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Research status and prospects for bioactive compounds of *Chlorella* species: Composition, extraction, production, and biosynthesis pathways

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ABSTRACT

Chlorella species have gained considerable attention in the past decades due to their capacity to synthesize valuable bioactive compounds in large quantities. While most research has focused on their use in biofuel production, limited consideration has been given to their nutritional applications. This critical review aims to present the latest advancements in the cultivation of *Chlorella* sp., highlighting the biosynthesis, extraction, and potential application of high-valued bioactive compounds in *Chlorella* cells. *Chlorella* sp. can be cultivated in four distinct modes (photoautotrophic, heterotrophic, mixotrophic, and photoheterotrophic conditions) to support their growth and metabolism accumulation. The typical high-valued bioactive compounds within *Chlorella* cells are carbohydrates, proteins, lipids, and pigments, which possess various nutritional properties and potential health-promoting benefits. This review also focuses on the carbon and nitrogen metabolism within *Chlorella* cells that underlie the biosynthesis of these bioactive compounds. Additionally, it extensively discusses various extraction methods employed to obtain these valuable compounds for their utilization and commercialization. These findings offer a comprehensive understanding of the biochemistry and physiology of *Chlorella* sp. The current challenges and further prospects for the biomass and derived byproducts of *Chlorella* sp. are also presented. Overall, this review provides critical insights that can drive the advancement and optimization of industrial production systems, paving the way for the widespread utilization and commercialization of *Chlorella*-derived bioactive compounds.

1. Introduction

Microalgae have attracted considerable interest in recent years due to their ability to synthesize valuable biological substances, including proteins, carbohydrates, fatty acids, and lipids (Cai et al., 2021; Li et al., 2023; Zhou et al., 2018). Among the wide range of microalgae studied, *Chlorella* species have emerged as particularly promising sources of commercially significant compounds (Cai et al., 2021), and have become one of the most extensively studied and cultivated worldwide. The biomass productivity of *Chlorella* sp. was observed to range from 0.08 to 0.42 g/L/day in BG-11 medium at the laboratory scale, which was significantly higher than that of other microalgae including *Scenedesmus* sp., *Diplosphaera* sp., and *Spirulina* sp. (Bezerra et al., 2022; Ferro et al., 2019; Lai et al., 2019; Nordin et al., 2016; Zhang et al., 2020). Furthermore, *Chlorella* sp. cultivated in the culture medium can synthesize a greater quantity of bioactive compounds, including carbohydrates (10 %-24 %), proteins (15 %-20 %), and lipids (15 %-20 %), compared to other microalgae species (Chong et al., 2019; Guo et al., 2015; Zhang et al., 2019b).

Nevertheless, the growth rate and contents of bioactive compounds accumulated in *Chlorella* when cultivated in culture media are insufficient for industrial production. To address this issue, external carbon and nitrogen sources have been applied to the cultivation system to enhance *Chlorella* sp. growth and metabolite accumulation. The addition of glucose in BG-11 medium from 2.5 g/L to 20 g/L resulted in a 378 % enhancement in biomass concentrations. Similarly, increasing the sodium nitrate concentration from 0.5 g/L to 1.5 g/L resulted in a 39 % enhancement in biomass concentrations and a 43 % enhancement in

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protein content (Chen et al., 2023). Wastewater from municipal, agricultural, and industrial sources acts as a nutrient reservoir, providing substantial quantities of nitrogen and carbon for microalgal growth. Zhang et al. (2020) reported that *C. vulgaris* could efficiently utilize the ammonium and phosphorus in real piggery wastewater for protein and lipid production (29 %-58 %) while improving wastewater quality. These findings underscore the ability of *Chlorella* sp. to thrive and produce bioactive compounds under diverse conditions, positioning it as a valuable source of high-value compounds. Consequently, *Chlorella* sp. emerges as a promising candidate for a variety of commercial applications across various industries.

The low bioavailability of these bioactive compounds found in green algae can be largely attributed to the inherent poor digestibility of the cells due to the presence of cellulose-based cell walls (Bernaerts et al., 2019). Thus, multiple techniques have recently been proposed for extracting biological substrates from microalgae, including mechanical, chemical, and biological approaches (Ranjith Kumar et al., 2015). Nevertheless, only a limited number of reviews have focused on extraction technologies. Several reviews have summarized cultivation technologies for producing biological compounds in microalgal cells (Zheng et al., 2022), and evaluated their physiological properties and nutritional value (Koyande et al., 2019). Some reviews have discussed the carbon and nitrogen metabolism in microalgal cells (Perez-Garcia et al., 2011; Su, 2021). However, comprehensive reviews focusing on *Chlorella* sp. are lacking.

The nutritional and pharmaceutical properties of compounds derived from *Chlorella* cells have been extensively studied. For example, *Chlorella*-derived carotenoids have applications as food additives, antioxidants, natural food dyes, and vitamin supplements (Ambati et al., 2019). The lutein and zeaxanthin produced by *Chlorella* strains have been linked to protection against age-related macular degeneration (Ambati et al., 2019). Additionally, *Chlorella* oil contains beneficial fatty acids and could potentially act as an important source of omega-3 fatty acids (Barta et al., 2021). The significant levels of amino acids in *Chlorella* cells suggest the possibility of protein supplementation (Cai et al., 2022a). Various extraction techniques, such as ultrasound, enzymatic or chemical treatment, and mechanical processes, have been employed to isolate valuable compounds from *Chlorella* sp. (Hildebrand et al., 2020; Kulkarni and Nikolov, 2018).

So, how can the unique bioactive compounds in *Chlorella* sp. transform industrial practices and contribute to innovative solutions in health, nutrition, and environmental sustainability? This review seeks to answer this question by comprehensively evaluating *Chlorella* sp. from several critical perspectives: 1) identifying the biological compounds present in *Chlorella* cells; 2) elucidating their applications in various industries; 3) summarizing the extraction methods for these biological compounds; 4) discussing the carbon and nitrogen metabolism within *Chlorella* cells. By synthesizing the latest research findings on *Chlorella*-derived compounds, this review not only underscores the vast potential of these microalgae but also highlights promising avenues for future investigation. The insights derived from this study have the potential to catalyse transformative advances and establish *Chlorella* sp. as an indispensable cornerstone in the pursuit of sustainable and valuable bioproducts.

2. Biochemical composition and high-value compounds

2.1. Proteins and amino acids

Chlorella sp. contains a wealth of proteins ranging from 19 % to 65 % on a dry weight basis (Table 1). Nearly 20 % of *Chlorella* proteins serve as structural support and aid in transportation by connecting to the cell wall. Approximately 50 % of the proteins are intracellular, which perform enzymatic functions, while the remaining 30 % are secreted extracellularly (Coronado-Reyes et al., 2022). The nutritional quality of *Chlorella* sp. is determined by its amino acid profile, which includes large quantities of essential and non-essential amino acids such as Arginine and Histidine (Table 2). These findings have promoted interest in *Chlorella* sp. as a potential substitute for animal-derived proteins due to its ability to synthesize crucial amino acids for human nutrition (Chen et al., 2022).

Nevertheless, the thick, rigid, and cellulosic cell walls of *Chlorella* sp. present a barrier to digestibility, ultimately leading to reduced bioavailability. To improve digestibility, advanced processing methods have been developed for *Chlorella* protein. Depending on the protein content and purification, *Chlorella* protein can be processed into various forms including protein hydrolysates, protein concentrates and isolates, bioactive peptides, and whole-cell proteins (Soto-Sierra et al., 2018). Among these protein products, bioactive peptides are highly desirable due to their ability to enter the human bloodstream directly through the intestinal barrier and exhibit various appealing biological activities. Ko et al. (2012) demonstrated the anti-oxidative activity of bioactive peptides from *C. ellipsoidea* protein, produced by pepsin. The hendeca-peptide produced from *C. vulgaris* also showed an excellent ability to inhibit angiotensin I-converting enzyme, thereby providing a promising avenue for the alleviation of hypertension (Sheih et al., 2009). Additionally, *Chlorella* proteins also present various other biological benefits, including anti-inflammatory, anti--hypercholesterolemic, and immunomodulatory effects (Ko et al., 2012; Soto-Sierra et al., 2018).

Despite the nutritional and pharmaceutical benefits of *Chlorella* proteins, the protein content and amino acid profile vary significantly under different growth conditions. This variability is influenced by environmental conditions, strain variability, and cultivation modes. Key environmental factors affecting protein yield include nitrogen and carbon sources, and micronutrient availability. For example, *C. sorokiniana* cultivated with nitrate showed a higher protein content (29.1 %, w/w) compared to urea (20 %, w/w) and ammonium chloride (16.2 %, w/w) (Lai et al., 2019). An increase in the initial nitrate level from 0.25 g/L to 1.25 g/L enhanced protein content from 42.8 % (w/w) to 63.4 % (w/w) in *C. vulgaris* (Xie et al., 2017). Conversely, increasing ammonium concentrations from 2 mM to 36 mM reduced protein content by 38.7 %, with further increases having a negligible effect. These findings indicate that appropriate nitrogen supplementation is important for protein

Table 1
Protein content in *Chlorella* species.

Cultivation conditions	Microalgae species	Culture medium	Carbon source	Nitrogen source	Biomass (g/L)	Protein (%)
Autotrophic	*C. vulgaris* FSP-E (Lai et al., 2019)	BG−11	CO_2	$NaNO_3$	4.09	19.40
	C. sorokiniana (Lai et al., 2019)	BG−11	CO_2	NH_4Cl	4.23	19.20
Heterotrophic	C. sorokiniana SU−9 (Chen et al., 2023)	BG−11	Glucose	$NaNO_3$	4.14	40.30
	C. vulgaris FACHB−8(Xie et al., 2017)	Se medium	Glucose	$NaNO_3$	2.90	44.30
	Chlorella sp. MBFJNU−17 (Xiao et al., 2022)	HA-SK medium	Glucose	Urea	24.15	59.75
	C. vulgaris (Xu et al., 2021)	Endo medium	Glucose	NH_4Cl	232	37.30
	C. sorokiniana GT−1 (Jin et al., 2021)	Endo medium	Glucose	KNO_3	247	37.70
	C. pyrenoidosa (ATCC75668) (Safafar et al., 2016)	Wastewater	-	NH_4-N	1.75	65.20
	C. vulgaris (SAG 211–81) (Safafar et al., 2016)	Wastewater	-	NH_4-N	1.51	55.20

"-" means not reported.

Table 2
Amino acid compositions in *Chlorella* species (n.d. indicates not detected).

Amino acids (g/100 g protein)		Cultivation conditions		Soybean meal (Chen et al., 2010)	Menhaden fish meal (Li et al., 2004)
		Heterotrophic (Xie et al., 2017)	Autotrophic (Xie et al., 2017)		
Essential amino acids	Arginine	14.36	10.02	3.41	4.10
	Histidine	5.58	2.67	1.26	1.90
	Leucine	4.99	3.10	3.58	4.70
	Valine	3.82	2.35	2.17	3.20
	Lysine	3.49	2.06	2.87	5.20
	Phenylalanine	3.13	2.49	2.38	2.60
	Isoleucine	2.61	1.60	2.09	n.d.
	Methionine	0.83	0.17	0.66	2.10
	Threonine	0.65	0.32	1.83	2.70
	Tryptophan	n.d.	n.d.	n.d.	2.10
Non-essential amino acids	Glutamic acid	12.53	5.45	8.26	8.50
	Proline	9.19	2.45	2.38	3.20
	Aspartic acid	5.67	2.96	5.43	5.80
	Glycine	3.45	2.41	1.71	4.90
	Serine	3.02	1.99	2.09	2.50
	Alanine	2.36	1.68	2.01	4.30
	Tyrosine	1.81	1.52	1.75	n.d.
	Cysteine	n.d.	n.d.	n.d.	0.60
Total		77.49	43.24	43.88	58.40

accumulation in *Chlorella* sp. cells. CO_2 concentrations are also important to protein production, and the optimal CO_2 concentration for *Chlorella* sp. has been identified as 5 % (Lai et al., 2019). Conversely, the addition of iron at 12 mg/L resulted in a reduction of protein content by 38.2 % in *C. vulgaris* (Lai et al., 2019), underscoring the importance of balanced environmental factors for maximizing protein production in microalgae species.

Furthermore, other environmental factors such as light intensity and temperature also affect protein synthesis. An increase in temperature from 20 °C to 30 °C led to a 21.7 % reduction in protein content in *C. minutissima* MACC-452 (Ördög et al., 2016). A reduction in light intensity was observed to enhance protein content by 19.9 % when the intensity decreased from 33.75 to 8.44 μmol/m^2/s (Freitas et al., 2017). These findings underscore the significance of operational parameters in protein production. Furthermore, strain variability has been shown to impact protein content. For instance, *C. pyrenoidosa* was reported to produce more protein (65 %) than *C. vulgaris* (55 %) when cultivated under identical conditions (Safafar et al., 2016). Higher protein content was also reported in *C. minutissima* MACC-452 (48.7 %) compared to the other strains (MACC-438 and MACC-728) (Ördög et al., 2016). The selection of an appropriate strain is crucial for protein production.

Significant discrepancies in protein production were also observed across different cultivation modes. In autotrophic conditions, where *Chlorella* sp. assimilates CO_2 and utilizes inorganic sources of carbon and nitrogen, the protein content is generally lower (around 19 %) (Lai et al., 2019; Zhou et al., 2023). In contrast, heterotrophic cultivation has been demonstrated to promote protein synthesis in *Chlorella* sp. due to the presence of abundant carbon and nitrogen sources and diverse assimilation pathways (Lai et al., 2019; Safafar et al., 2016; Xiao et al., 2022). For instance, adding glucose to the BG-11 medium enhanced protein content from 19.2 % to 37.3 % (Table 1) and total amino acid content from 43.3 g/100 g protein to 77.5 g/100 g (Table 2). The total amino acid content is significantly higher than that of fish meal (58.4 %) and soybeans (43.88 %) (Table 2), indicating the potential value of protein derived from *Chlorella*. When cultivated in wastewater with high levels of organic carbon, *Chlorella* sp. achieved a protein content exceeding 50 % (Table 1), which can be attributed to the appropriate carbon-to-nitrogen ratio in the wastewater. Cai et al. (2022a) demonstrated that a lower carbon-to-nitrogen ratio is significant in protein production, particularly when the ratio falls below 12:1. It was also highlighted that intracellular nitrogen has a greater impact on protein synthesis in these conditions. Conversely, protein yields were observed to decrease significantly with a carbon-to-nitrogen ratio exceeding 12:1. Therefore, heterotrophic conditions with an optimal carbon-to-nitrogen ratio are favourable for the synthesis of proteins and amino acids in *Chlorella* sp.

Although proteins and amino acids in *Chlorella* cells exhibit essential functions, high costs of cultivation technology and unstable yields have limited the market potential. Therefore, understanding these factors is crucial for optimizing cultivation strategies to maximize protein production in *Chlorella* sp., which significantly advances the commercial viability of *Chlorella*-based protein. Furthermore, there is considerable concern regarding the potential transfer of contaminants, including heavy metals, pathogens, and xenobiotics, from wastewater to the extracted microalgal products during the cultivation process (Alvarez-Gonzalez et al., 2023). To mitigate this risk, the purification step is crucial, as it employs highly efficient separation technologies designed to remove these contaminants and ensure that the final product is both pure and safe for consumption (Markou et al., 2018). In addition, screening and selection for microalgae strains with inherent resistance to contaminants can be employed to further reduce the contamination risks. By integrating rigorous purification processes with the use of robust microalgae strains, it is possible to achieve high-quality products that meet safety standards and are suitable for consumption.

2.2. Carbohydrates

Carbohydrates play a crucial role in *Chlorella* cells, which fall into two categories, reducing sugars and polysaccharides. Reducing sugars include monosaccharides and disaccharides, while polysaccharides consist of complex carbohydrates, including starch, cellulose, exopolysaccharides, and intracellular polysaccharides (Bernaerts et al., 2019; Zhou et al., 2018). Starch granules, which are mainly composed of amylose and amylopectin and stored in the chloroplasts of *Chlorella* sp., serve as the primary energy and carbon reserves. Cellulose functions as a vital structural component in the cell walls (Chen et al., 2013; Mehariya et al., 2021). *Chlorella* sp. reportedly contained 30–40 % of the linear α-glucan amylose (Ferreira et al., 2020). Starch, synthesized in the cells, is used as the primary carbohydrate source, making *Chlorella* sp. a promising feedstock for bioethanol production. A higher carbohydrate content in *Chlorella* cells has been demonstrated to enhance the efficiency of their conversion into bioethanol (Chen et al., 2013). Exopolysaccharides are complex biopolymers composed of diverse monosaccharides, such as glucose, galactose, and mannose (Zhang et al., 2019a). These biopolymers are commonly secreted into the extracellular environment or deposited as a protective layer around microbial cell

walls (Gu et al., 2024; Li et al., 2022). Studies have shown that exopolysaccharides possess significant antioxidant properties, demonstrating a high inhibition rate of 71.4 % on DPPH radicals and 77.5 % on hydroxyl radicals (Zhang et al., 2019a). Additionally, in vitro tests have demonstrated the antitumor activity of exopolysaccharides on colon cancer cell lines (Zhang et al., 2019a). These findings underscore the potential of exopolysaccharides as valuable bioactive compounds with significant antioxidant and anticancer properties.

Furthermore, the intracellular polysaccharides present in *Chlorella* cells provide various biological benefits including antioxidant activity, as evidenced by in vivo and in vitro studies (Qi and Kim, 2017; Yu et al., 2019). For instance, the intracellular polysaccharides extracted from *C. vulgaris* exhibited high antioxidant activity in both in vitro and in vivo tests (Yu et al., 2019). Additionally, in vivo tests demonstrated that *Chlorella*-derived intracellular polysaccharides significantly prolonged the lifespan of *Nematodes* from 460 min to 500 min under oxidative stress induced by hydrogen peroxide (Yu et al., 2019). The bioavailability of bioactive polysaccharides in *Chlorella* cells is limited due to the cellulose-based composition of their cell walls, which hinders digestion in the gastrointestinal tract (Bernaerts et al., 2019), requiring further research into innovative methods to enhance the bioavailability of *Chlorella* polysaccharides for more effective utilization in various applications.

Carbohydrate yield in *Chlorella* cells can vary depending on specific growth conditions, which is a crucial factor to consider, with carbohydrate contents typically ranging from 10 % to 50 % of the dry biomass (Chen et al., 2013; Ho et al., 2013). To produce carbohydrates from *Chlorella* cells in a cost-effective manner, a nutrient limitation strategy is recommended. The availability of nitrogen significantly influences carbohydrate accumulation in *Chlorella* cells. Under nitrogen-sufficient conditions, cells contain carbohydrates comprising 10–20 % of their dry weight (Cheng et al., 2017). However, nitrogen starvation or deprivation substantially promotes carbohydrate accumulation. Ho et al. (2013) found that a 4-day nitrogen starvation increased carbohydrate content from 12 % to 51 %. Nitrogen and sulfur limitation caused a marked increase in the accumulation of starch (30.5 mg/L) and carbohydrate (146.0 mg/L) (Chong et al., 2019). Similarly, phosphorus starvation also led to an increase in carbohydrate accumulation across LED light conditions, with substantial carbohydrate content observed at 75.9 % (Li et al., 2019). Additionally, carbohydrate production in *Chlorella* cells can be enhanced by regulating other culture conditions (e.g., carbon sources and pH values).

The quantity and types of carbon sources can affect carbohydrate synthesis in cells. Increased CO_2 levels appear to stimulate carbohydrate production in *Chlorella* sp. Li et al. (2015) found that cultivating these strains in a 30 % CO_2 condition led to a 30 % improvement in carbohydrate content over those grown in 10 % CO_2. Additionally, when cultivated under mixotrophic or heterotrophic conditions, cells exposed to 1 % glucose showed higher levels of carbohydrate accumulation compared to those exposed to 1 % acetate or glycerol in the growth medium (Liang et al., 2009). Maintaining pH levels at 7.0 or 7.5 facilitated the accumulation of carbohydrates to 47 %-49 % in *C. vulgaris* JSC-6 in autotrophic culture, which was twice as high as that at pH 6.5 (25 %) (Cheng et al., 2022). Shifting from photoheterotrophic to mixotrophic conditions also increased carbohydrate contents from 26 % to 50 % at pH 7.5 (Cheng et al., 2022). These techniques demonstrate the potential for manipulating carbohydrate production in *Chlorella* sp. by regulating culture conditions and stress factors during cell growth and reproduction. Optimizing these conditions offers the promise of enhanced carbohydrate yields from large scale.

2.3. Lipids and fatty acids

Chlorella sp. can accumulate significant quantities of lipids, making them oleaginous. The lipid content in *Chlorella* cells ranges from 10 % to 50 % of dry biomass (Dragone, 2022). Based on their functions, lipids can be classified into storage lipids and structural lipids (Hamed, 2016). The primary storage lipids, triglycerides, are commonly synthesized through photosynthesis and stored within the cells. They can be further utilized to generate fatty acids by transesterification (Dragone, 2022). Structural lipids, including phospholipids and sterols, constitute vital components of the cell structure (Levasseur et al., 2020).

Chlorella-derived fatty acids are generally classified as saturated, monounsaturated, and polyunsaturated. Polyunsaturated fatty acids (PUFAs) in *Chlorella* cells are valuable lipid components due to the abundance of long-chain ω-3 and ω-6 PUFAs, which are important in promoting human health. Long-chain PUFAs are beneficial for cancer prevention, as well as cardiovascular and hypertensive diseases (Barta et al., 2021; Breslow, 2006; Yang et al., 2014). Moreover, ω-3 long-chain PUFAs are crucial for brain and vision development in infants (Barta et al., 2021). Owing to the critical function of PUFAs in human health, *Chlorella*-derived PUFAs can serve as a wholesome nutritional supplement for the food and pharmaceutical industries. Furthermore, incorporating PUFAs-rich microalgae into animal feeds enhances the immunity of animals and effectively reduces overuse of antibiotics and medicine (Yang et al., 2020). Consumption of microalgae-derived PUFAs by animals also results in an increase in PUFA content, consequently improving the quality of meat (Ponnampalam et al., 2016). Vitor et al. (2023) demonstrated that feeding with microalgae oil resulted in a notable enhancement in n-3 PUFA levels, from 32.7 to 42.9 mg/g total fatty acids, in the muscle tissue of lamb.

The health benefits of the PUFAs found in *Chlorella* cells are well-documented, driving researchers to explore optimal methods for large-scale cultivation of *Chlorella* sp. The productivity of lipids and fatty acids within *Chlorella* sp. is influenced by a multitude of factors (Mehariya et al., 2021; Zhou et al., 2021, 2022). For instance, lipid production can vary considerably among different species of *Chlorella* sp. In a study conducted by Ördög et al. (2016), it was observed that *Chlorella* sp. MACC-728 demonstrated a higher lipid productivity rate of 96 mg/L/day compared to *C. minutissima* and *Chlorella* sp. MACC-438, which exhibited rates of approximately 70 mg/L/day. Strategies like genetic engineering and co-cultivation play a significant role in enhancing lipid production in *Chlorella* sp. Yan et al. (2019) demonstrated that overexpression of malic enzyme in genetically engineered *C. protothecoides* achieved a 2.8-fold enhancement in total lipid content. Similarly, the fatty acids in the co-culture system of *Ettlia* and *Chlorella* were enhanced by 53.2 % (Rashid et al., 2019).

Environmental factors such as temperature, light intensity, nutrient concentrations, and salinity also affect lipid production. Lower temperatures (20 ℃) led to an enhancement in lipid productivity and fatty acid percentage for *Chlorella* sp. MACC-438 and *C. minutisisma*, which indicates the effects of temperature on the lipid content and fatty acid profile (Ördög et al., 2016). Additionally, nitrogen levels in the culture medium are important in determining lipid accumulation. The microalgae *Chlorella* sp. MACC-438, *Chlorella* sp. MACC-728, and *C. minutisisma* showed the highest lipid contents when cultivated in 3 % nitrogen concentrations, compared to 1 % and 10 % nitrogen concentrations (Ördög et al., 2016). However, two-stage cultivation revealed that nitrogen starvation for 4 days following a shift from nitrogen-rich conditions could enhance lipid accumulation by 1.2 times, while concurrently reducing the proportion of linolenic acid (C18:3) (Nayak et al., 2019). Thus, it is essential to exercise caution when controlling nitrogen levels to achieve the greatest possible lipid yield.

Moreover, increasing the concentration of carbon sources improves lipid productivity. For example, lipid productivity of *C. vulgaris* cells during mixotrophic culture increased threefold from 12.6 mg/L/day to 52.9 mg/L/day in soil extract medium, by increasing the glycerol concentrations from 1 g/L to 10 g/L (Kong et al., 2013). Similarly, cultivating *C. vulgaris* in domestic wastewater containing glycerol with increased glycerol concentrations from 6.1 mM to 50 mM led to a 58 % promotion of lipid accumulation (Cabanelas et al., 2013). These results illustrate that lipid synthesis is greatly enhanced by organic carbon

sources. Additionally, Guo et al. (2015) revealed that upping the light intensity to 320 μmol/m^2s in the medium led to a 30 % enhancement in lipid content and a three-fold rise in unsaturated fatty acids for *Chlorella* sp., compared to those grown at 80 μmol/m^2s. Yun et al. (2019) observed a 1.3-fold enhancement of lipid content in *C. vulgaris* cells under salinity stress of 30 mM NaCl. An adaptive laboratory evolution process for *C. vulgaris* in suboptimal concentrations of NaCl for 35 cycles also demonstrated that the evolved strains overproduced 2.2-fold more lipids than the parent strains (Varunraj et al., 2023). These studies indicate that optimizing growth conditions can effectively enhance lipid productivity in microalgae.

2.4. Pigments

Chlorella sp. contains chlorophyll, the most prevalent pigment found in the thylakoids, which can constitute up to 1–2 % of the dry weight (Hamdy et al., 2021; Khoshnamvand et al., 2021; Mary et al., 2021; Wang and Sheng, 2021). Furthermore, *Chlorella* cells also possess a substantial quantity of carotenoids, which can function as auxiliary pigments for photosynthesis (Hamdy et al., 2021). Carotenoids serve as photo-protectants to safeguard chlorophyll molecules against degradation from extreme light and oxygen exposure. In vitro and in vivo studies have demonstrated that microalgae carotenoids exhibit several benefits, including antioxidant and blood cholesterol regulation (do Nascimento et al., 2020; Silva et al., 2013).

The contents of chlorophyll and carotenoids in *Chlorella* cells are significantly influenced by a variety of growth conditions, including light intensity, nitrogen and phosphorus availability, and salt stress (Huang et al., 2021; Li et al., 2019). Enhanced light intensity from 40 to 140 μmol photons/(m^2•s) has been demonstrated to enhance carotenoid production by 2 folds, while simultaneously reducing chlorophyll content (Hamdy et al., 2021). A previous study demonstrated that increasing the phosphorus supply from 0.1 to 0.4 mg/L significantly facilitated the accumulation of chlorophyll and carotenoids (Huang et al., 2021). Similarly, an increase in nitrogen concentration from 1–2 mg/L resulted in a substantial enhancement in chlorophyll and carotenoid production (Huang et al., 2021). Moreover, salt stress could trigger carotenoid synthesis, resulting in a 3–10-fold increase in production (reaching up to 4.5 mg/L) while concurrently reducing chlorophyll-a production (Hamdy et al., 2021). These insights provide valuable guidance on strategies for chlorophyll and carotenoid production, which could pave the way for the industrial production of *Chlorella*-derived pigments.

Lutein is a key pigment found in *Chlorella* sp., which is a member of the carotenoid family (Zheng et al., 2022). It is responsible for capturing light, protecting it from light, and transferring energy as an accessory pigment. Owing to its antioxidant properties, lutein has shown a positive correlation with safeguarding against cardiovascular ailments and respiratory health (Chung et al., 2017; Leermakers et al., 2016). A diet rich in lutein can prevent early atherosclerosis and minimize the occurrence of age-related macular degeneration (Ma et al., 2012). Because of the good physiological effects, the global lutein market size was USD 362.1 million in 2022 and is estimated to grow at a CAGR of 5.7 % in the next 10 years (Pulidindi and Ahuja, 2023). However, the current production of lutein is generally sourced from *Tagetes erecta* L. and is limited by various factors, including seasonality, land availability, and labor expenses. The lutein content found in *Chlorella* sp. can reach up to 1800–6000 μg/g, a range of 6–20 folds higher than that in *Tagetes erecta* L. (300 μg/g) (Table 3). Within *Chlorella* sp., microalgae such as *C. sorokiniana*, *C. minutissima*, and *C. protothecoides* are found to contain higher lutein content. This indicates that *Chlorella* sp. provides the potential for lutein production.

Table 3
Comparison of lutein content in *Chlorella* species.

Species	Biomass (g/L/d)	Lutein (μg/g)	Reference
Tagetes erecta L.	-	300	(Cordero et al., 2011)
AuxenoChlorella protothecoides	2.48	4990.00	(Xiao et al., 2018)
C. minutissima	0.57	6050.00	(Dineshkumar et al., 2015)
C. sorokiniana MB−1-M12	2.76	5880.00	(Chen et al., 2018)
C. sorokiniana	0.99	1760.00	(Wagenen et al., 2015)
C. protothecoides	2.64	4580.00	(Shi et al., 2000)

"-" means not reported.

3. Extraction and separation technology for bioactive compounds

The extraction of bioactive compounds from *Chlorella* sp. is pivotal for harnessing its full potential in a range of applications. The efficiency of these extraction processes has a significant impact on the yield and purity of valuable compounds including proteins, polysaccharides, lipids, and pigments. Recent advancements have introduced novel techniques to improve these processes, addressing traditional challenges such as inefficiency, high energy consumption, and environmental impact.

3.1. Protein extraction

A variety of techniques have been employed for the separation of proteins, including ultrafiltration, precipitation, membrane separation, and high-performance liquid chromatography (Azmi et al., 2021). Ultrafiltration, which separates proteins based on their molecular weight (10–500 kDa), is notable for its gentle operation (Grossmann et al., 2019). Ultrafiltration preserves protein integrity and is applicable at both the laboratory and pilot scales. However, factors such as membrane resistance, biofouling, porosity, morphology, shear rate, and fluid dynamics exert an influence on the separation efficiency (Wang et al., 2019). On the other hand, precipitation is a cost-effective method for protein separation but is limited by its lengthy processing time and challenges with purity, making it less suitable for large-scale applications. High-performance liquid chromatography, while achieving high purity, is limited by its high cost and the extensive use of neutralization materials (Azmi et al., 2021). Overusing these neutralization materials can lead to a reduction in extraction efficiency (Azmi et al., 2021).

To reduce the processing costs associated with the extraction process and to enhance separation efficiency, innovative techniques such as liquid biphasic flotation and three-phase partitioning systems are emerging as promising alternatives. The liquid biphasic flotation system combines concentration, separation, and extraction into a single procedure (Koyande et al., 2020), offering high efficiency, ease of operation, and environmentally sustainable outcomes (Chew et al., 2019a). Three-phase partitioning, which relies on alcohol and salt concentrations, provides an efficient and cost-effective method for protein recovery. This method is suitable for both small and large-scale applications (Chew et al., 2019b, 2018). While these novel technologies show significant promise for future applications, it is imperative that ongoing advancements in optimised operational procedures remain a critical necessity. Future developments may also be directed towards the creation of hybrid systems that combine with other techniques, with the aim of enhancing both protein yield and purity.

3.2. Polysaccharide extraction

Conventional polysaccharide extraction methods from *Chlorella* sp. such as hot-water extraction, alcohol processing, and alkali extraction,

face challenges related to inefficiency and high energy consumption (Khanra et al., 2018; Liu et al., 2023). Moreover, the excessive use of solvents (such as alkali) in these methods negatively impacts the composition of *Chlorella* polysaccharides (Liu et al., 2023). Recent advancements have introduced innovative approaches, including microwave-assisted extraction, ultrasound-assisted extraction, and enzyme-assisted extraction (Liu et al., 2023; Silva et al., 2018). The application of microwave-assisted extraction facilitates the rapid disruption of the outer layer of the microalgae structure and enhances the mass transfer process. This results in higher extraction efficiencies over shorter periods, compared to conventional methods (Silva et al., 2018). However, the possibility of uneven heating and the potential degradation of polysaccharides must be considered. Enzyme-assisted extraction employs one or more enzymes to selectively disrupt the microalgae cell wall to release polysaccharides with high purity and yield (Zhao et al., 2018). However, this method is constrained by the high cost of enzymes and the necessity of precise control (e.g., pH and temperature). It is recommended that further research should focus on the development of green solvents or solvent-free extraction methods, with the aim of enhancing the efficiency of the extraction process and mitigating its environmental impacts.

3.3. Lipid extraction

The extraction process is crucial for lipids as it ultimately determines the final lipid yield. Numerous studies have documented various techniques for lipid extraction from *Chlorella* sp., including mechanical (e.g., expeller and press) and chemical methods (e.g., solvent extraction and supercritical fluid extraction) (Mubarak et al., 2015). Mechanical methods employed a mechanical force to rupture cell walls, thereby expelling the oil from the dry biomass. However, the practical and large-scale applications of these techniques are constrained by their high energy requirements. Chemical techniques typically use solvents such as chloroform, acetone, hexane, or methanol, to extract lipids through a process of repeated washing and percolation (dos Santos et al., 2015; Zhou et al., 2024, 2018). These solvents are toxic and flammable, and have negative effects on humans and the environment. In contrast, supercritical fluid extraction, utilizing CO_2 as the solvent under supercritical conditions, enables a safe and sustainable approach for isolating lipids from microalgae (Mubarak et al., 2015). This technique eliminates the need for poisonous solvents and ensures efficient lipid separation. Wetterwald et al. (2023) reported that supercritical fluid extraction from freeze-dried *C. vulgaris* at 40 °C and 200 bar yielded up to 16.2 wt% extract/dry biomass of extracted lipids, representing a significant advancement in this field.

3.4. Pigment extraction

The extraction of pigments from *Chlorella* sp. employs similar methods to those used for the extraction of other bioactive compounds, including supercritical carbon dioxide extraction, solvent extraction, and enzymatic extraction (Zheng et al., 2022; Zhou et al., 2021). These methods have a few common limitations, such as inefficiency and environmental concerns. Consequently, further studies should focus on optimizing extraction methods, developing eco-friendly solvents, and utilizing genetic engineering to enhance pigment yield and purity.

Overall, the advancement of extraction and separation technologies for bioactive compounds derived from *Chlorella* sp. is essential to fully exploiting the potential benefits. Ongoing research and development in innovative methods and sustainable practices will be crucial in addressing current limitations and improving overall efficiency, yield, and purity in various applications.

4. Cultivation of *Chlorella* species

4.1. Cultivation systems

Chlorella sp. naturally grows in lakes and rivers by utilizing CO_2 and light energy for photosynthesis. Since their potential to survive in extreme water environments (Zhou et al., 2021), a few open pond systems have been proposed for microalgae cultivation since the 1950s, including natural, raceway, circular, and inclined systems (Suparmaniam et al., 2019) (Fig. 1). Among these systems, raceway ponds appear to be the most popular open cultivation systems for commercial practices because of their simple and cost-effective design (Zou et al., 2020). The raceway ponds are designed with inexpensive materials such as concrete or ramming earth, lined with white plastic (Suparmaniam et al., 2019). These ponds are approximately 0.3 m deep to maximum exposure to sunlight and promote the growth rates of microalgae (Gorgich et al., 2021). Zou et al. (2020) documented that *C. vulgaris* could grow rapidly in BG-11 medium in the lab-scale raceway pond, and biomass concentration was up to 200 mg/L at day 10, with 0.89 g/m^2/day biomass productivity.

Despite their low cost for construction and maintenance, open ponds have some drawbacks, including rapid water evaporation, biological contamination, and difficulty in temperature control (Gorgich et al., 2021; Li et al., 2022). During the cultivation of microalgae, large amounts of water are consumed due to the rapid evaporation, which leads to an increased water footprint and maintenance costs (Li et al., 2022). Furthermore, biological contaminants in open ponds include a variety of microscopic organisms (i.e., grazers, fungi, bacteria, etc.) co-existing with *Chlorella* sp. and competing for available resources (Lam et al., 2018). The serious biological contamination in feed and food gives rise to safety concerns. Moreover, the activities of birds and other animals can contaminate the environment where microalgae grow. Additionally, the biomass yield is greatly affected by climatic conditions and seasonality, which is difficult to control in open pond systems. Therefore, novel cultivation systems are required to address these disadvantages.

Closed culture systems, such as photobioreactors (Fig. 2), provide controlled environments and reduce the possibility of contamination to improve microalgal yields. Flat-panel and tubular photobioreactors are widely recommended as closed culture systems for commercial-scale production. They also reduce land occupation (Suparmaniam et al., 2019). Tan et al. (2021) documented that *C. pyrenoidosa* cultivated in a tubular photobioreactor on a pilot scale achieved 1.8–2.1 g/L of biomass concentrations. Despite the higher biomass yields, the costs of building and operating photobioreactors are higher than open ponds. The biofilms on the tubular photobioreactors can be a barrier to sunlight reaching algal cells. Moreover, the accumulated oxygen in the photobioreactors may inhibit microalgae growth (Tan et al., 2021).

To address these constraints, novel photobioreactors have been recently proposed. A bubble-column photobioreactor equipped with a self-rotating bubble-driven mixer was designed by Naira et al. (2020), which demonstrated an improvement in light utilization efficiency and achieved a 33 % improvement in biomass productivity under natural sunlight, compared to the conventional bubble-column photobioreactor. To further enhance biomass productivity, additional configurations such as agitation or mixing, degassing, and automated control systems are also incorporated into photobioreactors. In a tubular photobioreactor, Tan et al. (2021) employed a diaphragm pump with a flow velocity of 0.35–0.40 m/s, resulting in a maximum biomass productivity of 1.0 ±0.05 g/L/day during the summer when cultivated outdoors. The diaphragm pump could provide the necessary agitation or mixing to prevent cell sedimentation and ensure optimal light distribution.

Moreover, it is crucial for photobioreactors to possess gas exchange capabilities to maintain optimal dissolved oxygen concentrations. This can be achieved through the use of degassers, which play a key role in ensuring optimal oxygen concentrations within the system (Tan et al.,

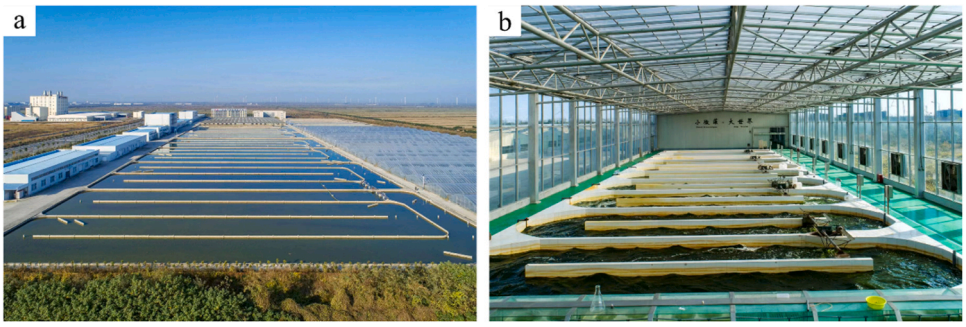

Fig. 1. The open ponds used for the cultivation of *Chlorella* species at Dongtai City, Jiangsu Province, China (pictures were provided by Dongtai Cibainian Biotechnology Co., Ltd).

Fig. 2. The photobioreactors used for the cultivation of *Chlorella* species at Xiamen City (a), Fujian Province, China, and Beijing City (b), China, respectively (pictures were provided by Shanghai Guangyu Biological Technology Co., Ltd).

2021). Membrane-based photobioreactors have also been developed for degassing purposes, which reduce operational costs (Luo et al., 2017). Additionally, automated control systems have been integrated into photobioreactors to monitor environmental parameters, including temperature, pH, and CO_2 concentration. This enables the consistent exposure of microalgae to optimal growth conditions, thereby enhancing productivity and efficiency (Naira et al., 2020). These innovations underscore the potential for advancing photobioreactors to significantly improve the efficiency and scalability of microalgae cultivation.

4.2. Cultivation modes and cell growth

Chlorella sp. can be grown in four main ways: photoautotrophic, heterotrophic, mixotrophic, and photoheterotrophic cultivation. *Chlorella* sp. consumes CO_2 as a carbon source and light for energy to produce bioactive compounds via photosynthesis. This process is widely recognized as autotrophic. Autotrophic growth results in less severe contamination problems than other cultivation methods (Chen et al., 2011). Autotrophic cultivation using either natural or artificially illuminated environments for biomass production has been the prevailing commercial method (Parniakov et al., 2018).

Heterotrophic cultivation involves *Chlorella* sp. that uses organic compounds as both carbon and energy sources, thereby circumventing the limitation of light in biomass production. Heterotrophic cultivation of *Chlorella* sp. is commonly conducted in fermenters where organic substances, including glucose, galactose, and lactose, are provided (Abreu et al., 2022). This procedure reduces the likelihood of biological contamination and improves biomass concentration (Cai et al., 2021). Kim et al. (2013) showed that the growth of *C. sorokiniana* in heterotrophic cultures was nearly three times higher when fed with glucose (1.8 of OD_{540}) compared to autotrophic conditions (0.6 of OD_{540}). However, adding organic carbon sources to cultures increases production expenses, despite the excellent growth performance of the microalgae. For cost reduction, researchers have developed more economical substrates, such as starch, for microalgae biomass. Cai et al. (2021) demonstrated that broken rice hydrolysate was suitable for biomass and pigment production in *C. Vulgaris*, resulting in a 90 % cost reduction, compared to the use of culture medium.

Mixotrophic cultivation allows microalgae to grow photographically, heterotrophically, or both, using inorganic carbon (CO_2) and organic compounds as carbon sources for their growth. In mixotrophic conditions, microalgae growth was over two times higher (1.7 of OD_{540}) compared to autotrophic conditions (0.6 of OD_{540}) (Kim et al., 2013). The proportion of carotenoids in the total pigments increased significantly to 38 % in mixotrophic cultures, compared to 20 % under photoautotrophic conditions (Caporgno et al., 2019), suggesting a promising potential for carotenoid production for human food and cosmetics.

Photoheterotrophic cultivation is a process where light serves as an energy source, and organic compounds also act, simultaneously, as a carbon source (Abreu et al., 2022). The primary distinction between photoheterotrophy and mixotrophy is the light. However, photoheterotrophic cultivation results in high biomass accumulation and significant lipid production. Tekin et al. (2021) observed a 1.4-fold improvement in microalgae growth and a 2.1-fold enhancement in lipid concentration in *C. vulgaris* with 0.5 g/L carrot pomace sugar under photoheterotrophic conditions compared with photoautotrophic conditions.

5. Biosynthesis pathway of high-valued bioproducts in *Chlorella* species

5.1. Carbon metabolism

5.1.1. Inorganic carbon sources

Under autotrophic conditions, microalgae typically convert CO_2 into organic compounds in the chloroplast by utilizing the byproducts of the light reactions (adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH)) via the Calvin cycle (Su, 2021). Carbon fixation, reduction, and ribulose 1,5-bisphosphate (RuBP) regeneration are the main processes involved in the Calvin cycle (Su, 2021).

During the initial phase, light energy photons are absorbed by chlorophyll pigments, and electrons are released in the thylakoid membranes of the chloroplast. With the transfer of electrons, adenosine diphosphate (ADP) and $NADP^+$ are transformed into ATP and NADPH (as shown in Eq. (1)) (Kong et al., 2021). In the second phase, CO_2 is taken into the *Chlorella* cells through diffusion and subsequently converted to produce two molecules of 3-phosphoglycerate through the capture by ribulose-1,5-bisphosphate carboxylase oxygenase (Vuppaladadiyam et al., 2018). The efficiency of photosynthetic carbon fixation is primarily limited by RuBisCo (Hartman and Harpel, 1994). The enzyme RuBisCo catalyzes the phosphorylation of 3-phosphglycerate to 1,3-bisphosphoglycerate and then to 3-glyceraldehyde phosphate. A series of reactions then takes place to form ribulose 5-phosphate (Vuppaladadiyam et al., 2018). Ribulose 5-phosphate is responsible for restoring and regenerating RuBP, which can repeatedly participate in the Calvin cycle. Through these various steps described above, the Calvin cycle can gradually convert one molecule of carbon dioxide into one molecule of glucose over six cycles (Eq. (2)) (Kong et al., 2021). This fully illustrates the primary pathways and mechanisms for CO_2 fixation during photosynthesis.

$$2H_2O + 3\,P + 2NADP^+ + 3ADP + light \rightarrow 2\,H^+ + O_2 + 2NADPH + 3ATP \quad (1)$$

$$3CO_2 + 6H^+ + 6NADPH + 9ATP \rightarrow C_3H_6O_3 + 8\,P + 3\,H_2O + 6NADP^+ + 9ADP - phosphate \quad (2)$$

5.1.2. Organic carbon sources

In heterotrophic conditions, the photosynthesis of *Chlorella* sp. differs substantially from autotrophic photosynthesis, which relies on organic carbon in the culture medium to grow. The primary carbon sources available are glucose and acetate.

(1) Glucose assimilation

In heterotrophic *Chlorella* cultures, glucose results in considerably higher growth and respiration rates when compared to other substrates, like organic acids, sugar phosphates, monohydric alcohols, and sugar alcohols (Griffiths et al., 1960). It is probably due to glucose having a higher energy content per mole (2.8 kJ/mol) than other substrates, e.g., acetate (0.8 kJ/mol) (Boyle and Morgan, 2009).

Glucose in cells can be phosphorylated to generate glucose-6-phosphate. This process requires the crucial catalytic roles of hexokinase and Mg^{2+}, while ATP provides energy and phosphate (Jiao et al., 2018). The main carbohydrate metabolism processes under heterotrophic growth conditions are Embden-Meyerhof-Parnas (EMP) pathway and the Pentose Phosphate Pathway (PPP) (Gao et al., 2021). However, *Chlorella* cells cannot metabolize glucose during anaerobic conditions due to limited energy released from glucose metabolism and/or low lactate dehydrogenase levels (Droop, 1974). Approximately 1 % of glucose is retained as free glucose by microalgae, with over 85 % absorbed and transformed into oligosaccharides (predominantly sucrose at nearly 50 %) and polysaccharides (primarily starch at almost 30 %) (Tanner, 2000). Moreover, the PPP pathway takes precedence in glucose metabolism during dark environments, while the EMP pathway is preferred under light conditions and in the cytoplasm (Rie et al., 2019).

(2) Acetate assimilation

Acetate (or acetic acid), an inexpensive and widely available substrate from numerous industry applications, is a widely used carbon source for microalgae (Cai et al., 2022b). Under aerobic and dark conditions, microalgae cells absorb acetate through the monocarboxylic/proton transporter protein, which can facilitate the transportation of monocarboxylic molecules across the cell membrane (Perez-Garcia et al., 2011). With the aid of acetyl-COA synthetase, the inside acetate is converted into acetyl coenzyme A (CoA) with coenzyme A. It can be further oxidized through two pathways: (1) glyoxylate cycle to produce malate and (2) tricarboxylic acid cycle to produce citrate, generating carbon skeletons and energy simultaneously (Cai et al., 2022b; Perez-Garcia et al., 2011).

Microalgae that assimilate acetate typically have a glyoxylate cycle pathway, which allows them to integrate acetyl groups from acetyl-CoA into carbon skeletons. This process requires isocitrate lyase (EC 4.1.3.1) and malate synthetase (EC 2.3.3.9) (Fig. 3). When *Chlorella* sp. is cultivated in a culture medium containing acetate, these enzymes can be triggered. Isocitrate lyase, the primary enzyme in the glyoxylate cycle, exhibits increased activity in cells after a 24-hour exposure to acetate in the absence of light (Harrop and Kornberg, 1966; Perez-Garcia et al., 2011). Conversely, the synthesis of isocitrate lyase is suppressed by glucose when exposed to light (Perez-Garcia et al., 2011). The utilization of sodium or potassium salt as a substrate raises the pH of the culture medium. It is probably due to the residue Na^+ or K^+ reacting with anions (e.g., hydroxyl ions), resulting in the formation of alkalis (Huang et al., 2016). Therefore, to ensure optimal growth of *Chlorella* sp., the culture medium should be neutralized or adjusted to suitable pH levels by adding acids. Additionally, acetate concentrations should be maintained at low levels through fed-batch as high levels of acetate are toxic to microalgae cells.

It has been shown that heterotrophic growth can be achieved by using glycerol as a growth substrate. Glycerol can function as an osmoregulator and diffuse into cells. Upon glycerol metabolism, it is first phosphorylated and subsequently oxidized to pyruvate, which then enters the tricarboxylic acid cycle. Despite its possibility as a carbon source, there has been limited exploration of glycerol metabolism under heterotrophic conditions. Researchers have examined the potential of cultivating microalgae through heterotrophic methods using organic carbon sources, such as lactose, sucrose, lactic acid, and ethanol. These indicate that microalgae exhibit poor capability in utilizing such organic carbon sources (Tian et al., 2020).

5.2. Nitrogen metabolism

Nitrogen is most crucial in *Chlorella* sp., excluding carbon, hydrogen, and oxygen, which account for 1–10 % of its dry weight. Inorganic nitrogen consumed by microalgae cells can be synthesized into amino acids. Adequate nitrogen sources enhance the biomass production of *Chlorella* sp., while high concentrations of nitrogen hinder lipid accumulation (Zhang et al., 2023). A wide range of nitrogen sources, including ammonium (NH_4^+-N), nitrate (NO_3-N), yeast extract, urea, and amino acids, can also be assimilated by *Chlorella* cells (Fig. 4) (Cao et al., 2018; Kumar and Bera, 2020; Zhang et al., 2020).

5.2.1. Ammonium assimilation

Ammonium is recognized as the preferred nitrogen source for microalgae (Zhang et al., 2020). When ammonium is mixed with other nitrogen substrates, microalgae use alternative nitrogen substrates only after all the ammonium is depleted. This is likely due to its less energy requirement for uptake (Hellebust and Ahmad, 1989; Lachmann et al., 2019).

Ammonium is transported across the cell membrane by ammonium transporters that facilitate uptake and assimilation. These transporters are found in both the chloroplast membrane and plasma membrane

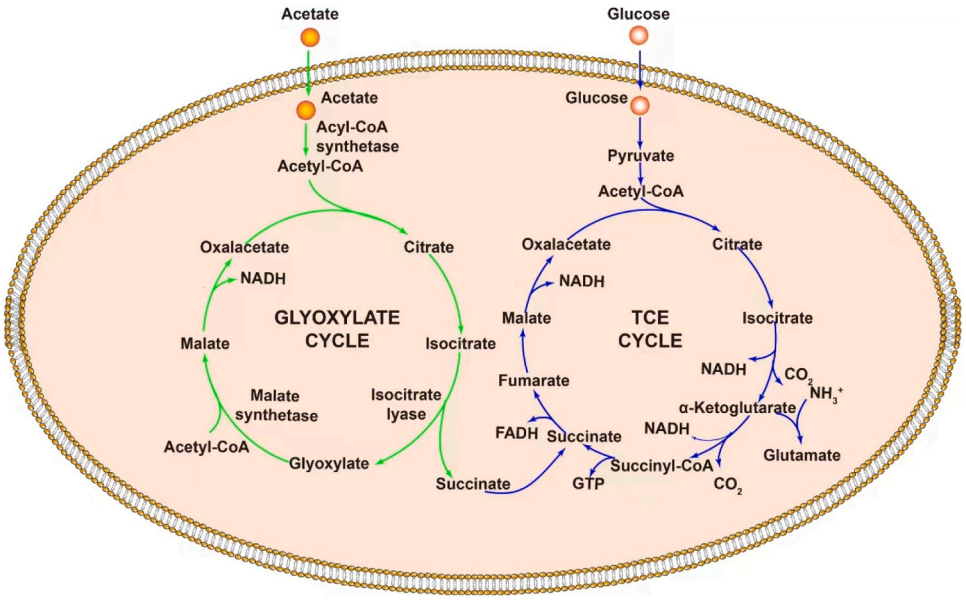

Fig. 3. Metabolism pathway of acetate and glucose in *Chlorella* species.

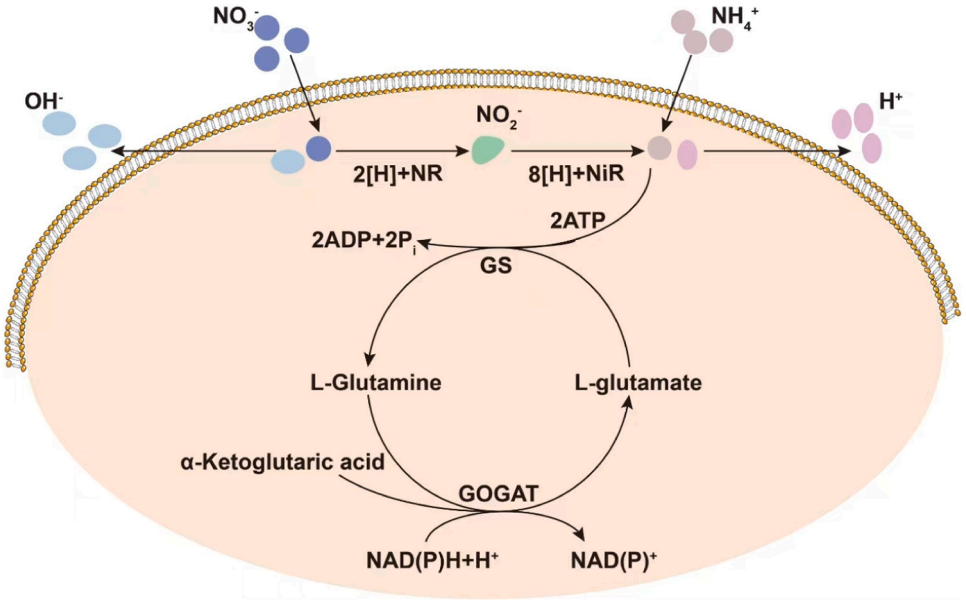

Fig. 4. Metabolism pathway of nitrogen in *Chlorella* species.

(Hellebust and Ahmad, 1989). Two pathways are involved in ammonium assimilation: glutamate dehydrogenase (Inokuchi et al., 2002), and glutamine synthetase (GS)/glutamate synthase (GOGAT) (Perez-Garcia et al., 2011). Glutamate dehydrogenase was initially considered the primary enzyme responsible for ammonia assimilation (Hellebust and Ahmad, 1989). Whereas, it has been considered to play a limited role. When free ammonia in the cell reacts with α-ketoglutarate, glutamate dehydrogenase catalyses the reaction to form glutamate via reductive amination. However, recent studies have shown that glutamate dehydrogenase exhibits a low affinity for ammonia and solely performs at high ammonia concentrations to mitigate the toxicity of ammonia. Recent research has shown that the GS/GOGAT pathway is key to ammonia assimilation (Fig. 4). Glutamine synthetase initially converts NH_4^+ into glutamine, which is then converted into two glutamate molecules by glutamate synthase in combination with α-ketoglutarate. Finally, aspartate is produced from glutamate and oxaloacetate through aspartate aminotransferase. Asparagine synthetase is an enzyme that aids in transforming aspartate and glutamine into asparagine and glutamate. These amino acids act as building blocks for many biological molecules, such as nucleotides, chlorophyll, polyamines, and alkaloids (Inokuchi et al., 2002; Perez-Garcia et al., 2011).

Although ammonium salts can promote microalgal growth within suitable pH ranges, their assimilation leads to medium acidification that limits microalgal proliferation (Zheng et al., 2019). Buffer solutions have conventionally been employed to counteract pH decline. However, buffers themselves can negatively impact microalgal physiology and increase cultivation expenses due to large volumes. For these reasons, an alternative approach has been proposed. This involves precisely regulating pH during microalgal cultivation using alkaline reagents instead of buffer solutions. This innovative approach ensures pH stability in a cost-efficient manner without causing any adverse impact on microalgal metabolism or viability. In neutralizing acidification resulting from

ammonium assimilation, alkaline reagents support optimal microalgal growth and biomass productivity in the culture system.

5.2.2. Nitrate assimilation

Nitrate is one of the nitrogen sources that typically affects microalgal growth and metabolism (Pozzobon et al., 2021). Cha et al. (2011) found that the addition of 0.18 mM nitrate increased the cell density to 0.95×10^7 cells/mL) and saturated fatty acids to 86.3 % in *C. sorokiniana*. The nitrate is assimilated by being transported into the cells where it is reduced to nitrite by the nitrate reductase. The nitrite enters the chloroplast to undergo further conversion into ammonium by nitrite reductase (Fig. 4), which is ultimately integrated into the GS/GOGAT cycle. Several factors, including light and nitrogen source availability, can influence nitrate reductase activity. Notably, the assimilation of nitrate is an energy-demanding process that is primarily light-dependent. Nitrate reductase activity is induced by light at the protein level, resulting in faster nitrate assimilation by microalgae in nitrate media under light conditions, compared to dark conditions (Crawford, 1995). In addition, nitrate reductase activity is induced by nitrate, when used as the sole nitrogen source, but is inactivated by ammonium (Sherman and Funkhouser, 1989). This provides further evidence for microalgae preference for ammonia.

5.2.3. Assimilation of organic nitrogen sources

Microalgae can utilize several organic nitrogen sources for growth under heterotrophic conditions, including urea, glycine, glutamate, glutamine, asparagine, arginine, citrulline, and allantoin (Huang et al., 2021). These organic nitrogen compounds support microalgae growth in both light and dark conditions. Urea, for instance, is an economical and readily available nitrogen source for microalgae cultivation. Some *Chlorella* sp. can utilize urea as their only source of nitrogen. During the assimilation process, the DUR3 transporter facilitates the initial transportation of urea into the cell, and subsequently, urea is hydrolysed into ammonium and CO_2 by enzymes, such as urea carboxylase and allophanate hydrolase (Maitz et al., 1982; Kanamori et al., 2004; Pinton et al., 2016).

Microalgal growth and physiological characteristics differ significantly between organic and inorganic nitrogen mediums. For example, in a urea medium, *C. variabilis* exhibits comparable lipid content (16.4 %) and lipid productivity (4.2 mg/L/day) to that in a nitrate medium (15.2 % and 4.2 mg/L/day, respectively). By contrast, these values were significantly higher than those in an ammonium medium (Altın et al., 2018). The highest cell concentration (1.59 g/L) was obtained in the ammonium medium, which was nearly 6-fold higher than that in the urea medium (Altın et al., 2018). Moreover, a higher abundance of various metabolites of *C. sorokiniana* was identified in the urea medium, compared to the nitrate medium, where alanine increased by 370 %, serine by 350 %, valine by 180 %, myo-inositol by 190 %, glyceric acid by 230 %, and glutamic acid by 220 % (Ribeiro et al., 2020). In nitrate medium, the growth of *C. pyrenoidosa* enhanced by 110 % and 63 % by adding aspartic acid, and arginine, respectively, while protein content was stable in the cells (Zhang et al., 2015). Therefore, the choice of nitrogen source significantly impacts the growth, lipid content, and metabolic synthesis of microalgae, highlighting the importance of understanding and optimizing nitrogen utilization in microalgal cultivation for diverse applications.

6. Harvest process of *Chlorella* sp

The harvesting process is the second stage following microalgae production, involving the separation or detachment of microalgae from its growth medium (Singh and Patidar, 2018). A few harvesting technologies have been demonstrated to be effective and are in widespread use, including centrifugation, flotation, membrane filtration, and flocculation (Javed et al., 2019; Najjar and Abu-Shamleh, 2020). These methods are fundamental to the efficient concentration and collection of microalgae, enabling subsequent processing and utilization in various applications.

Centrifugation is a widely recognized and effective method for harvesting microalgae. It exploits the differences in particle size and density among the medium components through the application of appropriate rotational speeds (Najjar and Abu-Shamleh, 2020). This technique enables a rapid increase in biomass concentration and achieves high harvest efficiency in a relatively short period of time. Studies have shown that the majority of microalgae species achieved 80–90 % recovery within 2–5 min of centrifugation (Javed et al., 2019). Despite its effectiveness, the centrifugation process has certain drawbacks. It is an energy-intensive process with high operational maintenance costs, making it less ideal for large-scale operations (Najjar and Abu-Shamleh, 2020). Additionally, there is a risk of cell damage due to the mechanical stress involved (Singh and Patidar, 2018). These factors highlight the need for careful consideration when choosing centrifugation as a harvesting method for microalgae.

Flotation is a gravity-based separation process that utilizes air or gas bubbles to facilitate the transportation of suspended microalgae to the liquid surface, where they can then be collected through a skimming process. The combination of flotation with a flocculant, which destabilizes the cells, has led to flotation becoming a prominent method for microalgae harvesting. This method provides several advantages, including high overflow rates, short detention periods, a minimal operational footprint, and the production of concentrate with greater thickness than that obtained through sedimentation (Laamanen et al., 2016). These benefits make flotation one of the most economically viable methods for microalgal harvesting (Sharma et al., 2014). Flotation processes can be categorized into different types based on the method of bubble production, including froth flotation, dispersed air flotation, dissolved air flotation, and electrolytic flotation (Roy and Mohanty, 2019). Each of these techniques is tailored to meet specific operational requirements and offers varying levels of efficiency and effectiveness in microalgae recovery.

Froth flotation typically utilizes conventional surfactants containing a single hydrophobic group, which often leads to low harvesting efficiency. To overcome this limitation, a novel Gemini surfactant, N,N′-bis (cetyldimethyl)-1,4-butane diammonium dibromide (BCBD), has been developed (Huang et al., 2019). This utilization of this advanced surfactant significantly enhanced the flotation process, improving recovery by 21.4 % and enrichment ratio by 22.9 % (Huang et al., 2019). Dispersed air flotation is a process where gas is introduced into a cell suspension, often with added surfactants, to generate foam with bubble sizes of 700–1500 μm. This foam facilitates the recovery of microalgae cells. Although this process consumes relatively low energy (0.003–0.015 kWh/m^3), the use of expensive equipment and the occurrence of significant pressure drops to generate the necessary bubbles make it a costly endeavour (Alhattab and Brooks, 2020). The higher lipid content in microalgae cells harvested via dispersed air flotation compared to centrifugation (Coward et al., 2014) makes this method particularly suitable for harvesting microalgae for low-value products like biofuels. Dissolved air flotation differs from dispersed air flotation in that it requires greater energy inputs (0.1–0.3 kWh/m^3) due to the necessity of pressurization to produce microbubbles that transport suspended particles to the reactor surface (Alhattab and Brooks, 2020). The air bubbles generated in this process range from 10 to 100 μm in size, with an average size of 40 μm (Roy and Mohanty, 2019). The flotation efficiency of dissolved air flotation has been reported to exceed 94 % under various operational conditions (Leite et al., 2019). In contrast, electro-flotation, another energy-intensive process, is primarily effective at the bench scale and in marine environments (Roy and Mohanty, 2019). Consequently, it is unlikely to be a suitable process for harvesting *Chlorella*.

Membrane filtration is a technique used to separate microalgae from a culture medium by employing a membrane under conditions of gravity, pressure, or vacuum force. A few filter assemblies have been

utilized for microalgae harvesting, including microfiltration (0.1–10 μm), macrofiltration (10 μm), dead-end filtration, ultrafiltration (0.02–0.2 μm), tangential flow filtration, vacuum filtration, and pressure filtration (Singh and Patidar, 2018). This process can be conducted either continuously or discontinuously without the necessity for chemical inputs, resulting in the production of highly concentrated and minimally disrupted microalgae (Mkpuma et al., 2022; Singh and Patidar, 2018). One of the key advantages of membrane filtration is its low energy requirement compared to other harvesting methods. However, the high cost of the membrane and the tendency for fouling limit its widespread application in microalgae harvesting. To solve these challenges, anti-fouling strategies, including membrane cleaning and optimization of filtration configuration have been developed for *Chlorella* harvesting (Mkpuma et al., 2022). These advancements have markedly improved the efficiency and practicality of membrane filtration, making it a more viable option for microalgae harvesting in diverse settings.

Flocculation is widely used as an economic step in the microalgae harvesting process, as it effectively concentrates diluted microalgal suspensions into a thick slurry. Flocculation can be classified into three main categories: chemical, physical, and biological flocculation (Roy and Mohanty, 2019; Singh and Patidar, 2018). Chemical flocculation is typically achieved by the addition of flocculants, which can be either inorganic (such as multivalent metal salts, ammonia, etc.) or organic flocculants (such as chitosan, poly γ-glutamic acid, etc.) (Labeeuw et al., 2021; Vu et al., 2020). The efficiency of chemical flocculation ranges from 60 % to 99 %, depending on various factors including the types and dosages of flocculant used, as well as the specific microalgal strains involved (Labeeuw et al., 2021). Physical flocculation can be achieved through methods such as ultrasound, electro-flocculation, and magnetic separation. Compared to chemical flocculation, physical flocculation has the advantage of preventing contamination from chemical additives. Particularly, electro-flocculation is considered a promising option for scale-up, due to its non-species-specific nature, cost-effectiveness, and ease of control (Lucakova et al., 2022; Singh and Patidar, 2018). Biological flocculation involves the use of bio-flocculants produced by microorganisms. Recent research in this area has focused on the incorporation of flocculating species like bacteria and other microalgae species to enhance the process (Roy and Mohanty, 2019). Among these three flocculation methods, biological flocculation stands out as particularly promising for microalgae harvesting, due to its safe and eco-friendly characteristics. This method aligns well with sustainable practices, making it an attractive option for large-scale applications.

7. Challenges and prospects

Recent developments for *Chlorella* sp. show substantial potential for its bioactive compounds. However, several challenges need to be addressed to fully exploit these benefits.

• The selection of dominant *Chlorella* strains

The production of bioactive compounds varies between *Chlorella* strains (Lai et al., 2019). However, the whole-genome sequence of *Chlorella* sp. has not yet been fully determined, which leads to unclear functional roles for many genes and their associated metabolic pathways. Therefore, future studies are crucial to deciphering the whole-genome sequence of *Chlorella* sp. This endeavour could enable the identification and characterization of genes responsible for key metabolic functions, thereby facilitating targeted genetic modification and improvement. For example, advanced genetic engineering techniques can be employed to develop *Chlorella* strains with desirable traits, such as increased production of high-value bioactive compounds and improved extractability. Such targeted approaches have the potential to significantly improve the efficiency and yield of bioactive compounds.

• Economic and efficient medium source

The use of a chemical-supplemented culture medium typically increases the overall cost of microalgae cultivation, while also resulting in low biomass productivity and limited production of bioactive compounds (Table 1 and Table 3). This hinders its industrial scalability. In contrast, food industry byproducts such as brewery wastewater and hydrolysate from cassava residues provide a rich source of nutrients for *Chlorella* sp. growth (Cai et al., 2021, 2022b; Yan et al., 2024). By incorporating food industry byproducts into *Chlorella* sp. cultivation, it becomes possible to effectively utilize these waste streams for bioactive compound production. This sustainable approach not only enhances the economic viability of microalgae cultivation but also makes a significant contribution to environmental sustainability. Consequently, *Chlorella* cultivation emerges not only as a method for bioactive compound production but also as a key component of sustainable industrial practices to achieve long-term environmental and economic balance.

• Efficient photobioreactors

Current photobioreactors frequently encounter challenges in efficiency utilizing light energy and optimizing mass transfer rates, thereby limiting the metabolic process essential for microalgal growth (Tan et al., 2021). These inefficiencies also undermine the potential to achieve maximum biomass yields and production of bioactive compounds. Therefore, researchers need to actively explore innovative strategies to optimize photobioreactor configurations. These include advances in the optimization of light distribution, the improvement of energy efficiency through sophisticated bioreactor designs, and the management of mass transfer rates to ensure optimal nutrient delivery and waste removal (Luo et al., 2017; Naira et al., 2020). Additionally, the adoption of cost-effective, durable materials alongside modular designs represents a pivotal shift towards reducing initial capital investment and ongoing operating costs, making advanced photobioreactors more accessible on an industrial scale. This dual approach not only improves the economic viability of advanced photobioreactors, but also facilitates their scalability for large-scale industrial applications. By overcoming these technical and financial barriers, the integration of advanced photobioreactor technologies promises to revolutionize microalgae cultivation practices.

• Efficient and low-cost harvesting of *Chlorella* biomass

Current harvesting technologies often involve costly and energy-intensive processes, which hinder widespread adoption and scalability. Thus, efficient separation technologies are essential. The development of dominant Chlorella strains engineered for extracellular secretion of specific high-value products is being encouraged. By eliminating costly extraction procedures, this innovation could streamline the harvesting process. Subsequently, simultaneous microalgae cultivation and continuous harvesting of high-value compounds could be achieved through the integration of advanced membrane systems. These advances will make Chlorella-based products more economically viable by improving operational efficiency and reducing production costs.

8. Conclusion

Chlorella sp. is typically grown under photoautotrophic, heterotrophic, mixotrophic, or photoheterotrophic conditions to accumulate biomass. Open ponds and photobioreactors are the primary systems used to cultivate *Chlorella* sp. Within *Chlorella* cells, carbon and nitrogen metabolic processes lead to the synthesis of high-value bioactive compounds, including carbohydrates, proteins, lipids, fatty acids, and pigments. However, *Chlorella* strains and various environmental factors such as light, temperature, and culture medium significantly impact biomass production and the accumulation of bioactive compounds. These factors are discussed in detail in this review. The bioactive compounds produced by *Chlorella* sp. exhibit nutritional properties and potential health benefits, making them suitable for use in food, pharmaceutical, and cosmetic products. Due to their extensive benefits, various extraction techniques have been developed to obtain these bioactive compounds, which are also highlighted in this review. Despite the promise of *Chlorella* sp., current technology faces several challenges in the efficient production of *Chlorella* biomass and its bioactive compounds, such as the high cost of the medium source, inefficient

photobioreactors, and suboptimal biomass harvesting technologies. Therefore, future investigations are required to overcome these limitations to achieve the full potential of *Chlorella* sp. as a sustainable and economically viable source of bioactive compounds.

CRediT authorship contribution statement

Yilin Fang: Writing – original draft, Conceptualization. **Yihui Cai:** Supervision, Resources, Methodology. **Qi Zhang:** Writing – review & editing, Funding acquisition, Conceptualization. **Roger Ruan:** Writing – review & editing, Methodology. **Ting Zhou:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization.

Declaration of Competing Interest

The corresponding author, Ting Zhou is a guest editor for the journal Process Safety and Environmental Protection, but has had no access to, or involvement in, the peer review process for this paper or its handling by the journal at any point.

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