the aqueous phase compared to the HPH (49.0%). Then an ultrafiltration/diafiltration (UF/DF) was performed on the supernatant to recover water soluble proteins. The enzymatic treatment with combination of cellulase and mannanase was applied to promote the recovery of lipids from *Nannochloropsis* sp [39]. The extraction yield increased from 40.8% for untreated microalga to over 73% for enzymatically treated microalga. The enzymatic treatment resulted in significant changes in the chemical composition, thermal behaviour and also in an increase of the crystalline-to-amorphous cellulose ratio. SEM images also revealed dramatic changes in cell morphology and extensive cell damage.

Mechanical methods

The cell walls of microalgae represent the most important barrier to target bio-molecules extraction. Nowadays, the different mechanical methods are used to disrupt the microalga cells [22]. They are based on the application of shear forces, hydrodynamic cavitation, hydraulic pressing, grinding or cryogenic grinding. Note that grinding, and cryogenic grinding have very high efficiency, but these methods are time consuming and rather expensive [42]. The highly efficient bead milling and HPH or French press disrupters and high shear mixer (HSM) can be applied to highly concentrated microalga suspensions [43, 44]. The applications of HPH to *Nannochloropsis* sp. suspensions have shown high efficiency for pigments extraction [45]. Hydraulic pressing has been shown to be an effective method for the disruption of *Nannochloropsis oculata* strain [46]. The effects were noticeably enhanced for the liquid nitrogen treated samples.

Other physical methods

To intensify the extraction of valuable bio-molecules from microalgae, the various physical methods involving thermal and pressure treatments (steam explosion, hydrothermal liquefaction, freeze drying, using of pressurized fluids and super-/sub-critical solvents), and alternative treatments by ultrasound, microwaves, pulsed electric fields and high-voltage electrical discharges were tested [20, 22]. The extraction efficiency of the specific method depends on the microalga characteristics (cells size, shape and structure), liquid media composition (types of solvent, suspension concentration), and processing conditions (temperature, pressure, presence of agitation, centrifugal, electric fields, etc).

Thermal-pressure assisted extraction

Subcritical and supercritical fluids The subcritical (S_bFE) and supercritical (S_pFE) fluid extraction techniques are recognized as eco-friendly and powerful tools to replace traditional extraction methods. Applications of these techniques for recovery of the valuable compounds of microalga biomass have several advantages as compared to the conventional organic solvent extraction techniques. The current state of such applications has been recently reviewed [47].

In S_bFE the hot water (100–374 °C) under pressure (10–60 bar) is frequently used as extraction reagent. The variation of temperature and/or pressure allowed fine regulation of polarity of water that affects the extraction efficiency of the subcritical solvent. Water is considered as extremely polar solvent at ambient conditions, but, it could be used for solubilization and partitioning of moderately polar and non-polar compounds at subcritical conditions. In some experiments, a pressurized ethanol was used as extracting agent in

S_bFE . The S_bFE technique can be classified as safe, “green” and rapid. The existing studies of S_bFE reported different subcritical solvents used for microalga species (for a review see [48, 49] and references therein):

- Pressurized water. Extraction of antioxidant and antimicrobial bio-molecules from microalgae *Spirulina platensis* and *Haematococcus pluvialis*;
- Pressurized ethanol. Extraction of carotenoids from microalgae *Haematococcus pluvialis* and *Dunaliella salina*;
- Pressurized propane in the presence of ethanol (200 bar/80 °C). Extraction of fatty acid methyl esters from microalga *Nannochloropsis oculata*.

In S_pFE , the fluid in the supercritical state is used. This fluid has the viscosity and diffusivity that are similar to a gas and the density and solvation properties that similar to a liquid. The characteristics of the supercritical fluid can be also fine regulated by changing the temperature and/or pressure. The better transport properties supercritical fluid allows obtaining the faster processing (lower extraction times) and higher extraction yield. CO_2 is an inert, non-toxic, environmentally friendly and inexpensive fluid, which has been widely used as a supercritical fluid due to its small critical temperature (31.3 °C) and pressure (72.9 bar). However, CO_2 has low polarity and it is not very effective for the extraction of polar compounds. To overcome this problem the small quantity of polar additive to CO_2 (for example, CO_2 + ethanol, or n-hexane as co-solvents) can be used. Among other supercritical agents the water (101.1 °C/217.6 bar), ethene (10.1 °C/50.5 bar) and ethane (32.4 °C/48.2 bar) can be mentioned. The S_pFE is a widely recognized as green extraction technique and the supercritical solvents are generally recognized as safe that is also an important advantage.

The S_pFE has been applied to obtain different bio-molecules from several microalga species. For example, the recovery of long-chain hydrocarbons (i.e. 25–31 carbon atoms) from *Botryococcus braunii* cells, carotenoids from *Chlorella vulgaris*, β -carotene from *Dunaliella salina*, γ -linolenic acid (GLA) from *Arthrospira (Spirulina) maxima*, lipids and GLA from *Cyanobacterium Spirulina platensis*, lipids, tocopherol, and polyunsaturated fatty acids (PUFAs) from *Nannochloropsis oculata* and *Tetraselmis suecica* were tested. The main attention was paid to the optimization of temperature, pressure and content of subcritical agent for attaining the maximum extraction yield (for a review see [48] and references therein). For example, for S_pFE -(CO_2) the highest recovery of PUFAs from *Nannochloropsis oculata* and *Tetraselmis suecica* were 40 °C/207 bar and 40 °C/483 bar, respectively. Commonly, the water content of microalga samples is rather high and the preliminary drying

step is usually required for the extraction of lipids. For $S_pFE-(CO_2)$ extraction, it is suggested that the water content should be less than 20% wt to achieve the best performance. Note that markedly accelerated kinetics of lipid extraction and increased extraction yield for $S_pFE-(CO_2)$ technique can be obtained using a preliminary drying or lyophilisation of raw biomass, but these procedures are rather expensive.

In general, an application of S_pFE to recover components from microalgae is rather effective and results in high-quality extracts [47]. However, commercial applications of S_pFE are still impeded owing to the high cost and scale up problems [50].

Hydrothermal liquefaction Hydrothermal liquefaction (HTL) is a thermal depolymerisation process that can be used to convert the wet microalga biomass directly into biocrude oil under elevated temperatures and pressures [51]. In this process, the biomass is thermally cracked. Water at these conditions promotes formation of the high H/C ratio biocrude oil. The HTL technique applied at optimal conditions (350 °C/175 bar) allowed effective extraction of lipids from *Nannochloropsis salina* with a yield of 34–46%. After HTL, the algae residue contained a high quantity of proteins. Efficiency of HTL of *Nannochloropsis oceanica* in different solvents at mild conditions (<250 °C) has been tested [51]. The addition of alcohols (e.g., ethanol) allowed significant improving bio-oil yield (up to ~60%) comparable to the obtained at more severe operating conditions without alcohols. The HTL technique at 350 °C was applied to extract biocrude oil from the microalgae species *Nannochloropsis gaditana* (marine) and *Scenedesmus almeriensis* (freshwater) [52].

Ultrasound assisted extraction

Ultrasound assisted extraction (UAE) technique is based on perturbation of biomass by ultrasonic waves with the frequency in the range from 20 kHz to 1 MHz that produce bubble cavitation phenomena, intensive shear forces, erosion and breakdown of particles, development of macro-turbulences and micromixing. These perturbations can shatter the cell walls, improve penetration of solvent inside biomass and accelerate diffusion. Nowadays, UAE is widely applied in food processing industry [53].

UAE in-situ transesterification of microalgae slurry to extract lipids and convert to biodiesel has been tested. The optimal values of ultrasonic power, reaction time, concentrations of methanol and chloroform in oil to maximize fatty acid methyl ester content and exergy efficiency were evaluated. The mechanism of ultrasonic disruption in suspensions of five strains of microalgae with different sizes and cell wall compositions (including *Nannochloropsis* sp.) was studied [54]. The most significant cell disruption

and a small difference between species were observed during the initial seconds of sonication. At longer exposure times, differences between species became more pronounced. The UAE of lipids from several microalga species (*Chlorella* sp., *T. suecica* and *Nannochloropsis* sp.) has been examined [55]. The cell disruption efficiency correlated well with sonication energy consumption. For freshwater *Chlorella* sp. with rigid cell walls the lipids were easily released to the aqueous phase whereas for other species *T. suecica* and *Nannochloropsis* sp. the cells retained the membrane lipids after the disruption. The UAE was tested for the optimization of lipid extraction from microalga *Nannochloropsis gaditana* in the temperature interval between 50 and 60 °C [56]. For the conventional extraction, the best data were obtained using chloroform/methanol mixtures. UAE with methanol gave comparable fatty acid (FA) w/w% from dried microalgae. The cavitating tube protocol was preferred as it afforded better process control. The UAE method (booster horn, 20 kHz, 1000 W), with water as a solvent, was applied to extract lipids from fresh *Nannochloropsis oculata* biomass [19]. After extraction, the oil/water emulsion was demulsified using the saline solution and centrifugation step. Finally, water and oil were separated into two distinct phases that simplified the oil recovery. SEM analysis had shown that external structure of the surface of the cells was modified after UAE. Cells were smaller and their parietal system and cell walls were damaged. During 30 min of extraction, the oil recovery continuously increased with temperature increases from 1 to 35 °C. The maximum oil recovery at optimum conditions (1000 W ultrasonic power, 30 min extraction time and dry weight content at 5.0%) was around 0.21%. The UAE process implied less solvent consumption, and a marked reduction in treatment time and temperature compared to conventional extraction.

The UAE of lipids from raw microalga *Nannochloropsis oculata* was studied for different mixtures of solvents were studied (see [57] and references therein). For example, the combination of the Folch method using chloroform/methanol (2:1, v/v) with ultrasounds allowed almost complete extraction of microalga fatty components. Application of UAE with pure and binary solvents allowed non-selective extraction of lipids, carbohydrates and proteins [58]. The extraction efficiency was optimal at 2/1 proportion of hexane/isopropanol and ultrasound frequency of 50/60 Hz. The UAE of phenolics and pigments from microalga *Nannochloropsis* sp. has been tested [57]. The maximum recovery of phenolics and chlorophylls was found after UAE (400 W, 5 min) + binary mixtures of solvents (water-DMSO and water-EtOH) at 25–30% + microalga concentration (10%). The novel technique combining simultaneous UAE and enzymatic hydrolysis treatment was used for the extraction of reducing sugars from microalga *Chlamydomonas*

mexicana with improved yield by fourfold as compared with the UAE pretreatment under optimum conditions [59].

The effectiveness high-frequency focused ultrasound (HFFU, 3.2 MHz, 40 W) and low-frequency non-focused ultrasound (LFNFU, 20 kHz, 100 W) techniques for the disruption of *Nannochloropsis oculata* has been compared [60]. HFFU treatment was more energy efficient as compared with LFNFU. Moreover, the combination of high and low-frequency treatments was even more effective than single frequency treatment. The effectiveness of a continuous ultrasonic flow system (2 kW) for the disruption of *Nannochloropsis oculata* has been studied [61]. Cell recirculation was found beneficial to cell disruption. Nile red stained lipid fluorescence density and cell debris concentration in treated systems treatments increased up to 56.3% and 112%, correspondingly, compared to the control.

Microwave assisted extraction

Microwave assisted extraction (MAE) technique is based on selective interactions of ionic and polar components with high-frequency electromagnetic energy (300 MHz–300 GHz). The mechanism of microwave action may be rather complex. An application of microwave power and an increase of a temperature inside biomass samples can cause the separation of bio-molecules from active sites, acceleration of the solvent diffusion and dissolution of bio-molecules in solvent.

Nowadays, the MAE technique is widely used to facilitate the extraction of different bio-molecules from microalgae (for a review see [62] and references therein): pigments (fucoxanthin, lutein, chlorophylls and carotenoids) from *Cylindrotheca closterium* and *Dunaliella tertiolecta* (25–100 W/3–15 min, acetone), lipids from *S. obliquus* (hexane) and *Nannochloropsis* sp. (biodiesel as co-solvent in ethanol) [63]. Hydrothermal microwave processing (HMP) under controlled irradiation was applied as effective pretreatment for hydrothermal liquefaction and extraction of lipids and phytochemicals from three strains including, *Nannochloropsis oculata*, *Chlorogloeopsis fritschii* and *Pseudochoricystis ellipsoidea* [64]. The novel technique combining simultaneous cooling and microwave heating was used for the production of biodiesel from *Nannochloropsis* sp. and *Tetraselmis* sp. with improved yield (83.33% and 77.14%, correspondingly) as compared with the control methods [65]. The different methods (Hara and Radin, Folch, Chen and Bligh, and Dyer) of extraction of lipids from *Nannochloropsis* sp. and *Tetraselmis* sp. combined with conventional heating and microwave irradiation were tested [66]. The lipid yields for *Tetraselmis* sp. and *Nannochloropsis* sp. were highest for Hara and Radin (8.19%), and Folch (8.47%) methods combined with microwave irradiation.

Several investigations were devoted to the using of microwaves for the direct transesterification (DT) of the *Nannochloropsis* biomass into a biodiesel. Using microwave and ultrasound radiation with the aid of a SrO catalyst was applied for the DT (a one-stage method) of the *Nannochloropsis* biomass into biodiesel [67]. It was concluded that the applied method appears to be the most simple and efficient method of the DT. The wet biomass of *Nannochloropsis* sp. was converted to biodiesel using DT by the combination of microwaves and ionic liquids (IL). The highest percentage of cell disruption (99.73%) and biodiesel yield (36.79% per dried biomass) after 15 min of simultaneous extraction-DT using microwave irradiation and 1-ethyl-3-methylimidazolium methyl sulphate was obtained [68]. The DT approach applied for converting of wet biomass *Nannochloropsis salina* into crude biodiesel under microwave-mediated supercritical ethanol conditions [69] and in the presence of methanol and combined alkali/acid catalyst [70]. The biodiesel produced by such microwave-mediated method has high-quality characteristics compared with water bath heating DT.

Pulsed electric energy assisted extraction

Pulsed electric fields Pulsed electric fields (PEF) extraction technique is based on the possibility of damage of cell membranes under the action of high electric fields. These fields pierce the membranes and they become permeabilized temporary or permanently with the formation of pore inside them. Remarkably, that effective electroporation can occur with the application of short duration pulses (from several nanoseconds to several milliseconds) with relatively high electric field strength (from 100 to 300 V/cm up to 300 kV/cm). Nowadays, the PEF are widely used for the improvement of different processing steps with raw biomaterials (plant food materials, by-products, and biosuspensions), e.g., in pressing, drying, freezing, osmotic treatment and extraction procedures.

In recent years, many successful examples of PEF application for the enhancement of extraction of different valuable components from microalgae were demonstrated. PEF-assisted extraction of C-phycoyanin from cyanobacterium *Arthrospira platensis*, pigments from microalga *Chlorella vulgaris*, proteins, carbohydrates and phenolics from microalga *Nannochloropsis* sp., cytoplasmic proteins from microalga *Nannochloropsis salina* and *Chlorella vulgaris*, and lipids from microalga *Auxenochlorella protothecoides* have been recently tested (see, [71] for a recent review). In many cases, it was supposed that observed effects reflect the cell membrane permeabilisation.

For the extraction of water-soluble hydrophilic components (ionics, carbohydrates, TPC, proteins) the aqueous media, or mixed solvents and pH regulation have been

used. The several cell disruption methods (PEF, UAE, HPH, enzymatic and others) were tested on *Nannochloropsis* sp [45, 72]. PEF-assisted extraction allowed recovery of ionic solutes, amino-acids and small water-soluble proteins of microalga *Nannochloropsis* sp [45]. HPH and bead milling were the most efficient (50% release of total proteins w/w) with low energy input [72]. Enzymatic treatment was less efficient (35% proteins, w/w) and also required low energy input. However, PEF treatment was neither successful for protein release (10% proteins, w/w) nor energy-efficient. PEF-assisted extraction allowed recovery of carbohydrates and small ionic solutes of microalga *Chlorella* up to 39% and 75%, respectively [73]. For better efficiency of PEF-assisted extraction, the use of (1) the binary mixture of organic solvent and water or (2) adjustment of extracellular media conductivity or (3) application more potent cell disruption techniques (like HPH or ultrasonication, etc.) were tested.

PEF-assisted extraction of different bio-molecules (total chlorophylls, carotenoids, proteins and phenolics) from microalga *Nannochloropsis* spp. using the mixture of organic solvents (dimethyl sulfoxide, DMSO and ethanol, EtOH) and water has been tested [74]. Two-stage PEF-assisted ($E = 20$ kV/cm) extraction procedure involved extraction in water at the first step and extraction in a binary mixture at the second step. Applied two-step procedure allowed efficient extraction of proteins at the first step with a better extraction of pigments and other high-added value bio-molecules at the second step.

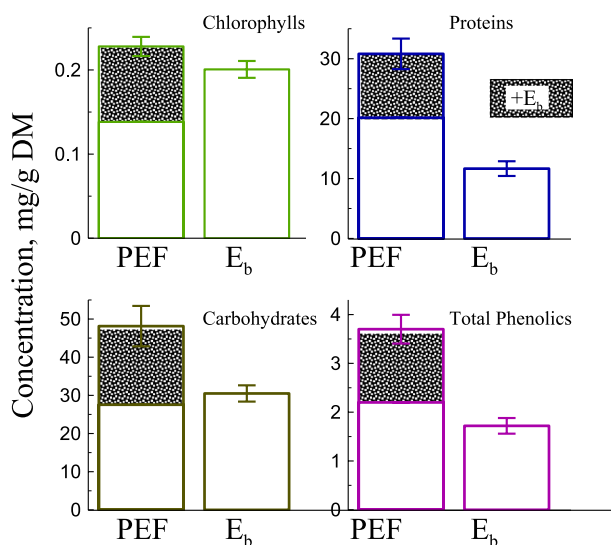


Fig. 4 Concentration of chlorophylls, proteins, carbohydrates and total phenolics extracted from microalga *Nannochloropsis* sp. The data are presented for extracts, obtained after PEF treatment, and aqueous extraction in the basic medium E_b . The effects of supplementary aqueous extraction $+E_b$ are also shown. Adapted from [75]

PEF treatment was used as a preliminary step of pH-assisted aqueous extraction from microalga *Nannochloropsis* sp [75].

Figure 4 shows the concentration of chlorophylls, proteins, carbohydrates and phenolic compounds in water extracts, obtained using PEF, and conventional aqueous extraction in the basic medium E_b . The efficiency of extraction of various components, stimulated by PEF treatment, was comparable with that obtained for aqueous extraction in a basic medium. However, supplementary basic extraction at pH = 11 ($+E_b$ is shown as dashed section of bars) after the PEF treatment allowed a noticeable increase in the concentrations of all components in the extracts.

Thus, the PEF pre-treatment has an excellent potential as a preliminary step of aqueous extraction of *Nannochloropsis* sp. components. Moreover, PEF technique allowed selective extraction of some pure proteins that were different from the proteins extracted after conventional extraction. The discovered effects have shown the advantages of PEF application in a normal medium (pH 8.5) and basic medium supplementary extraction (pH 11) for selective extraction of different intracellular components, especially proteins.

Impact of PEF for the extraction of cytoplasmic proteins from microalgae *Nannochloropsis salina*, *Chlorella vulgaris* and *Haematococcus pluvialis* was also demonstrated [76]. PEF-assisted extraction of a C-phycoyanin (a pigment-protein complex) from the fresh biomass of cyanobacterium *Arthrospira platensis* was studied [77]. The results evidenced the PEF's potential for selective extraction of these compounds and higher purity of obtained extracts.

PEF was also applied for the extraction of chlorophylls and carotenoids from microalgae. Due to the hydrocarbon structure, these pigments are hydrophobic substances, soluble only in organic solvents, oils and fats, and practically insoluble in water. For example, the chlorophyll molecules have the hydrophilic ring with magnesium in the centre and the long hydrophobic tails. Chlorophylls can be dissolved easily in acetone, and alcohol, but they have low solubility in alkanes (such as hexane and butane) and are practically insoluble in water. However, the complexes of chlorophyll binding molecules can be dissolved in water. The effects of PEF protocol and temperature (10–40 °C) on the extraction efficiency of pigments (carotenoids, chlorophylls) and Lutein (carotenoid) from *Chlorella vulgaris* were compared (see [78] and references therein). Higher temperature increased the sensitivity of microalga cells to irreversible electroporation. It was demonstrated that irreversible “electroporation” required electric field strengths of order ≥ 4 kV/cm and ≥ 10 kV/cm for pulse durations in the millisecond and microsecond ranges, respectively. Moreover, the induction period was observed and the extraction yield of carotenoids was significantly increased for the extraction applied after 1 h of the PEF treatment.

The PEF-assisted extraction of hydrophobic intracellular lipids from microalgae requires application of organic solvents or strong mixtures to penetrate the cell wall and outer membranes. The green solvent (ethyl acetate) used as supporting solvent allowed significant improvement of the lipid recovery for PEF-assisted extraction from microalga *Ankistrodesmus falcatus* [79]. In the absence of PEF, the extraction efficiency for ethyl acetate was lower (83–88%) than that of chloroform. Focused-pulsed (FP) assisted extraction applied for microalga *Scenedesmus* yielded 3.1-fold more crude lipid and fatty acid methyl ester (FAME) (using hexane over control) after recovery in different solvent mixtures [80]. FP assisted extraction also increased the FAME-to-crude-lipid ratio for all tested solvents.

The effects of PEF treatment on lipids recovery from microalga *Auxenochlorella protothecoides* were tested in several works [81, 82]. The evaluated lipid content for this microalga is rather high (30–35% of cell dry weight). PEF treatment (at 23–43 kV/cm, 52–211 kJ/kg) was applied to \approx 10% aqueous suspension and after extraction of water-soluble cell components during the first step, the lipid extraction from residual biomass was applied using 70% ethanol as solvent at the second step [81]. The proposed extraction procedure from the wet biomass had the comparable efficiency with extraction from dry biomass. The proposed PEF assisted extraction of lipids from PEF-treated wet biomass is economically expedient, because the energy requirements for the PEF-assisted extraction (1.5 MJ/kg DW) is lower compared to the required drying energy (7 MJ/kg dw). In other work [82], PEF (at 10 kV/cm, 150 kJ/kg) was applied to concentrated biomass (10% w/w solids) as pre-treatment

prior to organic solvent extraction of lipids in the triple mixture of water/ethanol/hexane (1: 18: 7.3, v/v/v). Experiments were performed with mixotrophic and autotrophic cultures. For PEF untreated the extraction yield was up to 10% of total lipid content. PEF treatment enabled to recover 92% (mixotrophic), and 72% (autotrophic) of the evaluated lipid content after 2 h of extraction, and 97% (mixotrophic), and 90% (autotrophic), after 20 h of extraction.

In general, the PEF treatment is rather “gentle” in the sense that it can be applied in a non-thermal mode with an insignificant elevation of temperature. Commonly, the direct effects of PEF on the cell walls and disruption of them are marginal. The PEF treatment did not alter protein, pigment, lipid and fatty acid compositions. The PEF-assisted extraction techniques can be applied in highly selective modes for the extraction of non-degraded ionic components, phenolic compounds, proteins, pigments and lipids from microalgae. These techniques show promising perspectives for industrial upscaling. However, the extraction efficiency of this technique for high molecular weight and hydrophobic components may rather low. Moreover, in practical application the thorough optimisation of PEF treatment protocols, temperature, pH and supporting solvents are required for different species of microalgae.

High voltage electrical discharges High voltage electrical discharges (HVED) are commonly applied to aqueous wet biomass using needle-plane electrode geometry and typically, such treatment accompanies by different processes, including the electrical breakdown, propagation of streamer, bubble formation, and cavitations, light emission,

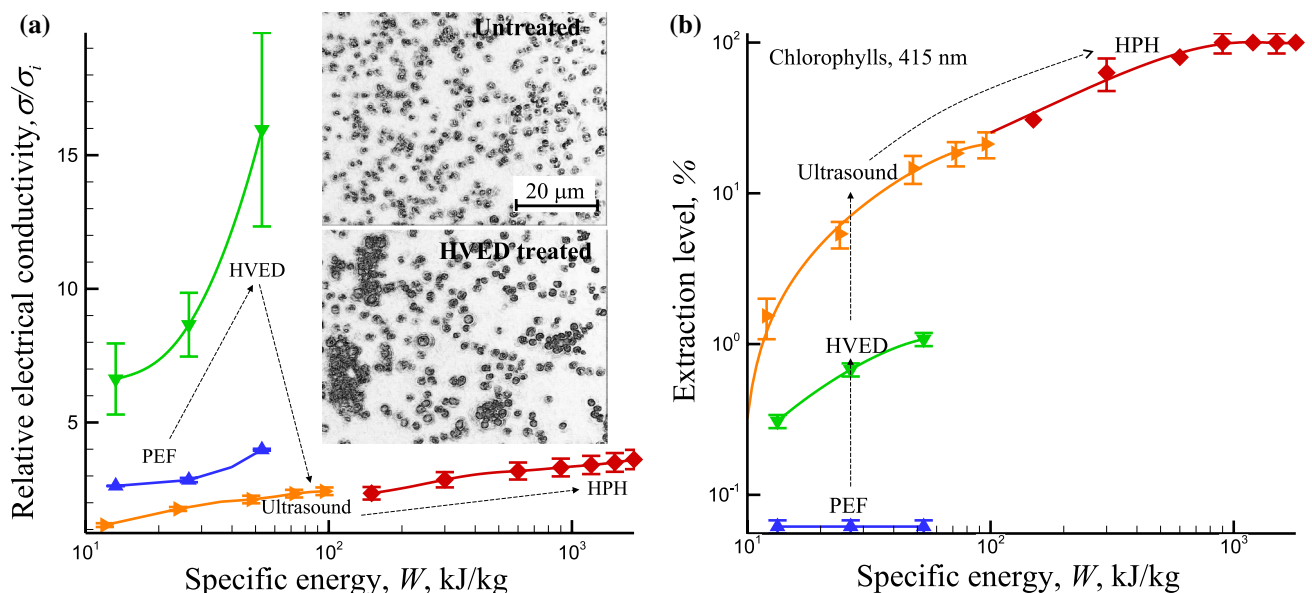


Fig. 5 Ratio of electrical conductivities σ/σ_i after and before treatment **a** and extraction level of chlorophylls versus the specific energy W **b**. Insert **a** shows the micro-photos of untreated and HVED treated microalga *Nannochloropsis* sp. Adapted from [45]

appearance of localized regions with high pressure, formation of shockwaves, and acoustic waves. Important effects of HVED on wet biomass may include electroporation as well as additional thermal and mechanical stresses [71].

Recently, the HVED-assisted aqueous extraction of ionics, amino-acids, proteins and pigments from *Nannochloropsis* sp. was also tested [45]. Figure 5 shows the effects of different techniques (PEF, HVED, UAE and HPH) on aqueous extraction of ionic components (1) and chlorophylls (2) from microalga *Nannochloropsis* sp. The extraction steps were applied sequentially, using the order PEF→HVED→UAE→HPH, and after each extraction, the supernatants were replaced by the distilled water. HVED-assisted extraction allowed noticeably increase recovery of ionic components (Fig. 5a). The order of treatment applications is shown in Fig. 5 by dashed arrows. After application of the first PEF and HVED steps, the next sequential UAE and HPH steps gave rather small additional input to the extraction. However, pulsed electric energy steps (PEF and HVED) were ineffective for recovery of chlorophylls (Fig. 5b) and gave only $\approx 1\%$ of the recovery. The noticeable recovery of chlorophylls was only obtained after application of sequential steps UAE→HPH. It is interesting that the sedimentation and microscopic analyses evidenced that microalga cells after the HVED treatment were highly agglomerated (see inset to Fig. 5a) [45]. This phenomenon can be explained by changes in surface charge of the microalga *Nannochloropsis* sp. that results in the loss of the stability of the suspension.

In general, HVED treatment has shown very promising results for the enhancing extraction of nutritional and bioactive compounds (ionics, polyphenols, proteins, and pigments) from different microalga species. The process is simple, fast, can be energetically efficient and combined with other extraction techniques. However, the scaling up to industrial scale and continuous flow HVED treatment systems still presents various problems. Moreover, HVED treatment can produce a large quantity of active radical species, ozone, and provoke the fragmentation of solid particles suspended in the biomass and intensive damage of cell walls.

General discussion and comparison of efficiency of different techniques

Cell disruption effectiveness and selectivity bio-molecules extraction

The sustainability of biofuel production and high value products largely depends upon efficient extraction of the bio-molecules. Cell disruption effectiveness was found to differ according to the microalga species, cell wall strength, and disruption methods. An ideal extraction method should be

more selective towards extraction of specific microalga products and simultaneously minimize the co-extraction of contaminants [22]. Therefore, it is important to find appropriate cell disruption methods to improve the extraction effectiveness. The disruption effectiveness of different techniques on microalga *Nannochloropsis* sp. in terms of disrupted cells, recovery of pigments, proteins and lipids, as well as other compounds were summarized in Table S1 (Supplementary Material).

Cost-effectiveness

Microalgae cell disruption technique often acquires high energy input. Cost-effectiveness in cell disruption is related to several factors such as energy consumption per kilogram of dry weight, dry biomass concentration of treated microalga suspensions, time to obtain reasonable disruption yields and so on [22]. Most of the researchers reported current cell disruption techniques such as UAE, MAE and S_pFE as cost-effective technologies for bio-molecules extraction from microalgae. However, generalization is very complicated due to different operating conditions in varied methods and many unknown factors in different microalga species. Comparison between treatments is also complicated because of the lack of knowledge concerning the relation between the extraction yield and the energy input. A varied energy consumption of 0.1–1500 kJ/kg was obtained for these cell disruption techniques (Table S2, Supplementary Material).

Benefits and limitations

Conventional cell disruption methods are hindered by longer treatment time, large toxic solvent requirements and production process with difficulties in scaling-up. Compared with conventional methods, the use of emerging techniques allowed the recovery bio-molecules avoiding toxic solvent, high temperature and treatment time. Most of them are potential for scale-up and have been used for commercial application. The main benefits and drawbacks of varied cell disruption techniques are summarized in Table S3 (Supplementary Material).

Conclusions

Microalgae *Nannochloropsis* sp. present a diverse collection of various species that have different contents of proteins, pigments, lipids, phenolics and other important constituents. The proposed conventional and recently developed extraction methods have different advantages and disadvantages in terms of efficiency, the selectivity of extraction, maintenance and operation costs, energy consumption, processing time, degradation of compounds, and possibility of scaling up to

industrial scale. The combination of different methods and development of optimized treatment protocol could give new roads for highly selective extraction techniques with reduced treatment time, consumed energy, and other useful benefits, when seen from a techno-economic point of view.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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