Algal Research 82 (2024) 103638

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Protein extracts from microalgae and cyanobacteria biomass. Techno-functional properties and bioactivity: A review

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ARTICLE INFO

Keywords:
Microalgae
Cell disruption
Purification techniques
Protein yield
Techno-functional properties
Bioactive peptides

ABSTRACT

Microalgae and cyanobacteria are photosynthetic and unicellular organisms that contain considerable amounts of proteins, lipids, carbohydrates, and polyunsaturated fatty acids, among others, with applications in the cosmetic, pharmaceutical, and food industries. These microorganisms can accumulate protein up to 70 % of total biomass depending on the microalgal strain, hence they have been regarded as an alternative protein source for the future. Microalgal proteins have important applications such as emulsifying, foaming, and gelation properties, which are important for the determination of quality and texture of foods. Some microalgal peptides possess important bioactivity with many health-benefit effects. Therefore, to maximize the production of proteins from microalgae and cyanobacteria, many protein extraction procedures have been studied to increase the economic return. They have been tested towards higher protein yields at low energy cost, the preservation of protein native properties, and lower cell debris. This later is fundamental to facilitate the subsequent purification processes so that the overall cost can be reduced. The aim of this work is to review some cell disruption processes for the extraction of protein from microalgae and cyanobacteria, considering that this step is crucial for the overall process due to the high rigidness of microalgal cell covering, which can hamper the release of proteins. It also aims at reviewing the purification techniques after cellular disruption, from conventional to more recent approaches, and finally addresses the antioxidant, antidiabetic, antihypertensive, antibacterial and other bioactive properties of microalgal protein hydrolysates and peptides.

1. Introduction

Microalgae are unicellular organisms with the highest photosynthetic efficiency in nature, reaching values between 10 and 20 %, while most terrestrial plants show efficiencies of 1 to 2 % [1]. Approximately 30,000 species of microalgae exist, each possessing high nutritional value due to their rich content in proteins, lipids and minerals, which make them a product of high interest with the potential for incorporation into the human diet [2–6]. Cultivation of microalgae is fairly straightforward. They do not compete for arable land, require only nutrients, can grow in non-potable water, and can be cultivated in both open or closed systems [7,8]. To avoid contamination, these microorganisms are often cultivated under extreme conditions, such as high pH or salinity, which minimize the presence of bacteria and other contaminants competing for nutrients in the cultivation medium [8].

The human population is expected to increase to 9 billion people by 2050, creating a greater need for food and other resources, requiring a 70 % increase in food production [2,9–11]. Keeping up with the food production requirements is challenging, and traditional crops and animal proteins cause environmental impact [1,2,11]. The culture conditions of microalgae significantly affect their final composition, and the cultivation of these microorganisms offers several advantages over traditional food production methods. The traditional cultures rely on limited resources, such as arable land, freshwater and nutrients, while

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https://doi.org/10.1016/j.algal.2024.103638
Received 28 May 2024; Received in revised form 8 July 2024; Accepted 26 July 2024
Available online 29 July 2024

microalgae can be cultivated in non-arable land, and can use seawater or wastewater [12,13]. Additionally, microalgae fix a high quantity of CO_2, producing about half of the global oxygen levels [2].

Proteins are a major component of human diet, and most of the protein we consume comes from meat. Due to the environmental impacts of meat production, there is a need to replace animal protein with plant protein, as well as to advocate for alternative diets and protein sources [2,9,10]. The microalgae proteins possess a well-balanced amino acid composition, including many essential amino acids required for an adequate diet. The amino acid score (AAS) of microalgae is similar to that of traditional protein sources, such as eggs [4,9,14,15]. The Cyanobacteria *Arthrospira platensis* Gomont has a protein content of 50 to 70 % in dry matter, which is close to the 70 to 76 % found in meat, and is even higher than alternative sources like soybeans, which have around 40 % protein [16,17]. Microalgae are already used as raw material in several industries, such as food, medical, cosmetic and biofuel industries. Due to the tough microalgae cell wall, the cost of extracting protein or lipid fraction is high. It was suggested to find mild cell disruption techniques to extract both fractions separately, reducing overall costs [18]. Due to the presence of bioactive compounds, microalgae biomass has several applications in biotechnology, cosmetics and biofuel production [2,19].

Proteins are a major fraction of microalgae biomass. The protein composition depends on the cultivation conditions, and a key challenge is the extraction of these biomolecules maintaining their native form, which is responsible for their functionality [20]. Microalgae have poor digestibility. The proteins in the biomass are extracted, and then used as food supplements or added to food products to increase their nutritional value [12]. The first step in protein extraction involves cell lysis or cell disruption, which breaks the tough cell wall of microalgae and exposes the intracellular components. Some examples of cell disruption techniques are bead milling, enzymatic treatment, ultrasounds, high pressure homogenization, and pulsed electric field [21,22]. After disruption of cells, purification steps are needed to separate the proteins from other compounds present in the lysate, and a usual first step is the centrifugation of the crude extract to separate the soluble proteins from the cellular debris [23]. Further purification/isolation steps can be applied, depending on the degree of purity desired for the protein extract, and such steps include precipitation techniques, *e.g.* isoelectric point or ammonium sulphate precipitation, dialysis and chromatography [23].

To enhance the appeal of microalgae, there has been a growing focus on extracting proteins from the biomass and applying their techno-functional properties to existing food products. Foaming, emulsion and gelation are some of the desirable properties. These are responsible for giving food products their texture, consistency, and flavour retention, and some examples of applications are dairy products, and baked goods [17,19,24]. Peptides derived from microalgae proteins possess bioactive properties, such as antioxidant, anti-inflammatory, anti-tumour, antimicrobial, antihypertensive and immunostimulant [25–27]. Peptides are chains of 2 to 20 amino acids that can be generated through chemical hydrolysis and enzymatic hydrolysis [25]. The advantage of using bioactive peptides is that they can be quickly administered. Antioxidant peptides can prevent cellular damage caused by reactive oxygen species (ROS), helping in tissue proliferation and wound healing [27].

In this review, the current extraction and purification techniques of proteins from microalgae are explored. Among the available techniques, bead milling, high pressure homogenization, pulsed electric field, ultrasounds, enzymatic and chemical treatment are discussed. This review analyses the potential of microalgae proteins to be used in food products due to their techno-functional properties, and looks at current bioactive properties of peptides obtained from microalgae proteins.

2. Methodology

This review was conducted using Google Scholar database, searching for articles written in English, since 2018. The primary focus was about the production of protein extracts from microalgae and cyanobacteria and its applications. Four major topics were selected for discussion, including cell disruption processes, protein purification, techno-functional properties of proteins, and bioactive peptides. Nine main keywords included *Spirulina platensis*, *Chlorella vulgaris*, *Haematococcus pluvialis*, cultivation, cell disruption, protein extraction, purification, function, and functionality. As secondary keywords, gelation, emulsification, foaming, water-holding capacity, and oil-holding capacity were used. Another set of secondary keywords were also employed, including microalgae, *Arthrospira platensis*, *Chlorella vulgaris*, *Haematococcus pluvialis*, protein extraction, bioactive peptides, and hydrolysates. Articles containing the words 'seed', 'fuel', 'biofuel', 'plant', and 'nanoparticles' were excluded during the search. The software used for bibliographic management was Mendeley for Windows (v1.19.8). Relevant references found in the research articles gathered from the database were considered, independently of the year.

3. Cell disruption methods for the release of microalgal proteins

The production of microalgal proteins at a biorefinery level ranges from microalgae cultivation to final purification procedures to obtain the desired products (Fig. 1). During cultivation, the effect of shear stress during mixing (mechanical mixing, aeration, and pumping) on microalgae cells must be considered to minimize cell mortality and achieve the highest growth rates and cell viability [28]. Robust microalgae are utilized in mass culturing since they can withstand the shear forces. In general, green algae, such as Chlorophyta *Chlorella vulgaris* Beijerinck, 1890, and Chlorophyta *Haematococcus pluvialis* Flotow, 1844, are more resistant than cyanobacteria [28].

The nature of the microalgae cell walls is important to consider not only for the cultivation stage, but also for the downstream processing. They are quite diverse, presenting significant differences between species [29,30]. Cell walls can greatly vary in the molecular composition and the nature of the molecular linkages between the constituents, both factors shaping the overall structure [30]. Generally, microalgae cell wall is more resistant than that of cyanobacteria [31]. Among the commercially available species, *Haematococcus pluvialis* in the aplanospore phase is characterized by a very thick trilaminar cell wall constituted of cellulose and sporopollenin, making the alga remarkably resistant against sheer and disruption forces [31,32]. *Chlorella vulgaris* also possesses a rigid, unilamellar cell wall composed of cellulose and glucosamine, but it lacks sporopollenin [28,33,34]. Heterokontophyta *Nannochloropsis* sp. are also known for their rigid cell wall composed of

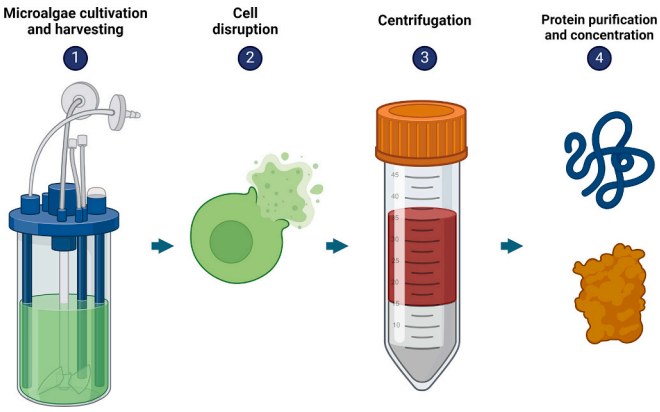

Fig. 1. Production of microalgal proteins. The process initiates with microalga cultivation and concludes with the final purification steps. Cell disruption is necessary to enhance the release of intracellular proteins, and it is crucial due to the rigid cell covering of microalgae. Scheme adapted from the template "4-Step Sequence Panels (Layout 4x1)" retrieved from *BioRender Templates*.

cellulose and hemicellulose [30,31]. *Arthrospira platensis* possesses a peptidoglycan cell wall, primarily composed of murein, and is weak due to the absence of cellulose [31,35,36]. Another marketed microalga species is the Chlorophyta *Dunaliella salina* (Dunal) Teodoresco, 1905, which lacks a cell wall. However, this alga is mainly used in research and in the industry due to its high capacity for β-carotene production, with scarce studies dedicated to protein production [37]. The composition and thickness of microalgal cell wall is also influenced by the environment and growth conditions applied [29,34].

Cell wall disruption is of crucial importance since it can greatly increase the costs of the entire process [38]. This step involves the breakage of the cell covering to release the intracellular components into the extraction media [39]. The method employed for the destruction of the cell wall highly influences the quality and yields of the final products [40]. The rigidness of the cell wall and the location of the desired component inside the cells must be considered to determine which method is the most adequate [41], since some microalgal cell walls are thick and resistant, and the proteins can be encountered in some organelles or even bound to the cell membrane, thus making the disruption process challenging [42]. An adequate cellular degradation step should release the desired products at high yields, preventing any degradation and contamination of the components to be isolated. It should be energetically efficient, fast, easy to operate, and should not complicate the subsequent purification steps [43,44]. Several methods to degrade the cell covering have been utilized and they can be classified as mechanical, physical, chemical and enzymatic methods [45]. Table 1 presents protein yields obtained with six different cell disruption methods from several studies, while Fig. 2 displays a bar graph for better visualization of the results. Table 2 lists some advantages and disadvantages of the techniques discussed.

A direct comparison between the studies is difficult since the protein extraction procedures vary and cannot be standardized. The environmental and growth conditions during cultivation affect the rigidity of the cell wall [29,34]. Therefore, the same species cultivated under different environments might exhibit variable resistance to disruption forces, resulting in different rates in the release of intracellular components. The features of the equipment used for the disruption process are also important. The analytical technique used for protein determination also influences the results, since each technique has its own limitations. For instance, total nitrogen analysis, *e.g.* Kjeldahl method, tends to overestimate the protein content since this technique also measures non-protein nitrogen, and often the conversion factors are not accurate [46]. Spectrophotometric techniques such as Lowry and Bradford methods are used for soluble protein determination, and are affected by interfering substances that can be present in the extracts, overestimating the protein content [46]. Therefore, the results presented in Table 1 highlight general trends, aiming at addressing current challenges in protein production from microalgae.

3.1. Pulsed electric fields

Pulsed electric field (PEF) technology involves the exposure of cells to high-intensity electric field pulses. This causes the accumulation of electrical charge on the cell membrane, which induces an elevated transmembrane potential [47,48]. As a result, cell membrane conductivity and permeability increases due to the formation of pores across the lipid bilayer, enhancing the release of intracellular compounds to the extraction media [49,50]. Cell disruption and extraction efficiency using PEF increases at higher electric field intensity, energy input, treatment time, and temperature [51–53]. The polarity of the pulses also plays an important role, with better protein and phycocyanin extraction yields using monopolar rather than bipolar pulses [54]. Post-treatment incubation is also important for PEF efficiency, since this step allows the components to diffuse out of the cells after being disrupted [55]. The primary advantage of PEF treatment is its selectivity [36]. This method promotes a mild cell disintegration process, releasing the intracellular components without the formation of cell debris, making the process clean and simplifying subsequent downstream purification steps [43,54,56,57]. Some studies showed higher purity in phycocyanin (CPC) extraction using PEF when compared to more disruptive methods, such as bead milling [36,53] and high-pressure homogenization [54], demonstrating its selectivity. PEF also allows the release of valuable compounds at low energy consumption [53], with specific energy inputs that can be lower than 1 $kWh \cdot kg^{-1}$ suspension [57,58]. However, PEF treatment is not suitable for protein release from most microalgae, as it results in poor yields (Table 1). Also, it usually requires a refrigeration system to avoid excessive heat [51,56,59,60], preventing protein denaturation due to thermal damage [4].

In this review, the highest protein yields with PEF treatment were reported for Cyanobacteria *Arthrospira platensis* biomass, with values of 100 % [36] and 37.4 % [54]. These results markedly exceed the yields obtained from *Chlorella vulgaris* [51,56,57], Chlorophyta *Neochloris oleoabundans* S. Chantanachat & H. C. Bold [58], and Heterokontophyta *Nannochloropsis* sp. biomass [59,60], with reported extraction yields below 10 %. Such differences can be attributed to variations in cell covering composition, as *Arthrospira platensis* cells have a weaker cell wall compared to the other species mentioned [31,36]. PEF treatment may be an adequate approach for protein release from species with weaker cell walls, allowing for promising yields through a clean and selective process. Such species could be *Arthrospira platensis*; Rhodophyta *Porphyridium cruentum* (S.F.Gray) Nägeli, 1849, which lacks a true cell wall and is covered by a layer of sulphurised polysaccharides [31]; and *Dunaliella salina*, which does not have a cell wall, although this species has not been studied for protein production [37]. Because of the weak cell wall electroporation mechanism [55], which cannot provoke major changes in the structure of microalgal cell covering [56], PEF treatment is ineffective for protein release from rigid cell wall microalgae (Table 1), even at higher energy inputs [39]. The permeabilization of cells by PEF was efficient at releasing high amounts of small ions, carbohydrates, and phenolic compounds, but it is not sufficient to release proteins due to their large molecular size [51,57]. Among the methods screened in this review, PEF was the least efficient method for this purpose.

3.2. Ultrasounds

Ultrasound-assisted extraction (US) has also been applied as a novel method to enhance the extraction of intracellular compounds from different materials, with demonstrated efficacy and selectivity for the recovery of certain compounds, such as antioxidants [61]. The technique is based on the phenomenon of cavitation that occurs in liquid media, due to the propagation of ultrasound waves [62]. The bubbles generated in the medium collapse near the cells, causing damage to the microalgal cell wall and enhancing the release of intracellular compounds into the extraction media [63]. A range of variables can affect the US extraction efficiency, such as frequency, amplitude, temperature, and treatment time, among others [12,38,64]. An advantage of the US treatment is the high extraction yields of valuable compounds [38]. When combined with other pretreatments, such as alkaline or enzymatic approaches, protein yields with values between 75 and 82 % [12,64,65], and roughly 95 % [66] could be achieved. In combination with maceration, freeze/thawing, and homogenization, there was a boosted allophycocyanin extraction yield of up to 93 % [67]. Another advantage is the clean extraction process that the method can offer, without cell debris production [44]. Additionally, the method requires low amounts of solvent, is fast, and compatible with water, making it an attractive option [38]. US treatment also has disadvantages. The method requires a cooling system to avoid temperatures above 35 °C, as it provokes protein denaturation, reducing extraction yields and bio-functionality [4,59,68,69]. Although higher ultrasonication amplitude, time, and energy input imply an enhanced protein yield, it might compromise the selectivity of the process due to greater cell disruption, leading to the

Table 1
Protein extraction yields obtained from cyanobacteria and microalgae biomass by different cell disruption methods.

Pretreatment	Experimental conditions	Microalgae and cyanobacteria	Protein yield (% of total protein)	Reference
Alkaline treatment	2 % (w/v) biomass concentration at pH 12. The mixture was stirred for 2 h at 40 °C. Lowry method for protein analysis	*Chlorella vulgaris*	47.3 % (corresponds to 26 % protein of dry weight)	[34]
	1 g freeze-dried biomass in 50 mL solvent at pH 12. The mixture was stirred for 1 h at 40 °C. Total Nitrogen Elemental Analysis for total protein (factors of 6.35, 6.27, 6.35, 6.28, and 6.25) and Lowry method for hydro-soluble protein analysis.	*Porphyridium cruentum* *Arthrospira platensis* *Chlorella vulgaris* *Nannochloropsis oculata* *Haematococcus pluvialis*	76.07 ± 1.48 67.72 ± 1.64 42.43 ± 0.52 31.56 ± 1.06 27.43 ± 0.49	[75]
	2 % (w/v) biomass concentration, pH 12. 2 h at 40 °C. Total Nitrogen Elemental Analysis for total protein and Lowry method for hydro-soluble protein analysis.	*Porphyridium cruentum* *Arthrospira platensis* *Chlorella vulgaris* *Nannochloropsis oculata* *Haematococcus pluvialis*	73.5 ± 1.2 53.4 ± 0.2 33.2 ± 0.0 31.1 ± 2.0 15.8 ± 0.1	[73]
	Maceration of the biomass suspensions at 170 rpm at 20 °C during 2 h and 18 h. pH 13.6. Total Nitrogen Analysis (factor 6.25)	*Chlorella vulgaris*	73.6 ± 1.2 % for 18 h.	[65]
	8 % *w/v* suspensions, pH 12. Total Nitrogen Analysis (Kjeldahl method, factor 6.27)	*Arthrospira platensis*	32.48 %	[12]
Enzymatic treatment	Enzymes used: Lysozyme, collagenase, trypsin, and autolysin. Autolysin + sonication treatment. (BCA assay for protein analysis)	*Chlamydomonas reinhardtii*	20 % 95 %	[66]
	5 % w/v Alcalase solution during 4 h, 50 °C and pH 8. Kjeldahl method (factor 5.20)	*Nannochloropsis gaditana*	35 ± 1 %	[90]
	5 % (*w/v*) Alcalase (per dry matter) at pH 8 and 50 °C during 4 h. Kjeldahl method (factor 5.20)	*Nannochloropsis gaditana*	35 ± 1 %	[29]
	Cellulase, mannanase, and the combination of both enzymes. pH 4.4, T: 53 °C. Total Nitrogen Analysis (factor 4.78).	*Nannochloropsis* sp.	20 % (either with individual or combined enzymes)	[78]
BM	Biomass suspensions of 25 g (dry weight)·L^{-1}, ZrO_2 beads at 2039 rpm, 25 °C, 0–10 min treatment. Bead milled biomass + enzymatic treatment, pH 7.4, 24 h (Lowry method)	*Chlorella vulgaris*	~40 % at 10 min of BM. 68 % with lipase enzyme at 45 °C	[41]
	A 100 g·L^{-1} suspension, 1 h treatment, ZrO_2 beads, a filling percentage of 65 %. Kjeldahl method (factor 5.20)	*Nannochloropsis gaditana*	53 ± 4 %	[29]
	25 kg (dry weight)·m^{-3} biomass, ZrO_2 beads, 25 % *v*/v filling. Modified Lowry method (DC™ Protein assay, Bio-Rad)	*Chlorella vulgaris*	40–45 %	[57]
	25–145 g (dry weight)·kg^{-1}, 1 mm ZrO_2 beads, 65 % v/v filling, speed rotations of 9–10 m/s. Modified Lowry method (DC™ Protein assay, Bio-Rad).	*Chlorella vulgaris*	32–42 % depending on the biomass concentration.	[69]
	1/13 (w/v) biomass in distilled water, 1–1.6 mm zirconium silicate beads, 1–60 min, 2500 rpm. Lowry method.	*Chlorella vulgaris*	96 % after 40 min	[34]
Stirred BM	10–1000 g·L^{-1} biomass, 0.8–1.25 mm ZrO_2 beads, stirring speeds of 1000–3000 rpm, filling volume of 83 %. Total Nitrogen Elemental Analysis (factor 6.25)	*Tetraselmis suecica*	96 % (1 mm beads, 2000 rpm, 100 g·L^{-1} biomass suspension)	[99]
PEF	40 kV·cm^{-1}, specific energies of 28–112 J·mL^{-1}, square pulses of 1 μs. Incubation step for up to 6 h after PEF. Modified Lowry method (DC™ Protein assay, Bio-Rad).	*Arthrospira platensis*	>100 % (56 and 112 J·mL^{-1}) after 30 min of incubation step. (when compared to BM extracted protein)	[36]
	10–30 kV·cm^{-1}, 20–100 kJ·kg^{-1}, 25 °C 20 kV·cm^{-1}, 100 kJ·kg^{-1}, 25–45 °C (Lowry method)	*Arthrospira platensis*	25.4 % (20 kV/cm, 100 kJ/kg) 37.4 % at 35 °C	[54]
	12 g (dry weight)·L^{-1} algae suspension, 10–30 kV·cm^{-1}, 20–100 kJ·kg^{-1} energy input, 25 °C. Lowry method.	*Chlorella vulgaris*	5.2 % (20 kV·cm^{-1}, 100 kJ·kg^{-1})	[56]
	15–60 g·L^{-1} biomass suspension, 30 kV·cm^{-1}, N: 2 and 10 pulses. Modified Lowry method (DC™ Protein assay, Bio-Rad).	*Nannochloropsis gaditana*	10 % ± 0.1 (60 g·L^{-1} suspension, 10 pulses)	[29]
	17.1 kV·cm^{-1}, pulse length of 5 μs, 25–65 °C. Modified Lowry method (DC™ Protein assay, Bio-Rad).	*Chlorella vulgaris*	4.4 % at 45 °C, and 4 % at 55°.	[57]
	Batch mode: 7.5 and 30 kV·cm^{-1}. Pulse lengths of 0.05–5 ms were applied each 5 s. Modified Lowry method (DC™ Protein assay, Bio-Rad).	*Neochloris oleoabundans*	13 %	[58]
	27 and 35 kV·cm^{-1}, total specific energy input of 50–150 kJ·kg^{-1}suspension. T = 20–25 °C. Modified Lowry method (DC™ Protein assay, Bio-Rad).	*Chlorella vulgaris*	8 % (35 kV·cm^{-1}, 100 kJ·kg^{-1}suspension)	[51]
	20 kV·cm^{-1}, 1–4 ms, 13.3–53.1 kJ·kg^{-1}suspension. Bradford method for protein analysis.	*Nannochloropsis* sp.	5.2 % (20 kV·cm^{-1}, 4 ms)	[59]
	E = 20 kV·cm^{-1}, n = 1–800 pulses, t = 0.01–8 ms. Total and extracted protein measured by BCA and Bradford method, respectively.	*Nannochloropsis* sp. *Phaeodactylum tricornutum* *Parachlorella kessleri*	3.1 ± 0.4 % 4.1 ± 0.3 % 1.8 ± 0.2 % (at Wmax ≈ 704 kJ·kg^{-1})	[60]
HPH	N: 1–3 passes, Pressures: 10–100 MPa, 10–200 kJ·kg^{-1}. Water as the solvent. Kjeldahl method (5.95 factor)	*Arthrospira platensis*	91 % (200 kJ·kg^{-1} dry biomass)	[74]

(*continued on next page*)

Table 1 (*continued*)

Pretreatment	Experimental conditions	Microalgae and cyanobacteria	Protein yield (% of total protein)	Reference
	10–100 g·L^{-1} desalted biomass concentration, 0–150 MPa. Total Nitrogen Elemental Analysis (factor 6.25)	*Tetraselmis suecica*	>80 % (40 MPa, 1 pass); ~90 % (50 MPa, 1 pass).	[82]
	2 % biomass concentrations in water, N: 2 passes, P: 2700 bar. Total Nitrogen Elemental Analysis for total protein (factors of 6.35, 6.27, 6.35, 6.28, and 6.25) and Lowry method for hydro-soluble protein analysis.	*Porphyridium cruentum* *Arthrospira platensis* *Chlorella vulgaris* *Nannochloropsis oculata* *Haematococcus pluvialis*	88.52 ± 1.17 % 76.02 ± 0.75 % 51.68 ± 2.03 % 49.50 ± 1.51 % 40.93 ± 1.97 %	[75]
	100 g·L^{-1} biomass concentration, N: 1 pass, 1500 bar of pressure. Kjeldahl method (factor 5.20)	*Nannochloropsis gaditana*	49 ± 1 %	[90]
	100 g·L^{-1} suspension, 300–1500 bar, N: 1 pass. Kjeldahl method (factor 5.20)	*Nannochloropsis gaditana*	50 % (1000 bar)	[29]
	N: 2–10 passes, P: 150 MPa, 150–1500 kJ·kg^{-1}. Water as the solvent. Bradford method.	*Nannochloropsis* sp.	91 % (150 MPa, 6 passes)	[59]
	2 % dry biomass concentration in distilled water, N: 2 passes, 2700 bar. Lowry method.	*Chlorella vulgaris*	66 %	[34]
	1 % of biomass suspensions in water, N: 1–10 passes, P: 400–1200 bar, 22 °C. Bradford method.	*Parachlorella kessleri*	71 % (10 passes at 1200 bar)	[63]
	12 g·L^{-1} (dry weight) suspensions, N: 1.10 passes, $P = 150$ MPa, 25 °C. Lowry method.	*Chlorella vulgaris*	54.1 % after 5 passes	[56]
	Samples were dissolved before and after HPH treatment at pH 7 and 12. HPH processing included 2 passes at a pressure of 2.7 kbar. Lowry method.	*Chlorella vulgaris*	98 % at pH 12 and after HPH (2 passes, 2.7 kbar)	[88]
	2 % biomass concentration in distilled water, $n = 2$ passes, $p = 2700$ bar. Total Nitrogen Elemental Analysis for total protein and Lowry method for hydro-soluble protein.	*Porphyridium cruentum* *Arthrospira platensis* *Chlorella vulgaris* *Nannochloropsis oculata* *Haematococcus pluvialis*	90.0 ± 2.4 78.0 ± 2.8 52.8 ± 0.6 52.3 ± 0.6 41.0 ± 3.7	[73]
HPP	Pressures of 0–600 MPa during 0.1 min, with pH values of 5.7–8.6, followed by extraction for up to 24 h. Lowry method.	*Arthrospira platensis*	~99 % with pressures >300 MPa, pH values between 7.1 and 8.6, and ≈2 h extraction after HP processing.	[93]
	100–600 MPa, 0–20 min at 20 °C. Solvents: dH_2O, phosphate buffer pH 6.8, 10 % NaCl. HPH is followed by aqueous extraction for up to 24 h. Lowry method.	*Arthrospira platensis*	94.2 % at 600 MPa using dH_2O as the solvent (120 min extraction after HP processing)	[92]
US	100 kg·m^{-3} biomass concentration, 24 kHz, 200 or 400 W of power input, US duration of 30–120 s. Water as the solvent. Kjeldahl method (5.95 factor)	*Arthrospira platensis*	87.8 % (100 kJ·kg^{-1} dry biomass)	[74]
	10-min US (100 % power) + 0.4 M HCl/0.4 M NaOH/Water. 10-min US (0–100 % power, 35 to 130 kHz) + lysozyme or protease treatment (1, 2, and 6 h) (Total Nitrogen Analysis, factor 6.25)	*Chlorella vulgaris*	76.6 ± 0.6 % with 0.4 M NaOH. It was <40 % in water. 82.1 ± 1.1 % with protease enzyme.	[65]
	Amplitudes 20–100 %, duty cycles 20–100 %, 10–50 min. pH 12. Kjeldahl method (factor 6.27)	*Arthrospira platensis*	76.83 % with 80 % of amplitude, 30-min US and 60 % duty cycle	[12]
	24 kHz, 200 W power, 50 % amplitude, US duration of 1–880 s. Deionized water as the solvent. Total and extracted protein measured by BCA and Bradford method, respectively.	*Nannochloropsis* sp. *Phaeodactylum tricornutum* *Parachlorella kessleri*	8.9 ± 0.4 % 11.4 ± 1.1 % 6.4 ± 1.3 % (at Wmax ≈ 704 kJ·kg^{-1})	[60]
	20 kHz, 50–200 W power. US time 60 min. Water 100 kHz, 30–120 W power. US time up to 60 min. Water. Total Nitrogen Elemental Analysis (factor 6.25).	*Tetraselmis suecica*	90 % (100 and 200 W, 60 min-US) 90 % (120 W, 60 min)	[38]
	US (amplitudes of 30 and 60 %, 10 and 30 min, 20 kHz) + alkaline treatment at pH 12. $T = 35$ °C. Kjeldahl method	*Arthrospira platensis*	81.86 % (amplitude of 60 %, 30-min US)	[64]
	2 % w/v biomass in distilled water. 30 min-US and 20 kHz. Total Nitrogen Elemental Analysis for total protein and Lowry method for hydro-soluble protein.	*Porphyridium cruentum* *Arthrospira platensis* *Chlorella vulgaris* *Nannochloropsis oculata* *Haematococcus pluvialis*	67.0 ± 0.9 47.1 ± 0.9 18.1 ± 0.0 13.5 ± 0.1 8.5 ± 0.0	[73]
	2 % of dry biomass in distilled water. 25 kHz. US times up to 30 min. Lowry method.	*Chlorella vulgaris*	≈16.4 % after 25 min (9 % of protein per DW biomass)	[34]

BM – Bead milling; PEF – Pulsed electric fields; HPH – High pressure homogenization; HPP – High pressure processing; US – Ultrasounds.

release of undesired components and the production of cell detritus [12,38,64,70]. Other drawbacks of US treatment include the fact that cavitation is localized only near the probes, leading to losses of acoustic energy and reduced extraction efficiency [68,71], and the energetic requirements that might hinder its scale-up [72].

When the ultrasounds technique was conducted alone, in water, protein yields were below 20 % for Chlorophyta *Chlorella vulgaris*, *Parachlorella kessleri* (Fott et Nováková) Krienitz et al., *Haematococcus pluvialis*, Heterokontophyta *Nannochloropsis* sp., *Nannochloropsis oculata* (Droop, 1955) Hibberd, 1981, and *Phaeodactylum tricornutum* Bohlin,

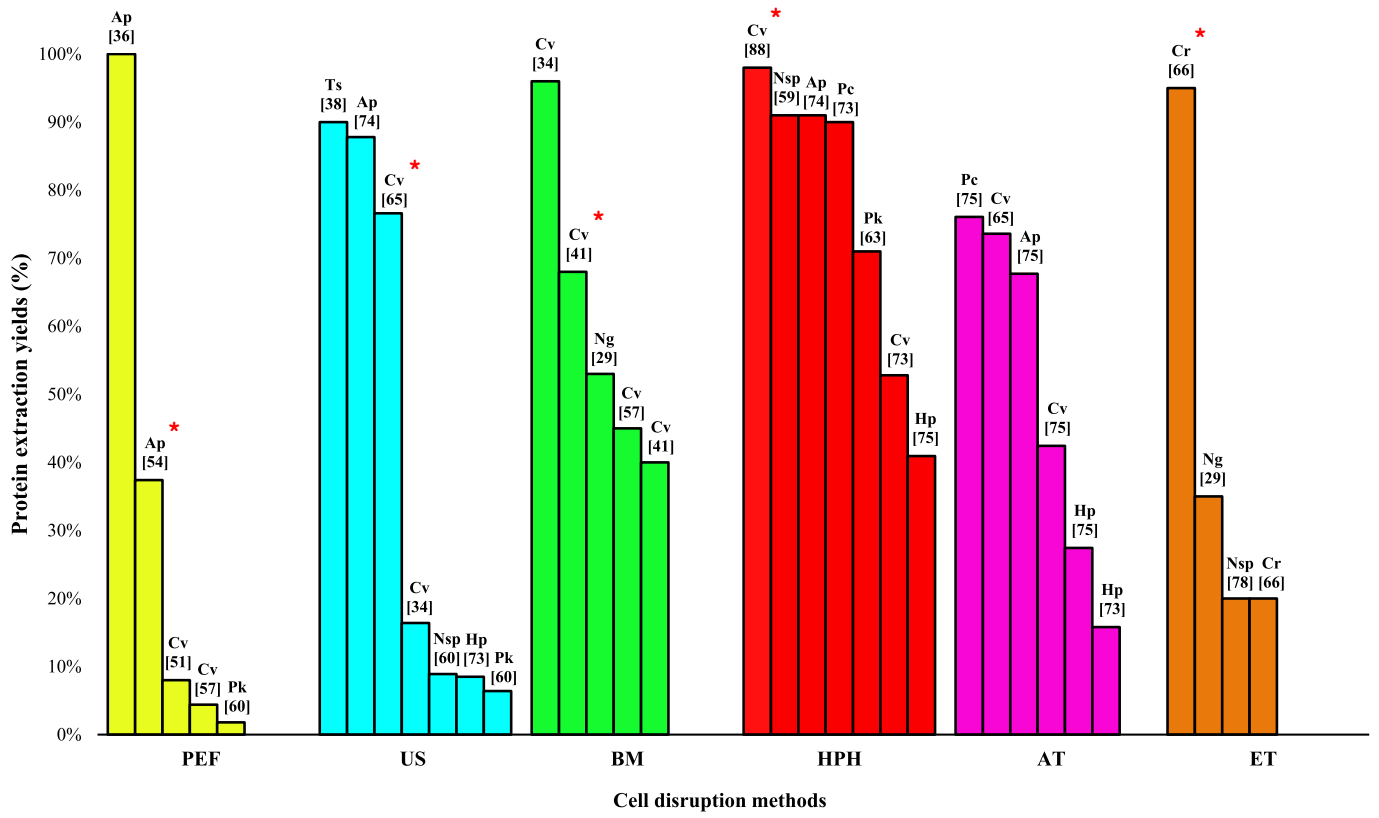

Fig. 2. Some protein extraction yields for the cell disruption methods discussed in this review. Cell disruption methods: AT – Alkaline treatment; BM – Bead milling; ET – Enzymatic treatment; PEF – Pulsed electric fields; HPH – High pressure homogenization; US – Ultrasounds. Microalgae species: Ap – *Arthrospira platensis*; Cr – *Chlamydomonas reinhardtii*; Cv – *Chlorella vulgaris*; Hp – *Haematococcus pluvialis*; Ng – *Nannochloropsis gaditana*; Nsp – *Nannochloropsis* sp.; Pc – *Porphyridium cruentum*; Pk – *Parachlorella kessleri*; Ts – *Tetraselmis suecica*. Asterisks (*) in red indicate combined approaches. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1897 [34,60,73] (Table 1). However, protein yields of 87.8 % [74] and 47.1 % [73] for *Arthrospira platensis*, 67 % for *Porphyridium cruentum* [73], and 90 % for Chlorophyta *Tetraselmis suecica* (Kylin) Butcher, 1959 [38] were also obtained in water. This could be attributed to the differences in cell wall composition and complexity between those microalgae. For instance, *Arthrospira platensis* and *Porphyridium cruentum* have a delicate cell wall in comparison to *Chlorella vulgaris* [31,73,75]. Similarly, *Tetraselmis suecica* had a weaker cell wall than that of *Chlorella vulgaris* [76]. Protein extraction from *Chlorella vulgaris* required the combination of the US treatment with alkaline conditions to enhance the yield [65]. Ultrasound itself was less effective than alkaline treatment. However, the combined approach is more efficient than both methods separately [64] (Table 1). Ultrasounds conducted in water could be a suitable approach for fragile cell wall microalgae and cyanobacteria, such as *Arthrospira platensis* and *Porphyridium cruentum*. High yields were obtained for these species, while avoiding alkaline conditions that could lead to protein denaturation [77], and the utilization of enzymes that increase the cost of the process [78]. Considering this, it is necessary to optimize US parameters to permeabilize the cell membrane rather than causing its destruction [55], in order to improve the selectivity of the ultrasound-assisted extraction. The method was also effective for robust microalgae in combination with alkaline and enzymatic treatments (Table 1).

3.3. High-pressure homogenization

High pressure homogenization (HPH) is a mechanical cell disruption method, and it is considered one of the most efficient approaches to enhance the release of intracellular substances [79,80]. In the homogenizer, cell suspensions are forcefully pumped through a small orifice inside high-pressure valves. Then, the cells are rapidly directed towards a low-pressure compartment, and this abrupt pressure change is responsible for the destruction of the cell covering [79,81]. The applied pressures and the number of passes highly influence the protein extraction yields and cell lysis [82,83]. The initial biomass is also important. Lower initial biomass concentrations have resulted in a higher cell lysis degree by HPH [83,84], which could enhance protein yields, although in another study it did not pose significant influence [82]. The main advantage of HPH is the high cell disruption efficiency, which enables high extraction yields of the intracellular products [56], including proteins (Table 1). Cell disruption rates of 91.8 and 99.9 % were obtained for microalgae belonging to the Chlorophyta *Haematococcus pluvialis* [85] and *Auxenochlorella protothecoides* (Krüger) Kalina & Puncochárová, 1987 [*Chlorella protothecoides*] [86], respectively. As disadvantages, the extraction assisted by HPH is not selective, resulting in the release of undesired cellular components into the medium, along with the production of cell detritus, complicating the downstream purification steps [54,59]. HPH was less selective than PEF treatment in extracting c-phycocyanin (CPC) from *Arthrospira platensis*, resulting in lower CPC purity for HPH extracts [54,87]. HPH treatment is a highly energetic method [59], and requires a refrigeration system to avoid protein denaturation because of the heat generated during the process [56], increasing the costs.

The best result among the HPH studies in this review reported a protein yield of 98 % from *Chlorella vulgaris* under alkaline conditions (pH 12) after 2 passes at 2700 bar [88]. Lower extraction yields between 51 and 66 % are displayed for the same microalgae (*Chlorella vulgaris*) in Table 1, when the HPH process was conducted with distilled water [34,56,73,75]. This suggests that HPH efficiency in protein extraction is enhanced under alkaline conditions for rigid cell wall microalgae.

Table 2
Advantages and disadvantages of the different cell disruption methods discussed.

Pretreatment method	Advantages	Disadvantages
Alkaline treatment	• Generally high cell disruption rates [44]. • High protein yields in combination with US and HPH treatments (Table 1). • Cheap and effective process using water [44].	• Possible denaturation and hydrolysis of proteins [77,103]. • Corrosion of reactors [68].
Enzymatic hydrolysis	• Mild cell disruption process [29,55,107]. • High selectivity [107]. • Low energy consumption [107].	• High cost of the enzyme [78,105]. • Enzyme specificity required for each microalga species [78]. • Low protein extraction yields when conducted alone [66,78] (Table 1)
PEF	• High selectivity [36,53]. • Mild cell disruption process (No cell debris) [54,56]. • Low energy requirements [53,57,58].	• Ineffective for rigid cell wall microalgae, showing protein yields below 10 % (Table 1). • Requires a refrigeration system [56,59].
BM	• High cell disruption rates (>90 %) [29,34,76]. • High protein extraction efficiency [34,99]. • Fast [68]. • Green process: avoids toxic solvents [99]	• Highly energetic method [34]. • Non-selective extraction with cell debris production [44,93]. • Requires a refrigeration system [29,34,69]. • Hard to scale-up [44].
HPH	• High cell disruption rates (>90 %) [85,86]. • High extraction yield of intracellular products [56], including proteins (Table 1) • Green process: compatible with water, avoiding toxic solvents [99].	• Highly energetic method [59] • Non-selective extraction, and cell debris production [54] • Requires a refrigeration system [56,59]
US	• High extraction yields [38]. • Enhanced protein extraction in combination with other techniques [12,65,66] • Low amounts of solvents, compatibility with water (green procedure), and fast [38] • Can offer a mild disruption process (no cell debris) [44]	• Requires a refrigeration system [38,59,60]. • High energy consumption [72]. • Cavitation process is localized, with losses of acoustic energy and diminished extraction efficiency [68,71]. • Low protein yields for rigid cell walls when conducted alone (Table 1)

BM – Bead milling; PEF – Pulsed electric fields; HPH – High pressure homogenization; US – Ultrasounds.

Table 1 displays promising protein yields, ranging from 76 to 91 %, for *Arthrospira platensis* [73–75], *Tetraselmis suecica* [82], and *Porphyridium cruentum* biomass [73,75], using HPH with 1–3 passes and distilled water as the solvent. These yields can be attributed to a less resistant cell wall on the part of *Arthrospira platensis*, *Tetraselmis suecica*, and *Porphyridium cruentum* compared to that of *Chlorella vulgaris* [31,36,73,75,76]. *Nannochloropsis* sp. microalgae also have a rigid cell wall [31,60,75,89]. For *Nannochloropsis oculata* and Heterokontophyta *Nannochloropsis gaditana* Lubián, 1982, protein yields between 49 and 52.3 % were obtained with 1–2 passes in distilled water [29,73,75,90] (Table 1). An extraction rate of 91 % was also reported for *Nannochloropsis* sp. in water after 6 passes [59]. In this case, the application of a higher number of passes could potentially explain the increased protein yield. HPH treatment is a feasible approach for the complete extraction of microalgal proteins. Using species with more fragile cell walls, such as *Porphyridium cruentum* and *Arthrospira platensis*, could increase the value of microalgal proteins by applying fewer passes, thereby reducing energy consumption, and by using water as the solvent which avoids basic conditions that could denature proteins [77] and increase the costs. The technique is effective for robust microalgae, but it may require more energy usage, including a higher number of passes, or the combination with alkaline treatment. The HPH parameters should be optimized to maximize the protein yields maintaining their quality, as pressures above 450 MPa can lead to protein denaturation [91–93].

3.4. Bead milling

Bead milling (BM) is another mechanical pretreatment [94] and has been used for lipid extraction from algal cells [45]. Within the chamber, beads collide with cells in a high-speed spinning system, causing damage to the cell structure [45,95,96]. Several factors affect the efficiency of cell disruption by bead milling, such as bead size, biomass load, residence time, feed rate, and stirring speed [97,98], among others. Cell disintegration and protein release rates were higher for robust microalgae such as *Chlorella vulgaris*, *Neochloris oleoabundans*, and *Nannochloropsis oculata* when smaller bead sizes (0.3–0.4 mm) were used [76,98]. For the species *Tetraselmis suecica* [76] and *Porphyridium cruentum* [98], beads of ~1 mm were needed to achieve greater disintegration rates, and this finding might be related to differences in the cell wall structure between the microalgae mentioned. Protein release rate for *Tetraselmis suecica* did not depend on the bead size [76]. Different outcomes were observed for the initial biomass concentration, as some studies reported higher yields with lower biomass loads, while others reported the opposite [69,97,99]. The primary advantage of BM treatment is the high cell disruption rate, with values of 95 to 100 % achieved for robust microalgae such as *Chlorella vulgaris* [34,76], *Nannochloropsis gaditana* [29], and *Tetraselmis suecica* [76], meaning that all the intracellular components can be released with this technique, including proteins. Also, BM treatment is fast [68]. The method has some drawbacks as well. The lack of selectivity and the production of cell debris affect the quality of the extraction and complicate the protein separation processes [44,93]. The method also requires a refrigeration system to prevent excessive heat [29,34,69], which could lead to protein denaturation [4]. BM is highly energetic [34], and difficult to scale-up [44]. A 96 % yield of protein extraction have been achieved using this technique [34,99], meaning that almost all the proteins can be obtained with this method. BM is comparable to HPH treatment in terms of cell disruption efficiency, as both techniques have shown values above 90 % [29,34,76,85,86], indicating that almost all intracellular products can be released using either method. However, further studies are required to determine which of these techniques is more adequate in terms of protein extraction. BM parameters, such as bead size, should be optimized according to the microalgal strain to be extracted to enhance the results. Bead milling procedure was significantly more efficient for protein release than PEF and US treatments (Table 1).

3.5. Alkaline treatment

The alkaline treatment is a chemical method to disrupt cell walls. Other options available are organic solvents, detergents, and acids [68], each functioning by different mechanisms in degrading the chemical linkages between the components of the cell wall. However, the alkaline treatment is the main chemical method used for microalgal cell wall disruption [44]. The alkaline approach involves exposing cells to strong alkaline conditions. Under these conditions, sodium hydroxide (NaOH) hydrolyses the ester linkages and hydrogen bonds that exist between the different components of the cell wall, thereby enhancing cell disruption and facilitating the release of microalgal compounds [44]. Furthermore, at higher pH values, the negative net charge of the proteins increases. Consequently, the electrostatic repulsions between proteins intensify, improving their solubility in aqueous media and enabling the extraction [4,100]. When performing a chemical method for protein extraction, it is crucial to consider the influence of the chemicals on the different protein assays to obtain a reliable quantification of protein. For instance, Lowry method is affected by ethanol, Tris-HCl buffer, uric acid, guanine,

xanthine, and sodium citrate buffer, among others [101,102]. For Bradford assay, the known interfering substances are SDS, Triton X-100, detergents, alkali buffers, and NaOH [102]. In the case of alkaline cell disruption treatment, Lowry method rather than Bradford method should be the assay of choice for protein determination since it is carried out under alkaline conditions. The alkaline method can effectively disrupt rigid microalgal cell walls and enhance protein recovery due to their increased solubility at higher pH [44]. Another advantage is the low cost of the process, particularly when using water instead of organic solvents. [44]. Possible aggregation and denaturation of proteins at high pH values, and alkali-caused corrosion of the reactors are some of the disadvantages of the technique [68,77,103].

Protein yields of 73.5 % and 76.07 % for *Porphyridium cruentum*; 53.4 and 67.72 % for *Arthrospira platensis*; and 33.2 to 47.3 % for *Chlorella vulgaris* (Table 1) were obtained after 1–2 h of treatment. Therefore, the extraction efficiency also depends on the characteristics of the microalgal cell wall [31,34,73]. A yield of 73.6 % for *Chlorella vulgaris* after 18 h of treatment was reported in a study [65], which showed an increased yield over time. Alkaline approach in combination with other techniques such as US and HPH treatment has been employed to improve the extraction of proteins from microalgae, obtaining yields above 75 % [12,64,65,88]. The alkaline treatment was more effective than US and PEF techniques for protein release (Table 1), indicating that promising yields can be achieved with low energy requirements, making the technique economically feasible. The properties and bioactivities of the desired proteins could be compromised under strong alkaline conditions due to protein denaturation [77,103], thus performing the experiments in milder conditions could be more advantageous.

3.6. Enzymatic-assisted extraction

Enzymatic-assisted extraction is another approach to disrupt cells. Promising results in lipid extraction from microalgae using enzymatic treatment have been reported [104]. Enzymes operate by degrading specific components of the microalgal cell walls, such as proteins, hemicellulose, polysaccharides, among others, enhancing the release of products into the extraction media [78]. Several enzymes can be utilized to degrade the cell walls, such as cellulases, which degrade the β-1-4 glycosidic bonds found in cellulose [78,105]. Lipases catalyse the hydrolysis of triglycerides, and proteases catalyse the hydrolysis of peptide bonds of proteins [41]. The enzymatic activity highly depends on the temperature, pH, and enzyme concentration [106]. Enzymatic treatment is considered a gentle cell disruption method which avoids cell detritus production and preserves the properties of the cellular bioproducts [29,55,107]. The selectivity of the enzymatic reaction, and the low energy consumption are other advantages of this method [107]. However, the high costs of enzymes might impose an important obstacle for its industrial scalation [78,105]. Another drawback is the significant diversity in the nature of microalgal cell walls among species, requiring the selection of specific enzymes to target particular compounds present in the cell walls [78,107], thereby complicating its applicability in the industry.

In this review, protein yields of up to 35 % were reported in Table 1, suggesting that the total release of proteins was not possible using enzymatic cell wall disintegration in any of these studies. The method clearly improved the extraction yields in combination with other techniques, including bead milling and ultrasounds treatments [41,65,66]. Further research on the identification of suitable enzymes for each commercially available microalgae is recommended to extend the applicability of enzymatic treatment [78,107]. This requires more information on the structural features of the microalgal cell walls, which is very limited for most species [30].

4. Protein purification

In this review, 'purification' has been defined as the step conducted subsequently to the cell disruption process, aiming to recover the protein released into the medium. Various purification techniques have been addressed, ranging from conventional to novel approaches. Techniques such as centrifugation, precipitation, and dialysis, have been applied as the first steps of protein purification, and the process might finalize with of ion-exchange or gel filtration chromatography [23]. After cell disruption, the crude extract is usually centrifugated to obtain the supernatant containing the water-soluble proteins and separate the insoluble cell debris [108].

Isoelectric protein precipitation is a commonly employed method to recover proteins from the supernatant. Protein solubility increases at pH values significantly different from the isoelectric point (IEP) because of the net electric charge induced on the molecule surface [109]. At the isoelectric point, the electrostatic charges in the molecule are balanced, making the proteins electrostatically neutral, which favours the protein-protein interactions. Uncharged proteins become insoluble and precipitate in the media when the pH of the solution and the IEP are equal [12,100,108]. The IEP approach can be used in the selective proteins precipitation due to their distinctive properties (different IEP and solubility) [39]. Other advantages are the low cost of the process, its applicability in scale-up, and is generally known for its mild conditions, retaining the properties of the proteins. [100]. This is not always the case, as protein denaturation can occur upon precipitation, leading to lower yields throughout the purification process [108,110]. Different IEP values have been reported for microalgal proteins according to the species being studied. For *Arthrospira platensis*, protein IEP values around 3.5 have been documented [35,108,111]. For *Haematococcus pluvialis*, protein IEP values between 5 and 7 [110]. Recovery yields above 60 % were reported in some studies after protein IEP precipitation from *Arthrospira platensis* and *Chlorella vulgaris* [12,88,112]. In another study, there was only a 13.6 % recovery of protein isolate from *Arthrospira platensis* biomass after IEP precipitation, which was attributed to protein denaturation [108].

Protein precipitation with ammonium sulphate ($(NH_4)_2SO_4$) is a common practice to fractionate protein from microalgal crude extract. This technique is advantageous because it prevents protein denaturation due to its low solubilization heat of $(NH_4)_2SO_4$, and is inexpensive [55]. It can be used for the selective precipitation of phycocyanin (CPC). At 25 % saturation with $(NH_4)_2SO_4$, other proteins precipitate while CPC remains soluble. Then, CPC can then be precipitated at 50 % saturation of $(NH_4)_2SO_4$ [113]. During the c-phycocyanin purification from *Arthrospira platensis*, a recovery yield >80 % with purity ratios (A_{620}/A_{280}) over 1.5 have been obtained after precipitation with ammonium sulphate [113,114], enabling the obtention of food-grade purity protein [93]. A degree of phycocyanin purification above 90 % from *Arthrospira platensis* biomass using 60 % of $(NH_4)_2SO_4$ was showed [115]. After ammonium sulphate precipitation as a preliminary step in protein purification, dialysis followed by ion-exchange chromatography have been applied to further increase the purity of c-phycocyanin, achieving final values over 4.4 [113,114]. C-phycocyanin products with a purity ratio >4.0 are classified as analytical grade [116]. These results suggest that ion-exchange chromatography, as the final step in protein purification, is indeed an adequate approach to yield pure microalgal proteins. However, these purification techniques often involve multiple steps, leading to yield losses throughout the process resulting in a lower concentration of the final protein product [117]. They can be time-consuming, and might increase the production costs due to equipment requirements and other related expenses [72,118]. Different techniques have been developed in order to overcome important drawbacks from conventional techniques [72].

Filtration techniques have been employed for the separation, purification, and concentration of proteins [119], and consist in the separation of molecules based on differences in their molecular weight or charge by using membranes of different pores [120]. Several factors determine the efficiency of ultrafiltration technique, such as the material and the pore size of the membrane, the physicochemical properties of

the solution to be filtered, the flow velocity in the system, and the pressure applied, among others [121]. The advantage of ultrafiltration techniques is their mild operating conditions, as they do not require the use of chemicals that could potentially alter the properties of proteins [121]. They can be easily scaled-up to concentrate large extract volumes, and are also attractive due to their low energy requirements [118,121]. Nevertheless, the formation of cake layers on the membrane, resulting from the accumulation of proteins, can negatively impact in the results, avoiding the elimination of small molecular contaminant through the membrane [118]. The cake layer influences the permeation flux depending on the properties of the proteins, and its affinity to the solvent [119]. Ultrafiltration in combination with microfiltration or diafiltration have been applied for the c-phycocyanin purification from *Arthrospira platensis* extracts, allowing retention indexes above 90 % and purity values >1.0 by using membranes with different cut-off values [118,121,122]. These outcomes suggest that the obtention of food-grade phycocyanin is possible by ultrafiltration being a method more suitable for industrial scale up.

Aqueous two-phase system (ATPS) is another technique used in protein purification. It is a liquid-liquid extraction procedure in which the molecules to be separated distribute differently between two immiscible aqueous solutions, according to their polarity and affinity to both phases [123–125]. These systems typically consist of polymer/polymer, polymer/salt, or alcohol/salt combinations [126]. Recently, ionic liquids (ILs) have been used as an alternative to polymer or alcohol utilization [127,128]. The salt solution forms the lower phase, while the polymer, alcohol, or ionic liquid (IL) layer constitutes the upper phase, where the target product accumulates [23,125,127,129]. Variables such as protein recovery (protein obtained in the top phase compared to the total protein in the crude extract) and separation or extraction efficiency (the concentration of proteins in the lower phase when compared to the initial value in the same phase) are normally measured to evaluate the technique performance [130]. The separation performance is significantly influenced by various factors, including the type of salt and the molecular weight of the polymer, their concentrations, the pH, and the microalgal biomass load [125,131–133]. Higher pH values are required to solubilize proteins and to induce a net negative charge on the protein molecule [134]. Then, the salting-out capacity of the anions in the bottom phase favours the migration of the proteins towards the top phase in polymer/salt systems, promoting protein separation [128,134]. Higher salt concentrations normally diminish protein solubility in the bottom phase, prompting their migration to the upper polymer or alcohol phase [135]. Different salt compounds have been used, such as sodium citrate, ammonium sulphate, among others, showing different salting-out capabilities [132]. However, high salt concentrations might cause protein denaturation, thus diminishing protein recovery yields in the top phase [129]. Lower molecular weight polymers enhance the protein partition to the top phase, because of more free volume available for protein solubilization there [134]. Positive features of the technique are the mild conditions it operates, the high recovery rates, the potential for scaling up to industrial level, process simplicity, energy efficiency, and short times of processing [117,127,136]. It also has disadvantages, as ATPS involves the use of environmentally harmful solvents in large quantities. Another drawback is the lower purity of the proteins obtained due to the presence of salts [121]. Protein recoveries superior to 70 % have been reported to *Arthrospira platensis* and Chlorophyta *Chlorella sorokiniana* Shihira & R.W.Krauss crude extract, using the ATPS approach [132,134,135]. Extraction efficiencies for proteins and c-phycocyanin above 80 % were documented for *Chlorella vulgaris*, *Neochloris oleoabundans*, *Tetraselmis suecica*, and *Arthrospira platensis* [127–129], evidencing that the technique can effectively separate the proteins from other compounds. The experimental parameters should be optimized to facilitate phase formation, enhance protein recovery, and improve separation efficiency in the top phase, while simultaneously minimizing protein denaturation.

Liquid biphasic flotation (LBF) is a novel approach that has also been used for protein separation, and it combines ATPS with solvent sublation (SS) system [137]. The performance of this technology is governed by ATPS parameters, such as polymer/alcohol molecular weight and type of salt utilized, their concentrations, the pH of the solution, and the biomass load [130,138]. An acetonitrile/sugar system was proposed as an alternative to polymer or alcohol/salt systems, as high salt concentrations might affect the properties of the proteins, whereas sugar solutions have minimal effect on the isolated proteins [94]. The recovery yields in the LBF approach might be enhanced by the adsorption of proteins in the lower phase onto the surface of gas bubbles generated by a sintered glass disk installed at the bottom of the system. Proteins adsorbed on the ascending bubbles are delivered to the top phase, where they accumulate [137,139]. The air flotation effect is another variable that might play an important role. The LBF system combines the advantages of ATPS with an enhanced separation efficiency due to solvent sublation (SS) [139]. By employing this approach, c-phycocyanin recovery yields above 90 % were obtained in the top phase from *Arthrospira platensis*, with purification index of 3.49 when compared to the crude extract [117]. Protein recoveries of roughly 90 % with extraction efficiencies >80 % from *Chlorella vulgaris* and *Chlorella sorokiniana* microalgae were reported [94,135,138].

Three phase partitioning technique (TPP) has been applied for protein separation [140–142]. The system is usually created by mixing the crude extract, containing the desired proteins, with aqueous antichaotropic salt solution, and the subsequent addition of t-butanol or another aliphatic alcohol [143,144]. This mixture separates into three phases, with the top and the bottom phases consisting of the alcohol and salt layers, respectively, and the intermediate phase containing the proteins to be isolated [144,145]. The separation of the components is governed by a combination of factors, such as osmolytic, osmotropic, and isoionic precipitation, with salting-out effect [142,143,146]. In addition to the type of salt and its concentration, as well as other variables, the slurry/t-butanol ratio is a parameter that influences the performance of this technique [22]. Unlike ATPS system, TPP technology offers the advantage of simultaneous separation of more than two components, with non-polar compounds, such as lipids and pigments, accumulating in the organic top phase; polar compounds, including carbohydrates, in the aqueous bottom phase; and proteins in the intermediate phase, where they precipitate [124,145]. Other advantages are the simplicity of the technique, its scalability, and efficiency [72]. The recovery yield of phycobiliproteins from *Porphyridium cruentum* decreased when slurry/t-butanol ratios varied from 1:0.5 to 1:2, due to protein denaturation at higher alcohol concentrations. More specifically, there was a decrease from 74.49 to 25.82 % for b-phycoerythrin [22]. However, an increase in protein recovery was observed as the concentration of t-butanol increased from 1:0.5 to 1:1.5 slurry/t-butanol ratio, in experiments with Chlorophyta *Auxenochlorella pyrenoidosa* Molinari & Calvo-Pérez, 2015 [*Chlorella pyrenoidosa*] [147]. Protein or phycobiliprotein recovery yields between 50 and 90 % were reported by some of the studies screened in this review for *Chlorella vulgaris* [72,148], *Chlorella thermophila* [124], *Chlorella pyrenoidosa* [147], *Chlorella* sp. [149], and Rhodophyta *Porphyridium cruentum* and *Porphyridium purpureum* (Bory) K.M.Drew & R.Ross, 1965 microalgae [22]. Some of these studies, performed the TPP procedure in combination with enzymatic, ultrasounds, and microwaves treatments, with some of them reporting an improvement in the results, when compared to TPP alone [72,148].

5. Techno-functional properties of proteins

Microalgae have great nutritional value due to their high protein content and other beneficial compounds. The protein levels of microalgae are comparable to those of traditional sources like egg, meats, and milk, but their smell, taste and texture are off putting to most consumers [24,45,150,151]. Microalgae have a tough cell wall, with a high resistance against mechanical forces, and this results in a low digestibility of

microalgae biomass [24,97,151,152]. Thus, a current focus is the extraction of the protein fraction in an attempt to improve the digestibility and to enhance their techno-functional properties [97]. The proteins of microalgae and cyanobacteria possess useful techno-functional properties such as protein solubility, water and oil holding capacity (WHC and OHC, respectively), foaming capacity and others. These properties are essential for providing desirable characteristics to food products during preparation, transformation, and storage (Fig. 3) [4,108,150,153]. The techno-functional properties of proteins are derived from their physicochemical characteristics such as molecular size, net charge, and amino acid composition [151]. There has been great interest in the techno-functional properties of proteins derived from microalgae due to an increased awareness of many consumers, who prioritize sustainable and natural ingredients. Microalgae could replace artificial and animal-based proteins, which results in more food products that comply with vegetarian/vegan diets. Microalgae contain many valuable compounds with benefits for human health [13,16,151,154]. Proper evaluation of all techno-functional properties is an essential step to avoid unnecessary expenses [19]. A commonly used method to determine whether the techno-functional property arises from the proteins in microalgae or from another compound in the extract involves a simple test. If the introduction of proteases hinders the techno-functional property under investigation, it indicates that the proteins in the microalgae are responsible for this property. On the contrary, if the functionality of the extract persists despite the addition of proteases, it suggests that the proteins are either not responsible or not solely responsible for the observed techno-functional property [155].

5.1. Protein solubility

Solubility is a key factor for the application of proteins in food products. Adding less-refined extracts enhance the techno-functional properties. The proteins from a crude extract can interact with other compounds, such as polysaccharides. For instance, glycoproteins improve protein solubility over a wide pH range [108,150,151]. The protein solubility directly affects the ability of proteins to be used as emulsion, foaming or gelation agents. The solubility is affected by the physicochemical characteristics of the protein such as amino acid composition and the native/denaturation state of the protein [153]. This property is also influenced by several external factors, including, pH, salt or ions concentration, ionic strength, pre-treatment method, and temperature. The microalgae proteins are also affected by the interaction with other biomolecules such as pigments [12]. The pH is a key factor in protein solubility as each protein has an isoelectric point, defined as the point where the pH neutralizes the proteins net charges, reducing the interactions with water and thus favouring the protein-protein interactions, leading to aggregation and subsequent precipitation. The further away the pH is from the isoelectric point, the higher is the protein solubility. Certain pre-treatment methods can break hydrophobic and hydrogen bonds, leading to increased solubility [6,24,86,150,153,156]. Protein hydrolysis increases solubility in acidic conditions, leading to the breakdown of insoluble aggregates, the reduction of molecular weight, and the exposure of more hydrophilic groups [24].

5.2. Water holding capacity (WHC) and oil holding capacity (OHC)

Water holding capacity refers to the ability of the protein to hold its own and added water during the force's application. This property plays a major role as a thickening agent affecting the viscosity and texture of sauces, soups, meat products, and baked doughs [12,157]. Furthermore, quality attributes, including taste and texture, affect the yield of meat products, meaning that good WHC of proteins is economically desirable [6]. Oil holding capacity is another crucial techno-functional property of proteins. Various food properties rely on the interaction of proteins and lipids. OHC is a particularly important property for the cold meat industry, as the proteins serve as a bridge between the fat and water. OHC is responsible for improving the mouthfeel, flavour, and shelf life of foods [6,12,24].

5.3. Gelation

Gelation is a techno-functional property, essential to produce dairy products, tofu, and jellies. Protein gels are formed because of the denaturation and unfolding of proteins. This process exposes non-polar groups, thereby causing the aggregation of protein clusters and ultimately forming a spatial gel network [3,155]. Gel formation is affected by electrostatic forces, thus the presence of ions or the pH during gel formation are important factors. Protein gels form a 3D network, capable of entrapping a liquid phase, with resistance against mechanical

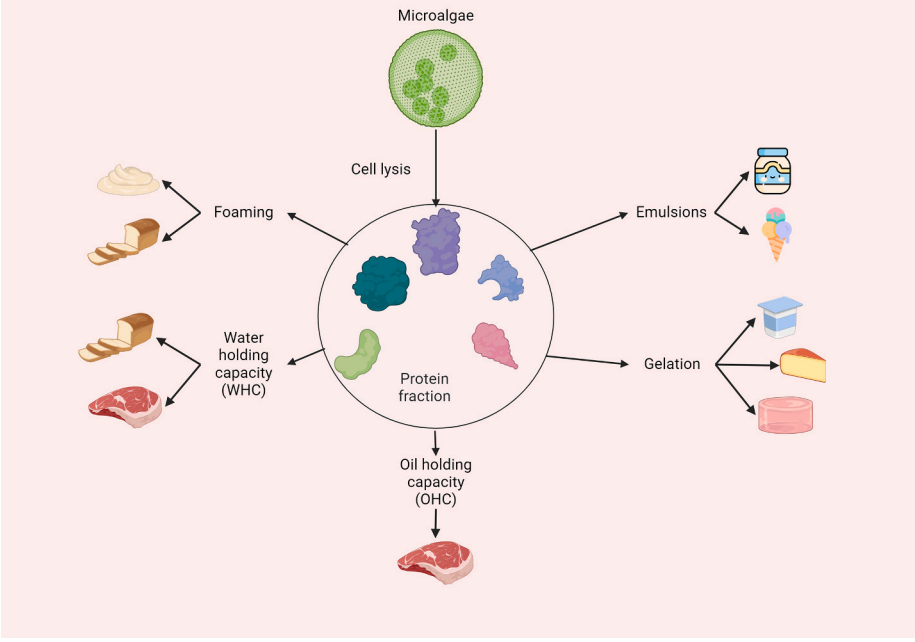

Fig. 3. Techno-functional properties of microalgae proteins and their applications. Scheme elaborated in *Scientific Image and Illustration Software | BioRender.*

forces over a given time. Protein gels can be formed using various techniques, including physical methods such as heat and pressure as well as chemical methods like isoelectric point precipitation, enzymatic treatment, and the addition of salts [155,158,159]. The formation of protein gels involves the partial unfolding of proteins, leading to the aggregation of protein clusters. These clusters of unfolded proteins, trough intra- and intermolecular forces, then form the 3D matrix of the gel, effectively retaining lipids and other compounds within it [155]. External factors such as pH, pressure, temperature, the presence of enzymes, and ionic strength play crucial roles in the formation and stability of protein gels [3,154,155,158,159]. For example, macroscopic gel formation occurred only when the pH was 6.5 [17], since at lower pH levels the proteins were not soluble, highlighting the role of solubility in protein gel formation [3].

5.4. Emulsion capacity

Emulsions are blends of two immiscible liquids, each occupying a distinct phase. Typically, one liquid is aqueous, while the other is oil-based [19]. Regarding the emulsion capacity, two distinct factors are evaluated, namely emulsifying activity index (EAI) and emulsifying stability index (ESI). Emulsion capacity is affected by the concentration and type of protein present. The presence of other compounds may also influence the emulsion capacity. For instance, polysaccharides may improve the emulsion capacity [12]. Proteins are appropriate emulsion agents as they can stabilize the interface between an aqueous phase and an organic or oil phase. Emulsion capacity is defined as the amount of oil that can be dispersed into an emulsifier without the occurrence of destabilization over a given time period [153]. Proteins create visco-elastic layers at the link between the two phases, preventing the coalescence and aggregation of emulsion droplets [13,108,151]. A protein must be water soluble and be adsorbed in the oil-water interface to form an emulsion [19]. Both crude extracts and purified extracts are capable of forming and stabilizing emulsions, although a higher degree of purification resulted in an enhancement of techno-functional properties. The stability of emulsions is affected by a diverse set of factors, including but not limited to pH, droplet size, net charge, and viscosity [6]. Partial unfolding of proteins improved the stability of emulsions due to the exposure of the hydrophobic groups [6,16].

5.5. Foaming properties

Foams consist of a gas dispersed within a thin continuous layer, which holds the gas in a liquid or solid phase. This property is especially important in baked goods, such as bread. Foam affects the visual appeal of foods and the texture, including the smoothness and lightness. This property allows the of-flavours to volatize which in turn improves palatability [6,12,153]. Foam capacity is heavily influenced by the surface hydrophobicity, structural stability, ligand binding, and molecular flexibility [13,153]. These characteristics of proteins are a result of the drying method and the extraction technique. Ultrasound assisted extraction improved the foaming capacity due to the exposure of the hydrophobic groups [12,153]. Partial hydrolysis increased foaming capacity, with higher foaming observed under lower pH rather than alkaline conditions [24].

5.6. Techno-functional properties of microalgae proteins

Currently, a focus is being placed on finding alternatives for meat-based protein products. The main reason to switch from animal proteins to plant proteins is the environmental impact of meat protein production [11]. Another factor pushing plant-based products is the attempt to comply with vegetarian and vegan diets. Currently in the market, meat based proteins like whey, insect, egg and gelatine are used [16]. The meat alternatives are pea, soybeans, flaxseed, lupin, wheat, and rice proteins [6,10,11,16]. Edible insects contain a range between 30 and 65 % of proteins in dry biomass, while beans and soybeans have around 20 % and 40 % of proteins, respectively [11]. Microalgae produced under the right cultivation conditions can reach up to 70 % of protein content in dry biomass. Acknowledging the high protein content of microalgae and their ease of cultivation, due to non-competitive nature for arable land, further research needs to be placed on the techno-functional properties of microalgae proteins [3,14,159–161]. Extraction conditions, *e.g.* techniques applied, and other factors including pH, temperature, buffer solution, greatly affect the proteins techno-functional properties. Some works exploring different biomasses and treatment conditions are presented in Table 3. Partial hydrolysis of microalgae protein greatly increases their techno-functional properties, especially under acidic conditions. This process increases the solubility, foaming capacity, foaming stability, emulsifying capacity and emulsifying stability of proteins compared to the crude protein extract [24]. The techno-functional properties of proteins extracted from microalgae varied with pH [4,24]. The pre-treatment method also affects the techno-functional properties. In a laboratory scale, microalgae proteins possessed similar, or in some cases, better techno-functional properties than current meat alternative protein sources. The techno-functional properties of protein extracts from *Arthrospira platensis* were compared with soybeans, obtaining similar techno-functional properties for both untreated *Arthrospira platensis* extract and soybeans, while ultrasound *Arthrospira platensis* extract showed better results for these techno-functional properties [12]. Microalgae proteins are soluble over a wide range of pH and exhibit good emulsifying, foaming and gelling properties, which allows microalgae proteins to be a possible competitor with current meat alternative proteins [6,12,13,16,19,157,162]. Several factors, mainly economic and environmental, will influence the capability of microalgae proteins to be used in the market. Additionally, microalgae may be more advantageous in certain regions than others.

To obtain optimal techno-functional properties various factors need to be considered. The initial biomass is important, as it affects the protein content and the amino acid composition of proteins, thereby influencing their final 3D structure [152]. The pre-treatment method may cause partial denaturation of the proteins, as it increased some techno-functional properties [6,16]. External conditions such as temperature, pH, salt, and ions play a key role [12]. Purification steps must be addressed, although they are often unnecessary depending on the application of the final protein product. Purifications steps could reduce the techno-functional properties. A purified extract obtained by ethanol precipitation yielded better results than the aqueous counterpart. The increase in activity was attributed to denaturation of the proteins and protein-polysaccharide interactions [150]. Further studies are needed to evaluate the role of other biomolecules in the techno-functional properties of microalgae protein. For the protein product to be economically feasible, a balance between purity and techno-functional properties must be established based on the intended applications, as purification steps increase costs and can reduce techno-functional properties. In many cases, high purity is unnecessary if the product has adequate techno-functional properties.

6. Bioactive peptides

Bioactive peptides with *in vitro* activity have been obtained from a wide range of food sources, such as rice, cheese, eggs and meat [74]. Health-beneficial effects such as anticancer, antihypertensive, antioxidant, antimicrobial activities, among others, have been reported from these compounds [163]. Bioactive peptides are protein fragments composed of 2 to 20 amino acids which are part of the protein polypeptide chain [24]. Such peptides remain inactive within the primary structure of the native protein [164]. Upon fragmentation of the protein molecule, these peptides can be released and gain bioactivity, potentially offering health benefits for its consumers [165]. These biopeptides are obtained by protein hydrolysis, leading to the production of hydrolysates, which are complex mixtures of protein fragments with

Table 3
Techno-functional properties of microalgal proteins.

Biomass	Techno-functional property	Extraction Conditions	Observations	Reference
Arthrospira platensis *Isochrysis galbana*	WHC	Sonication in ultrapure water	*A. platensis obtained a WHC of* 2.25 g water·g^{-1} protein while *I. galbana* obtained 0.47 g water·g^{-1} of protein. Defatted flaxseed obtained a WHC of 6.39 g water·g^{-1} of protein.	[6]
	OHC		*A. platensis* obtained a OHC of 5.80 g oil·g^{-1} protein, *I. galbana* obtained 3.16 g oil·g^{-1} protein, compared with 1.23 g oil·g^{-1} of protein from the defatted flaxseed and whey protein obtained a OHC of approximately 1 g oil·g^{-1} of protein.	
	Foaming		Foaming stability was highest at pH 2 and 4 for both microalgae extracts.	
	Solubility		Minimum solubility of *A. platensis* was obtained at pH 4 (4.99 %) and highest at pH 12 (62.99 %). The minimum solubility of *I. galbana* was obtained at pH 2 (14.12 %) and the highest at pH 12 (19.15 %). Both Whey and defatted flaxseed had highest solubility at pH 12 with 100 % and 87 % respectively.	
	Emulsion		Both microalgae extracts displayed poor emulsion activity and stability when compared to the commercial controls however *Arthrospira platensis* exhibited an emulsion stability of 85.91 % in olive oil.	
Arthrospira platensis	Solubility	Sonication at 4 °C and pH 12 using distilled water as solvent.	Minimal solubility observed at pH 3 at 5.48 % and maximum solubility observed at pH 7 reaching 90.18 %	[12]
	Emulsion		Emulsion activity of ultrasound assisted extraction was lower than conventional extraction however the emulsion stability of the ultrasound assisted extraction was almost 5 times higher than conventional extraction method.	
	Foaming		The foaming capacity and stability of the ultrasound assisted extraction of *Arthrospira platensis* were double the values obtained by soybean proteins.	
	WHC		Ultrasound assisted extraction obtained 5.97 g of water·g^{-1} of protein, while the soybean protein extract obtained 11.11 g of water·g^{-1} of protein	
	OHC		Ultrasound assisted extraction obtained 7.6 g of oil·g^{-1} of protein, while the soybean protein extract obtained 4.15 g of water·g^{-1} of protein	
Arthrospira platensis	WHC	Sonication in pure water, adjusting to pH 10 with 1 N NaOH	4.97 g of water·g of protein^{-1}	[157]
Phaeodactylum tricornutum	Emulsion	Thermostatic circulator water bath using 25 % ethanol or water and two extraction temperatures, 40 °C and 80 °C	The extract obtained with 25 % ethanol and 80 °C resulted in the highest emulsion index and stability	[151]
Chlorella sorokiniana *Phaeodactylum tricornutum*	Emulsion	High-pressure homogenization in double distilled water.	*Chlorella sorokiniana* had good emulsifying properties at 1 wt% at pH ≥ 5 compared to *Phaeodactylum tricornutum* that needed 3.7 wt% and was stable at pH 7.	[162]
Arthrospira platensis	Emulsion	Sonication with hexane and adjusting pH to 9	The higher the concentration of the microalgae in wt%, the higher was the stability and the lower the mean droplet size. Most emulsions were stable for a storage period of 30 days.	[16]
Arthrospira platensis	Emulsion	Overnight hydration in phosphate buffer pH 7 at 4 °C and then homogenized in a microfluidizer	Regardless of purification degree, the proteins were able to form stable emulsions, however pure protein isolates yielded smaller emulsion droplets and the interface between the proteins and oil formed faster.	[108]
Tetradesmus obliquus	Emulsion	Biomass dissolved in distilled water and homogenized in a mechanical stirrer, pH adjusted to 10 and subjected to sonication.	*Tetradesmus obliquus* displayed good emulsifying properties producing stable emulsions over 28 days of storage using a concentration of proteins of 0.5 and 1 wt%.	[19]
Arthrospira platensis	Solubility	pH adjusted to 10 for cell disruption and the proteins were isolated by precipitation adjusting pH to 4.2	Partial hydrolysis led to a drastic increase in the solubility of the microalgae proteins.	[24]
	Emulsion		Both the emulsion activity index and the emulsion stability greatly increased after partial hydrolysis especially in acidic conditions	
	Foaming		Foaming capacity greatly increased after partial hydrolysis and the foaming stability increased under acidic conditions.	
	WHC		5.4 g of water·g^{-1} of protein obtained from the crude protein extract, while the trypsin hydrolysate obtained 5 g of water·g^{-1} of protein.	
	OHC		7 g of oil·g^{-1} protein obtained by the crude protein extract, while the alcalase hydrolysate obtained 6.5 g of oil·g^{-1} of protein.	

WHC – Water holding capacity; OHC – Oil holding capacity; wt - weight.

different molecular weight, such as oligopeptides, peptides, and individual amino acids [166]. These protein hydrolysates can be produced by different methods, including chemical hydrolysis of the native proteins, enzymatic digestion by proteases, or microbial fermentation [25]. Bioactive peptides are also produced naturally during the cooking or digestion of foods [74,165]. Among the approaches available, enzymatic digestion is the most common approach to obtain protein hydrolysates [74], since it is a more controlled process, allowing a better prediction of the resulting peptides [166]. Additionally, it only requires mild conditions during the reaction and does not employ organic harmful solvents [166]. Microalgal peptides possess bioactivity with several benefits for human health [25] (Fig. 4). This review mainly addresses the antioxidant, antidiabetic, and antihypertensive properties of the peptides derived from the biomass (Table 4), antibacterial properties (Table 5),

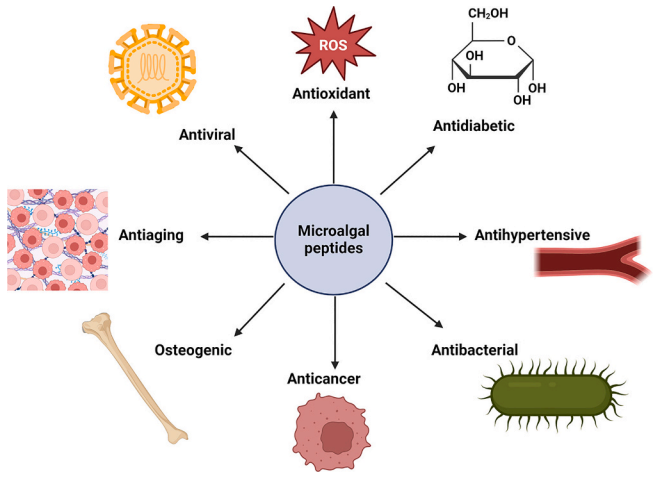

Fig. 4. Bioactive properties of microalgal peptides. These peptides are normally obtained by performing the enzymatic hydrolysis of proteins extracted from microalgal cells. Scheme elaborated in *Scientific Image and Illustration Software | BioRender*

and the osteogenic, anticancer, and antiaging activities (Table 6).

6.1. Antioxidant activity

The microalgal proteins antioxidant properties can be evaluated either *in vitro* or *in vivo*. In the *in vitro* assays normally the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and the Ferric Reducing Antioxidant Power (FRAP) methods or parameters are used [167]. Microalgal peptides obtained from Cyanobacteria *Arthrospira platensis*, *Arthrospira maxima*, and Chlorophyta *Chlorella vulgaris* have the capacity to scavenge the ABTS, DPPH, and hydroxyl radicals, to remove the nitric oxide (NO), and to promote the reduction of Fe^{3+} ions, among other antioxidant properties [24,27,167,168]. These activities increase with higher peptide concentrations and higher hydrolysis times [24,169–171]. The latter is likely attributed to the molecular weight of the released peptides [169], since the bioactive sequences become exposed. The presence of hydrophobic and antioxidant amino acids is crucial for the antioxidant activity [169]. Some of these amino acids can interact effectively with free radicals (DPPH, ABTS, OH•, *etc.*), by either donating protons or electrons to those radical species, and this feature depends on the intrinsic properties of those amino acids [169]. The specificity of protease used in the protein digestion is also important and influences the peptides' bioactivities [172], since it results in different amino acid sequences of the peptides released. After protein hydrolysis, the ABTS scavenging capacity of microalgae peptides increased when compared to non-hydrolysed protein from *Arthrospira platensis* [*Spirulina platensis*], Heterokontophyta *Nitzschia laevis* Hustedt, 1939, and *Chlorella vulgaris* [170]. The GMCCSR peptide possessed higher ABTS free radical scavenging activity than ascorbic acid, with IC50 values of 16.94 and 26.18 $\mu mol \cdot L^{-1}$, respectively. The same hexapeptide also showed DPPH free radical scavenging and ferric reduction activities, albeit with lower efficacy compared to ascorbic acid [167]. At the cellular level, with L929 and erythrocyte cells, microalgal peptides also exhibited scavenging capacity against reactive oxygen species (ROS) and enhanced the activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) [167]. These effects, namely ROS scavenging capacity and the improvement of SOD and CAT activities, were also demonstrated *in vivo* in *Caenorhabditis elegans*, in addition to the extension of the lifespan of the nematode treated with *Chlorella vulgaris* peptides [27]. The antioxidant activity of microalgae peptides can depend on the methods employed for protein extraction. Better ABTS radical scavenging capacity for PEF protein extracts compared to those obtained by bead milling [36] was obtained. As an explanation for this phenomenon, apoptosis in algal cells, induced by PEF treatment, led to enzymatic digestion by proteases within the cells during the apoptotic process, producing smaller protein fragments released to the medium afterwards, unlike bead milling, which is a high disruptive method, promoting the immediate release of the entire proteins to the media. Smaller peptides generally possess better bioactivity compared to that of the entire protein, and that explains the higher ABTS scavenging capacity from PEF extracts [36].

6.2. Antidiabetic activity

The antidiabetic properties of microalgal peptides were also assessed (Table 4). The antidiabetic activity deals with the inhibition of α-amylase, α-glucosidase, and dipeptidyl peptidase-IV (DPP-IV). The α-amylase occurs in the saliva and is released by the pancreas into the digestive system and catalyses the hydrolysis of polysaccharides into oligosaccharides. These are further hydrolysed into glucose by α-glucosidase enzyme in the intestine. Subsequently, the glucose molecules are absorbed through the intestinal epithelium and released into the bloodstream, increasing the glucose levels [173,174]. The inhibition of α-amylase and α-glucosidase activities allows the postprandial hyperglycaemia control [174]. The DPP-IV enzyme causes the deactivation of the incretin hormones, glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), which are responsible for stimulating high levels of insulin secretion [175]. Thus, the inhibition of the DPP-IV enzyme reduces postprandial glucose levels and can be adopted as an approach to deal with type 2 diabetes [176].

Eleven peptides were identified from *Arthrospira platensis* extracts with antidiabetic activity against the three enzymes previously mentioned. Three of those peptides with amino acid sequences GVPMPNK, RNPFVFAPTLLTVAAR, and LRSELAAWSR, were found to exhibit similar *in vitro* inhibitory properties on α-amylase, α-glucosidase, and DPP-IV enzymes when compared to synthetic drugs such as acarbose and sitagliptin [177]. Phycocyanobilin protein from *Arthrospira platensis* biomass exhibited a superior inhibitory interaction with α-amylase and α-glucosidase compared to acarbose by *in silico* studies [173]. These studies demonstrate the potential of microalgal and cyanobacteria peptides and proteins as possible substitutes of current antidiabetic drugs for these enzymes. Higher inhibitory potential on DPP-IV activity was shown for protein hydrolysates from *Porphyridium purpureum*, *Phaeodactylum tricornutum*, and *Arthrospira platensis* microalgae when compared to the non-hydrolysed protein extracts [74,178]. The hydrolysis time as well as the protease utilized for protein hydrolysis determine the bioactivity of the peptides generated [74]. Microalgal peptides from *Arthrospira platensis* and *Chlorella pyrenoidosa* have lower inhibitory potential on DPP-IV activity in Caco-2 cells than *in vitro* assays [26,175] (Table 4). Such difference is attributed to the existence of proteases on the membrane of the intestinal cells, which promote the disintegration of the ingested peptides before the release into the bloodstream, with the subsequent loss of bioactivity against the vascular DPP-IV enzyme [26,175].

6.3. Antihypertensive activity

Hypertension is a serious illness that provokes the death of 9 million people a year and occurs when the systolic and/or diastolic blood pressure are above 140 and 90 mmHg, respectively [179]. The hypertension condition increases the risk of kidney disfunction, stroke, and heart disease [180]. One of the approaches to treat hypertension disease is by administering Angiotensin-1-converting enzyme (ACE-1) inhibitors [181]. This enzyme is a central component of the renin–angiotensin system (RAS) which is involved in the regulation of blood pressure [180,181]. The renin enzyme is produced in the kidney when the blood pressure drops, and the enzyme hydrolyses the angiotensinogen hormone present in the bloodstream to produce angiotensin I [180]. Then,

Table 4
Antioxidant, antidiabetic, and antihypertensive properties of microalgae and cyanobacteria peptides.

Microalgae and cyanobacteria	Sample/peptide sequence	Bioactivity	IC50	Reference
Arthrospira platensis	GMCCSR peptide derived from trypsin hydrolysis, which was further synthesized after identification.	Antioxidant activity	ABTS free radical scavenging: 11.11 μg·mL^{-1} (16.94 μmol·L^{-1}) DPPH free radical scavenging: 24.22 μg·mL^{-1} (36.93 μmol·L^{-1}) FRAP assay: 152.49 μmol·L^{-1}	[167]
Arthrospira maxima	Alcalase 2.4 L hydrolysates. Isolated peptides of <3 kDa		DPPH scavenging activity: 0.83 ± 0.22 and 1.52 ± 029 mg·mL^{-1} (two fractions)	[168]
Chlorella vulgaris	Flavourzyme hydrolysates		Hydroxyl Radical Scavenging: 0.139 mg·mL^{-1} Superoxide Radical Scavenging: 0.323 mg·mL^{-1}	[27]
Arthrospira platensis	Pepsin hydrolysate Alcalase hydrolysate Alcalase hydrolysate		DPPH free radical scavenging of 68.6 % at 50 mg·mL^{-1} Hydroxyl radical scavenging of 77 % at 40 mg·mL^{-1} Nitric Oxide scavenging of 28 % at 50 mg·mL^{-1}	[24]
Arthrospira platensis	GVPMPNK RNPFVFAPTLLTVAAR LRSELAAWSR (Present in the protein extracts, and further synthesized)	α-amylase, α-glucosidase, and DPP-IV inhibition (antidiabetic activity)	236.2 μg·mL^{-1}, 151.5 μg·mL^{-1}, and 192.3 μg·mL^{-1}, respectively 1077.6 μg·mL^{-1}, 164.5 μg·mL^{-1}, and 181.2 μg·mL^{-1}, respectively 313.6 μg·mL^{-1}, 134.2 μg·mL^{-1}, and 167.3 μg·mL^{-1}, respectively.	[177]
Arthrospira platensis	Pepsin and trypsin hydrolysates (1–1.5 kDa)	DPP-IV inhibition	*In vitro*: 3.4 and 3.0 mg·mL^{-1}, respectively. In caco-2-cells: Inhibitions of 31.7 ± 5.8 % and 39.8 %, respectively, at 5 mg·mL^{-1}	[175]
Chlorella pyrenoidosa	Pepsin (CP) and trypsin (CT) hydrolysates (< 3 kDa)		*In vitro*: 63.7 ± 0.5 % (CP) and 69.6 ± 1.4 % (CT), respectively, at 5 mg·mL^{-1} In caco-2-cells: 38.4 ± 3.4 % (CP) and 42.5 ± 5.7 % (CT), respectively, at 5 mg·mL^{-1}	[26]
Arthrospira platensis	Alcalase hydrolysates		47 % inhibition on DPP-IV activity	[74]
Arthrospira platensis *Isochrysis galbana T-iso*	Protein extracts	ACE-I and Renin inhibition (antihypertensive activity)	91.04 % and 25 ± 7.7 %, inhibition, respectively, at 1 mg·mL^{-1} 95.34 % and 23.18 ± 2.16 % inhibition, respectively, at 1 mg·mL^{-1}	[6]
Arthrospira platensis	Alcalase hydrolysates	ACE-I inhibition Renin inhibition	0.49 ± 0.05 mg·mL^{-1} (270-min hydrolysis) 0.85 ± 0.09 mg·mL^{-1} (270-min hydrolysis)	[74]
Chlorella pyrenoidosa	Pepsin (CP) and trypsin (CT) hydrolysates (< 3 kDa)	ACE-I inhibition	*In vitro*: inhibition percentages of 84.2 ± 0.37 % (CP) and 78.6 ± 1.7 % (CT), respectively, at 1 mg·mL^{-1} In caco-2-cells: inhibition percentages of 61.5 ± 7.7 % (CP) and 69.9 ± 0.8 % (CT), respectively at 5 mg·mL^{-1}	[26]
Arthrospira platensis	Pepsin and trypsin hydrolysates (1–1.5 kDa)		*In vitro*: 0.1 ± 0.04 and 0.28 ± 0.03 mg·mL^{-1}, respectively. In caco-2-cells: 2.7 ± 0.3 and 2.8 ± 0.9 mg·mL^{-1}, respectively	[175]
Nannochloropsis oculata	3 kDa xylanase permeate fraction. Synthesized VYNKFPYTTQ Synthesized NKFPYTTQ (these two peptides were identified in the xylanase permeate fraction)		96.6 % of inhibition at 1 mg·mL^{-1} 81.68 % of inhibition at 1 mg·mL^{-1} 76.78 % of inhibition at 1 mg·mL^{-1}	[181]
Bellerochea malleus *Nitzschia* sp.	Papain hydrolysates		Inhibition of 84.35 % at 2 mg·mL^{-1} Inhibition of 61 % at 2 mg·mL^{-1}	[182]

DPP-IV – Dipeptidyl peptidase-4; ACE-I –Angiotensin-converting-enzyme.

angiotensin I is converted to angiotensin II by the action of ACE-I enzyme, which is characterized as a potent vasoconstrictor. The ACE-I enzyme also acts by inhibiting some vasodilator peptides [182,183]. Several synthetic drugs are available to inhibit the ACE-I enzyme, including Benazepril, Captopril, Enalapril, and Fosinopril. However, these are associated with side effects [181], thus the utilization of natural ingredients appears as an alternative to treat the disease.

Some studies demonstrated that microalgal peptides possess inhibitory action on ACE-I and Renin enzymes (Table 4). Two studies showed greater inhibitory potential on the ACE-I enzyme compared to renin inhibition, for protein extracts and protein hydrolysates from *Arthrospira platensis* and Haptophyta *Isochrysis galbana* Parke, 1949 *T*-iso [6,74]. These outcomes were justified by the higher substrate specificity of the renin enzyme compared to that of ACE-I [74], as the characteristics of inhibitory peptides for ACE-I are well known, unlike those for the renin enzyme [6]. *In vitro* inhibition percentages on ACE-I enzyme above 60 % at concentrations of 1 and 2 mg·mL^{-1} of protein fractions and hydrolysates were reported for Heterokontophyta *Nitzschia* sp., Heterokontophyta *Bellerochea malleus* (Brightwell) Van Heurck, 1885, Chlorophyta *Auxenochlorella pyrenoidosa* [*Chlorella pyrenoidosa*], Heterokontophyta *Nannochloropsis oculata*, Cyanobacteria *Arthrospira platensis*, and Haptophyta *Isochrysis galbana T-iso* (Table 4). The inhibitory percentage on ACE-I activity increases with the hydrolysis time and concentration of the peptides and/or hydrolysates [26,74]. 95 peptides in the 3 kDa peptide permeate from *Nannochloropsis oculata* after xylanase treatment were obtained, and eight of them were synthesized based on *in silico* analysis. Two of the synthesized peptides with amino acid sequences NKFPYTTQ and VYNKFPYTTQ displayed *in vitro* inhibition percentages of 76.78 and 81.68 %, respectively, at a concentration of 1 mg·mL^{-1}, which resulted to be higher than that of the synthetic drug captopril, that inhibited the ACE-I activity by 55.59 % at the same concentration [181]. ACE-I inhibition assays in caco-2-cells with protein

Table 5
Antibacterial effects of microalgae and Cyanobacteria peptides.

Microalgae	Sample/peptide sequence	Bioactivity	Performance	Reference
Dunaliella salina	63 kDa isolated protein <3 kDa peptide fraction from trypsin and chymotrypsin hydrolysis of the protein.	*E. coli*, *S. aureus*, and *H. pylori* inhibition	59.4 % and 42.9 % cytotoxicity for *E. coli* and *S. aureus*, respectively. MIC ($mgmL^{-1}$) values of 0.58, 0.81, and 0.175, for *E. coli*, *S. aureus*, and *H. pylori*, respectively.	[186]
Arthrospira maxima	<10 kDa peptide fraction from Pepsin hydrolysis	*B. subtilis*, *S. aureus*, *S. typhi*, *and E. coli* inhibition	MIC values ($mg{\cdot}mL^{-1}$) of 0.63, 0.63, 1.25, and 1.25, respectively.	[192]
Arthrospira platensis	Purified peptide KLVDASHRLATGDVAVRA after alkaline protease and papain hydrolysis	*E. coli* and *S. aureus* inhibition	MIC values ($mg{\cdot}mL^{-1}$) of 8 and 16, respectively.	[189]
Limnospira maxima	Purified pepsin fractions (Seven peptides found with KLENCNYAVELGK as the most abundant)	*E. coli* and *S. aureus* inhibition	Greater bacterial inhibition zones for *E. coli.*	[25]
Arthrospira platensis	*Arthrospira* protein (SP) Alcalase SP hydrolysate Pancreatin hydrolysates	*E. coli* and *B. cereus* inhibition	Greater inhibition zones for *B. cereus*. Greater inhibition zones for *E. coli.* No effects shown	[24]
Arthrospira platensis	Alcalase hydrolysates of sonicated *Arthrospira* protein	*E. coli* and *B. cereus inhibition*	Greater inhibition zones for *B. cereus*	[169]
Chlorella sorokiniana	Pepsin hydrolysate <5 kDa pepsin fraction after UF <10 kDa pepsin fraction after UF	*E. coli* and *S. aureus* inhibition	Greater inhibition zones for *S. aureus*. Greater inhibition zones for *E. coli*. Greater inhibition zones for *S. aureus*.	[188]

MIC - Minimum Inhibitory Concentration; UF – Ultrafiltration.

Table 6
Other bioactivities registered from microalgae and cyanobacteria peptides: anticancer, antiaging, anti-osteoporosis, and anti-covid19 effects.

Microalgae	Sample/peptide sequence	Bioactivity	Performance	Reference
Dunaliella salina	<3 kDa peptide from combined trypsin and chymotrypsin hydrolysis	SW480 cell line inhibition (anticancer activity)	70 % of inhibition after 72 h	[186]
Arthrospira platensis	GMCCSR peptide identified in trypsin hydrolysate, and further synthesized	Enhanced collagen production (antiaging activity)	16.5 % increase of collagen production in UVB-treated HSFs cells	[167]
Arthrospira maxima	Combined subtilisin A and pepsin hydrolysis	Collagenase inhibition (antiaging activity)	92.5 % inhibition at 75 $\mu g{\cdot}mL^{-1}$	[192]
Dunaliella salina	Combined pepsin and trypsin hydrolysis	Osteogenic activity (anti-osteoporosis effects)	Improvement of the bone mineral density and other osteogenic effects in OVX rats.	[194]
Arthrospira sp.	LDAVNR peptide retrieved from PDB databank	Anti-COVID19 activity (Spike protein inhibitor)	Binding energy of −113.456 $kcal{\cdot}mol^{-1}$	[195]

SW480 – Human colon adenocarcinoma cell line; HSFs – Human skin fibroblasts; OVX – ovariectomized.

hydrolysates from *Arthrospira platensis* and *Chlorella pyrenoidosa* showed reduced bioactivity compared to *in vitro* assays (Table 4), following the same trend observed in DPP-IV inhibition experiments [26,175]. The difference between the results obtained *in vitro* and in a cellular environment is attributed to the existence of proteases in the surface of intestinal cells, which degrade the ingested biopeptides, leading to a decrease in their bioactivities against ACE-I [26,175]. Papain hydrolysates from *Bellerochea malleus* could reduce the systolic blood pressure in hypertensive rats after five days of treatment either by oral administration or intraperitoneal injection [182]. This outcome proves that microalgal peptides can indeed resist gastrointestinal digestion and conserve their properties. However, more studies are needed to confirm their effectiveness *in vivo*. These studies prove that microalgal peptides are a potential source to treat hypertension in affected patients.

6.4. Antibacterial activity

In Asia, nearly 80 % of the population is infected with bacteria *Helicobacter pylori*, which causes ulcers and leads to other complications [184]. Moreover, the antibiotic resistance that many strains of bacteria have developed is a general concern worldwide [185]. Therefore, a need to develop novel antibacterial agents arises. Differences in the antimicrobial resistance among bacteria strains might be attributed to differences in their cell walls [186]. The nature of the microalgal peptides also contribute to the effectiveness on bacteria inhibition. The molecular weight of the peptide, their hydrophobicity, and cationic peptides play an important role on bacteria inhibition [169]. Lower molecular peptides exert a better antibacterial activity and are more likely to remain intact across the gastrointestinal barrier [25]. Cationic peptides are more effective in disrupting bacteria cells due to a high electrostatic affinity with anionic phospholipids and lipopolysaccharides present in the surface of the outer bacteria membrane [187–189], replacing cationic species present in those biomolecules, such as Mg^{2+} and Ca^{2+} [189]. This leads to a perturbation of the bacteria cell membranes [189], since the peptides move through the outer membrane and subsequently embed into the cytoplasmatic membrane, creating aggregates that resemble micelle complexes and initiating a disruptive process [190]. The process results in the incorporation of the peptide into the cytoplasm [188,189] due to the hydrophobicity, that helps the peptides to incorporate into the cell membrane, facilitating the disruption process of bacterial cells [191].

Protein extracts, protein hydrolysates, and peptides from Cyanobacteria *Arthrospira platensis*, *Arthrospira maxima*, *Limnospira maxima* (Setchell & N.L.Gardner) Nowicka-Krawczyk, Mühlsteinová & Hauer, 2019, Chlorophyta *Dunaliella salina*, and *Chlorella sorokiniana*, have exhibited inhibition growth action against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, *Helicobacter pylori*, *Bacillus cereus*, and *Bacillus subtilis* bacteria (Table 5). Generally, gram-negative bacteria are more resistant to antibacterial agents than gram-positive bacteria, as species *E. coli* (gram-negative) were more resistant against pepsin fractions of <5 kDa from *Chlorella sorokiniana* microalgae than *S. aureus* (gram-positive) [188]. This phenomenon is corroborated by other studies screened in this review, who also showed greater inhibition action by microalgae peptides against *B. cereus* and *S. aureus* (gram-positive) than *E. coli* (Table 5). However, this is not always the case, and the opposite outcome can occur. For instance, *Limnospira maxima* peptides obtained from pepsin hydrolysis showed higher antibacterial activity

against *E. coli* than *S. aureus* bacteria [25]. Other similar outcomes were shown in this review. Lower minimum inhibitory concentration (MIC, $mg{\cdot}mL^{-1}$) values were shown for *E. coli* bacteria compared to *S. aureus* bacteria, using a 63 kDa protein and its trypsin/chymotrypsin hydrolysates from *Dunaliella salina* [186], and a purified KLVDASHRLATGD-VAVRA peptide derived from alkaline protease/papain hydrolysis of *Arthrospira platensis* proteins [189]. Well-known antibiotics presented a better performance in inhibiting bacteria cultures than microalgal peptides [24,169,188]. For instance, the antibiotic oxytetracycline was more effective on *E. coli* and *B. cereus* inhibition than *Arthrospira platensis* peptides [169]. Penicillin antibiotic resulted to be more effective than *Chlorella sorokiniana* protein hydrolysates/peptide fractions on the inhibition of *E. coli* [188]. However, the studies cited above demonstrate the capacity of microalgae peptides against bacteria strains, and more studies should be encouraged in order to find alternatives to antibiotics because of the antibiotic resistance of microorganisms.

6.5. Other bioactivities

A limited number of studies exploring additional bioactivities are displayed in Table 6. The GMCCSR hexapeptide, derived from *Arthrospira platensis* trypsin hydrolysates and further synthesized after identification by MALTI-TOF-TOF, could restore collagen production in UV-treated human skin fibroblasts cells (HSFs) to normal production levels [167]. Anti-collagenase activity from *Arthrospira maxima* protein hydrolysates was reported [192]. Peptide fractions obtained from the hydrolysis with the combination of subtilisin A and pepsin enzymes (PHS) showed an inhibition rate of 92.5 % at 75 μg/mL, surpassing the synthetic inhibitor 10-Phenanthroline activity, which inhibited collagenase by 57.13 ± 1.9 % at the same concentration [192]. These two studies highlight the potential of microalgal peptides as antiaging agents. *Dunaliella salina* peptides, derived from combined trypsin and chymotrypsin hydrolysis, inhibited the proliferation of human colon cancer cells (SW480 cell line) in a dose- and time-dependent manner. The best results were obtained for <3 kDa peptide fractions, demonstrating a better inhibitory action on SW480 cells when compared to the non-hydrolyzed 63 kDa protein [186].

Complexes of microalgal protein hydrolysates from *Chlorella pyrenoidosa*, *Nannochloropsis oceanica*, *Arthospira platensis*, and *Dunaliella salina* with Ca^{2+} ions (peptides-Ca^{2+} chelates) reverted the osteoporosis effects on dexamethasone-induced osteoporosis zebrafish by inducing bone mineralization in those fish, demonstrating the osteogenic capacity of microalgal peptides [193]. Anti-osteoporosis effects of *Dunaliella salina* protein hydrolysates were also reported. The hydrolysates increased bone mineral density and exhibited other osteogenic effects in ovariectomized (OVX) rats. A novel ALVFQAQH peptide was identified and synthesized, and exerted the major effects in the hydrolysate from *Dunaliella salina*. This peptide was found to effectively reverse osteoporosis symptoms in dexamethasone-induced osteoporotic zebrafish [194]. The potential of 13 known *Arthrospira* sp. (formerly called *Spirulina* sp.) peptides to inhibit the COVID-19 spike protein was demonstrated *in silico*. Such peptides interacted effectively with the binding site of the viral protein. The LDAVNR peptide was the most effective against the spike protein of the virus, with a binding energy of −113.456 $kcal{\cdot}mol^{-1}$ and an interaction energy of −71.0736 $kcal{\cdot}mol^{-1}$ [195]. Despite the promising results achieved by molecular docking tools, *in vitro* and *in vivo* tests must be performed to confirm the effectiveness of *Arthrospira* sp. peptides in opposing the COVID-19 spike protein activity.

7. Conclusion

Along with other compounds of interest, microalgae and cyanobacteria can produce up to 70 % protein of the total biomass, making them a potential source of protein in the future to meet the high demand from a growing worldwide population. After microalgal cultivation and harvesting of the biomass, cell disruption is necessary to release the intracellular proteins for further purification, concentration, and obtention of the final product. The cell disruption step is crucial since it significantly contributes to the overall cost of the entire process due to the high rigidness of the microalgal cell wall, and this process might have implications on the subsequent protein purification processes as well as on the properties on the protein product. In general, high-pressure homogenization proved to be the most effective among the cell disruption methods addressed in this review, with cell lysis rates and protein extraction yields that could reach values of up to 99 % in some cases. However, other parameters such as the production of cell debris, the selectivity of the process, and energy costs must be considered to evaluate the adequacy of a cell disruption method for protein extraction. Regarding high-pressure homogenization, its scale-up to industrial levels might be hampered because of its tendency to produce high amounts of cell debris and the release of undesired components, complicating the subsequent protein purification processes, and due to its high energy requirements. Milder cell disruption methods might be suitable to obtain cleaner protein extracts with high yields, such as PEF treatment. This method has been demonstrated to effectively release proteins and phycocyanin from *Arthrospira platensis*, yielding promising amounts and exhibiting greater purities compared to those achieved with more disruptive methods such as HPH and bead milling. This technique proved to be energetically efficient in some studies. However, PEF treatment is not suitable for protein extraction from most microalgae species because of their tough cell wall. The weak cell electroporation mechanism of PEF proved insufficient for achieving satisfactory protein yields. For species such as *Chlorella vulgaris* and *Nannochloropsis* sp., protein extraction rates were reported to be below 10 % in some studies with PEF technique. Combining ultrasound with alkali or enzymatic treatment is also an effective approach for releasing proteins in high quantities, with extraction rates reaching values around 80 % in some studies. The alkali treatment might have negative implications in the properties of the final protein product because of the high pH values. The enzymatic treatment, despite being a mild cell disruption method, is difficult to scale up because of the costs of the enzymes and the specificity needed for each microalgae strain.

After the cell disruption step, proteins are often purified using diverse techniques. Centrifugation is used to obtain the supernatant with water-soluble proteins. Ammonium sulphate precipitation in combination with dialysis and ion-exchange chromatography led to a phycocyanin purity of 4.4 (analytical grade). Despite the high purity of the final product using this conventional approach, it entails high protein loss throughout the process and proved to be time consuming, making it economically inviable to scale up. Alternatives such as aqueous two-phase system (ATPS), liquid biphasic system (LBF), and three-phase partitioning (TPP) have been applied successfully, allowing high protein yields that can reach values of up to 90 %. The efficiency of these later methods is highly dependent on the type of salt and alcohol/polymer solution for the formation of the two or three phases, and also influence the protein partition between those phases. High concentrations of salts might denature proteins, and negatively affect their separation and final properties, and therefore it is a parameter that must be optimized. As an alternative, sugar instead of salt could be used because of the low influence of sugar compounds on the properties of proteins to be isolated. An advantage for the TPP technique is the separation of three different components (proteins, lipids, and carbohydrates) at the same time, increasing its economic return.

The techno-functional properties of microalgal proteins were explored in this review. Such properties are derived from the physicochemical characteristics of proteins such as the molecular weight of the molecule, its net charge, and its amino acid composition. Microalgal proteins possessed techno-functional properties including emulsifying, foaming, and gelation properties, which can affect the quality and texture of foods. The treatment of microalgae proteins influenced their techno-functional properties in some studies. With this regard, enzymatically hydrolysed or denatured proteins could enhance the

emulsifying and foaming properties of proteins derived from *Arthrospira platensis*, probably due to hydrophilic and hydrophobic interactions. The pH of the medium was also important. The method selected for cell disruption plays an important role on the techno-functional properties, and it was shown that the application of ultrasounds treatment improved the water holding capacity, emulsifying stability, foaming capacity, and stability of *Arthrospira platensis* proteins.

Peptides derived from microalgal protein extracts exhibit important bioactivity properties with health benefits for humans. The studies screened in this review showed antioxidant, antidiabetic, antihypertensive, antimicrobial, anticancer, antiaging, and osteogenic activities from microalgal peptides. Such peptides might be hidden within the whole protein molecule, thereby hindering their bioactivity due to structural issues. Among the different methods existing, enzymatic hydrolysis of proteins is the most adequate method to release the bioactive peptides because of the higher predictability of the protein fragments obtained, the mild conditions it requires, and the absence of harmful organic solvents during the treatment. The bioactivity of microalgal peptides depends on their molecular weight, hydrophobicity, and net charge. Overall, lower molecular weight peptides showed better antioxidant, antidiabetic, antihypertensive, and antibacterial properties, since the bioactive sequence becomes exposed to the target, and interacts more effectively with it. Low molecular weight peptides are also preferable because they can remain intact through the digestive tract, retaining their bioactive properties. Hydrophobic peptides were reported to interact better with oxidative radical species, and exert a greater antibacterial action as they can embed more effectively in the bacterial membranes, causing its disruption and subsequent bacterial cell death. The results are dependent on the protease used for protein digestion, since it determines the type of sequences to be released, as well as on the hydrolysis time. Both *in vitro* and *in vivo* bioactivities were documented by research studies, as well as at cellular level, highlighting the potential of microalgal peptides for future therapies against major diseases.

8. Future perspectives

Due to their high protein content, microalgae are excellent candidates to serve as a widespread protein source in the future, competing with current conventional foods such as egg, meat, soybean, among others. For this to happen, some important drawbacks must be overcome so that they can be released to the market. Microalgal biomass have poor digestibility, and protein extraction procedures on microalgae must be carried out to improve their value in the market. Regarding this, the microalgal cell wall constitutes an important barrier to the extraction process and further studies are encouraged to optimize cell disruption parameters to effectively disrupt the cells, aiming at high protein yields, low cell debris and contaminants, and high energy efficiency, so that the production of microalgal protein is economically feasible. The isolation procedures must be performed retaining the properties of the protein product and avoiding the utilization of harmful solvents. Another important drawback is the colour, smell, and texture of the microalgal food products because they might seem unappealing to most consumers. To overcome this, it is important to conduct market research on the protein products from microalgae, and further develop studies on how to surpass this aspect. The promising techno-functional properties of microalgae proteins constitute an opportunity to increase the microalgal protein value in the market, considering the growing awareness of the population for natural and sustainable protein products, and the development of adequate protein foods for vegetarian and vegan people. This review demonstrated the potentiality of microalgal peptides as therapeutic agents for different illnesses. While promising results have been achieved *in vitro* in numerous research studies, comparable to those of conventional drugs, we emphasize the necessity for further *in vivo* investigations to gain a more realistic evaluation, since some studies have demonstrated lower bioactivities of those peptides at the cellular level in Caco-2 cells compared to their *in vitro* results. This means that metabolic processes and other physiological effects must be considered when evaluating the therapeutic properties of algal peptides if they are to be released as pharmaceutical agents in the future.

CRediT authorship contribution statement

Emmanuel Nunes: Writing – original draft, Investigation. **Kilian Odenthal:** Writing – original draft, Investigation. **Nuno Nunes:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Tomásia Fernandes:** Writing – review & editing, Supervision, Funding acquisition. **Igor A. Fernandes:** Writing – review & editing, Project administration, Funding acquisition. **Miguel A.A. Pinheiro de Carvalho:** Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was financially supported by PhytoNutre project “Extensive processing of microalgae biomass for the extraction of proteins with nutritional, nutraceutical and pharmaceutical properties”, funded by EEA grants with reference PT-INNOVATION-0118. Also, thanks to ARDITI - Regional Agency for the Development of Research, Technology and Innovation for the support granted within the BIO-VALOR project, financed by PRODERAM 2020, Submeasure 16.2, Post-Doctoral Scholarship. This work is also supported by National Funds by FCT - Portuguese Foundation for Science and Technology, under the project UIDB/04033/2020 (doi:10.54499/UIDB/04033/2020).

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