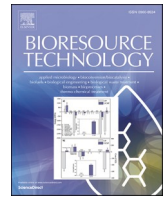




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Review

Microalgae-based carbohydrates: A green innovative source of bioenergy

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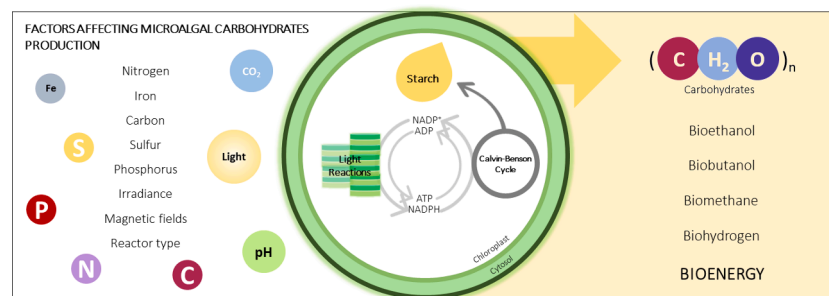
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HIGHLIGHTS

- Microalgae biomass is a promising source of third-generation of biofuels.
- Versatility of microalgae carbohydrates for biotechnological applications.
- Microalgal carbohydrate metabolism can be shifted to increase carbohydrate content.
- Integration of processes will lead microalgae-based carbohydrates to be feasible.

GRAPHICAL ABSTRACT



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ABSTRACT

Microalgae contribute significantly to the global carbon cycle through photosynthesis. Given their ability to efficiently convert solar energy and atmospheric carbon dioxide into chemical compounds, such as carbohydrates, and generate oxygen during the process, microalgae represent an excellent and feasible carbohydrate bioresource. Microalgae-based biofuels are technically viable and, delineate a green and innovative field of opportunity for bioenergy exploitation. Microalgal polysaccharides are one of the most versatile groups for biotechnological applications and its content can be increased by manipulating cultivation conditions. Microalgal carbohydrates can be used to produce a variety of biofuels, including bioethanol, biobutanol, biomethane, and biohydrogen. This review provides an overview of microalgal carbohydrates, focusing on their use as feedstock for biofuel production, highlighting the carbohydrate metabolism and approaches for their enhancement. Moreover, biofuels produced from microalgal carbohydrate are showed, in addition to a new bibliometric study of current literature on microalgal carbohydrates and their use.

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1. Introduction

In the current global scenario of rising energy demands and environmental degradation, sustainability has gained momentum and sparked the interest in biofuels as substitute and complementary bioenergy sources (Srivastava et al., 2020). According to the International Energy Agency (IEA), biofuel production is increasing worldwide, having reached a record level of 154 billion liters in 2018, with production volumes estimated to increase by 25% until 2024, surpassing 190 billion liters. However, to meet the goals proposed by the Sustainable Development Scenario (SDS), biofuel consumption in the transportation sector needs to almost triple by 2030 (International Energy Agency (IEA), 2020). For such an energy transition, it is essential to make use of renewables for the development and implementation of a less carbon-intensive energy system (Brennan and Owende, 2010).

Renewable carbon-fixing feedstocks, such as biomass, represent an effective strategy to fully develop and harness the potential of the bioenergy industry (Su et al., 2017). Algae and plant biomasses contribute significantly to the global carbon cycle by fixing carbon dioxide (CO₂) and producing oxygen (O₂) through photosynthesis (Silva et al., 2019). Biofuels produced from biomass show great potential as feasible and commercial alternatives for CO₂ mitigation (Ho et al., 2012).

Biofuels can be classified according to the origin of biomass (Vassilev and Vassileva, 2016). Several types of biomass can be used as feedstock to produce renewable fuels, including liquid (bioethanol, biobutanol, and biodiesel) and gaseous biofuels (biogas, biomethane, and biohydrogen) (Milano et al., 2016). Highly energetic conventional crops, such as sugarcane, soybean, corn, and sugar beet, are used to produce first-generation (1G) biofuels (Vassilev and Vassileva, 2016). The use of edible feedstocks, based mainly on sugar, starch, and oils, has generated much discussion regarding their impact on global food markets, food security, and land usage (Singh et al., 2018; Su et al., 2017). The drawbacks of 1G production stimulated the search for novel strategies, culminating in the development of second-generation (2G) biofuels, which are based on non-edible crops, mainly lignocellulosic biomass derived from industrial and agricultural residues (Lakatos et al., 2019; Su et al., 2017). However, cost-effectiveness and technological barriers related to pretreatment difficulties, biomass recalcitrance, and lignin removal still hinder the consolidation of large-scale 2G biofuel production (Brennan and Owende, 2010).

A potential solution to meet future energy demands lies in the use of microalgae and cyanobacteria (Vassilev and Vassileva, 2016). Microalgae-based biofuels have high technical viability, short harvest times, high biomass productivity relative to land area, low land usage (non-arable land), and high CO₂ uptake capacity, overcoming the difficulties of sustainable land usage, and technological efficiency associated with 1G and 2G production (Brennan and Owende, 2010; Vassilev and Vassileva, 2016). Biofuels derived from algal biomass, known as third-generation (3G) biofuels, have emerged as promising and attractive energy sources (Debnath et al., 2021).

Microalgae comprise a wide range of photosynthetic microorganisms that vary in shape, size, morphology, and habitat (Markou et al., 2012a). Applied phycology refers to eukaryotic microscopic algae (e.g., green algae, red algae, and diatoms) and oxygenic prokaryotic photosynthetic bacteria (e.g., cyanobacteria) as microalgae (Lakatos et al., 2019; Richmond, 2013). These green sources of bioenergy have simple growth requirements, mainly light, CO₂, nitrogen, and trace elements. Furthermore, microalgal cells exhibit high photosynthetic efficiency and ability to fix dissolved inorganic and gaseous carbon, resulting in fast growth rates (Costa and de Moraes, 2011; Salama et al., 2018).

Microalgae have attracted interest because of their versatility and zero emission balance: no additional CO₂ is formed for sequestration and transportation of atmospheric carbon into the metabolic cycle, yielding CO₂-neutral source of biofuels (Costa and de Moraes, 2011). Furthermore, they have robust adaptability and metabolism plasticity, being flexible genetic and environmental changes (Silva et al., 2018; Xiong

et al., 2017).

Microalgae have the ability to produce energetic compounds as storage molecules, such as lipids and polysaccharides that can be converted into a broad array of high-value bioproducts (Chew et al., 2017; Costa and de Moraes, 2011). These energetic compounds occur in different concentrations in biomass and may vary according to microalgal species and growth stage (González-Fernández and Ballesteros, 2012; Lakatos et al., 2019). Microalgal lipid content have been widely studied in biodiesel research (Ferreira et al., 2019). Nevertheless, other microalgal primary metabolite can also be used as substrate for biofuel generation, such as polysaccharides or whole-cell cultures. Lipids are the most energy-rich organic components, but carbohydrates are the most versatile fraction, being suitable for biotechnological conversion (Markou et al., 2012a). Microalgal polysaccharides have applications in the food, feed, and nutraceutical industry, and importantly as raw material for biofuel production and bioenergy generation (Costa et al., 2020; Silva et al., 2019)

In a biorefinery context, it is crucial to obtain maximum utilization of all microalgal macromolecules. The carbohydrate fraction has not yet been widely studied or explored for bioenergy and 3G biofuel production, even though it can be used to obtain several interesting compounds, such as bioethanol, biobutanol, biomethane, and biohydrogen, as Fig. 1 illustrates. This review provides an overview of microalgal carbohydrates, focusing on their use as bioenergy source for biofuel production, highlighting the carbohydrate metabolism and approaches for their enhancement, as well as the factors that influence polysaccharides accumulation, such as macro and micronutrients, culture conditions, reactor type, and operation mode. Moreover, biofuels produced from microalgal carbohydrate are discussed as possible compounds for bioenergy generation, in addition to a new bibliometric study of current literature on microalgal carbohydrates and their utilization.

2. Microalgal carbohydrates: Production and relevance

Carbohydrates are the main product of the photosynthetic pathway and have different physiological roles in microalgal development (Chen et al., 2013; Levasseur et al., 2020). Some species can increase their carbohydrate content and accumulate within plastids, a feature associated with the carbon fixation step in photosynthesis (Lam and Lee, 2015; Silva et al., 2019). The higher carbohydrate content, the higher the effectiveness and feasibility of their conversion to biofuels (Lakatos et al., 2019). Knowledge of microalgal metabolism and methods to alter biomass sugar profile and content is decisive for enhancing microalgae-based biofuel production (Chen et al., 2013; Radakovits et al., 2010).

2.1. Composition

Microalgae differ in carbohydrate quantity and composition (Greque De Moraes et al., 2016). Carbohydrates are found mainly as reserve polysaccharides within plastids and structural components, such as cell walls (Chen et al., 2013). The main reserve polysaccharide synthesized and stored by microalgae is starch, whereas cyanobacteria usually accumulate glycogen (Levasseur et al., 2020). The microalgal cell wall is composed of an inner layer (containing mainly cellulose) and an outer layer (containing pectin, agar, or alginate), nonetheless carbohydrate metabolism differs significantly between species (Chen et al., 2013).

Starch reserves serve for carbon and energy storage to support cell growth (Choix et al., 2014). Starch content in microalgae can be modulated through by manipulating growth conditions (Levasseur et al., 2020). Both starch and cellulose (found in the plastids and cell walls) can be converted into fermentable sugars, and the absence of lignin, found in plants, allows for fast and effective hydrolysis and sugar release, evidence of great potential of carbohydrate-rich microalgae as feedstocks for biofuel production (Silva et al., 2019).

Neutral sugar profile also depends on microalgal species, strain, and growth stage (González-Fernández and Ballesteros, 2012). The main

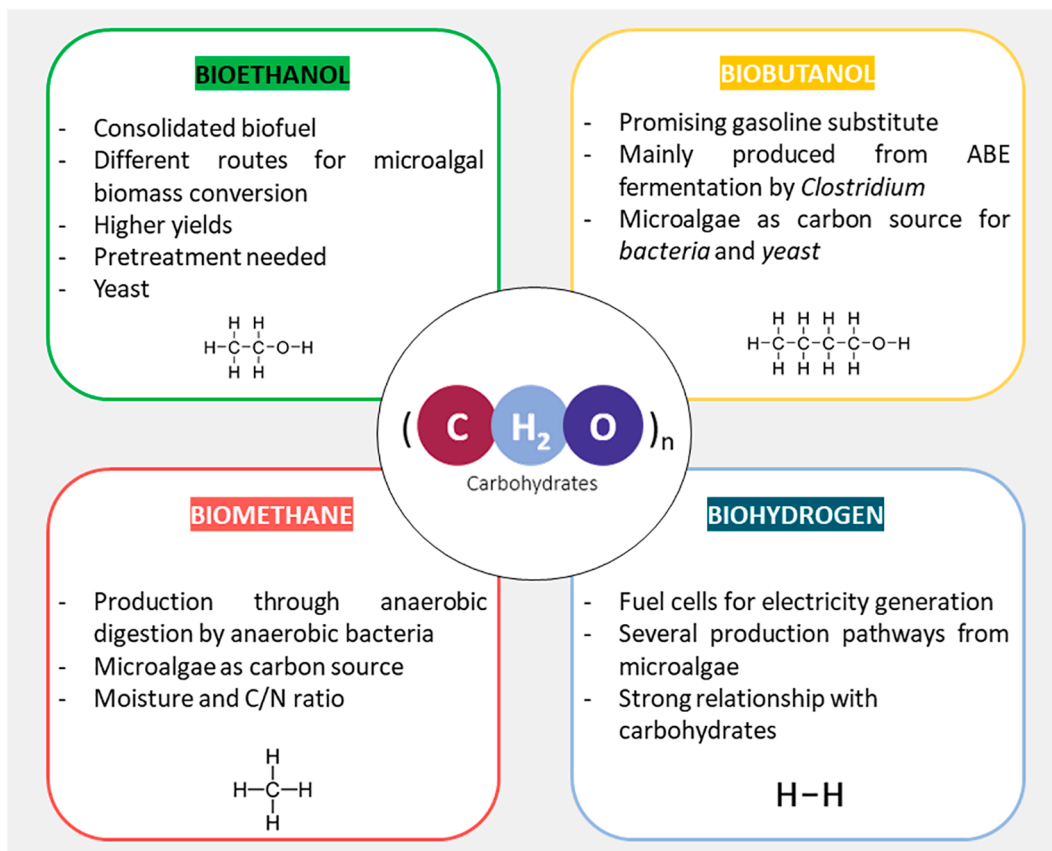


Fig 1. Potential pathways from microalgal carbohydrates to biofuels production for bioenergy generation.

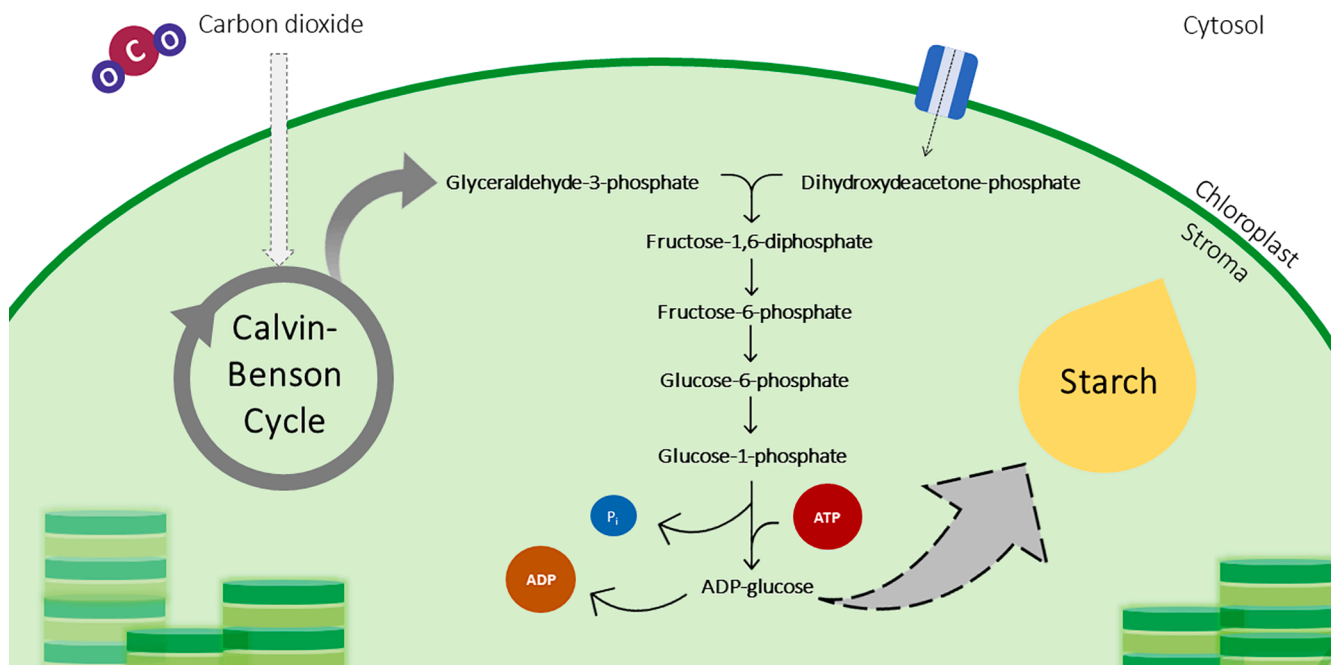


Fig 2. Metabolism of carbon assimilation and starch generation in microalgae.

monosaccharides are glucose, mannose, rhamnose, xylose, galactose, fucose, and arabinose (Harun and Danquah, 2011). Glucose is the major monosaccharide in *Chlorella vulgaris* biomass (Silva et al., 2018), *Chlorella* sp. KR1 (Lee et al., 2015), *Scenedesmus obliquus* (Miranda et al., 2012a; Silva et al., 2018), *Chlorella sorokiniana*, and *Scenedesmus almeriensis* (Hernández et al., 2015).

2.2. Metabolism

Carbohydrates are synthesized intracellularly by a series of complex reactions of photosynthesis (Lakatos et al., 2019; Levasseur et al., 2020). The photosynthetic pathway is the biochemical process that converts sunlight into energy-rich molecules, taking place in chloroplasts of photosynthetic eukaryotic microorganisms (Masojídek et al., 2013; Milano et al., 2016). Oxygenic photosynthesis is conventionally divided into light and dark reactions. In the first stage, light energy is harvested by pigment molecules (such as chlorophyll, carotenoids, and phycobilin) found in the membrane of thylakoids stored in chloroplasts. The excited electrons absorbed are carried through a series of acceptors in the electron transport chain, during which a water molecule is split into protons, electrons, and oxygen (released into the atmosphere). Protons are pumped across the thylakoid membrane, generating energy in the form of ATP and NADPH biochemical reductant power. These energy carriers are indispensable to meeting metabolic needs and are used in subsequent dark reactions, by which atmospheric CO₂ is reduced to carbohydrates. Carbon assimilation takes place in stroma (outside the thylakoid membrane), and sugar biosynthesis (or that of other compounds) occurs via a reaction mechanism called the Calvin–Benson cycle (Deviram et al., 2020; Markou et al., 2012a; Masojídek et al., 2013), as illustrated in Fig. 2.

The Calvin–Benson cycle has three basic phases toward carbohydrate production: fixation, reduction, and regeneration. In the first stage, CO₂ is added to a five-carbon sugar (ribulose 1,5-bisphosphate) in a reaction catalyzed by ribulose 1,5-bisphosphate carboxylase oxygenase (RuBisCo), forming two molecules of a three-carbon compound (phosphoglycerate). Then, phosphoglycerate molecules are converted to glyceraldehyde-3-phosphate (triose-P) at the expense of ATP and NADPH (Ran et al., 2019). One of the glyceraldehyde-3-phosphate molecules is used as substrate for carbohydrate formation and the other is maintained in the cycle, whereas ribulose phosphate is subjected to a series of reactions involving sugar combinations, being regenerated for further CO₂ fixation (Masojídek et al., 2013). Carbon assimilation reactions and carbohydrate biosynthesis occur inside chloroplasts, but prokaryotes synthesize carbohydrates in the cytosol (Markou et al., 2012a).

The fraction of glyceraldehyde 3-phosphate that is not used immediately as an energy source is converted into starch in chloroplast stroma. Starch is a high molecular weight D-glucose polymer linked by α -1,4 glycosidic bonds; it is synthesized and temporarily stored in chloroplasts as insoluble granules (Masojídek et al., 2013). Glyceraldehyde-3-phosphate condenses with dihydroxyacetone phosphate to form fructose 1,6-bisphosphate, a starch precursor. By a series of reactions Glucose 1-phosphate is produced as the starting material for starch synthesis (Chen et al., 2013; Nelson and Cox, 2004).

Starch biosynthesis requires three steps: glucose activation, chain elongation, and chain branching. The glucose activation mechanism is the most important, as it is converted to nucleoside-diphosphate-glucose (ADP-glucose), which acts as the glycosyl donor responsible for starch chain elongation. The enzyme ADP-glucose pyrophosphorylase catalyzes the formation of ADP-glucose by condensation of glucose 1-phosphate with ATP (Choix et al., 2014; Ran et al., 2019). Starch synthase then transfers glucose residues from ADP-glucose to preexisting starch molecules (Fig. 2) (Nelson and Cox 2004; Chen et al. 2013).

ADP-glucose synthesis is the limiting step, directly affecting starch formation (Chen et al., 2013; Nelson and Cox, 2004). One of the investigated strategies for promoting starch accumulation in microalgae

is the regulation of ADP-glucose pyrophosphorylase (Choix et al., 2014; Radakovits et al., 2010). Omics studies focused on microalgae are essential for integrating knowledge about carbohydrate accumulation metabolism and its regulation on a molecular level, paving the way for biofuel production (Chen et al., 2013; Choix et al., 2014; Nagappan et al., 2020; Radakovits et al., 2012). Likewise, cultivation techniques, operation mode, nutrients, and reactor types are crucial for increasing microalga carbohydrate content and will be further discussed.

3. Factors affecting microalgal carbohydrate production

Biochemical composition of microalga biomass is determined by different factors, such as microalgal species, light intensity, agitation, pH, temperature, nutrient composition, and CO₂ concentration (González-Fernández and Ballesteros, 2012). Macronutrient (nitrogen, phosphorus, sulfur, potassium, and carbon) and micronutrient (iron and manganese) concentrations influence photosynthesis, altering carbon fixation and allocation, microalgal metabolism, and biomolecule accumulation, (Khan et al., 2018; Solís-Salinas et al., 2021).

3.1. Macronutrients

3.1.1. Nitrogen

Nitrogen is a fundamental component of primary metabolism and an essential nutrient for microalgal growth (Chen et al., 2013). According to González-Fernández and Muñoz (2017), nitrogen is required for the synthesis of biomolecules, such as proteins, DNA, and pigments. Low nitrogen concentrations shift the photosynthetic metabolism of proteins toward carbohydrate and lipid accumulation.

Freitas et al. (2017) cultivated *Chlorella minutissima* in a raceway photobioreactor in BMM medium with 50% reduction in nitrogen source concentration, addition of pentose, and 33.75 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, affording a carbohydrate content of 60.3%. Stress environments associated with nitrogen limitation likely altered biomass biochemical composition, stimulating carbohydrate accumulation (Chen et al., 2013; González-Fernández and Ballesteros, 2012; Solís-Salinas et al., 2021).

Braga et al. (2018) obtained biomass with 49.3% carbohydrate by cultivating *Spirulina* sp. LEB 18 in Zarrouk medium with 90% reduction in nitrogen source (2.25 g L⁻¹ NaNO₃) and addition of CO₂ (0.3 vvm for 5 min) and NaHCO₃. Ho et al. (2012) demonstrated that cultivation of *S. obliquus* CNW-N with 10% nitrogen limitation in Detmer's medium afforded a carbohydrate content of 46.7%.

3.1.2. Organic carbon

The effectiveness of varying carbon sources on carbohydrate accumulation depend on microalgal species and culture conditions (Maia et al., 2020). According to Borowitzka et al. (2016), carbohydrate storage represents one of the main carbon reserves in chlorophytes. Sugars (monosaccharides and disaccharides) are the most common organic carbon sources, being efficiently consumed for carbohydrate accumulation in cells (Posten and Chen, 2016).

The combination of organic carbon source and nitrogen reduction in culture media proved to be beneficial for carbohydrate accumulation. Costa et al. (2019) argued that the choice of culture medium is essential not only for biomolecule accumulation but also for cost-effectiveness; nutrients are responsible for 15% to 25% of total production costs. (de Freitas et al., 2019) found that pentose addition (5%) and 50% nitrogen reduction in *C. minutissima* culture resulted in 58.6% carbohydrate in biomass.

Salla et al. (2016) reported an increase in the carbohydrate content of *Spirulina* sp. LEB 52 cultivated in Zarrouk medium (diluted to 20%) added with 25% whey protein residue: a carbohydrate productivity of 60 mg L⁻¹ day⁻¹ was achieved. In the study of Margarites et al. (2017), a carbohydrate yield of 54% was achieved by cultivating *Chlorella homospaera* in Bristol's medium supplemented with glucose, 50% less nitrogen, and 20% more NaCl; under these culture conditions, the

carbohydrate content increased by 20% by comparison with the control.

3.1.3. Phosphorus

Phosphorus is essential for metabolic processes, its limitation in culture medium is an effective way to stimulate microalgal metabolism and induce the synthesis of the biomolecule of interest, such as carbohydrate accumulation, in response to intracellular stress (Li et al., 2018; Ran et al., 2019). As highlighted by Posten and Chen (2016), the source of phosphorus influences the production of nucleic acids, cell membranes, and ATP.

Juneja et al. (2013) reported that cultivating microalgae in nitrogen- and phosphorus-deficient medium leads to a reduction in protein and pigment (chlorophyll *a*) levels and an increase in carbohydrate content. Markou et al. (2012a) demonstrated that reduction of phosphorus concentration (K_2HPO_4) and variations in light intensity influenced *Arthrospira platensis* SAG-2.99 cultivation in Zarrouk medium. A carbohydrate content of 59.6% was obtained by using 10 mg L^{-1} phosphorus (98% K_2HPO_4 reduction), regardless of light intensity. The same microalga was cultivated in batch and semi-continuous processes with different phosphorus concentrations ($10\text{--}50\text{ mg L}^{-1}$). In batch cultures, carbohydrate content increased by 83.5% and 80.7% in culture media containing 10 and 20 mg L^{-1} phosphorus, respectively, compared with the control (500 mg L^{-1} phosphorus) (Markou et al., 2012b).

3.2. Micronutrients

3.2.1. Iron and sulfur

Microalgal growth and biochemical composition are also affected by micronutrient concentration. Iron is needed for the growth of all phytoplankton, performing essential metabolic functions in the photosynthetic system (Dragone et al., 2011). He et al. (2010) increased carbohydrate content of *Alexandrium tamarense* ATHK01 biomass at 15 days of cultivation by 3-fold by supplementing culture medium with iron. Rizwan et al. (2017) investigated the effects of different iron components (ferric chloride, ferrous chloride, ferric EDTA, ferrous EDTA, ferric ammonium sulfate, and ferrous ammonium sulfate) on *Dunaliella tertiolecta* cell growth and carbohydrate content. Of the six iron sources, ferrous ammonium sulfate was the most effective in enhancing carbohydrate accumulation (55%) in 48 h of cultivation.

Reductions in nutrient concentrations generate stress in microalgae, altering the metabolic strategy. Thus, microorganisms synthesize metabolites to increase survival (Richmond, 2013). Brányiková et al. (2011) cultivated *C. vulgaris* CCALA 924 in mineral medium with limited sulfur, nitrogen, and phosphorus, affording biomasses with carbohydrate contents of 60%, 38%, and 55%, respectively. The carbohydrate content of *Tetraselmis subcordiformis* biomass was increased by 54% with the use of Walne medium under nitrogen starvation and 62.1% sulfur reduction (Yao et al., 2012). Overall, studies have shown that different microalgal species increase carbohydrate or starch accumulation when grown under stress conditions with nutrient limitation (Table 1).

Table 1

Carbohydrate and starch contents of different microalgal species.

Microalgal species	Starchcontent (%)	Carbohydrate content (%)	Cultivation medium	Reducednutrients	Reference
<i>Chlorella pyrenoidosa</i> CCAP 211/8D	–	41	Watanbe	Iron and nitrogen	(Illman et al., 2000)
<i>Chlorella vulgaris</i> CCAP 211/11B	–	55	Watanbe	Iron and nitrogen	(Illman et al., 2000)
<i>Chlorella vulgaris</i> P12	41	–	OGM	Iron and nitrogen	(Dragone et al., 2011)
<i>Chlorella zofingiensis</i>	66.7	66.9	BG-11	Nitrogen	(Zhu et al., 2014)
<i>Neochloris aquatica</i> CL-M1	–	50.5	BG-11	Phosphorus and nitrogen	(Wang et al., 2017)
<i>Chlorella sorokiniana</i> SLA-04	–	20–23	BG-11	Calcium and nitrogen	(Hanifzadeh et al., 2018)
<i>Tribonema minus</i>	–	26.6	BG-11	Phosphorus and nitrogen	(Wang et al., 2019)
<i>Monoraphidium</i> QLZ-3	–	19.1	Walnut shell extracts	Phosphorus and nitrogen	(Dong et al., 2019)
<i>Scenedesmus obliquus</i> BR003	–	62.5	L4-m	Nitrogen	(Amorim et al., 2020)
<i>Chlorococcum humicola</i>	60	–	Chu 10	Sulphur and phosphorus	(Narchonai et al., 2020)

3.3. Culture conditions

3.3.1. Influence of CO₂ and pH

Carbohydrate production from microalgae may contribute to minimizing greenhouse gas emissions, given that atmospheric CO₂ can be used as a source of inorganic carbon in the photosynthetic process (Khan et al., 2018; Maia et al., 2020). A variety of microalgal strains can absorb high concentrations of atmospheric CO₂ and other components from flue gas and entrap these gases in cellular structures to produce biomolecules (Duarte et al., 2017). Modification of inorganic carbon concentrations in microalgal culture media was shown to increase the efficiency of carbohydrate production (Moraes et al., 2016). Inorganic carbon is essential for the control of culture medium pH, influencing microalgal growth by altering carbon availability, nutrient absorption, and biomolecule synthesis (Li et al., 2020). According to Khan et al. (2018), pH is an important factor influencing microalgal growth; most species tend to grow well in the pH range of 6 to 8.7.

In a study by Moraes et al. (2016), *Spirulina* sp. LEB 18 efficiently accumulated carbohydrates when cultivated in Zarrouk medium added with monoethanolamine and CO₂. An increase of 26% in carbohydrate content under a high CO₂ flow rate (0.3 vvm) was observed. Braga et al. (2019) assessed the effects of injecting CO₂ at 0.3 vvm for 1 or 5 min combined with thermoelectric fly ashes (0, 120, and 160 ppm) and a 10% reduction in nitrogen concentration in carbohydrate accumulation in *Spirulina* sp. LEB 18. Addition of thermoelectric fly ashes at 120 and 160 ppm and CO₂ injection for 1 min resulted in the highest carbohydrate contents, 63.3% and 61%, respectively.

3.3.2. Irradiance

Carbohydrate accumulation in microalgae is related to their photosynthetic system, which is divided into light and dark phases. Irradiance is an important factor in microalgal cultivation, acting directly on the photosynthetic apparatus, influencing growth and carbohydrate production (González-Fernández and Ballesteros, 2012; Maia et al., 2020). The effect of irradiance on microalgal cultures can be classified into three categories: light limitation, light saturation, and light inhibition. These effects differ according to species (Ho et al., 2012). Although microalgal growth increases as a function of light intensity, varying with temperature and species, the growth rate is maximum at light saturation (Juneja et al., 2013). Irradiance may affect medium pH, inducing changes in stromal NADPH concentrations, possibly influencing the activity of a key enzyme (phosphoglucomutase) involved in the photosynthetic pathway and starch synthesis. Therefore, variation in light intensity during cultivation may regulate carbohydrate synthesis (Table 2) (Levasseur et al., 2020).

Gifuni et al. (2017) evaluated carbohydrate and starch contents of different *Chlamydomonas* species cultivated in bold basal medium under an irradiance of $220\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$. Two species exhibited excellent results, *Chlamydomonas moewusii* and *Chlamydomonas oblonga*, with total carbohydrate contents of 72.8% and 72.6% and starch contents (DM basis) of 44.6% and 37.7%, respectively, after 7 days of cultivation. Some authors, such as Gifuni et al. (2018) and Janssen (2016), reported

Table 2
Physical stress factors used for carbohydrate and starch production by microalgae.

Microalgal species	Physical stress factor	Condition	Carbohydrate content (%)	Cultivation medium	Reference
<i>Chlorella ellipsoidea</i>	pH	6	69.2	MBL	(Khalil et al., 2010)
<i>Scenedesmus sp</i>	pH	8	61.2	Wastewater	(Posadas et al., 2015)
<i>Scenedesmus obliquus UTEX 393</i>	pH	6.7	55.4	Bold 3 N	(Singh et al., 2019)
<i>Scenedesmus obliquus CNW-N</i>	Irradiance	420 $\mu\text{mol m}^{-2} \text{s}^{-1}$	38	DM	(Ho et al., 2012)
<i>Chlorella vulgaris</i>	Irradiance	150 $\mu\text{mol m}^{-2} \text{s}^{-1}$	59	LC Oligo	(Chia et al., 2015)
<i>Chlamydomonas moewusii</i>	Irradiance	220 $\mu\text{mol m}^{-2} \text{s}^{-1}$	72.8	BBM	(Gifuni et al., 2017)
<i>Chlamydomonas QWY37</i>	Irradiance	750 $\mu\text{mol m}^{-2} \text{s}^{-1}$	63	Wastewater	(Qu et al., 2020)
<i>Chlamydomonas reinhardtii</i>	Temperature	23 °C	52.3	Mineral medium	(González-Fernández et al., 2016)
<i>Parachlorella kessleri QWY28</i>	Temperature	30 °C	43	BG-11	(Qu et al., 2019)
<i>Tribonema sp</i>	Temperature	25 °C	38	BG-11	(Huo et al., 2020)
<i>Micractinium IC-44</i>	Temperature	28 °C	38	BBM	(Sorokina et al., 2020)
<i>Spirulina sp</i>	Magnetic field	30 mT	30.3	Zarrouk	(Deamici et al., 2016)
<i>Chlorella minutissima</i>	Magnetic field	30 mT	60.5	MBM	(Menestrino et al., 2020)
<i>Chlorella fusca</i> LEB 111	Magnetic field	25 mT	34.2	BG-11	(Deamici et al., 2021)

that 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ is the theoretical maximum irradiance for carbohydrate accumulation, given that intensities higher than 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ can impair the accumulation of storage molecules, as light energy is directed toward protein synthesis and cell growth.

According to González-Fernández and Ballesteros (2012) and Maia et al. (2020), an increase in irradiance levels may cause photoinhibition and inhibit microalgal growth. As a result, triacylglycerides may accumulate. These molecules act as electron dissipators in culture media, reducing carbon availability for carbohydrate and protein synthesis.

3.3.3. Temperature

In bioprocesses, temperature is an important factor for microorganism cultivation. In microalgal cultures, the higher the temperature, the higher the growth rate, until a limit is reached, after which cells can be injured by oxidative damage (González-Fernández et al., 2016). Studies showed that the optimum temperature for microalgal growth ranges from 18 to 35 °C, depending on the species (Bernard and Rémond, 2012; Debnath et al., 2021). Temperature variations play an important role in carbohydrate synthesis, as they influence enzymes responsible for carbohydrate accumulation, such as starch synthase and sucrose synthase (Ho et al., 2014).

Qu et al. (2019) optimized carbohydrate production by *Parachlorella kessleri* QWY28 grown in BG-11 medium at different temperatures (20–35 °C). Carbohydrate production enhanced at 30 °C (43%), being considered the ideal temperature for carbohydrate accumulation for this microalgae (Table 2). Huo et al. (2020) assessed different temperatures (25–35 °C) for the cultivation of the filamentous microalga *Tribonema sp.* in BG-11 medium. Cultures at 35 °C suffered oxidative damage, inhibiting cell growth. Maximum carbohydrate accumulation (43%) was achieved in 9 days at 25 °C.

As described by Bernard and Rémond (2012), cultivation of microalgae below the ideal growth temperature may result in limited carbohydrate yields. On the other hand, overheating the culture may lead to cell death. As highlighted by Daneshvar et al. (2021) and Ho et al. (2013a), there is no consensus on the ideal temperature for microalgal cultivation. Thus, strategies that vary temperature conditions to achieve maximum carbohydrate production should be investigated.

3.3.4. Magnetic fields

The effect of magnetic fields (MF) on microalgal cultures has been studied since the last decade. It is known that the response to different MF intensities and exposure times differs according to microalgal species (Santos et al., 2017). When MF is applied to a biological system, the effect may be null, negative, or positive, possibly involving changes to metabolism, growth, and cell composition in response to stress caused by the magnetic effect (Huo et al., 2020). MF application is a non-toxic, low-cost alternative to increase cell growth and production of compounds of interest in bioprocesses, depending on the time and method of application (Abbas et al., 2021).

Menestrino et al. (2020) cultivated *C. minutissima* in Bristol's modified medium for 12 days for carbohydrate production. The interaction of three variables (reduction of nitrogen content, pentose addition, and MF application) was assessed, and the highest carbohydrate content (60.5%) was achieved in response to stress caused by the permanent MF applied during cultivation (Table 2). Deamici et al. (2016b) evaluated the biological effects of different MF intensities (30 and 60 mT) and application times (24 h day⁻¹ and 1 h day⁻¹) on *Spirulina sp.* cultivation. Use of a permanent MF of 30 mT for 15 days afforded a maximum carbohydrate content of 30.3%, 133.2% higher than the control (without MF application).

Microalgae are formed by a complex biochemical system. MF may alter the activities of free radicals, proteins, and enzymes as a cell defense mechanism against this stress factor (Santos et al., 2017; Zieliński et al., 2021). The response of microalgae to MF application needs to be explored, as the effects on microorganism cultivation are not linear and need to be understood and clarified at a molecular level (Menestrino et al., 2020).

In general, the production of biomolecules of interest by microalgae is not related to a specific factor, rather to a combination of various factors, as described above. Thus, to obtain high carbohydrate productivity in microalgae cultivation, factors such as strain and optimal growth conditions should be considered first, followed by technological approaches, including nutrient starvation, irradiance, and MF applications.

3.4. Operation mode

Different cultivation strategies, including different modes of operation and manipulation of nutrient availability in culture media, can be used to achieve high production of biomass and other compounds of interest. The most common operation modes are batch, semi-continuous, continuous, and two-stage (Brennan and Owende, 2010). The use of these operations can impact and favor cell growth, CO₂ fixation capacity, and nutrient absorption (Ho et al., 2013a).

da Rosa et al. (2015) produced macromolecules by *Spirulina sp.* LEB 18 in semi-continuous process with nutrient recycling and chemical absorbent addition, obtaining enhanced carbohydrate accumulation by comparison with control. Qu et al. (2020) achieved a carbohydrate productivity of 944 mg L⁻¹ day⁻¹ with *Chlamydomonas sp.* QWY37 cultivated in swine wastewater under semi-continuous conditions. Ho et al. (2013a,b) cultivated *S. obliquus* CNW-N in batch, semi-continuous, and continuous processes, using CO₂ as the carbon source and nitrogen starvation. Semi-continuous and continuous modes contributed to the increase CO₂ fixation rate (1988.6 mg L⁻¹ day⁻¹) and carbohydrate productivity (467.6 mg L⁻¹ day⁻¹).

Two-stage cultivation operation mode it is a scalable approach, even though requires high biomass levels for the transition from the first to the second stage. To overcome this issue, Nayak et al. (2020) optimized

a continuous two-stage culture of *Chlorella* sp. HS2 in photobioreactors, with nitrogen depletion in the first stage and phosphorous supplementation at the beginning of the second stage. This strategy resulted in a 3.8-fold increase in biomass yield and a 5.5-fold increase in carbohydrate productivity. [Zhu et al. \(2014\)](#) also performed two-stage cultivation to increase carbohydrate content and starch accumulation in *Chlorella zofingiensis*, with nitrogen depletion in the second stage. Biomass, carbohydrate, and starch productivities increased ($699 \text{ mg L}^{-1} \text{ day}^{-1}$, $407 \text{ mg L}^{-1} \text{ day}^{-1}$, and $268 \text{ mg L}^{-1} \text{ day}^{-1}$, respectively) after 5 days of cultivation. Thus, the continuous two stage cultivation coupled with nutrient depletion strategy is a promising alternative to increase biomass productivity on a large scale.

Variations in operation mode can be carried out together with changes in culture systems, categorized into autotrophic, heterotrophic, or mixotrophic ([Perez-Garcia et al., 2011](#); [Zhou et al., 2020](#)). These strategies in combination are used to optimize bioprocesses, resulting in increased concentration of components of interest for biofuel production, mainly microalgae carbohydrates. Autotrophic cultivations use light energy and inorganic carbon as the main carbon source and therefore yield and growth rate depends on photosynthetic rate. This type of culture can prevent contamination by bacteria and fungi because of the absence or low content of organic carbon in culture media ([Zhou et al., 2020](#)).

Heterotrophic cultivation uses organic compounds as the sole carbon source in the absence of light energy. Microalgae can assimilate organic carbon molecules, such as glucose ([Morais et al., 2021](#)), glycerol ([Hu et al., 2020](#)), acetate ([Li et al., 2020](#)), and other carbon sources for biomass synthesis ([Zhou et al., 2020](#)). Generally, heterotrophic cultivation tends to be cheaper than autotrophic cultivation and is easier to maintain on a large scale, however, few microalgal species can grow under fully heterotrophic conditions ([Perez-Garcia et al., 2011](#)). Moreover, excess organic carbon may inhibit cell growth and the lack of light may result in the non-production of some metabolic intermediates, hindering the use of this cultivation mode.

Mixotrophic cultivation is an interesting strategy for achieving high biomass production, growth rate, productivity and secondary metabolite synthesis via photosynthesis ([Li et al., 2018](#); [Verma et al., 2020](#)). Compared with other cultivation methods, mixotrophic culture can be applied to a wide variety of species and carried out in different facilities; furthermore, luminosity limitations can be overcome ([Perez-Garcia et al., 2011](#)). Autotrophic, heterotrophic, and mixotrophic systems were used for the cultivation of *A. platensis*, *Nannochloropsis* sp., and *Spirulina* sp. NCIM5143. The effects of culture medium composition (carbon, nitrogen, and vitamin concentrations) were investigated using an experimental design. The optimization of these variables increased biomass concentration under mixotrophic conditions by comparison with other cultivation modes and the control ([Verma et al., 2020](#)).

3.5. Reactor type

Microalgae can be cultivated indifferent types of reactors for enhanced productivity and, consequently, macromolecule concentration, aiming to increase the production scale. Several systems are used for cultivation, being classified as open (raceway ponds) or closed (tubular photobioreactors) ([Zhou et al., 2020](#)).

Open culture systems consist of oval-shaped channels with closed circuits and paddlewheels that allow continuous circulation and mixing of culture media while avoiding sedimentation. Raceway ponds operate under natural conditions, are widely used for large-scale production, and are more accessible in terms of construction and maintenance costs compared with photobioreactors. They are generally used for microalgae-based biofuel production (bioethanol, biodiesel, and biomethane) but are not suitable for pharmaceutical or food purposes, because of the greater risk of contamination and water loss by evaporation ([Chisti, 2007](#); [Ugwu et al., 2008](#)). Nonetheless, these systems allow for less control of growth conditions, temperature, and gas-liquid

mass transfer rates. Despite that, raceways are widely used for large-scale microalgae cultivation because of their low cost. [Ashokkumar et al. \(2019\)](#) cultivated *Synechocystis* sp. in an open raceway pond in semi-continuous mode for 40 days with municipal wastewater as culture medium for bioethanol production from extracted lipid residues (high carbohydrate content). Biomass and bioethanol yields were 2.2 g L^{-1} and 0.186 g g^{-1} , respectively. Production costs have been estimated at US\$2–3 per kilogram of biomass, suggesting potential for low-cost, integrated biofuel production from wastewater, in line with the bio-refinery concept.

Closed photobioreactors have been designed to overcome the problems related to open ones. These are closed systems in which the media circulates through a transparent array of tubes or plates from a central reservoir, allowing light to pass through. Thus, closed photobioreactors allow greater process control, minimize water evaporation and contamination risks, favor CO_2 and O_2 mass transfer, and can be designed for small areas with low solar irradiance. Various studies have shown that photobioreactors are more efficient in terms of microalgal yield by comparison with open systems because of the greater control over operating conditions. However, such systems tend to be more expensive than open raceway ponds ([Gupta et al., 2015](#); [Miranda et al., 2012b](#)).

Stirred tanks and vertical or horizontal tubular reactors are the main types of photobioreactors. They can be used with sunlight or artificial light sources. Vertical (airlift, bubble column, and flat plate) and horizontal tubular photobioreactors are more common in microalgae cultivation because of their high surface area, allowing cells to capture more light energy. Horizontal photobioreactors are the most common for large-scale processes because of the variability in configurations and arrangements ([Zhou et al., 2020](#)). Despite being scalable, these systems require temperature control and gas mixing; fouling can occur in internal pipeline surfaces, generating high operational costs ([Miranda et al., 2012b](#)).

Pseudoneochloris marina was cultivated in an airlift photobioreactor and a carbohydrate concentration of 53.77% was achieved under a light intensity of $364 \mu\text{mol m}^{-2} \text{ s}^{-1}$ at 36°C , demonstrating the potential of this microalga for large-scale biofuel production ([Gonçalves et al., 2019](#)). [Miranda et al. \(2012b\)](#) designed a vertical tubular photobioreactor system for biomass and sugar production by *S. obliquus*. A bubble column with continuous artificial illumination and a closed-loop reactor with natural light/dark cycles were evaluated; the reactors afforded an overall sugar production of 0.081 and $0.153 \text{ g eq}_{\text{glu}} \text{ L}^{-1}$, respectively. Such an increase in sugar concentration with the use of the closed photobioreactor under light/dark cycles was attributed to nitrate depletion after 22 days of cultivation.

4. Biochemical conversion of microalgae-based carbohydrates for biofuels

Carbohydrates from microalgae find many applications in biofuel production for bioenergy exploitation, but that of bioethanol is the most promising. [Fig. 3](#) shows a bibliometric map of terms that appear in the literature associated with microalga carbohydrates and a network of the top author keywords that appear five times or more. Other parameters of analysis are showed in the [Supplementary material](#) (see [supplementary material](#)). As expected, lipids, fatty acids, and biodiesel are terms associated with microalgal carbohydrates, given that biodiesel production from microalgae is widely explored because of the advantages these microorganisms offer over other feedstocks. According to biorefinery concept, microalgae-based biofuel production is promising, as it is possible to extract lipids from microalgae and use the remaining carbohydrate fraction.

[Fig. 3](#) also depicts that this line of research has gained attention over the years. Studies have focused on the biorefinery concept and microalgae area used for wastewater treatment followed by fraction exploitation. The goal has changed over time, from understanding the factors

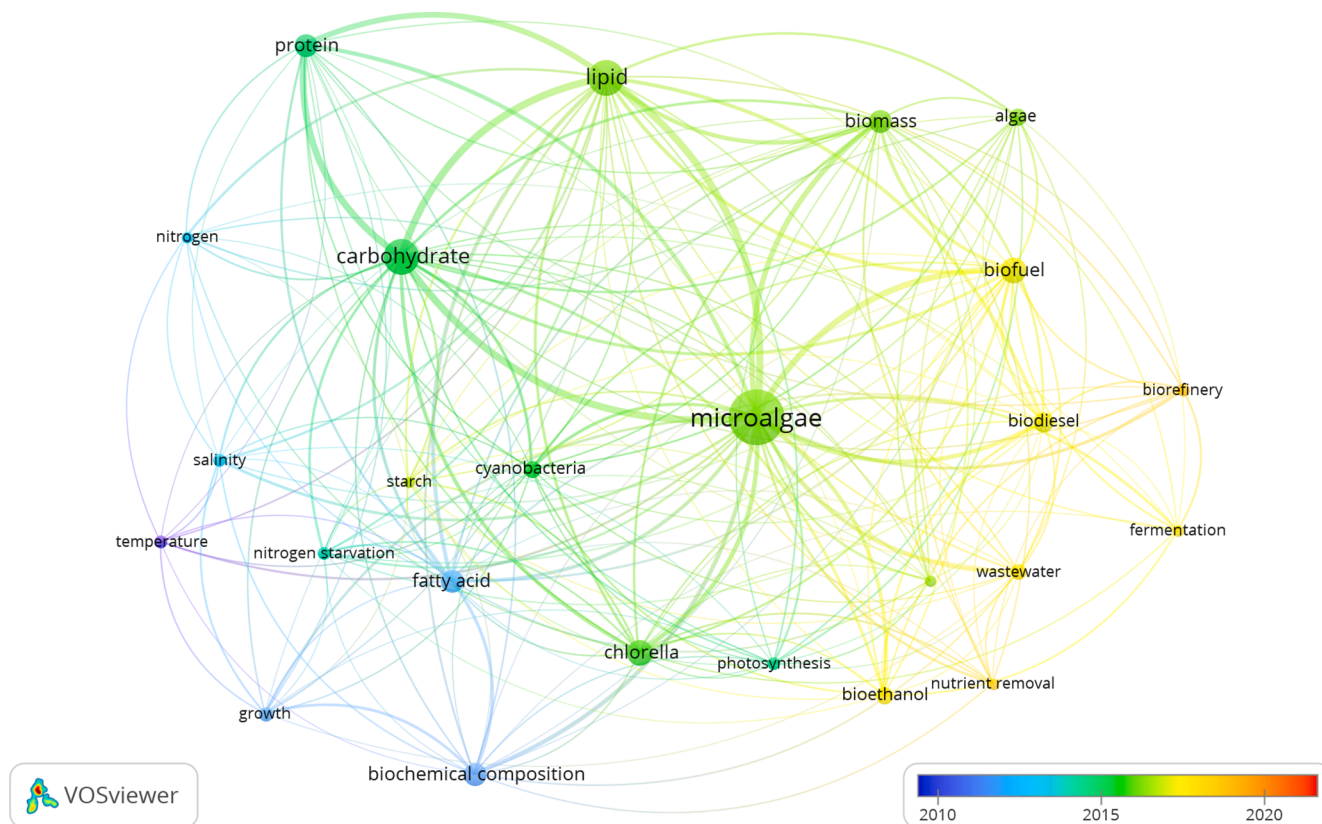


Fig. 3. Network overlay visualization of the top 25 most frequently used author keywords. Keyword size is proportional to frequency of occurrence, and link thickness is proportional to how often keywords appear together. Article and review papers were retrieved from a Web of Science Core Collection database search for “microalgae” and “carbohydrate” from 1991 to 2021 in the topic field (which includes title, abstract, author keywords, and keywords plus). A total of 1193 results were processed using VOSviewer® software, and 25 keywords with a frequency equal or superior to 5 are exhibited.

influencing carbohydrate accumulation and cell growth (salinity, temperature, nitrogen starvation) toward optimization biofuel production, such as bioethanol by fermentation. *Chlorella* is the most widely used microalgal genus, although a wide range of microorganisms have been investigated and even combined to achieve high carbohydrate yields.

It is important to highlight that the terms enzymatic treatment and hydrolysis were not among the top keywords, showing a gap in this field and molecular level studies. Furthermore, several biofuel applications for microalgal carbohydrates have not been presented (e.g., methane, biobutanol, and biohydrogen production) as well as their application in food and nutraceuticals industry, representing opportunities for research.

4.1. Bioethanol

Currently, bioethanol is the most widely used biofuel worldwide (Lakatos et al., 2019). Production volumes are increasing because of the high demand of the transportation sector (Srivastava et al., 2020). Recently, 3G bioethanol has gained attention because of the ability of microalgae to accumulate high carbohydrate concentrations under stress conditions (Brar et al., 2021). As shown in Fig. 3, only recently has 3G bioethanol gained attention, needing optimization for industrial-scale production to fulfill gaps in the process.

Microalgal biomass can be converted into bioethanol via three pathways: Dark fermentation, Photofermentation and Fermentation of

Table 3

Pretreatments and fermentation conditions for bioethanol production from several microalgal species.

Microalgal species	Biomass pretreatment	Glucose released	Fermenting strain	Ethanol concentration	Reference
<i>Chlamydomonas reinhardtii</i> UTEX 90	Amyloglucosidase (AMG 300L) and α -amylase (Termamyl 120L)	44.70%	<i>S. cerevisiae</i> S288C	11.73 g L ⁻¹	(Choi et al., 2010)
<i>Chlorella</i> sp. TIB-A01	2% HCl and 2.5% MgCl ₂	90.74 g L ⁻¹ , 64.21%	<i>S. cerevisiae</i> Y01	22.60 g L ⁻¹	(Zhou et al., 2011)
<i>Pseudochlorella</i> sp. GU732422,	Ultrasonication at 50 °C for 30 min followed by cellulase	360 mg _{sugar} g ⁻¹ biomass ⁻¹	<i>S. cerevisiae</i> YPH499 immobilized in Ca-alginate (2%)	0.81 g L ⁻¹	(Ha et al., 2020)
<i>Scenedesmus obliquus</i> CNW-N	2.5% H ₂ SO ₄	52.90%	<i>Z. mobilis</i> ATCC 29,191	0.202 g g ⁻¹	(Ho et al., 2013a)
<i>Scenedesmus raciborskii</i> WZKMT	Cellulase, α -amylase and amyloglucosidase	58.03 g/L	<i>S. cerevisiae</i> NRRL Y-2034	79.38 g L ⁻¹	(Alam et al., 2019)
<i>Arthrospira platensis</i> SAG 21.99	HNO ₃ 0.5 N or H ₂ SO ₄ 0.5 N at 100 °C	–	<i>S. cerevisiae</i> MV 92,081	16.32% (g g ⁻¹) from 0.5 N HNO ₃ and 16.27% (g g ⁻¹) from 0.5 N H ₂ SO ₄	(Markou et al., 2013)
<i>Chlorella</i> sp. and <i>Scenedesmus</i> sp.	1% H ₂ SO ₄ at 121 °C for 15 min followed by α -amylase and cellulase	0.490 g glucose g ⁻¹ deoiled algal biomass ⁻¹	<i>S. cerevisiae</i>	0.145 g g _{deoiled algal biomass} ⁻¹	(Naresh Kumar et al., 2020)

pretreated microalgal biomass. However, for the first two routes to be viable, it is necessary to produce high amounts of ethanol, which is still a challenge, necessitating advanced genetic manipulation techniques, thus the last one is the most common and viable method (Lakatos et al., 2019). There are several reports on bioethanol production from microalgae, with different pretreatment methods and fermenting microorganisms (Table 3).

There is a wide range of biomass pretreatments, from physical methods, such as sonication and bead-beating (Miranda et al., 2012a), to chemical treatment with H₂SO₄, HCl (Lee et al., 2015; Silva et al., 2018), and NaOH (Hernández et al., 2015) and enzymatic hydrolysis (Shokrkar et al., 2017). Although sulfuric acid is still the most used chemical for pretreatment because of its high sugar recovery potential, enzymatic hydrolysis has emerged over the years as a promising green alternative with the potential to overcome economic issues related to this technology (Harun and Danquah, 2011). Cellulases (Hernández et al., 2015), amylases (Choi et al., 2010; Singh et al., 2018), pectinases (Lee et al., 2015), and their combinations (Shokrkar et al., 2017) are the most frequent enzymes used. Of note, there is a trend in combining pretreatment methods to maximize the recovery of reducing sugars (Alavijeh et al., 2020; El-Dalatony et al., 2016).

Several approaches have been used to achieve high ethanol concentrations: use of high-solid loadings (Ha et al., 2021), enzyme combinations (Silva et al., 2018), biocatalyst immobilization (Rempel et al., 2018), immobilization of fermenting yeasts (El-Dalatony et al., 2016), fed-batch enzymatic hydrolysis and fermentation (Alam et al., 2019), and simultaneous saccharification and fermentation ((Luiza Astolfi et al., 2020); El-Dalatony et al., 2016).

According to the biorefinery concept, other macromolecules could be extracted, and residues converted to biofuels (Lee et al., 2015). Use of other effluents (Hemalatha et al., 2019; Salla et al., 2016) or production of other biomolecules, such as biomethane (Rempel et al., 2019), biopeptides (Luiza Astolfi et al., 2020), and biopolymers (Naresh Kumar et al., 2020), is an effective way to integrate the bioethanol production in a cyclic way toward zero waste.

4.2. Biobutanol

Butanol is a four-carbon alcohol with four isomers, two of which (namely, *n*- and *iso*-butanol) are considered promising biofuels. With interesting physicochemical characteristics and advantages over ethanol, such as high energy density and lower vapor pressure, butanol has been described as a potential substitute for gasoline (Amiri and Karimi, 2019; Narchonai et al., 2020). Biological production of butanol (biobutanol) is a well-consolidated technique based on acetone-butanol-ethanol (ABE, ratio of 3:6:1) fermentation by *Clostridium* bacteria (Kushwaha et al., 2019). Over the years, several strains of *Escherichia coli* bacteria and *S. cerevisiae* yeast have been genetically modified to produce biobutanol. These microorganisms are more

industrially friendly but produce lower concentrations of butanol than *Clostridium* bacteria (Swidah et al., 2018).

Microalgal biomass can serve as a carbon source for biobutanol production by *Clostridium* species, as these microorganisms have the ability to ferment a wide variety of substrates by secreting hydrolytic enzymes (Martín-Juárez et al., 2017). Different microalgal species can be used to convert carbohydrates into biobutanol (Table 4), and several routes can be explored, but few studies have been able to achieve high titers (Alam et al., 2019).

Gao et al. (2016) performed ABE fermentation using lipid-extracted microalgae (*C. vulgaris* UTEX 2714) as substrate for *Clostridium saccharobutylicum* DSM 13864. Starch obtained after lipid extraction with hexane and detoxified was converted to 8.05 g L⁻¹ butanol. Additionally, the lipid fraction could be used for biodiesel production, reducing the cost of each individual process from a biorefinery perspective, where all biomass fractions should be exploited. More recent findings revealed that genetically modified yeasts are also able to convert biomass proteins into biobutanol. For instance, Ha et al. (2021) applied an engineered strain of *S. cerevisiae* S288C to convert the residual protein fraction from *C. mexicana* and *Chlamydomonas pittedmannii* to butanol, reaching yields of 3.8 and 3.9 g L⁻¹, respectively.

4.3. Biomethane

In addition to liquid fuels, microalgae-based carbohydrates can also generate gaseous bioenergy sources, such as biogas. Biogas with a high methane content can be produced by anaerobic digestion of microalgal biomass (Chen et al., 2013). Anaerobic digestion, the major biological process of conversion of organic matter into biogas, comprises several steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Brennan and Owende, 2010).

Through this simple and low-cost approach, it is possible to harness the photosynthetic energy fixed in cells to produce valuable biomethane gas (Cuellar-Bermudez et al., 2019; Park et al., 2020) for heat and power generation (Chew et al., 2017). The recovery of other macromolecules can add value to the approach and help achieve process integration (Chew et al., 2017). Rempel et al. (2019) demonstrated the use of residues resulting from bioethanol production from *Spirulina platensis* biomass to obtain biomethane. An energy potential of 13,945 kJ kg⁻¹ was achieved when the biomass was subjected to saccharification and biobutanol fermentation before biomethane conversion.

Moisture and C/N ratio are critical parameters for anaerobic digestion (Markou et al., 2012a). The optimal C/N ratio is reported to be about 20–30:1 (Milano et al., 2016). High C/N ratios can be obtained by manipulating microalgal culture conditions for carbohydrate accumulation (Markou et al., 2012a). Microalgal biomass with a high content of carbohydrates, especially simple sugars, such as glucose, facilitates anaerobic digestion and is a promising approach to enhance biomethane production.

Table 4

Pretreatments and fermentation conditions for biobutanol production from several microalgal species.

Microalgal species	Biomass pretreatment	Glucose released	Fermenting strain	Butanol concentration	Reference
<i>Arthrospira platensis</i> (NORDST.) GEITL strain rsemsu-1/02-P	Thermal treatment at 108 °C for 30 min	8.3 g L ⁻¹	<i>Clostridium acetobutylicum</i> strain B-1787 (immobilized)	64.2% (g g ⁻¹)	(Efremenko et al., 2012)
<i>Pseudochlorella</i> sp. GU732422,	Ultrasonication at 50 °C for 30 min followed by cellulase	1.8 g L ⁻¹	<i>S. cerevisiae</i> YPH499 immobilized in Ca-alginate (2%)	0.44 g higher-alcohols g ⁻¹	(Ha et al., 2020)
<i>Chlorella sorokiniana</i> CY1	2% H ₂ SO ₄ at 121 °C for 60 min, followed by 2% NaOH at 121 °C for 60 min	89.08 g L ⁻¹	<i>C. acetobutylicum</i> ATCC 824	3.86 g L ⁻¹	(Cheng et al., 2015)
<i>Chlorella vulgaris</i> UTEX 2714	Acid hydrolysates (2% H ₂ SO ₄)	32.44 g L ⁻¹	<i>Clostridium saccharobutylicum</i> DSM 13,864	8.05 g L ⁻¹	(Gao et al., 2016)
<i>Chlorella vulgaris</i> JSC-6	NaOH (1%), followed by H ₂ SO ₄ (3%). Cellulase and amylase from <i>Pseudomonas</i> sp. CL3	55.6 g L ⁻¹	<i>C. acetobutylicum</i> ATCC824	13.1 g L ⁻¹	(Wang et al., 2016)
<i>Neochloris aquatica</i> CL-M1	1% NaOH, followed by 3% H ₂ SO ₄ for 20 min at 121 °C	48.7 g L ⁻¹	<i>Clostridium acetobutylicum</i> ATCC824	12.0 g L ⁻¹	(Wang et al., 2017)

Cuellar-Bermudez et al. (2019) achieved high methane yields (251 mL CH₄ g⁻¹ COD_{in}) using *Pseudanabaena* sp. containing 23% carbohydrate as feedstock for anaerobic digestion. Park et al. (2020) optimized biomethane production by *Chlorella* sp. using response surface methodology. A high methane yield (302.22 CH₄ g⁻¹ COD) was achieved by anaerobic digestion of dilute acid-pretreated biomass. Including anaerobic digestion of microalgae biomass in a biorefinery concept for biofuel production could be promising, as it would allow to simultaneously treat wastewaters and expand microalgal commercialization.

4.4. Biohydrogen

Another gaseous biofuel and sustainable energy carrier is biohydrogen (H₂) (Srivastava et al., 2020). Biohydrogen has attracted much attention as a renewable energy source for its efficiency when used in fuel cells for electricity generation and its cleaner combustion, which releases H₂O and no carbon byproducts (Chen et al., 2013). This biogas has high energy content and can be generated by a broad array of biological routes, but the technology associated with its storage and application is still under development (Nagarajan et al., 2020; Srivastava et al., 2020).

Biohydrogen production can be performed either by microalgae or using microalgal biomass as feedstock in microbial processes (Markou et al., 2012a). Under dark conditions, auto-fermentation of carbohydrates releases energy, stored in carbohydrates and the excess reducing power can be used to form molecular hydrogen by the action of hydrogenases (Deviram et al., 2020; Markou et al., 2012a). Recently, genetic manipulation of hydrogenases in microalgae has improved the yield of biohydrogen production (Salama et al., 2018).

Alternatively, the dark fermentation route from microalgae-based carbohydrates represents a potential solution to reduce process costs in biohydrogen production. In dark fermentation, microalgal carbohydrates, such as starch and glucose, are fermented by strict anaerobes (Clostridiaceae family) or facultative anaerobes (Enterobacteriaceae family), producing H₂ (Chen et al., 2013; Nagarajan et al., 2020). The efficiency of biohydrogen production is closely related to the carbohydrate content of microalgae. The higher the carbohydrate content, the easier the conversion by anaerobic bacteria, and the better the efficiency of the biohydrogen production system (Markou et al., 2012a). Choosing the appropriate pretreatment and fermenting bacteria can improve the cost-effectiveness of biohydrogen production from microalgae (Nagarajan et al., 2020).

Singh et al. (2019) optimized the conditions for carbohydrate accumulation in *S. obliquus*, achieving 55.4%. The potential of defatted microalgal biomass—rich in carbohydrates—as substrate for dark fermentation was evaluated, and a cumulative hydrogen production of 68.9 mL g⁻¹ DCW was achieved. Chen et al. (2016) assessed three microalgal strains for their ability to accumulate carbohydrates and reported that *C. vulgaris* FSP-E displayed the highest carbohydrate productivity. *C. vulgaris* microalgal biomass was used as feedstock for biohydrogen production via a separate hydrolysis (acidic hydrolysis in 1% H₂SO₄) and fermentation process (*Clostridium butyricum* CGS5) and achieved a maximum H₂ yield of 2.87 mmol g⁻¹ and an H₂ production rate of 176.9 mL h⁻¹ L⁻¹.

5. Challenges, research needs and future directions

Microalgae-based carbohydrates are promising, viable, renewable, and available feedstocks for biofuel production to bioenergy generation. However, some challenges remain to be overcome for the consolidation of the use of bioenergy sources. The scalability of microalgal biomass production is limited by the insufficient technology, low biomass productivity and biomass fractionation (Costa et al., 2020; Salama et al., 2018). Technological advances in the cultivation, harvesting, and extraction of microalgae biomass are needed to reduce product costs, in addition to improvements in the downstream process. To bridge the

research gap between laboratory scale and pilot scale, it is mandatory to perform a cost analysis of each process and evaluate the whole biomass fractions exploitation, since the production of more than one biofuel from microalgal biomass is a very strong strategy to reinforce the versatility of carbohydrates.

The word defining the future outlook of microalgal carbohydrate-based biofuels is integration. For these biofuels to thrive and be commercially available, integration of steps in the production process is crucial. Based on a circular bioeconomy-technoeconomic and life-cycle analysis, in terms of profit, an integrated biorefinery gives high feasibility for biofuels production from microalgae (Rajesh Banu et al., 2020). The use of the polysaccharide fraction of microalgal biomass will be feasible from a techno-economic point of view within a biorefinery concept, where the extraction of high added value molecules (such as pigments, lipids, and peptides) can support the biofuel production.

Future trends include the following: (i) combined use of strategies to manipulate and increase carbohydrate content without compromising biomass growth, such as nutrient approaches with different modes of operation; (ii) coupling of pretreatment methods and exploitation of enzymatic hydrolysis; (iii) association of microorganisms to perform conversion and fermentation; (iv) co-culture of microalgae; and (v) integration of biomolecule extraction. Moreover, genetic modification can be used to increase the carbohydrate content of microalgal strains (e. g., through improvement of starch biosynthesis) and boost their conversion into biofuels. The so-called algomics techniques (genomics, proteomics, metabolomics) should be increasingly applied to improve microalgal strains for simultaneous production of biomass and carbohydrates (Brar et al., 2021).

6. Conclusion

This review has summarized technology approaches and new insights for microalgae-based carbohydrates as a source of biofuels for bioenergy exploitation. The manipulation of the main factors that affect carbohydrates accumulation and integration of parameters represent opportunities to enhance the cost-effectiveness of environmentally friendly microalgal carbohydrate-based biofuels. The main challenges involved in the carbohydrate utilization are the technology scalability and the biomass productivity. However, innovative technologies have been reported, providing new perspectives for biorefinery applications. The use of genetic techniques should be applied to improve microalgal strains for simultaneous production of biomass and carbohydrate and deliver techno-economical microalgal biofuels.

CRediT authorship contribution statement

Maria Augusta de Carvalho Silvello: Conceptualization, Writing – original draft, Writing – review & editing. **Igor Severo Gonçalves:** Writing – original draft. **Suellen Patrícia Held Azambuja:** Writing – original draft. **Sharlene Silva Costa:** Writing – original draft. **Pedro Garcia Pereira Silva:** Writing – original draft. **Lucielen Oliveira Santos:** Visualization, Writing – review & editing. **Rosana Goldbeck:** Writing – review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

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