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Extraction, functionality, and applications of *Chlorella pyrenoidosa* protein/peptide

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ABSTRACT

Chlorella pyrenoidosa (*C. pyrenoidosa*) has been widely used in commercial food and feed production for numerous years. Its high protein content and cost-effectiveness make it an attractive source of novel protein. With a focus on sustainable development and the search for green natural products, current research is dedicated to maximizing the utilization of *C. pyrenoidosa* protein (CPP) and peptide. Various techniques, such as the use of ionic liquids, freeze–thawing, ultrasonication, enzyme digest, microwaving are employed in the extraction of CPP. The extracted CPP has demonstrated antioxidant, anti-inflammatory, and bacteriostatic properties. It can also stimulate immune regulation, prevent cardiovascular disease, protect red blood cells, and even be used in wastewater treatment. Furthermore, CPP has shown some potential in combating obesity. Additionally, CPP is being explored in three-dimensional (3D) printing applications, particularly for the creation of biological scaffolds. It is also anticipated to play a role in 3D food printing. This review aimed to supply a comprehensive summary of CPP and *C. pyrenoidosa* peptide extraction methods, their functions, and practical applications in various industries. By doing so, it seeks to underpin subsequent research efforts, highlight current research limitations, and identify future research directions in this field.

1. Introduction

The growing global population is facing a crisis of limited arable land, and it hinders the development of traditional sustainable agriculture; how to meet the food needs of a large population and the rapid increase in protein demand are a couple of the problems that must be faced nowadays (Jones, 2016). Microalgae and their hard cell shell properties have attracted considerable attention given their wide applications in the agricultural, food, energy, and environmental industries. *Chlorella pyrenoidosa* (*C. pyrenoidosa*), which is rich in proteins, carotenoids, polysaccharides, and vitamins, has shown antioxidant, anti-inflammatory, anti-tumor, and anti-bacterial activities and immune-enhancing properties (Zhang et al., 2018). In the areas of nutritional supplements, fertilizers, and pharmaceuticals, by developing more and more relevant new product, it will contribute to meet differently pressing market needs. *C. pyrenoidosa*, which has high economic efficiency and low cross-contamination and more than 50% protein dry weight, is considered by most researchers as a promising new protein source (Wang and Zhang, 2013; Yadavalli et al., 2022). In addition to its high yield, its microalgal proteins can significantly reduce the consumption of resources (Katsimichas et al., 2023). *C. pyrenoidosa* has been used for commercial food and feed production on a large scale as early as the 1960s. *C. pyrenoidosa* protein (CPP) contains numerous essential amino acids and has a similar composition and ratio as that of feed products currently on the market (Cheng et al., 2020).

Compared with *Chlorella vulgaris*, the growth rate of *C. pyrenoidosa* is much faster. Also *C. pyrenoidosa* owns a higher protein nutrient value and a higher amino acid profile, meanwhile, *C. pyrenoidosa* is sensitive and responsive to exposure to toxic contaminants (Katsimichas et al., 2023). *C. pyrenoidosa* is classified in the Generally Regarded as Safe category by the U.S. Food and Drug Administration (Lisbôa et al., 2014); thus, *C. pyrenoidosa* is safe enough for food and pharmaceutical applications. The current growth requirements for *C. pyrenoidosa* in feed research are also lower than those for traditional crops, such as growth

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in saline water (e.g., seawater, wastewater) and nutrient-deficient land, lack of oxygen and constant light, etc. High-salinity wastewater with high NH4+ can be used as a nitrogen source for *C. pyrenoidosa* growth, which creates a positive cycle and increases the biomass of CPP (Wang et al., 2020).

In addition, an increasing number of studies have been focusing on the utilization of microalgae as feedstock for producing biodiesel to replace traditional sources of bioenergy production (e.g., food crops). Other findings consider *C. pyrenoidosa* as a third-generation bioenergy substance because of its extremely high photosynthetic efficiency. In 2018, the German Aerospace Center proposed a photobioreactor (PBR)-at-the-life-support-rack concept that uses *C. pyrenoidosa* as a protein source for a regenerative life support system that utilizes absorbed CO_2 for photosynthesis to provide sufficient oxygen (O_2) for astronauts; meanwhile, researchers are continuously exploring the stability of long-term cultures of microalgae in PBR under non-sterile conditions and the associated changes in performance (Keppler et al., 2018).

In recent decades, considerable effort has been exerted to clarify whether *C. pyrenoidosa* biocrude oil from microalga plants can be a substitute for fossil fuels. To achieve renewable energy sources, Peng et al. (2019) used organic solvents (i.e., methanol and ethanol) to increase the yield of this type of bio-oil, improve its stability, and reduce the oxygen content, which will greatly reduce the energy input and increase the energy recovery rate once it can be produced industrially, to achieve a significant reduction in production costs and a wide range of development prospects. The microalgal–bacterial symbiosis promotes the positive cycle of microalgal–bacterial growth and recycling of wastewater. During the production process, we need to be aware of the variability in the nutritional value of microalgae due to various external influences, such as pH, temperature, and light conditions. This is a point that needs special attention in the future industrial production process of related products and needs to be systematically explored (Muys et al., 2019).

However, *C. pyrenoidosa* is limited by the existence of hard cell walls and inner membranes of microalgae (Schwenzfeier et al., 2011), which prevents the full utilization of CPP functions. The significant impact on the bioavailability and digestibility of proteins also causes difficulties in the industrial production of CPP and *C. pyrenoidosa* peptides due to the reduced productivity and recovery rate. Therefore, the key to improving the extraction rate of CPP is to break the cell wall (Kubatka et al., 2015) and inner membrane to release high value-added nutrients while minimizing the damage of the extraction process to the nutritional value and maximizing its biological activity.

Considering the years 2014–2023, the co-citation and research trends based on "*Chlorella pyrenoidosa* protein/peptide" as keywords from were determined using CiteSpace software. The results indicated that "*C. pyrenoidosa*," "microalgae," "growth," "biomass," "cultivation," "vulgaris," "algae," "lipid production," "protein," and "accumulation" were the main keywords used in the last ten years (Fig. 1). Nutrition, phytoremediation, and biodiesel are important research trends for *C. pyrenoidosa* applications. The current literatures are still directed toward the application of *C. pyrenoidosa* itself, but the research aiming to discover specific active substances remains lacking. To break this research status quo, we implore future researchers to systematically clarify the active substances of *C. pyrenoidosa* to maximize the exploitation of *C. pyrenoidosa*. In this paper, the effective extraction methods of CPP were summarized, and the functionalities and applications related to CPP were clarified, providing new insights into the development of CPP. It helps to improve the future development of CPP applications for food industry, effectively increase the value of CPP applications, and meet the forecasted global protein demand.

2. Extraction methods

As mentioned above, the hard cytoplasmic shell poses difficulty in CPP separation. Traditional protein extraction methods are generally divided into four types: (1) mechanical extraction: use of shear, heat, etc. To destroy the cell structure; (2) chemical extraction: use of different chemical solvents to destroy the cell structure to extract target substances; (3) physical extraction: use of microwave or ultrasound and other ways to accelerate cell fragmentation to release active substances; (4) enzymatic method: use of various types of proteases permeability of the cell wall (Tan et al., 2020).

In general, the common extraction approaches to cell wall disruption include high-energy ultrasound, acid-base treatment, microwave, and a number of other techniques. However, these methods are inevitably for protein destruction, which can seriously affect the quality and purity of the extract. Other methods, including ultrafiltration, chromatography, and centrifugation, also present limitations. Ultrafiltration methods share the disadvantage of long processing time and membrane clogging, whereas chromatography and centrifugation are time-consuming purification methods. Herein, we summarized the previous extraction methods for CPP (Table 1). The extraction of CPP generally involves a series of innovative complex extraction methods and traditional extraction methods, such as alkali treatment, manual grinding, ultrasound, and so on, with excellent extraction rates (Table 2).

2.1. *Enzymatic hydrolysis*

Enzymatic hydrolysis is a mild and highly selective biological hydrolysis at low temperatures and does not cause protein denaturation. Gentle operating techniques often result in unexpected low extraction rates of target substances. Therefore, more efforts should be exerted to design insightful protocols combined with different cytolytic methods to achieve maximum protein extraction. Aqueous and spirituous soaking extraction was used for crude extraction of *C. pyrenoidosa* pigment–-protein complexes, with 62.83% extraction rate (Zhang et al., 2019). Zhang et al. (2018) presented a novel low-energy composite method (Fig. 2), in which enzymatic digestion of ethanol-soaked samples was followed by the application of ultrasonic and homogenization techniques to achieve an increased CPP extraction rate of up to 72.4%. The protein extraction rate presented in Fig. 2 was beyond that of most conventional protein extraction methods (Table 1).

2.2. *Alkali extraction method*

Although the enzyme-assisted extraction method has mild conditions and high extraction efficiency, its high cost hinders its practical application. In the meantime, alkaline extraction has been suggested as a favorable protein extraction method due to its simplicity in operation, without requiring high energy and cost investment. The rigid cell wall of *C. pyrenoidosa* is unstable under alkaline conditions. Thus, the cell wall becomes permeable, and the protein extraction rate is augmented. Meanwhile, alkaline solutions enhance protein solubility by promoting the dissociation of hydrogen from proteins and the separation of hydrogen bonds, which further improves protein extraction. However, extremely high alkali concentrations can, on the contrary, reduce protein extraction. Lu et al. (2019) optimized the alkali extraction method using a single-factor test and response surface methodology and extracted 722.70 mg/g CPP after 34.8 min at 7.9% NaOH concentration, 70 °C; the whole extraction process had no adverse effect on the amino acid composition.

2.3. *Three-phase partitioning (TPP)*

Waghmare et al. (2016) concluded that industrial extraction of CPP is a problem affected by cost, worker proficiency, and other factors. In this way a large-scale production of CPP would be greatly limited. They proposed a simple, rapid, and scalable method, that is, three-phase partitioning (TPP), to break through the limitations of traditional separation methods; they provided a mixed three-phase system consisting of an upper phase (non-polar component of the solvent phase), a lower

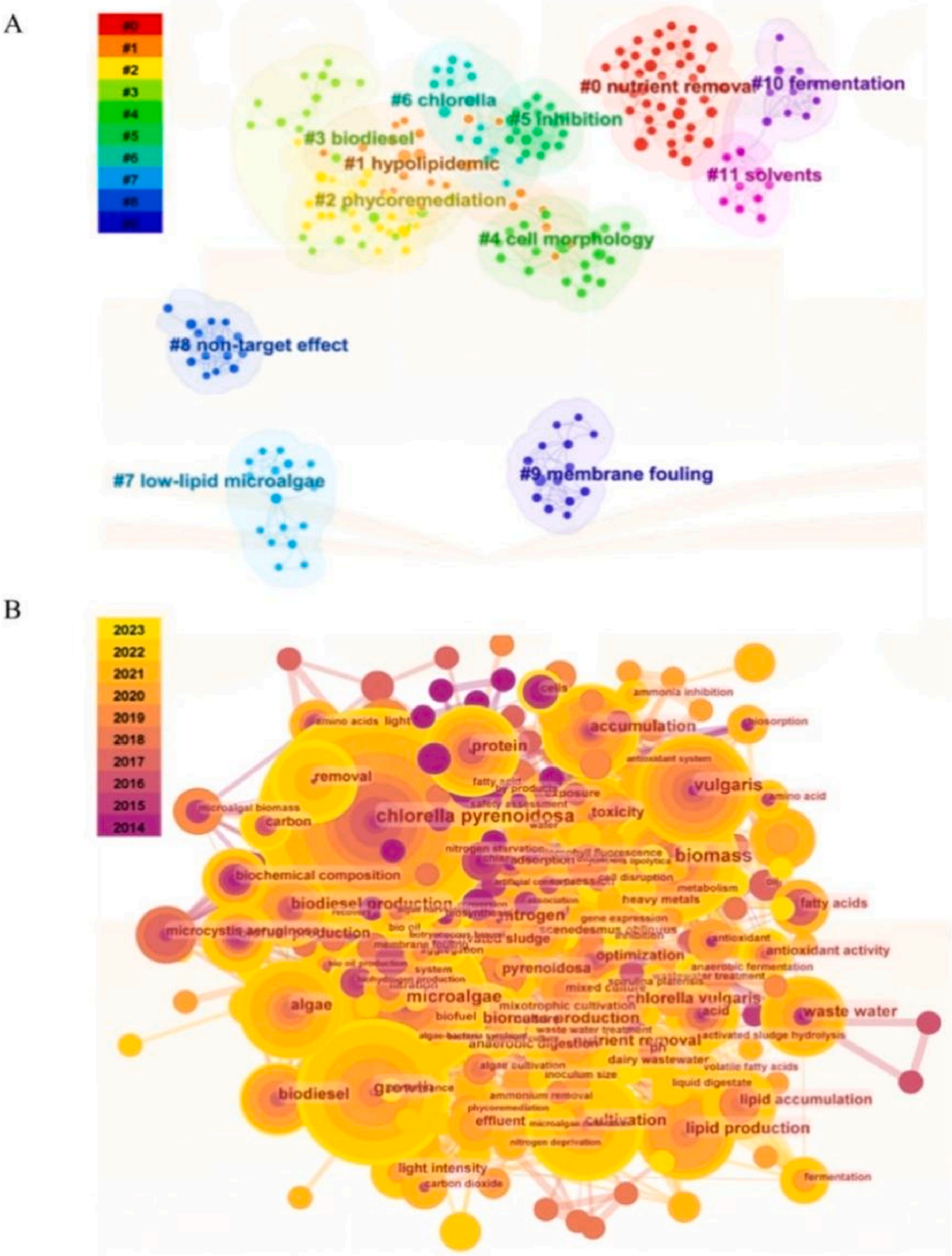

Fig. 1. Keywords and research trend analysis based on CPP/peptide (2014–2023) using CiteSpace software.

Table 1
Comparison of protein extraction rates of several cell disintegration methods.

Methods	Extraction rate	Optimal conditions	Advantages and disadvantages	References
Milling, centrifugation	7.56%	Bead milling for 30 min, with the pump speed of 1.5 L/min, centrifugation for 30 min at 4 °C, and 40,000 g	The heat generated during the grinding process can cause protein denaturation, and spending more time.	Schwenzfeier et al. (2011)
Milling, centrifugation, ion exchange chromatography, and decolorization by precipitation at pH 3.5	64.00%	Bead milling for 30 min, with the pump speed of 1.5 L/min; centrifugation for 30 min at 4 °C and 40,000 g; extensive dialysis; adjustment of pH to 3.5; centrifugation for 10 min; adjustment of the pH to 7.6	The solubility of soluble proteins above pH 5.5 reaches 100% and does not contain algae specific colors.	Schwenzfeier et al. (2011)
Alkali treatment	33.20%	Adjustment of the pH to 12, stirred for 2 h, centrifugation at 10,000 g for 10 min at 20 °C, adjustment of pH to 3, centrifugation again, and neutralization of the pellet with 0.01 M NaOH	Enhanced permeability of cell walls, thereby improving protein extraction efficiency.	Safi et al. (2014)
Freeze thawing	3.20%	Dissolution in distilled water, frozen at −20 °C for 30–100 min and thawing; centrifugation at 8000 rpm for 30 min	Low extraction efficiency.	Wang and Zhang (2012)
Freeze thawing and ultrasonication	22.90%	Dissolution in distilled water, centrifugation at 8000 rpm for 30 min; ultrasonication for 6 s, and centrifugation again	Composite methods can improve protein extraction efficiency.	Wang and Zhang (2012)
Ultrasonication	16.00%	Dissolution in distilled water, ultrasonication for 6 and 9 s interval; centrifugation at 4 °C and 8000 rpm for 30 min	Destroy protein structure.	Wang and Zhang (2012)
Ionic liquids (ILs)	12.10%	Mixing 2 M cationic and anionic moieties, incubation for 10 min at 100 °C, cooling, centrifugation at 6000 rpm and 4 °C for 20 min	Low energy consumption, green and safe. Strong thermal stability and high dissolution rate.	Wang and Zhang (2012)
Low-temperature high-pressure cell breakage methods	45.78%	Dissolution in distilled water at 6 °C and 160 MPa and centrifugation at 8000 rpm and 4 °C for 30 min	May cause protein denaturation.	Wang and Zhang (2012)
Aqueous and spirituous soaking extraction	62.83%	Soaking in ethanol 11.0% (67 h)	Safety and green.	Zhang et al. (2019)
Three-phase partitioning (TPP)	78.10%	Soaking in t-butanol (pH 6) for 20 min using the combination of Stargen™ and Carezyme™	Fast, simple, and capable of achieving industrial scale production.	Waghmare et al. (2016)
60% ethanol-soaking extraction	27.79%	Dissolution in distilled water, soaking for 67 h at the liquid-to-solid ratio of 27:1	Low extraction efficiency.	Zhang et al. (2018)
Distilled water-soaking extraction	28.52%	Soaking in ethanol 11.0% for 67 h at the liquid-to-solid ratio of 27:1	Low extraction efficiency.	Zhang et al. (2018)
60% ethanol-soaking, enzyme digestion, ultrasonication, and homogenization extraction	72.41% ± 1.40%	Soaking in 11% ethanol for 67 h; cellulase hydrolysis for 3 h at pH 5.0; ultrasonication at a power of 1000 W for 26 min; homogenization extraction at 8000 rpm for 10 min	Innovatively propose composite processing methods and continuously optimize extraction conditions until achieving maximum improvement in CPP extraction rate.	Zhang et al. (2018)
Simultaneous dual-frequency divergent ultrasound-assisted extraction (SDFUE)	52.36 ± 0.31%	Ultrasonication at 45 °C for 20 min (360 W) and alkali concentration of 3%	Improved thermal stability of CPP and improved efficiency of traditional single frequency ultrasound extraction.	Lian et al. (2021)
Microwave-assisted TPP (MWTPP)	63.20%	Microwave time of 120 s (100 W), microwave duty cycle of 80%, and microalgal biomass concentration 0.5 % w/w	Improve TPP extraction efficiency, economy, and environmental protection.	Chew et al. (2019)

phase (polar component of the aqueous phase), and an intermediate precipitation–protein layer. The addition of an appropriate amount of organic solvent butanol can increase the buoyancy of the precipitated CPP and thus achieve selective protein precipitation (Waghmare et al., 2016). TPP has demonstrated successful utilization in purifying a variety of proteins, for example, extracting peroxidase from orange peel (Vetal and Rathod, 2015), peroxidase from bitter melon (Panadare and Rathod, 2017), and so on, which significantly improved the protein extraction rate to 78.1%. The process can be optimized by continuous adjustment of the solution parameters to achieve the concentrated extraction of the target protein. This finding suggests that the extraction of CPP proteins on an industrial scale is feasible for the future market. The protein extracted by this method has a good foaming stability and great advantages for future applications in the food industries and chemical industries. Cell rupture is a prerequisite in the whole protein extraction process. Microwave is a cell fragmentation method that has been applied in industrial-scale production, and its principle is to use the thermal effect of microwave energy to cause an increase in temperature and pressure to induce cell rupture, which releases functional active ingredients. Chew et al. (2019) proposed MWTPP, and the extraction of relevant proteins from *Chlorella vulgaris* under optimized process conditions showed a nearly threefold increase in the extraction rate compared with that obtained with TPP alone (63.2%). However, this technique has not been applied to the extraction of CPP, which may require subsequent studies to further improve the extraction rate of CPP.

2.4. Ionic liquids (ILs) and low-temperature high-pressure cell breakage methods

Ionic liquids (ILs), which are known as molten salts at normal temperature, are organic liquids composed entirely of ions with a melting point below 100 °C. ILs have been recognized as a new type of “green solvent” with the advantages of low volatility, viscosity, and vapor pressure. They can be used to complete the whole protein extraction process with low contamination, low energy consumption, and sufficient safety for laboratory personnel. ILs also have strong thermal stability and high dissolution rate. These characteristics have led researchers to note the potential of ILs as an ideal method for biological extraction (Tan et al., 2020). The extraction of active substances from microalgae also involves the combination of ILs with microwave- and ultrasound-assisted techniques (Rodrigues et al., 2018; Wahidin et al., 2018). This also further improves the extraction efficiency of active substances from *C. pyrenoidosa*. Safi et al. (2014) showed that the high-pressure-treatment cell-crushing technique resulted in higher protein content compared with other methods, such as chemical treatment, ultrasound, and manual milling. Wang et al. (2012) innovatively

Table 2
CPP and *C. pyrenoidosa* peptide functions, mechanisms, and their applications.

Category	Function	Mechanisms	Applications	References
CPPH-Ca	CPP calcium chelate	Increasing the expression of calcium binding protein D9k, calcium pump and other related genes, and the abundance of intestinal flora	Calcium supplements	Hua et al. (2019)
C. pyrenoidosa	Photosynthesis	Absorbing nitrogen sources from wastewater as nutrients	Wastewater recycling; Develop biodiesel	Keppler et al. (2018)
CPP Pigment Complex	Antioxidant	Increasing the activity of superoxide dismutase; inhibition of high reactive oxygen species levels caused by hydrogen peroxide	antioxidant	Cherng et al. (2010)
CPPH	Emulsify	Adsorption of proteins and polypeptides at the oil water interface reduces surface tension, increasing solubility	Emulsifier	Liu et al. (2022)
C. pyrenoidosa pigment protein complex; Chlorella-11 peptide	Anti-inflammation, anti-aging	Inhibition of TNF- α and IL-6, Inhibition of endothelial cell adhesion molecule production	Treatment of chronic inflammatory related cardiovascular diseases	Zhang et al. (2019)
CPP	Immunoactivity	Increasing the phagocytic ability of macrophages, and natural killer cell activity, lymphocytes undergo transformation	Immune Enhancement Products	Zhang et al. (2018)
C. pyrenoidosa peptide	Antihypertensive	Targeted inhibition of ACE and DPP-IV by CP polypeptides	Treatment of cardiovascular diseases	Li et al. (2021)
CPP; symbiotic culture of *C. pyrenoidosa* and Yarrowia lipolytica	Anti-hemolysis	Y. lipolytica can stimulate the formation of superoxide dismutase and chlorophyll *a-b* binding proteins	Antihemolytic drugs	Wang et al. (2019)
CPP hydrolyzed by alkaline protease	Anti-obesity	Activating AMPK pathway, inhibiting lipid synthesis and metabolism, inactivating adipocyte specific proteins	Weight loss products	Kubatka et al. (2015)
CPP	Bacteriostat	Inhibition of pathogenic bacteria from animals and plants	Bacteriostatic products, or can be combined with 3D printing	Chen et al. (2005)
CPP	Rich nutrition, biocompatibility	–	3D printing	Xia et al. (2020)

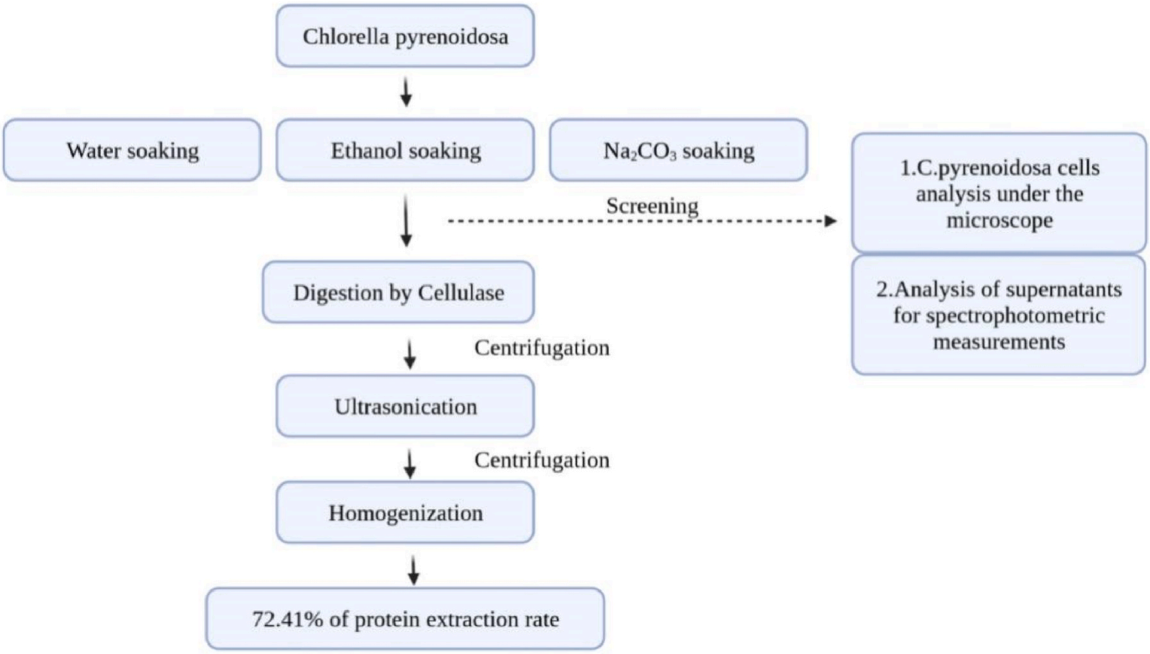

Fig. 2. Schematic of protein extraction method steps (Zhang et al., 2018).

used ILs and low-temperature and high-pressure cell breakage to extract CPP for the first time and showed that the low-temperature and high-pressure cell-crushing method can improve the protein extraction rate by 2–15 times compared with traditional extraction methods (e.g., ultrasonic treatment); however, the extraction rate of ILs was dissatisfactory. Therefore, more investigation is needed for the extraction of CPP by ILs, which is still a gap in the current research. The development of auxiliary techniques may improve the extraction efficiency of ILs for CPP.

2.5. Simultaneous dual-frequency divergent ultrasound-assisted extraction (SDFUE)

Compared with the traditional hot-water method for protein extraction, ultrasound-assisted method is currently widely used to enhance the extraction efficiency in plant-based protein extraction in the current market due to its short processing time and low cost. To resolve the problems of conventional ultrasound, Lian et al. (2021) proposed a greener method to improve the CPP extraction rate to 52.36% ± 0.31% using multi-frequency ultrasound-assisted extraction, which can form more than five times more bubbles due to better cavitation efficiency compared with conventional single-frequency ultrasound, which significantly shortens the extraction duration and is environmentally friendly. This greener method effectively reduces the extraction time and is also environmentally friendly due to the reduced consumption of organic solvents. Moreover, the subsequent protein characteristics may change due to alterations in the structure of the protein by ultrasound treatment; several experiments have demonstrated that dual-frequency ultrasound treatment improves thermal stability and increases the rate of protein solubilization (Fig. 3) (Cheng et al., 2017; Lian et al., 2021).

2.6. Enzymatic hydrolysates and calcium-chelating active peptide

While CPP has extensive applications in human and animal nutrition, the direct absorption of proteins in humans and animals is restricted. To improve the digestibility of proteins, we recommend the intake of enzymatic hydrolysates, which also solves the nutritional intake problem of certain protein-intolerant people, which shows the potential of *C. pyrenoidosa* peptides in the future advancements of nutritional supplements and health foods. Three proteases, namely, trypsin, papain, and viniferase, were used, and the results indicated that viniferase had the highest degree of hydrolysis (18.31%) (Wang and Zhang, 2012). Previous trials and clinical studies have demonstrated that *C. pyrenoidosa* can be consumed for hypotensive and hypoglycemic purposes, but a limited number of research has been conducted on the effects of *C. pyrenoidosa* peptides. The research gap was filled by exploring the potential of *C. pyrenoidosa* multifunctional peptides for hypotensive and hypoglycemic applications for the first time. The findings revealed that *C. pyrenoidosa* peptides are likely to target angiotensin-converting enzyme as well as dipeptidyl peptidase-IV activity. They used pepsin and trypsin to hydrolyze CPP and attained the hydrolysis degrees of 18.7% and 35.5%, respectively. In addition, several peptides (Pep7 and Pep10) exhibited good GI tolerance in gastric digestion stability assay, which indicated that *C. pyrenoidosa* peptides may be the most meaningful substances for future development of drugs for cardiovascular disease prevention (Li et al., 2021).

Calcium deficiency is one of the most common causes of childhood and elderly susceptibility. A number of calcium supplements are available on the market, but all have significant side effects, such as the tendency to cause calcium phosphate deposits. To improve the absorption and maintain the principle of the least possible side effects, scholars must consider the following. Hua et al. (2019) used ultraviolet and infrared spectroscopy to investigate the binding mechanism of CPP hydrolysate calcium chelation and investigate the role of *C. pyrenoidosa* calcium-chelating active peptide to fill the research gap; the experimental results showed that CPP hydrolysate-calcium chelate can improve bone mineral density and increase bone mineral content, and an upregulation of related gene expression, such as calcium-binding protein-D9k (CaBP-D9k), calcium pump, etc., was observed. The intestinal flora composition of the experimental rats was also analyzed, and the results showed an increased abundance of *Lactobacillus* and thick-walled bacteria compared with the blank control group; this finding presented the potential of the calcium chelate anti-inflammatory development of CPP as a novel calcium supplement.

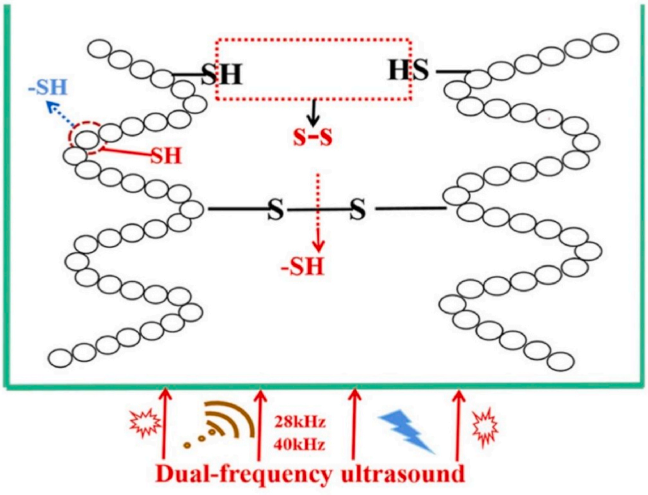

Fig. 3. Effect of SDFUE treatment on CPP (cited from (Lian et al., 2021) with permission).

3. Functions and applications

3.1. Antioxidant and emulsification

Emulsions are non-homogeneous systems containing two liquids. In the food industry, oil-in-water emulsions (O/W emulsions, e.g., milk) are commonly used to prepare emulsions. Several commercial emulsifiers are often added in industry to maintain the long-term stability and increase the nutritional value of emulsions. In the presence of substances in the aqueous phase of emulsions that promote lipid oxidation, several proteins and protein hydrolysates exhibit good emulsifying properties and antioxidant capacity. CPP and *C. pyrenoidosa* peptides have shown good antioxidant capacity in early studies (Cherng et al., 2010). Chen et al. (2016) initially extracted CPP using enzymatic digestion and observed that CPP scavenged hydroxyl radicals, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and superoxide anion radicals at the rates of 96.5%, 71.97%, and 93.68%, respectively, which indicates that CPP has a good antioxidant activity. In addition, pigment CPP can scavenge free radicals in the body and has a significant antioxidant capacity. Nevertheless, the emulsification capability of it has only been minimally investigated in existing studies. Therefore, to enhance the stability and nutritional value of emulsions, Liu et al. (2022) explored the feasibility of applying CPP and *C. pyrenoidosa* peptides in this field. The results showed that the hydrolysates can achieve up to 100% free-radical scavenging rate of DPPH and 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid). The hydrolysates increased the superoxide dismutase activity by 5–10 times and inhibited the high reactive oxygen species levels induced by hydrogen peroxide, which can be achieved by inhibiting lipid oxidation in the oil phase. The antioxidant capacity of CPP derivatives and its emulsifying capability should be further demonstrated.

In addition, prior art low-viscosity O/W emulsions usually had disadvantages, such as instability, narrow range of application, or strict composition selection. Low-viscosity and cold-processable O/W emulsions, such as sufficiently stable highly polar oils, are an example vegetable oils commonly used in commercial products, but they are currently unavailable on the market. According to prior art studies, tremendous difficulties are encountered in the formulation of O/W emulsions with low viscosity and the storage stability required for commercially available products. Whether CPP can be applied for this purpose and solve the problems now faced in production requires further exploration in future studies.

3.2. Anti-inflammatory and anti-aging

Stimulated by external environmental influences, such as light radiation, oxidation, and inflammation, cells respond with a systemic biological stress response: leukocytes and macrophages combine to fight inflammation and attempt to repair cellular damage. In addition, more pieces of evidence show that enhanced inflammatory signaling stimulates the production of oxygen and thus oxidative stress. This process is also known as cellular aging. Limited prior research has examined the anti-inflammatory activity of microalgal proteins; Zhang et al. (2019) investigated the anti-inflammatory and anti-aging effects of *C. pyrenoidosa* pigment–protein complex (PPC) and observed that CPP can inhibit the production of inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin-6 and the inflammatory mediator nitric oxide (NO), in addition to increasing the activity of antioxidant enzymes and inhibiting the upregulation of nuclear factor (NF)-κB, which causes DNA damage and activates cellular pro-inflammatory factors. As shown in Fig. 4, PPC can slow down aging through regulating NF-κB and peroxisome proliferator-activated receptor signaling in the brain and gut and p53/p21 pathways in the gut. The anti-aging goal of the experimental target was achieved. This finding suggests that researchers can develop PPC for future anti-aging products and the treatment of diseases, such as acute inflammation. Cherng et al. (2010) observed that *C. pyrenoidosa* peptide can effectively reduce the production of TNF-α and interleukin-6 in macrophages, which demonstrates the anti-inflammatory capability of this peptide. The anti-inflammatory effect of the peptide was investigated again by Shih et al. (2013), and the data showed that the peptide is a very effective inhibitor of endothelial cell adhesion molecules and can maintain the permeability of endothelial cells under the stimulation of pro-inflammatory cytokines. This result demonstrates the potential of *C. pyrenoidosa* peptide in the development of products for the treatment of cardiovascular diseases associated with chronic inflammation, such as the prevention of atherosclerosis.

3.3. Immune activity and antibacterial activity

C. pyrenoidosa and its active substances have immunomodulatory functions. The main principle of action is the increased phagocytic capacity of macrophages by the stimulation of CPP, which leads to the transformation of lymphocytes, enhancement of the viability of natural killer cells, and gradual increase in the number of lymphocytes based on the body immunity that is improved. A previous study that used CPP in long-term feeding of mice revealed that the increased viability of natural killer cells can regulate the immunomodulatory function of mice. However, most of the studies on immunoreactive microalgae focused on *Chlorella vulgaris*, and systematic and rigorous research on the immunoreactivity of CPP, which is mostly attributed to the active component of arabinogalactan rather than CPP, is lacking. Perhaps, future studies can reveal the structural complexity of *C. pyrenoidosa*-based immunomodulators by deep excavation of the immunoreactive capability of CPP. The extracted CPP have been tested by circular-paper-sheet method for antibacterial assays, and the results showed that CPP exhibited a certain degree of inhibition against several plant and animal pathogenic bacteria (Chen et al., 2005; Hsu et al., 2006; Kralovec et al., 2007; Suárez et al., 2005).

3.4. Hypotension and anti-hemolysis

*C. pyrenoidosa*s with high protein, low fat, low sugar, but rich in a variety of natural plant vitamins have been developed and applied in the field of weight loss and lipid-lowering health care medical supplies. Kubatka et al. (2015) also confirmed the positive effect of *C. pyrenoidosa* on lipid metabolism in rats. Fat accumulation in the body involves several key components, including central regulatory mechanisms, food intake, absorption, digestion, etc. If attempting to target the obesity problem, we can act on several of these components, for example, by suppressing appetite, reducing food intake, and promoting energy expenditure. The chemical inhibition of pancreatic lipase by CPP hydrolyzed by alkaline protease was higher than that by the rest of the proteases, and the polypeptide chains with molecular weight <5 kDa showed a stronger lipase inhibition ability of up to 75.73%. After the above screening, the leu-leu-valu-val-try-pro-thrn-gln-arg (PP1) polypeptide chain with a high lipid scavenging capability was selected for the subsequent investigation to explore the mechanism of action on lipid metabolism, the results showed that PP1 inhibited lipid accumulation by activating the energy metabolism pathway AMP-activated protein kinase, inhibiting lipid anabolism, and enhancing catabolism. As a natural food-derived substance, *C. pyrenoidosa* has fewer side effects on the human body than purely chemically synthesized drugs and is safer and more reliable. Overall, *C. pyrenoidosa* peptides have the potential to become effective lipid metabolism-regulating and weight loss drugs. Special food therapy will become a popular field of research in the future, and *C. pyrenoidosa* peptide will have a wide prospect in related development and application and lay a certain foundation for the development of weight loss products.

3.5. Treating wastewater

C. pyrenoidosa is highly adaptable to the environment, including harsh environments; thus, it has been considered an ideal biosorbent for contaminated dyes in wastewater and has been used as an economical and environmentally friendly approach of wastewater treatment (Xia et al., 2020). Zhou et al. (2020) used the cultivation of *C. pyrenoidosa* in municipal wastewater and achieved not only 91% degradation of nitrogenous material but also significantly increased the biomass of *C. pyrenoidosa* to 0.35 g/L. Wang et al. (2023) used *C. pyrenoidosa* to

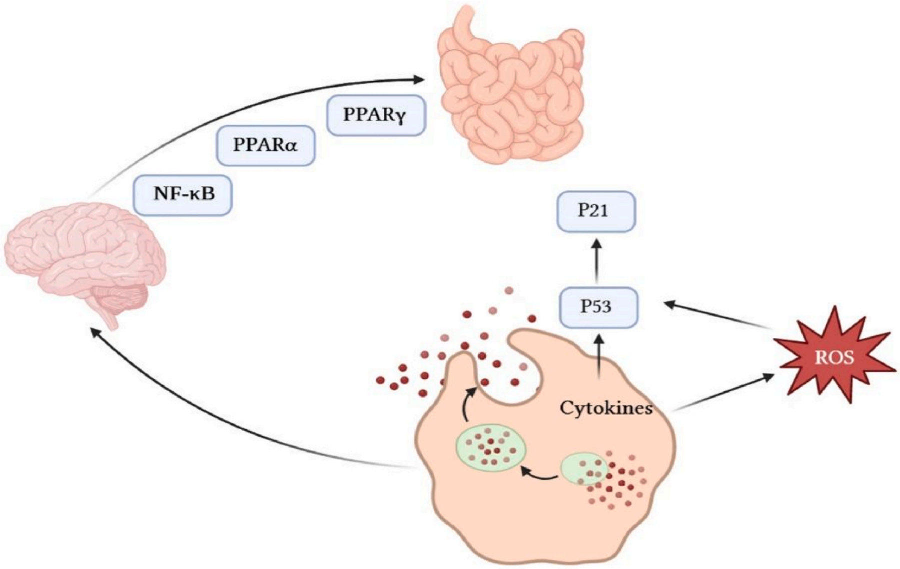

Fig. 4. Envisioned mechanism of action for the brain and intestine (reproduced from (Zhang et al., 2019) with permission).

treat aquaculture wastewater, successfully removed 85.15% of total phosphorus, 96.96% of total nitrogen, 88.53% of chemical oxygen demand, and 98.01% of total ammonia nitrogen removal efficiency, and the treated sewage can meet the discharge requirements. Fig. 5 shows the mechanism of wastewater treatment by *Chlorella pyrenoidosa*. Therefore, large-scale applications of *C. pyrenoidosa* in wastewater treatment are highly likely to solve the problem of wastewater recycling and degradation. In addition, *C. pyrenoidosa* can be cultivated on non-cultivated lands, which alleviates the crisis of limited arable land and may attract the interest of researchers.

3.6. CPP in three-dimensional (3D) printing

The 3D printing technology is a very popular emerging manufacturing process in the biomedical and food fields; it is based on the principle of forming 3D objects by stacking layers of printing materials (Yang et al., 2021). The key to 3D printing technology is dependent on the configuration of bio-inks. Acetic acid-polycaprolactone solution is the most widely used ink configuration, and to meet the market demand and the corresponding sustainable green development, scientists have gradually explored various composite biomaterials to achieve more personalized needs. Current research is mainly focused on the application of *C. pyrenoidosa* in 3D printing. Microalgae can photosynthesize to produce oxygen. Wang et al. (2022) used living microalga-laden hollow fibrous scaffolds, using the advantages of 3D printing for accurate and personalized different shapes; combined with the capability to photosynthesize *C. pyrenoidosa*, these scaffolds (Fig. 6) an oxygenate irregularly shaped wounds, which helps in angiogenesis and accelerates wound healing. In a study by Xia et al. (2020), a 3D-printed composite biomaterial scaffold–polylactide–poly (butylene adipate-co-terephthalate)-immobilized biomass of *C. pyrenoidosa* was a high-quality material for dye removal with 92.66% adsorption of methylene blue, which also proves that this technology can be considered for future applications in large-scale wastewater treatment. In addition, the functional groups on the *C. pyrenoidosa* structure were employed to adsorb heavy metals (e.g., lead), and the results showed that after 48 times of reuse, the lead removal rate can still reach 56.3%. These findings show that the product has evident advantages in wastewater treatment and sample separation (Lan et al., 2022).

Thus far, researchers have been interested in various types of high-quality proteins with good biocompatibility, non-immunoreactivity, biodegradability, etc. As composites for bioink configurations, such as whey protein, chitosan–lactalbumin complexes, soy protein isolate, carrageenan, and vanillin; bovine-serum-protein-based protein gel and other raw materials have been shown to be feasible for practical applications (de Castro et al., 2017; Narupai et al., 2021; Phuhongsung et al., 2020; Ye and Chen, 2018). One study showed that the anti-inflammatory properties of *Chlorella vulgaris* in bioinks significantly improved the printability and structural stability of the products (Uribe-Wandurraga et al., 2021). Xia et al. (2020) also conducted further investigation on immobilized *C. pyrenoidosa* to provide guidance for future industrial dye wastewater treatment; however, the research remains limited to the addition of *C. pyrenoidosa* itself, and the application of CPP and *C. pyrenoidosa* peptides in 3D printing technology is still less studied. The addition of more materials in the ink can improve the nutritional value. Thus, we can presume that CPP is not only a sufficiently safe and environmentally friendly non-toxic natural protein raw material but is also a substance with high nutritional value, anti-oxidant function, and anti-inflammatory effect. As a result, CPP in the food field has been widely used. For future research, we will explore more suitable applications of 3D/4D food printing. We can use the CPP configuration related to bio-ink through continuous adjustment of parameters and optimization of the process to improve printing stability and other performances. However, relevant research and systematic in-depth investigation are lacking. Given the emerging era of 3D/4D printing technology, the application prospects of CPP in this direction must be explored. This research has great potential to meet the personalization and special requirements in the food processing field, and taking full advantage of the role of CPP may have great commercial value in the future market.

To summarize, we have compiled an intuitive summary table of the functions of CPP and its related applications. Clinical and preclinical studies have explored natural substances with antitumor effects. It was demonstrated for the first time that *C. pyrenoidosa* has significant antitumor effects on experimental breast cancer in vitro and in vivo (Kubatka et al., 2015). This result was reflected in the pro-apoptotic effect of *C. pyrenoidosa* on tumor cells and inhibition of tumor cell proliferation and neovascularization. They concluded that the antitumor activity of *C. pyrenoidosa* is mainly derived from carotenoids, which are abundantly found in *C. pyrenoidosa*. Whether CPP has significant antitumor effects still needs to be proven in subsequent studies.

4. Challenges and prospects

Considering that the nutritional value and safety of microalgae are influenced by the origin and different batches, and that they may change during cultivation and subsequent processing, in the future, researchers in the field of industrial application of microalgae can invent an accurate and systematic method to determine the degree of nutritional variability

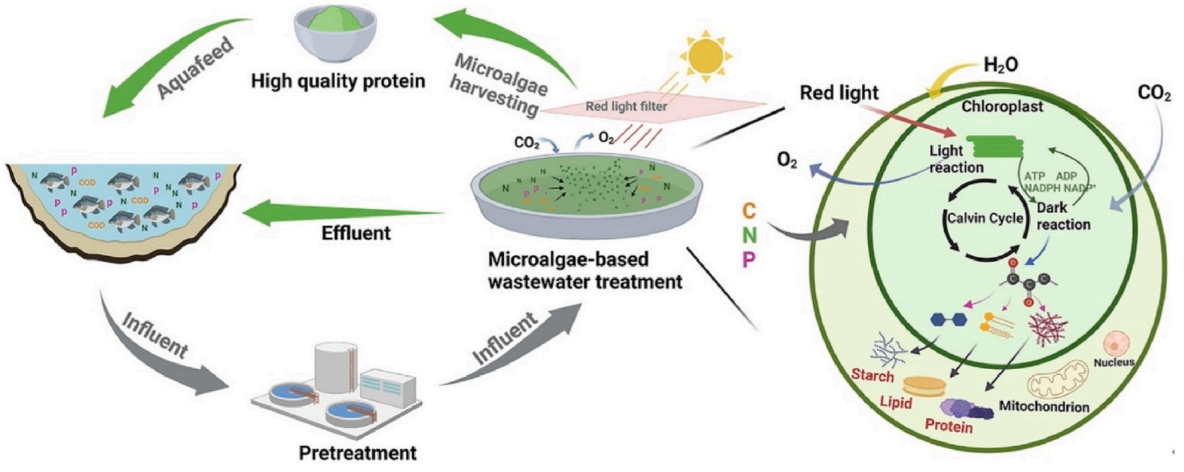

Fig. 5. Mechanism of wastewater treatment by *Chlorella pyrenoidosa* (Wang et al., 2023).

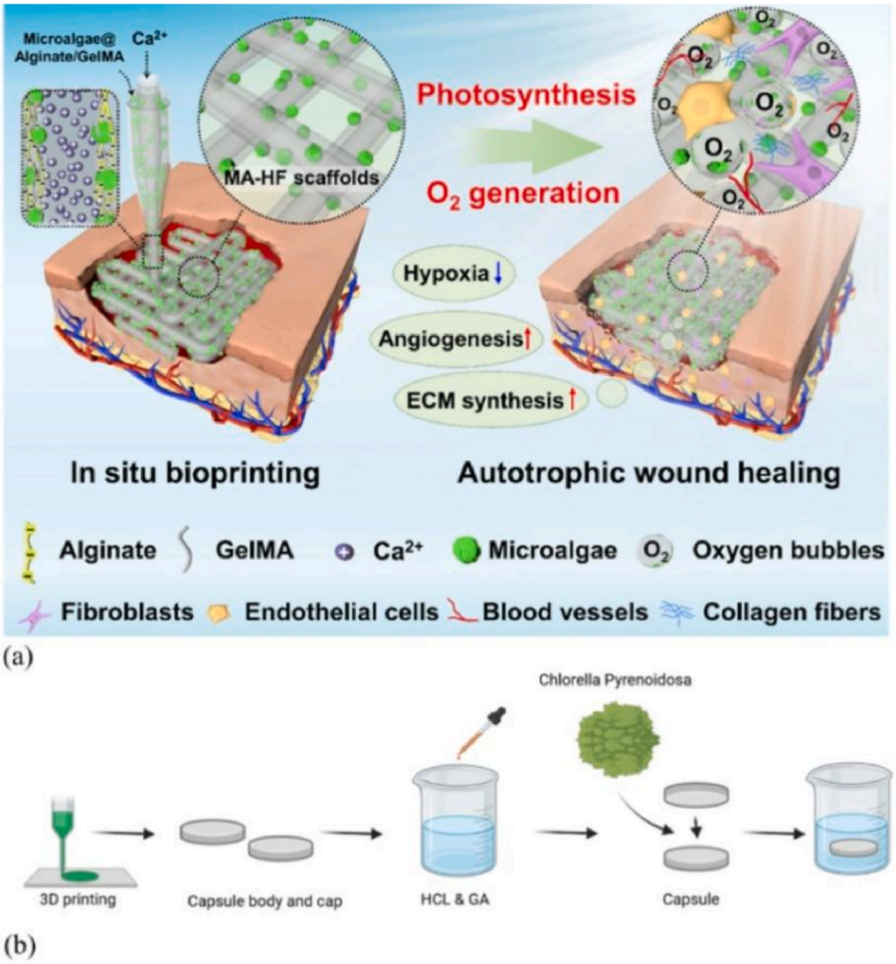

Fig. 6. (a) In situ 3D bioprinting of live photosynthetic scaffolds for autotrophic wound healing (Wang et al., 2022); (b) preparation process of 3D-printed cylindrical capsule structure and schematic of immobilized *C. pyrenoidosa* adsorption experiment.

and detect the contaminants involved in the cultivation of microalgae, such as pesticides and antibiotics. The nutritional quality and safety of microalgal products can be ensured to the greatest extent possible. As a processor, the dosage that is safe for consumption can be analyzed and determined based on precise data measurement to avoid exceeding the limits of various substances required by food safety laws, which helps in increasing consumer confidence.

The economic viability of *C. pyrenoidosa* as a food ingredient is considered when it is used in industrial production; Song et al. (2018) used heavy ion mutant to replace glucose with value-added protein using inexpensive sweet sorghum juice; this mutant has significant implications for the future development of related functional foods as it not only substantially reduces the cost of production but also promotes the increased biomass of positive-cycle CPP. However, this research had limited access to mutants, and future researchers need to explore multiple mutagenesis methods and mutant species. Advances in cultivation technology complemented by improvements in artificial strains will further facilitate the use of CPP for the development of new functional foods in the future.

In the future health food and bio-medicine, combining CPP and various probiotics to form a unique composite material is a promising research direction. Thus, we encourage researchers to further explore this idea. Thus far, we have found tens of thousands of different strains with various functions of probiotics and screened for strains that have the same function as CPP to combine into a new type. A patented strain of *Lactiplantibacilus plantarum*, type Lp90, can achieve weight control and effectively reduce cholesterol levels in the body. Will this probiotic and CPP produce a better weight loss effect? Does the combination of *Bifidobacterium animalis* subsp. *lactis*, a patented strain with immune boosting properties, have a stronger immune boosting effect or does it have an antagonistic effect that reduces functionality? Once researchers explore the functional properties of CPP and probiotic composites, a major breakthrough will be achieved in the field of health food and dietary therapy. In addition, although *C. pyrenoidosa* peptide is a promising regulator of lipid metabolism, the current study's discussion of its mechanism is incomplete. Whether the peptide molecule can target lipase in the body's internal cellular action is unknown. Thus, future researchers should conduct in-depth scientific studies on the structure of the peptide and its inhibition of lipase in vivo to facilitate its application in medical health food in the future. It will broaden the future consumer market of *C. pyrenoidosa* peptide and meet the demand of a considerable number of consumers for safe, healthy, green, and natural weight loss products. In addition, for the application of CPP in 3D printing technology, we still need to conduct more systematic research to explore the most suitable process, which will be a major breakthrough in the realm of biomedical engineering and food in the future and will fill the research gap and have a broad development prospect.

CRediT authorship contribution statement

Qiming Wu: Conceptualization, Literature collecting, Writing – original draft, Revision, Funding acquisition. **Yuchen Ma:** Conceptualization, Literature collecting, Writing – original draft, figure, Revision. **Lanxin Zhang:** Conceptualization, Literature collecting, Writing –

original draft, figure, Revision. **Jing Han:** Conceptualization, Writing – original draft, figure. **Yanan Lei:** Conceptualization, Literature collecting, Writing – original draft. **Yi Le:** Conceptualization, Literature collecting, Writing – original draft. **Caoxing Huang:** Conceptualization, Writing – original draft, figure. **Juntao Kan:** Conceptualization, Writing – original draft, Revision, Supervision, Funding acquisition. **Caili Fu:** Conceptualization, Writing – original draft, Revision, Supervision, 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

No data was used for the research described in the article.

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