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Challenges and opportunities to increase economic feasibility and sustainability of mixotrophic cultivation of green microalgae of the genus *Chlorella*

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ABSTRACT

Green microalgae belonging to the genus *Chlorella* have long been used as health foods and additives for human consumption, as well as animal feed. These photosynthetic microorganisms have also found application in the bioremediation of wastewater and bio-mitigation of CO₂. In the last decades, *Chlorella* microalgae have attracted considerable attention due to their great potential as feedstock for production of biofuels and high-value products. This review explores key aspects related to the cultivation of different *Chlorella* species under mixotrophic conditions. Common organic compounds and cheap substrates used for mixotrophic microalgal growth are presented. Bottlenecks and strategies to increase economic feasibility and sustainability of mixotrophic cultivation of *Chlorella* for future commercial production are also discussed. In this sense, the use of wastewater as a nutrient source for microalgae cultivation seems to be a promising alternative to improve the sustainability and economic feasibility of microalgae production systems. However, since wastewater presents a complex composition, methods to minimize the risk of contamination should be developed. Additionally, the screening of novel mixotrophic species of *Chlorella* capable of both assimilating a wide range of organic compounds from wastewater and accumulating high content of molecules of interest (e.g., lipids) would be highly recommended.

1. Introduction

Chlorella is a genus of unicellular eukaryotic green microalgae living in both aquatic and terrestrial environments. These microalgae have been cultivated at commercial scale in Taiwan and Japan since the early 1960s to produce nutritional supplements and healthy foods, owing to their high content of proteins, vitamins and minerals [1]. *Chlorella* biomass also finds application in aquaculture as feed supplement to enhance the skin pigmentation of ornamental fish or to improve the immunity and growth of fish and crustaceans, among others [2,3]. Some species of *Chlorella* have the ability to accumulate large amounts of starch under stressful culture conditions (e.g., nitrogen starvation), while others synthesize neutral lipids in the form of triacylglycerol (TAG) instead; thus, the biomass of these microalgal species has been considered as a promising feedstock for producing bioethanol or biodiesel, respectively [4–6]. Moreover, *Chlorella* can be used to remove soluble organic compounds and other nutrients (e.g., N and P) from industrial and municipal effluents [7,8]. Current research is targeting

other novel potential biotechnological applications of *Chlorella* in different industrial sectors such as: pharmaceutical, cosmetics as well as human and animal nutrition [9–12].

However, although several companies, research institutes and governments have invested in microalgae cultivation for many years, at present the commercial production of biofuels and high-value products from these photosynthetic microorganisms is still limited. The economic viability of producing microalgal biomass remains a major hurdle to its commercialization [13–15]. Photoautotrophic growth in open ponds using sunlight as an energy source and inorganic carbon (e.g. CO₂) as a carbon source is commonly used for culturing microalgae, but it has a number of drawbacks, including low biomass productivities and extended culture periods [16]. This mode of cultivation cannot be justified for commercial production of valuable products since the high costs of extraction and purification of intracellular metabolites could heighten due to the low cell concentrations [17]. Consequently, significant attention has been given to mixotrophic processes as a feasible alternative to overcome the limitations of pure photoautotrophic

Abbreviations: TAG, triacylglycerol; TS, thin stillage; FAMES, fatty acid methyl esters; COD, chemical oxygen demand; BOD, biological oxygen demand; TEA, techno-economic analysis; LCA, life-cycle assessment; LCEE, life cycle energy efficiency; FER, fossil-energy requirements; GWP, global warming potential.

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cultures. In the mixotrophic cultivation mode, an organic carbon source is supplemented in addition to inorganic carbon for growth [18,19]. Some species of *Chlorella* have been reported to achieve higher specific growth rates and biomass yields under mixotrophic conditions than under photoautotrophic conditions [20]. In mixotrophic growth both the photo-assimilation of CO₂ and oxidative assimilation of organic carbon sources proceed independently; therefore, this mode of cultivation offers the possibility of greatly increasing the microalgal cell concentration, and hence the volumetric productivity. Cultivation of microalgae under mixotrophic conditions may be applied for the production of high-value, low-volume chemicals in order to reduce the cost of downstream processing [17].

The novelties of this review are highlighting the effect of different organic carbon sources on metabolite production by *Chlorella* strains under mixotrophic cultivation conditions and defining strategies that has practical implications for the development of microalgae-based systems at large scale. A comprehensive review of pure compounds and potential, low-cost substrates used as organic carbon source in mixotrophic cultures of *Chlorella* is provided. The benefits, challenges and strategies adopted to address the limitations of culturing mixotrophic microalgae are also discussed. Additionally, a discussion on environmental and economic indicators is presented to help identify conditions that could lead to sustainable microalgae-based production systems.

2. Nutritional modes of microalgae

Microalgae are capable of using different nutritional strategies centred around the two main types of nutrition, namely, autotrophy and heterotrophy [21].

Autotrophs are those microorganisms that require inorganic compounds (e.g., CO₂ or bicarbonates) as a source of carbon. These microorganisms are known as photoautotrophs (photolithotrophs) when they obtain their chemical energy by the absorption of light energy through photosynthesis. If autotrophic microorganisms oxidize inorganic compounds for energy, they are referred as chemoautotrophs (chemolithotrophs). Heterotrophic (organotrophic) microorganisms obtain their principal source of carbon for growth from organic compounds, either by taking up dissolved substances (osmotrophy) or by the ingestion of living or dead particles (phagotrophy). They are known as photoheterotrophs (photo-organotrophs) if they require light as a source of energy, or as chemoheterotrophs (chemo-organotrophs) if they obtain their energy needs through the oxidation of organic compounds. Some microalgae are also mixotrophic (amphitrophic), capable of combining autotrophy and heterotrophy (Fig. 1). Under mixotrophic conditions, both organic compounds and CO₂ are necessary for growth [22].

The relative contribution of autotrophic and heterotrophic mechanisms to cell growth of mixotrophic species varies along a gradient of microalgae whose dominant nutritional mode is phototrophy, to those for which heterotrophy is the dominant mechanism. Some mixotrophic

microalgae can be mainly photosynthetic and occasionally utilize organic carbon sources [23].

3. Characteristics of mixotrophic cultivation of *Chlorella*

Three major cultivation modes can be used to produce microalgal biomass, namely photoautotrophic, heterotrophic and mixotrophic. These cultivation conditions have a significant influence on the growth characteristics and composition of microalgae.

Cultivation of microalgae under photoautotrophic conditions has been the most common mode of microalgal growth, performed both in photobioreactors and open ponds [24]. However, photoautotrophic cultivation has difficulty achieving high biomass concentration since light penetration is inversely proportional to the turbidity of the culture medium. Light requirements increase as cell density increases because mutual shading reduces exponentially the penetration of light into the medium [25]. Therefore, net photosynthesis cannot take place in deep regions of microalgal cultivation systems at high cell densities due to the strong attenuation of light. The reduction in light intensity becomes even more pronounced when using raw wastewaters which have high turbidity and contain particulate matter [26].

Several studies have demonstrated that cultivation of mixotrophic microalgae results in greater biomass concentration when compared to photoautotrophic growth [27–29]. For instance, the highest cell concentration (1.325 g L⁻¹) of *C. sorokiniana* under mixotrophic cultivation supplemented with 10 g L⁻¹ glucose was 4.2 times of that under photoautotrophic conditions [28]. As organic substrates are assimilated in mixotrophic cultures, cell growth is not strictly dependent on photosynthesis and hence, light is no longer a limiting factor for microalgal growth; thus, photo-limitation can be reduced in mixotrophic cultivation systems at low and moderate light intensities. Supplementing photosynthetic cultures with an organic carbon source provides an extra source of both energy and reduced carbon, which stimulate the production of biomass while simultaneously reducing biomass losses associated with respiration [30]. Mixotrophic cultivation may not only increase biomass production, but also improve lipid content which is of great importance for the production of microalgal biodiesel [28].

Compared with heterotrophic cultivation that solely relies on organic compounds, mixotrophic culture achieves higher biomass productivities when the same organic C source is supplied. Employing 1% w/v glucose as organic substrate, the highest biomass concentration of *C. minutissima* (0.216 g L⁻¹) cultivated mixotrophically was 2 times of that obtained under heterotrophic cultivation [29].

3.1. Improvement of microalgae growth rate under mixotrophic conditions

The mixotrophic growth of microalgae is characterized by a complex relationship between the photoautotrophic and heterotrophic metabolic activities. In mixotrophic cultures of *Chlorella* under light limited conditions, both the photosynthetic mechanism and the oxidative assimilation of the carbon source can proceed simultaneously and independently. Some studies [31,32] demonstrated that the specific growth rate of *Chlorella* cells cultured mixotrophically was approximately equal to the sum of the growth rates obtained in the heterotrophic and photoautotrophic processes. However, it has also been reported that the specific growth rate of certain *Chlorella* species (e.g. *C. vulgaris* UAM 101) cultured in mixotrophic conditions exceeded the sum of photoautotrophic and heterotrophic growth rates [33]. This result indicates that synergistic effects of photosynthesis and aerobic respiration may occur under mixotrophic cultivation of some strains of *Chlorella*.

The enhancement of photosynthetic and respiratory activities in mixotrophic cultures of *Chlorella* could probably be explained by the photosynthetic re-utilization of CO₂ released from aerobic respiration [34]. It has been reported [35] that the biomass concentration of the

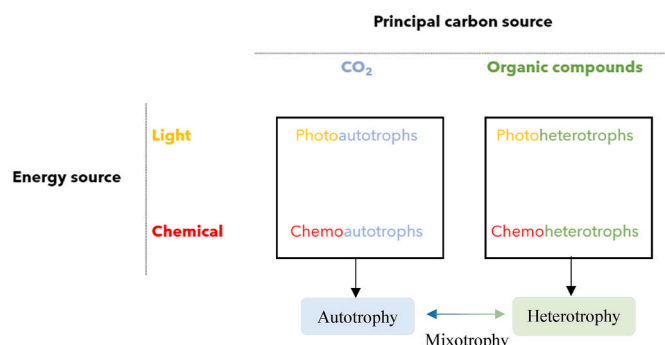


Fig. 1. Classification of nutritional categories for microalgae.

marine *Chlorella* sp. cultivated under mixotrophic conditions (1.41 g L^{-1}) with 2 g L^{-1} glucose was higher than the sum of those obtained from photoautotrophic (0.39 g L^{-1}) and heterotrophic (0.50 g L^{-1}) cultivation modes. The improved growth of mixotrophic cells was likely due to the positive effect of glucose on photoautotrophic metabolism and/or to the positive effect of light on glucose assimilation.

On the other hand, for some *Chlorella* species (e.g., *C. pyrenoidosa*), it was found that mixotrophic cell growth rates surpassed the photoautotrophic growth rates only at moderate light intensities (e.g., around 400 lux) [36]. Under high light intensities (e.g., those exceeding the intensity of saturation), the mixotrophic and photoautotrophic growth rates of that microalga tend to equalize, implying that no benefit might be derived by using an organic carbon source. Light intensity as low as 5 W m^{-2} (approx. 633 lux for sunlight) can hinder the uptake of organic carbon in mixotrophic cultures of *Chlorella* due to inhibition of synthesis of the glucose transport system on the cell membrane [34]. Nevertheless, the light-induced reduction of organic matter assimilation under such adverse conditions depends on the algal strain. Therefore, in outdoor microalgal culture systems where high light intensity of sunlight is used, only those species of *Chlorella* that are no sensitive to photo-inhibition of the organic carbon uptake would be suitable for mixotrophic cultivation [37].

4. Organic compounds as carbon sources

Many organic compounds have been investigated for microalgae culturing under mixotrophic conditions. Carbohydrates (e.g., glucose), acetate and glycerol have been the most common organic compounds used for the growth of mixotrophic species of *Chlorella* (Table 1).

Several studies demonstrated that glucose is the best organic compound for growing *Chlorella* cells mixotrophically [27,67,68]. However, the effectiveness of organic carbon sources in supporting cell growth of mixotrophic microalgae is species- and strain-specific.

Under mixotrophic conditions, *C. pyrenoidosa* attained higher cell densities on glucose ($9.7 \times 10^7 \text{ cells mL}^{-1}$) than on galactose ($7.5 \times 10^7 \text{ cells mL}^{-1}$) or fructose ($5.8 \times 10^7 \text{ cells mL}^{-1}$), when 5.0 g L^{-1} of each sugar was added to Bold's Basal Medium (BBM). Furthermore, monosaccharides were more effective as organic carbon source for mixotrophic growth of *C. pyrenoidosa* than disaccharides (sucrose, maltose and lactose) or starch [20]. Similarly, the supplementation of an anaerobically digested starch wastewater with 8.0 g L^{-1} glucose, fructose and galactose increased the final biomass concentration of *C. pyrenoidosa* to 4.03 g L^{-1} , 3.73 g L^{-1} and 3.67 g L^{-1} , respectively, after two weeks of mixotrophic cultivation [69]. A different study found that monosaccharides had more significant growth-promoting effect on *C. pyrenoidosa* than disaccharides (cellobiose, maltose and sucrose) at different light intensities (1080 and 1800 lux); and, among the hexoses, galactose gave the highest mixotrophic cell growth, followed by glucose and fructose [70]. The different efficiencies by which exogenous hexoses are assimilated by *Chlorella* cells could be related to the induction of a monosaccharide- H^+ symport that allows the energy-dependent transport of the sugars into the cell across the plasmalemma [71].

Studies exploring the mixotrophic metabolism of *C. regularis* showed that microalgal cells cultured in medium containing acetate showed higher specific growth rate (0.45 h^{-1}) than those cultivated on glucose (0.37 h^{-1}) or ethanol (0.29 h^{-1}) [49]. In mixotrophic cultures of *C. vulgaris*, the highest specific growth rate (1.22 d^{-1}) and biomass productivity ($0.2 \text{ g L}^{-1} \text{ d}^{-1}$) were obtained by using glucose, followed by sucrose, glycerol and acetate [68].

The optimum concentration of a given organic compound that maximizes mixotrophic growth of *Chlorella* microalgae depends on the strain in addition to the culture conditions used. For instance, the highest growth rate (3.4 d^{-1}) and biomass yield (0.82 g/g) of the strain *C. sorokiniana* UTEX 1602 grown mixotrophically at $25 \text{ }^\circ\text{C}$ and light intensity of $100 \mu\text{mol/m}^2 \text{ s}$, was obtained with the supplementation of 4 g L^{-1} of glucose [54]. On the other hand, it was found that the specific

Table 1
Organic compounds used for mixotrophic growth of selected *Chlorella* species.

Species	Organic compound	Concentration (g L^{-1})	References
<i>C. minutissima</i>	glucose	18.0 [100 mM] *	[38]
		10.0	[39]
	glycerol	9.8 [100 mM] *	[38]
	methanol	0.4–39.6 [0.05–5.0% v/v]	[40]
		*	
<i>C. protothecoides</i>	succinate	14.0 [100 mM] *	[38]
	acetate	4.1–20.5	[41]
	glucose	10.0	[42]
		16.6	[43]
<i>C. pyrenoidosa</i>		3.0–15.0	[41]
	glycerol	4.1–20.4	[41]
	acetate	5.0–10.0	[44]
		2.0–10.0	[45]
	fructose	0.5–12.0	[20]
	galactose	0.5–12.0	[20]
	glucose	0.4	[46]
		0.5–12.0	[20]
		10.0	[47]
		5.0	[48]
<i>C. regularis</i>	glycerol	6.3–12.6 [0.5–1.0% v/v] *	[44]
	lactose	0.5–12.0	[20]
	sucrose	0.5–12.0	[20]
	starch	0.5–12.0	[20]
	acetate	3.3–9.8 [40–120 mM] *	[31]
		1.39	[49]
<i>C. sorokiniana</i>	ethanol	1.0	[49]
	glucose	1.0	[49]
	acetate	1.0–8.0	[50]
		6.0	[51]
	glucose	6.0	[52]
		0.5–2.0	[53]
		2.0–20.0	[54]
		1.0–50.0	[50]
		2.5	[55]
		5.0–25.0	[28]
<i>C. vulgaris</i>	glycerol	2.3 [25 mM] *	[56]
	acetate	1.0–3.0	[57]
		4.1–20.5	[58]
	glucose	1.0	[59]
		5.0–20	[60]
		5.0–25	[61]
		3.0–15.0	[58]
	glycerol	2.3 [25 mM] *	[56]
		1.8 [20 mM] *	[62]
		2.0–10.0	[63]
	1.0–10.0	[64]	
	4.1–20.4	[58]	
	xylose	4.5 [30 mM] *	[65]
		3.0 [20 mM] *	[62]
<i>C. zofingiensis</i>	glucose	5.0–40.0	[66]

[] * = original concentration as reported in the study.

growth rate (1.11 d^{-1}) and biomass productivity ($1.18 \text{ g L}^{-1} \text{ d}^{-1}$) of a different strain (FACHB-275) of *C. sorokiniana* were significantly improved under mixotrophic cultivation at $25 \text{ }^\circ\text{C}$ and 1500 lux, with the addition of 20 g L^{-1} of glucose [72]. Similarly, the mixotrophic growth of *C. vulgaris* at $25 \text{ }^\circ\text{C}$ under illumination of 2500 lux was enhanced with increasing glucose concentration from 1 to 20 g L^{-1} . Maximum values of specific growth rate and biomass productivity achieved with a glucose content of 20 g L^{-1} were 1.12 d^{-1} and $0.37 \text{ g L}^{-1} \text{ d}^{-1}$, respectively [68]. However, a different study [73] demonstrated that under mixotrophic conditions, 10 g L^{-1} glucose provided the fastest growth rate of *C. vulgaris* compared with those at 20, 50 and 100 g L^{-1} .

High concentrations of the organic carbon source may also exert an inhibitory effect on the mixotrophic growth of *Chlorella* cells. It was reported that concentrations of glucose above 100 g L^{-1} , or glycerol above 2% v/v, significantly inhibit the biomass productivity of *C. vulgaris* cultivated mixotrophically [73]. The addition of 5% v/v methanol at the beginning of the mixotrophic cultivation of *C. minutissima* was found to be toxic to the microalgae, leading to a quick

death of the culture [40].

5. Low-cost organic carbon substrates

In addition to the pure and consequently expensive organic compounds, there is potential for using much cheaper sources of organic carbon, such as: wastewaters, wastes and by-products from agro-industrial sectors. Municipal and agro-industrial wastewaters, molasses, crude glycerol from biodiesel production and corn powder hydrolysate have been utilized as organic substrates for mixotrophic cultivation of several species of *Chlorella* [74–76]. Wastewater resulting from domestic, agricultural or industrial activities contains different organic and inorganic compounds of natural and anthropogenic origin that serve as nutrients for microalgae. The main organic components of wastewater include proteins, carbohydrates, lipids, volatile acids and amino acids [77]. Several studies have suggested that wastewater can be used as a nutrient source for microalgae cultivation and this could significantly improve the sustainability and economic feasibility of microalgae production systems at large scale [78–80].

However, even though wastewater can provide some essential nutrients for microalgal cell growth, it is quite different from common culture medium. The major difference between wastewater and defined (synthetic) culture media is the high complexity of wastewater in terms of composition [81]. The concentration of nutrients, such as carbon, nitrogen and phosphorus present in wastewater, varies over a wide range. Table 2 gives an overview of different types of wastewaters and their content of COD, N and P.

The efficiency of nutrient removal in microalgal-based systems is mainly determined by the characteristics of the wastewater and microalgae species. The overall wastewater composition affects nutrient uptake, existing an optimal C:N:P ratio, which differs between microalgae species. Moreover, nutrient uptake also depends on environmental factors that affect microalgae growth such as light intensity, temperature, pH, turbidity and colour, among others [89].

Three types of effluents obtained from the different stages of domestic wastewater treatment have been evaluated as media for cultivating microalgal cells mixotrophically. These different streams involve primary clarifier effluent, effluent from secondary treatment tank and centrate. Centrate, the liquid generated from the thickening process of activated sludge, has been considered as a suitable substrate for the cultivation of mixotrophic microalgae due to its high content of organic matter [15]. In this sense, the growth rate and biomass yield of *Chlorella* sp. cultivated in autoclaved and raw centrate were comparable to those obtained from standard cultivation media [90]. On the other hand, AlMomani and Örmeci [82] found that although centrate had higher concentrations of nitrogen, phosphorus and COD than the primary and secondary effluents (Table 2), the growth rate of *C. vulgaris* was significantly higher in primary effluent (0.61 d^{-1}) with a C:N:P ratio of 24:5:1 than those obtained in centrate (0.52 d^{-1}) and secondary effluent (0.25 d^{-1}). According to Woertz et al. [91], a suitable C:N:P for optimum growth of microalgae is about 50:8:1. Other study [92] suggested N:P ratio, ranging between 8:1 and 45:1, is an important macronutrient

Table 2
Composition of different wastewaters used for microalgal-based systems.

Source of wastewater	COD (mg L ⁻¹)	TN (mg L ⁻¹)	TP (mg L ⁻¹)	References
Primary effluents	242	41	10	[82]
Secondary effluents	59	63	26	[82]
Centrate	601	137	200	[82]
Piggery	7232	3500	117	[83]
Poultry processing	4020	305	35	[84]
Dairy	12,000	2400	480	[85]
Olive oil washing	1362	7	2	[86]
Soybean processing	5320	107	23	[87]
Starch	58,560	1492	154	[88]

requirement for freshwater microalgae cultivation. Therefore, not only the concentrations but also the ratios of C, N and P in wastewater are likely to affect microalgae growth.

It is also worth noting that a major constraint of using wastewater as a source of nutrients for microalgae production is that it reduces the range of biomass applications because the produced biomass could be contaminated by various pollutants that are present in the wastewater. Therefore, microalgae produced on wastewater should mainly be used for the production of biofuels rather than food or feed applications [93]. High removal efficiencies of nutrients from centrate were found when *C. kessleri* and *C. protothecoides* were cultivated under mixotrophic conditions aiming simultaneous wastewater treatment and biodiesel production [37]. A recent study reported that textile wastewater treatment and lipid accumulation through the mixotrophic cultivation of immobilized *Chlorella* strains in alginate beads resulted in NH₄⁺-N, COD and colour removal efficiencies as high as 90%, 75% and 60%, respectively [94]. Immobilization of *Chlorella* cells within natural biopolymers, such as alginate, could potentially overcome microalgae harvesting issues at commercial scale by preventing biomass from sticking on external surfaces.

Mixotrophic microalgae belonging to the genera *Chlorella*, *Chlamydomonas* and *Scenedesmus* were also capable of growing on wastewater and poultry litter extract (PLE) and produced higher biomass concentration than that achieved on freshwater-based enriched growth media (BG11) [29]. Agro-industrial wastes such as soy whey and dry-grind ethanol thin stillage (TS) were also evaluated as organic carbon substrates in mixotrophic cultures of *C. vulgaris*. Both the biomass productivity ($2.5 \text{ g L}^{-1} \text{ d}^{-1}$) and lipid content (43%) of microalgal cells grown mixotrophically on TS were higher than those obtained on modified basal medium containing glucose [95].

Hydrolysed cheese whey (the liquid by-product remaining from cheese production) has been used as a source of glucose and galactose to reduce the costs of mixotrophic cultivation of *C. vulgaris*. Specific growth rate (0.43 d^{-1}), final biomass concentration (3.58 g L^{-1}) and biomass productivity ($0.75 \text{ g L}^{-1} \text{ d}^{-1}$) under mixotrophic conditions were found to be 3.5, 2.9 and 7.5 times higher respectively than those obtained under photoautotrophic conditions [16].

6. Strategies for circumventing limitations of mixotrophic cultivation

While mixotrophic cultivation offers several benefits over autotrophic and heterotrophic microalgae cultures, some challenges still need to be overcome to make this cultivation regime feasible at commercial scale.

One of the major limitations of mixotrophic cultures is their vulnerability to undesired contamination by other heterotrophic microorganisms (e.g., bacteria, fungi, rotifers, ciliates, zooflagellates). On the one hand, the presence of some of these microorganisms (e.g., bacteria) could be beneficial to facilitate the conversion of biological carbonaceous material in wastewaters [96]. It was reported that almost 90% of BOD from unsterilized secondary wastewater was reduced by a co-existing *C. vulgaris* – bacteria system under mixotrophic conditions, whereas no substantial reduction in BOD concentration was recorded for sterile secondary wastewater treated by *C. vulgaris* alone [97]. In that cases, co-culturing of natural communities of microalgae and other microorganisms should be considered and developed in order to create stable consortia that perform in a predictable manner during wastewater treatment [7].

On the other hand, microalgae can also be strongly outperformed by competing organisms that reproduce rapidly, resulting in low microalgal biomass yields, reduced accumulation of metabolites of interest, or total culture loss. Methods for avoiding this type of undesired contamination include sterilization and operation under sterile conditions, use of excessive inoculation concentration as well as multi-stage culture systems; however, all these methods will increase production costs at large

commercial scale [27].

The traditional approach to mitigating contamination threat is to use axenic monoalgal cultures in closed cultivation systems. In general, mixotrophic growth of *Chlorella* microalgae has been conducted under sterile conditions using enclosed photobioreactors such as flat plate, bubble columns and tubular photobioreactors at laboratory or pilot-scale [98,99]. Enclosed photobioreactor systems offer the advantages of microalgae cultivation under controlled conditions, preventing the invasion of undesirable microorganisms. The addition of organic substrates can be carried out in a controlled way, allowing much higher areal productivities [100]. However, these culture systems are generally costly to build and operate, and are technically very difficult to scale-up, mainly due to the challenges in maintaining optimum cultivation conditions at large scale [99]. *Chlorella* was successfully cultivated under mixotrophic mode in a 10-L outdoor enclosed tubular photobioreactor, sterilized previously with 2–6% v/v hydrogen peroxide solution [98]; but, axenic microalgal cultivation could not be maintained when the culture volume was scaled-up to 300 L [101].

Some strategies have been proposed in order to address the contamination risks caused by using organic carbon nutrients in non-axenic environments. The use of certain organic carbon substrates (by addition or already found in the culturing medium) having bactericidal effects represents an alternative approach that could limit potential microbial contamination in non-sterile mixotrophic cultures. In this context, acetate may be a good choice as organic carbon source; continuous supplementation of acetate in small quantities may reduce the chance of contamination in *Chlorella* cultures [12]. The inhibitory effect of acetate on bacterial growth has been associated with the destabilization of homoserine transsuccinylase, the first enzyme responsible for the biosynthesis of methionine, an essential amino acid [102]. Acetate is also a non-fermentative substrate and does not support anaerobic growth of bacteria [103]. Methanol has also been proposed as a relatively cheap organic substrate that could maintain both the sterility of the mixotrophic culture and high biomass concentration of *Chlorella* sp. in long-term open pond systems [104]. Nevertheless, the addition of organic carbon sources is usually stopped at night, because microalgae may be outcompeted by fast-growing heterotrophic bacteria in the dark [101].

Attempts to minimize the risk of contamination have also been directed towards the development of sequential photoautotrophic-mixotrophic cultivation systems. In these systems, microalgae are initially cultivated under photoautotrophic mode until reaching a relatively high cell density before switching to mixotrophic cultivation by the addition of an organic carbon source. In this way, the high biomass concentration before the addition of the organic carbon source will enable the microalgae to outcompete the initial low concentration of the contaminant [105]. Marchello et al. [106] studied the biochemical composition of *C. sorokiniana* in photoautotrophic-mixotrophic cultures performed by growing the microalgae under photoautotrophic mode during 4 days, followed by 6 days of mixotrophic cultivation using 1.0 g L⁻¹ glucose as the organic carbon source. It was found that the sequential photo-mixotrophic cultures showed the highest total carbohydrate concentration (0.026 pg μm⁻³) when compared to the mixotrophic (0.009 pg μm⁻³) and photoautotrophic cultures (0.007 pg μm⁻³) after 5 days. At the end of the experiment, no differences in cell densities among the three cultures were observed.

Another important challenge that hinders the development of mixotrophic culturing of microalgae at industrial scale is the increasing energy expenses and general costs by adding an organic carbon source [107]. For instance, the cost of glucose has been estimated to be about 80% of the total medium cost [108], making mixotrophic cultivation systems economically unfeasible. Thus, there is need to drastically reduce the cost of the organic substrate. Many cheap organic carbon sources such as crude glycerol from biodiesel production, acetate from anaerobic digestion, and sugars from agricultural and industrial wastes offer great promise for the cultivation of *Chlorella* microalgae

mixotrophically [75]. The potential use of detoxified lignocellulosic hydrolysates as source of glucose and acetate should be further explored, in spite of the few *Chlorella* strains capable of efficiently metabolizing xylose (a pentose sugar also found in great amount in these hydrolysates) [109].

From an economic and environmental perspective, coupling wastewater treatment with mixotrophic cultivation of microalgae represents a win-win strategy that could decrease the cost of *Chlorella* biomass production by removing nutrients from the waste stream and reducing the use of water.

7. Metabolites of interest and applications

Chlorella microalgae can produce a wide range of valuable metabolites based on their composition. These include lipids, carbohydrates, pigments, proteins and vitamins, which can be used as specialty chemicals and bulk commodities in different industrial sectors (e.g., cosmetics, pharmaceuticals, nutraceuticals, biofuels and aquaculture).

7.1. Lipids

Lipids are constituents of microalgae which serve as membrane components, storage products, metabolites and energy sources. They can be classified according to their polarities, which depends on the non-polar (lipophilic) carbon chains (fatty acids) and the polar (hydrophilic) moieties (carboxylic groups, alcohols, etc.). The major part of the non-polar lipids (neutral lipids) of microalgae are triglycerides and free fatty acids, whereas the polar lipids are essentially glycerides in which one or more of the fatty acids has been replaced with a polar group, for instance phospholipids and glycolipids [110]. Triglycerides can be extracted from microalgal cultures and transesterified to produce fatty acid methyl esters (FAMES), which are one of the primary molecular components of biodiesel [111].

The lipid content and fatty acids profile in microalgae depend on the species and are affected by several culture conditions such as: light intensity periods, nutritional mode, temperature, pH, nutrient deficiency, salinity, and even the concentration of the organic carbon source supplied in heterotrophic/mixotrophic cultures [112,113]. Final biomass concentration, biomass productivity, lipid content and lipid productivity of several species of *Chlorella* cultivated mixotrophically with different organic substrates are summarized in Table 3.

A number of studies have demonstrated that mixotrophic cultivation of microalgae leads to increased lipid productivities. It is worth noting that the higher lipid productivity under mixotrophic growth conditions as compared to photoautotrophic conditions can result from the combined increases in biomass productivity and lipid content, or from increased biomass productivity at relatively constant lipid content. For some *Chlorella* strains and under certain culture conditions (e.g., *C. sorokiniana* cultivated in secondary municipal wastewater supplemented with glycerol [30]), there is no positive effect of mixotrophic culture on cell lipid content; thus, the increase in lipid productivity is mainly due to increases in biomass productivity [105].

In the case of *C. protothecoides* cells grown mixotrophically with 10 g L⁻¹ glucose, it was found that the rate of lipid accumulation was 2.9 times of that under photoautotrophic conditions. Mixotrophic cells also showed higher lipid content (10.3%) than to those in the photoautotrophic culture (5.9%) [42]. On the other hand, it was reported that there were no significant differences in the lipid content of *C. protothecoides* cells (about 25%) under mixotrophic cultivation with 15 g L⁻¹ glucose in comparison with that under photoautotrophic mode [41]. However, as biomass productivity of microalgae grown under mixotrophic conditions was about 4.5 times of that obtained in photoautotrophy, the lipid productivity of *C. protothecoides* grown mixotrophically turned out to be higher than that obtained in the photoautotrophic culture. From an analysis of the carbon flux distribution in *C. protothecoides*, it was revealed that supplementation with 10 g

Table 3
Biomass content, biomass productivity, lipid content and lipid productivity of *Chlorella* species under different mixotrophic culture conditions.

Species	Organic carbon source	CO ₂ (%)	Light intensity	Photoperiod – light:dark (h)	Final biomass concentration (g L ⁻¹)	Biomass productivity (mg L ⁻¹ d ⁻¹)	Lipid content (%)	Lipid productivity (mg L ⁻¹ d ⁻¹)	References
<i>C. protothecoides</i>	glucose (15 g L ⁻¹)	0.04	NA	16:8	4.1	1360	14.1	190	[41]
	glycerol (20.4 g L ⁻¹)	0.04	NA	16:8	2.3	780	17.5	140	[41]
	acetate (20.5 g L ⁻¹)	0.04	NA	16:8	4.4	1450	17.9	260	[41]
<i>C. pyrenoidosa</i>	acetate (10 g L ⁻¹)	0.04	40.5 μmol/m ² s	24:0	0.87	121	13.4	16.2	[44]
	glycerol (1% v/v)	0.04	40.5 μmol/m ² s	24:0	0.33	47	16.8	7.9	[44]
<i>C. sorokiniana</i> <i>Chlorella</i> sp.	glucose (20 g L ⁻¹)	0.04	1500 lux	24:0	11	1178	49.4	582	[72]
	molasses (5 g L ⁻¹)	3	70 μmol/m ² s	12:12	1.55	70	30	21	[114]
<i>C. vulgaris</i>	sucrose (0.5 g L ⁻¹)	10	4300 lux	NA	0.45	28.2	35.5	10	[115]
	molasses (1.44 g L ⁻¹)	0.04	65 μmol/m ² s	16:8	2.71	118	30	35.4	[74]
	wastewater + glycerol (2 g L ⁻¹)	0.04	100 μmol/m ² s	12:12	0.77	56	12.5	7	[116]
	effluent rich in VFA	0.04	100 μmol/m ² s	12:12	4.23	433	12.9	55.7	[117]
	glycerol (1 g L ⁻¹)	0.04	2500 lux	12:12	0.62	154.2	8.2	12.6	[64]
	glycerol (5 g L ⁻¹)	0.04	2500 lux	12:12	2.13	533.3	8.4	44.8	[64]
	glycerol (10 g L ⁻¹)	0.04	2500 lux	12:12	2.16	539.6	9.8	52.9	[64]
	wastewater + 6.1 mM glycerol	2.5	174 μmol/m ² s	12:12	1.09	54.3	21.7	11.8	[118]
	wastewater + 12.5 mM glycerol	2.5	174 μmol/m ² s	12:12	1.00	46.5	24.3	11.3	[118]
	wastewater + 25 mM glycerol	2.5	174 μmol/m ² s	12:12	1.80	107.7	13.4	14.4	[118]
wastewater + 50 mM glycerol	2.5	174 μmol/m ² s	12:12	1.92	118.5	15.7	18.6	[118]	
hydrolysed cheese whey (5 g L ⁻¹ glucose + 5 g L ⁻¹ galactose)	2	70 μmol/m ² s	24:0	3.58	750	33.7	253	[16]	

NA = Not available.

L⁻¹ glucose recalibrated the metabolism towards lipids and downstream metabolites, rather than carbohydrate accumulation [42]. This result clearly shows the importance of understanding the links between central carbon fluxes and synthesis of metabolites in microalgae.

The lipid content of *C. sorokiniana* mixotrophically cultivated on glucose improved significantly when compared to that obtained under photoautotrophic cultivation. The addition of 5 up to 15 g L⁻¹ glucose led to a substantial increase in the accumulation of lipids, but the lipid content declined at glucose concentrations higher than 20 g L⁻¹ [28]. Lipid production of *Chlorella* sp. was also compared under photoautotrophic, heterotrophic and mixotrophic cultivation [115]. Experimental results showed that, by adding 0.5 g L⁻¹ sucrose, lipid content (35.5%) and lipid productivity (0.01 g L⁻¹ d⁻¹) under mixotrophic conditions were higher than those in the heterotrophic and autotrophic cultures.

Lipid composition of some mixotrophic *Chlorella* species could also be affected when compared to that obtained under autotrophic cultivation. When glucose was supplemented as an organic carbon source for mixotrophic cultivation of *C. protothecoides*, microalgal cells accumulated storage neutral lipids rather than membrane polar lipids, while the composition of fatty acids changed from polyunsaturated to saturated and monosaturated, which favours the production of biodiesel [42]. An improvement of the neutral lipids content and higher concentrations of saturated (e.g., C16:0 and C18:0) and monosaturated fatty acids (e.g., C16:1 and C18:1), was also observed for mixotrophic cultivation of *C. pyrenoidosa* when acetate concentration was increased up to 10 g L⁻¹ [45].

7.2. Carbohydrates

Microalgae synthesize and accumulate a wide range of carbohydrates that serve two main purposes: they act as energy and organic carbon storage products inside microalgal cells and as structural components in cell walls [119]. Some *Chlorella* species are able to accumulate large amounts of carbohydrates (e.g., starch) which can be used as substrates for the production of biofuels such as bioethanol, biohydrogen and biobutanol through anaerobic fermentation or biogas through anaerobic digestion [120,121]. Several studies have confirmed that the productivity of microalgal carbohydrates can be improved in mixotrophic cultures as compared to photoautotrophic cultures. For instance, the mixotrophic cultivation of *C. sorokiniana* with 2 g L⁻¹ acetate resulted in higher carbohydrate content (47.4%) and carbohydrate productivity (377 mg L⁻¹ d⁻¹) than those obtained under photoautotrophic growth (35.2% and 221 mg L⁻¹ d⁻¹, respectively) [50]. Using acetate as organic carbon source in mixotrophic cultures of *C. vulgaris* also increased the productivity of microalgal carbohydrate (498.5 mg L⁻¹ d⁻¹) when compared with that achieved in photoautotrophy (365.8 mg L⁻¹ d⁻¹) [57]. Compared with photoautotrophic cultivation, the carbohydrate content of *C. pyrenoidosa* cultivated mixotrophically with 0.5–5.0 g L⁻¹ glucose was improved by 103.2–266.5% [20].

7.3. Photosynthetic pigments

All photosynthetic microorganisms accumulate organic pigments for

harvesting light energy. The major classes of pigments found in microalgae are: chlorophylls, carotenoids, and phycobilins. These pigments have received great attention since they exhibit several beneficial biological activities such as anti-carcinogenic, anti-inflammatory, antioxidant, anti-obesity, anti-angiogenic and neuroprotective [122].

The accumulation of photosynthetic pigments in microalgal cells cultivated under mixotrophic conditions depends on the relative influence of the photoautotrophic and heterotrophic metabolisms. At high cell densities, light becomes limiting and the photoautotrophic metabolic activity is reduced in comparison to that of heterotrophic mode. Under this condition, the cellular content of some photosynthetic products decreases significantly. In this sense, the mixotrophic cultivation of *C. sorokiniana* supplemented with acetate led to a decrease in the content of different pigments (e.g., chlorophyll, lutein, fucoxanthin and zeaxanthin, among others) compared to that achieved in the photoautotrophic culture [123]. It has been reported that the decline in chlorophyll concentration in mixotrophic cells reduces the dependence on light. Therefore, lower content of chlorophyll in microalgae may relieve photo-inhibition [124]. Moreover, since polar biomolecules such as chlorophyll interfere in the transesterification process [125], such reduction in chlorophyll content may also improve the efficiency of biodiesel production from lipid-rich microalgae.

Although primary pigments (e.g. chlorophylls and primary carotenoids) may be affected by mixotrophic culture conditions, it was also found that the accumulation of secondary carotenoids (including astaxanthin) in *C. zofingiensis* was favoured with increasing glucose concentrations, up to 30–40 g L⁻¹ [66].

8. Sustainability of mixotrophic cultivation of *Chlorella* vs autotrophic cultivation

Sustainability indicators have gained an increased importance as a tool to demonstrate the technological, economic, environmental and social impact of a process technology, being also a useful instrument for decision-making [126]. The evaluation of the sustainability of biofuel and high-value compound production systems from mixotrophic cultivation of *Chlorella* microalgae can be determined from an economic standpoint through Techno-Economic Analysis (TEA) as well as considering a set of suitable metrics via Life-Cycle Assessment (LCA). LCA is an effective methodology that allows to analyse (and compare) processes, services or products from an environmental perspective [127].

In this context, a study on sustainability indicators (e.g., Life-Cycle Energy Efficiency - LCEE) for the production of biodiesel by microalgae has suggested that the cultivation of *C. vulgaris* under mixotrophic conditions in raceway ponds with a glucose content of 1 g L⁻¹ and without salinity stress presents a beneficial net energy ratio when compared to its autotrophic cultivation [128]. Significant savings in the Fossil-Energy Requirements (FER) and Global Warming Potential (GWP) indicators were also found in a comparative LCA study on biodiesel production from *C. vulgaris* cultivated under autotrophic and mixotrophic conditions using a hybrid system which couples airlift tubular photobioreactors with raceway ponds, in a two-stage process. The mixotrophic growth of *C. vulgaris* with the addition of glucose as organic carbon source led to net savings in GWP and FER approaching 76% and 75%, respectively, relative to fossil derived diesel. The improvement in the environmental impact of this pure glucose-based microalgae cultivation system for biodiesel production was mainly attributed to the lower energy demands in biomass processing resulting from the highly dense cultures. The main bottlenecks identified included the energy requirement for cultivation and drying of the microalgae as well as embodied burdens associated with construction materials and nutrient supply [129]. A recent LCA study highlighted the environmental benefits obtained when *C. vulgaris* was cultivated using cheese wastewater as C source and digestate as N source in comparison to microalgae cultivated in autotrophic mode [130]. According to the authors, up to 60% of

CO₂ emissions were decreased by conducting microalgae growth on recovered carbon and nitrogen in comparison with the autotrophic cultivation.

Besides the environmental and social aspects, the development and commercialization of sustainable mixotrophic microalgal systems for biofuel production must take into account a detailed economic assessment. Although some studies have evaluated the economic viability of biofuel production from *Chlorella* strains cultivated in wastewater, comprehensive economic evaluations of the mixotrophic growth of microalgae compared to cultivations under autotrophic or heterotrophic mode at an industrial scale have not been reported so far.

A techno-economic study on the production of bio-oil, syngas, biochar and a phosphorus-rich co-product from *Chlorella* sp. grown on centrate from municipal wastewater demonstrated that the cost of microalgal bio-oil (US\$ 2.23/gallon) can be competitive to that of crude oil [131]. Moreover, it was reported that the co-product utilization as fertilizer had a crucial impact on the economic feasibility of the microalgal biofuel production system. In fact, diverting a fraction of biomass for production of high-value co-products offers a feasible path towards the economic parity between microalgal biofuels and other fuel options. On the other hand, the higher the capital costs of the cultivation system (e.g., increased investment in costly photobioreactors), the greater the amount of biomass that should be diverted to achieve economic parity, thus no longer making the microalgae cultivation system to function primarily as a platform for biofuel production [132].

These examples clearly show that although mixotrophic growth of *Chlorella* strains has potential to improve environmental and economic aspects of microalgal biofuel production, more research is needed to demonstrate the sustainability of this cultivation system. Specifically, TEA and LCA need to be performed using data from experimental mixotrophic cultivation trials including cheap organic carbon sources and cultivation systems that favours high biomass production at large scale. In addition, specific high-value co-products should be identified and valorised to achieve higher profits.

9. Conclusions

Cultivation of *Chlorella* species under mixotrophic conditions offers the possibility of greatly increasing microalgal biomass and accumulation of valuable intracellular metabolites but, to date, examples of successful facilities using this culture regime at commercial-scale are scarce. Challenges associated with utilization of cheap organic substrates and risk of culture contamination at an industrial level should be further addressed in order to increase the opportunities for scaling up mixotrophic culturing of *Chlorella*. Integration of microalgal biomass production with wastewater treatment will improve the economic feasibility of commercial scale cultivation of *Chlorella* species. Moreover, as the composition of wastewater is complex and diverse, the screening of novel mixotrophic species of *Chlorella* capable of both assimilating a wide range of organic compounds from waste streams and accumulating high content of molecules of interest (e.g., lipids) while withstanding other contaminants, is highly recommended.

Future trends of the biofuel and high-value compound production from *Chlorella* microalgae cultivated under mixotrophic conditions should address the sustainability issue in an integrative approach by evaluating the environmental, social and economic aspects of the process at a large scale.

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Credit author statement

Giuliano Dragone: Conceptualization, Investigation, Writing –

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Declaration of competing interest

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