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Trends on *Chlamydomonas reinhardtii* growth regimes and bioproducts

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Abstract

The green microalga *Chlamydomonas reinhardtii* is a model microorganism for several areas of study. Among the different microalgae species, it presents advantageous characteristics, such as genomes completely sequenced and well-established techniques for genetic transformation. Despite that, *C. reinhardtii* production is still not easily commercially viable, especially due to the low biomass yield. So far there are no reports of scientometric study focusing only on *C. reinhardtii* biomass production process. Considering the need for culture optimization, a scientometric research was conducted to analyze the papers that investigated the growth regimes effects in *C. reinhardtii* cultivation. The search resulted in 130 papers indexed on Web of Science and Scopus platforms from 1969 to December 2022. The quantitative analysis indicated that the photoautotrophic regime was the most employed in the papers. However, when comparing the three growth regimes, the mixotrophic one led to the highest production of biomass, lipids, and heterologous protein. The production of bioproducts was considered the main objective of most of the papers and, among them, biomass was the most frequently investigated. The highest biomass production reported among the papers was 40 g L^{-1} in the heterotrophic growth of a transgenic strain. Other culture conditions were also crucial for *C. reinhardtii* growth, for instance, temperature and cultivation process.

KEYWORDS
biomass production, *Chlamydomonas reinhardtii*, heterotrophic, mixotrophic, photoautotrophic, scientometric study

1 | INTRODUCTION

Chlamydomonas reinhardtii is a green, biflagellate, unicellular microalga, considered a model organism in cellular and molecular biology studies.[1–3] *C. reinhardtii* has three genomes (nucleus, mitochondria, and chloroplast) which already are fully sequenced and can be genetically transformed by well-developed techniques.[4–8] This knowledge set up microalgae as an alternative platform for heterologous expression of high-value products, such as

Abbreviations: GFP, green fluorescent protein; JCR, Journal Citation Reports; TAG, triacylglycerol; VFP, Verde fluorescent protein.

biopharmaceuticals.[9–11] Additionally, microalgae, including *C. reinhardtii*, are considered models to understand lipid metabolism.[12–14] Despite its potential, microalgae are still not widely used in the biotechnology industry due to low biomass yield. To overcome this problem and make this process profitable it is critical to invest in optimizing cultures conditions.

It is well known that culture medium composition and nutrient concentration can influence microalgae' biomass accumulation and/or composition. As an example, the marine diatom *Phaeodactylum tricornutum* grown in nitrogen-deficient medium resulted in higher lipid rates than cells grown in nitrogen-sufficient medium, although lack of nitrogen affected cellular growth.[15] Nitrogen deficiency also increased lipid synthesis in the microalga *Dunaliella tertiolecta*, and its growth rate was negatively impacted by iron, cobalt, magnesium, and molybdenum deprivation.[16] Likewise, chlorophyll production is also impacted by growth conditions, as light, macronutrient (carbon, nitrogen, and phosphorus), and micronutrients for several microalgae species.[17]

As a photosynthetic microorganism, *C. reinhardtii* can grow under light using only photosynthesis as the energy source (photoautotrophic growth). In this growth regime, an inorganic carbon source (for instance, carbon dioxide) can be supplied in the culture medium. Some microalgae species, including *C. reinhardtii*, can grow in the absence of light (heterotrophic growth). In this case, an organic carbon source (for instance, acetate) is used as energy source. Besides, mixotrophic growth can also be applied to *C. reinhardtii*, when both light and organic carbon are used as energy sources.[18]

Since the 1960s, scientometric research is used as a tool to statistically analyze and gather the scientific production advances.[19,20] The scientometric research also highlights which countries, authors, and scientific journals present the most relevant scientific contribution on a particular topic. Although *C. reinhardtii* is considered a model microorganism, so far, there is no scientometric study focused on this microalga species. Therefore, this work aims to carry out a scientometric research to investigate the influence of growth regimes (photoautotrophic, heterotrophic, and mixotrophic) on *C. reinhardtii* cultivation, as well as, analyze the production of its main bioproducts, and the carbon and nitrogen sources used for this microalga cultivation.

2 | METHODOLOGY FOR PAPERS SEARCH AND SELECTION

The search was conducted on December 05, 2022, on the Web of Science (WoS) and Scopus databases, which are well-known throughout the world scientific community. The keywords terms were: "*C. reinhardtii*" AND ("phototrophic" OR "photoautotrophic" OR "heterotrophic" OR "mixotrophic"). The keywords were searched within the titles, abstracts, and/or keywords. The search was not restricted by year to select the first papers indexed on the databases.

The initial search resulted in 434 publications on Scopus and 632 publications on WoS. Duplicate papers were discarded, and each title and abstract were evaluated to ensure the selection. Full text was read when necessary to confirm exclusions or selections. Papers in which *C. reinhardtii* was only cited in the abstract as an example, and papers which the growth regimes were not part of the study investigation were excluded from the search.

The following information were investigated: country of publication, scientific journal of publication, and its impact factor according to the 2021 Edition of the Journal Citation Reports (JCR), publication year, as well as the growth regimes, carbon and nitrogen sources used in each experiment of the paper. The country of publication was determined by the affiliation of the first author, as is frequently done in other scientometric researches.[21,22]

3 | DATA OF THE QUANTITATIVE ANALYSIS

After selecting the papers, the search on Scopus and WoS databases resulted in 130 research papers published from 1969 to 2022 (Figure 1A) (Table S1). All analyzed papers were published in English, since it is the main language for scientific communication worldwide.[23] Publications number started increasing in 2013 (5 papers), and the highest number was achieved in 2021 (18 papers). The number of published papers as open access or per subscription was constant over the years. Open access is an instrument to increase journal citations since the readers can access the papers without cost, and the researchers and external community are able to access scientific late information more easily.[24] On the other hand, this publication mode can be challenging for some authors given that open access papers require author's payment to be published.

The papers were published in 71 different scientific journals, grouped as: 5 journals with 5 or more published papers; 23 journals that published between 4 and 2 papers; and 43 journals with only 1 published paper. Table 1 presents a scientific journals list with at least three or more publications in the area, and their respective impact factor.

Algal Research is the scientific journal with the highest number of publications, with 12 published papers between 2013 and 2022. Algal Research's impact factor was 5.276
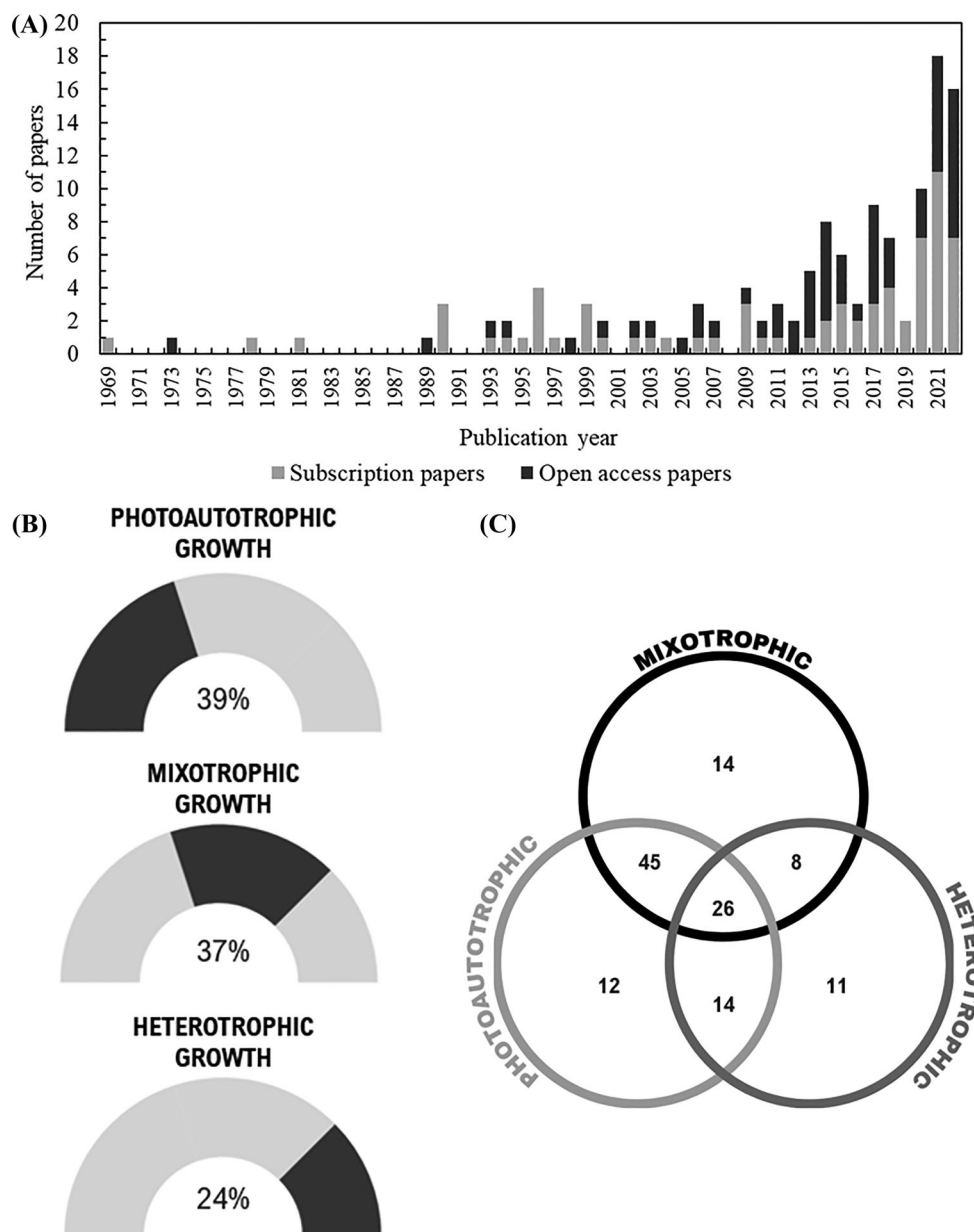
FIGURE 1 The number of published papers on the subject *Chlamydomonas reinhardtii* growth regimes in from 1969 to 2022 (A). Percentage (B) and number of papers (C) that conducted experiments with each growth regime.

according to the 2021 JCR. Only 8% of the scientific journals have impact factors above 10, whereas the majority of them (46%) have impact factor between 10 and 4, followed by 30% of the journals with impact factor below 4, and 16% of the journals do not have a registered impact factor in 2021. The impact factor is a measure based on the number of published papers and their citations.[25] As a matter of fact, fewer number of readers interested in the area cause a decrease of access and citation of the papers. Impact factor also depends on each journal publication area, for instance, the journals that publish general content may have a higher impact factor when compared to those that publish in more specific areas of study, as it is shown for Algal Research.[24]

Next, an analysis was carried out to determine the countries of publication according to the first author affiliation. The results reveal that the researches were developed in 29 different countries. The United States was the country involved with the highest number of publications (22 papers), followed by the United Kingdom (16 papers), and Russia (14 papers). A total of 9 countries were affiliated with 5 or more papers, 10 countries were affiliated with 4–2 papers, and 10 papers were affiliated with only 1 paper.

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TABLE 1 Scientific journals with three or more published papers in the area of growth regimes influence in *Chlamydomonas reinhardtii* cultivation.

Scientific journal	Number of papers	IF[a]
Algal Research	12	5.276
Plant Journal	6	7.091
Bioresource Technology	5	11.889
Journal of Applied Phycology	5	3.404
Plant Physiology	5	8.005
International Journal of Hydrogen Energy	4	7.139
Biotechnology for Biofuels	3	7.670
Frontiers in Plant Science	3	6.627
Journal of Bioscience and Bioengineering	3	3.185
Metabolic Engineering	3	8.929
Photosynthesis Research	3	3.429
Process Biochemistry	3	4.885

[a]Impact factor (IF): data according to the 2021 JCR.

4 | GROWTH REGIMES: ADVANTAGES AND APPLICATIONS

Among the growth regimes, the photoautotrophic growth is an attractive regime for microalgae cultivation once it can be performed using natural resources, such as sunlight and carbon dioxide (CO_2). Despite these advantages, the maximum biomass concentration can be negatively affected by the light penetration.[26]

The heterotrophic growth is an alternative regime in which light is not required. Not all microalgae species are able to grow heterotrophically, but when compared to the photoautotrophic growth, the heterotrophic growth is simpler to scale-up and can induce a high biomass accumulation.[27–30]

The mixotrophic growth is conducted with both organic and inorganic carbon sources. Therefore, it is interesting once cellular growth is not strictly limited by light intensity, and growth rate is still sustained while there is available organic carbon, independent of the light.[31,32]

A quantitative analysis showed that the photoautotrophic was the most investigated growth regime in *C. reinhardtii* cultivation (39% of the papers), followed by mixotrophic (37%), and lastly, heterotrophic growth (24%) (Figure 1B). In most papers (92), researchers cultivated *C. reinhardtii* in different growth regimes in order to compare the effects of each one. Among these papers, photoautotrophic and mixotrophic growth were the most compared (45 papers). Furthermore, 26 papers conducted a comparison between the three growth regimes effects on *C. reinhardtii* culture, 14 papers compared the effects of photoautotrophic and heterotrophic growth, and 8 papers compared the effects of heterotrophic and mixotrophic growth (Figure 1C).

The analyzed papers demonstrate that the growth regimes have significant effects on *C. reinhardtii* biomass and bioproducts accumulation due to the metabolism shifts. A computational study published by Boyle and Morgan[33] predicted that biomass, carbohydrates, and chlorophyll *b* production were higher in photoautotrophic growth. Total proteins concentration was higher in mixotrophic growth. Lipids and chlorophyll *a* concentrations were higher in heterotrophic growth. It is important to note that this trend was not commonly observed in the majority of the analyzed papers, which consequently highlight that bioproducts production is dependent on several culture conditions, beyond growth regimes.

The 130 selected papers were classified into eight different areas of study, according to their main objective (Figure 2A). The following criteria were adopted: (i) "bioproduct production," when the paper investigated or optimized one or more cellular bioproduct production; (ii) "cellular metabolism," when the paper addressed metabolic processes, culture condition effects, or enzymes activity; (iii) "computational analysis," when developing computational analysis, such as flux balance or metabolic modeling; (iv) "DNA replication process," when investigating regulation process or DNA replication alterations; (v) "gene expression profile," for studies on gene expression during different culture conditions or on the genes involved in primary metabolism; (vi) "mutant strain characterization," when investigating mutant strains; (vii) "nutrient removal," for studies on *C. reinhardtii* capacity of recycling nutrients from wastewater; and (viii) "photosynthesis or respiratory processes," when the paper investigated the photosynthesis or respiration processes.

The analysis points out that the bioproducts production was the objective of the majority of papers (48%). These
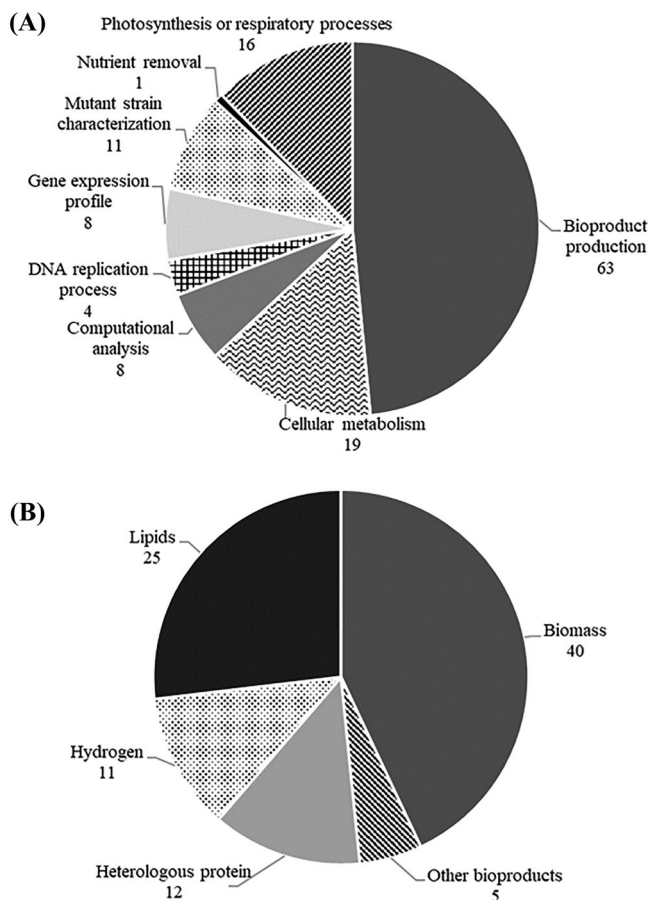
(A)

(B)

FIGURE 2 Classification of the analyzed papers according to their main objective (A), and classification of the papers grouped in "bioproduct production" according to the bioproduct targeted in the cultures (B).

papers were mainly developed with the goal of exploring *C. reinhardtii* growth or a specific bioproduct accumulation. Culture optimizations are desirable once they allow higher bioproducts accumulation, as well as costs reduction. In the next section, further analyzes were conducted with the 62 papers grouped in "bioproduct production" to assess the progress made in this area.

5 | EVALUATION OF THE PAPERS REGARDING BIOPRODUCTS PRODUCTION

Bioproducts are compounds extracted from organisms or the organism itself that have potential application for society, with economic and ecological advantages. Besides the biomass (whole microorganism), many important bioproducts can be extracted from it, such as carotenoids, lipids, proteins, vitamins, and minerals.[34–36] Further, the possibility of genome editing also expanded the range of bioproducts that can be obtained from microalgae, which

currently includes biopharmaceuticals and high valuable proteins.[11,37–39]

Changes in culture conditions affect cellular metabolic pathways, which can be an alternative to increase the concentration of a specific bioproduct. Several culture conditions, as nutrient availability, temperature, pH, light availability/intensity, and growth regimes have significant effects on microalgae growth.[40–42]

Papers related to bioproducts production were divided according to the target bioproduct in the *C. reinhardtii* cultivation. The main investigated bioproducts were categorized as: (i) biomass; (ii) carotenoids; (iii) heterologous protein; (iv) hydrogen; and (v) lipids (Figure 2B). More than half of the papers investigated more than one bioproduct. Therefore, these papers were accounted in more than one category.

5.1 | Influence of growth regimes in biomass production

Among the targeted bioproducts in *C. reinhardtii* production, biomass was the most frequently analyzed (40 papers) (Figure 2B). It indicates the culture's cellular density, expressed either in dry weight per volume (g L^{-1}) or cells number per volume (cell mL^{-1}). An analysis of the studies over the years demonstrated that from 1990 to 1999 the experiments were mostly conducted by heterotrophic growth. Over time, there was an increase in experiments using photoautotrophic and mixotrophic growth. This last one was more frequently applied for biomass production from 2017 to 2022.

This fact may be explained by the advantages previously described in the topic "Growth regimes: advantages and applications," as well as higher growth rates using mixotrophic growth when compared to the other two growth regimes. For instance, a *C. reinhardtii* transgenic strain cultivation achieved a maximum dry cell weight of 0.62 g L^{-1} in photoautotrophic regime in contrast to 1.23 g L^{-1} achieved in mixotrophic regime in the same 15 L "hanging-bag"-type photobioreactor.[43] Dry cell weight of the wild-type strain CC-124 was also higher in mixotrophic growth (2.15 g L^{-1}) when compared to heterotrophic (1.18 g L^{-1}) and photoautotrophic growth (0.27 g L^{-1}).[44] According to Young et al.[45] most of the microalgae species used in their study grew better mixotrophically than in the other two growth regimes.

Aiming to optimize the biomass production in the photoautotrophic growth, recently a new culture media named 6xP was developed by Freudenberg et al.[46] The photoautotrophic growth of a transgenic strain in this medium resulted in higher biomass production in the 6xP medium (20.49 g L^{-1}) than in the HSM medium

TABLE 2 Comparison of the highest biomass concentrations reported in the analyzed papers.

Strain	Growth medium	Cultivation process	Growth regime	Biomass concentration (g L^{-1})	Reference
Transgenic CR25	T10	Fed-batch	Heterotrophic	40	[48]
CC-1690	Modified TAP	Continuous	Heterotrophic	25.44	[49]
CC-2937 GFP	Modified TAP	Fed-batch	Mixotrophic	23.69	[51]
UV_6	6xP	Batch	Photoautotrophic	20.49	[47]
N-UVM4	6xP	Batch	Photoautotrophic	20	[46]
CS-51	CR-M1	Perfusion	Heterotrophic	9	[94,28]
–	HSM[a]	Continuous	Photoautotrophic	3.72	[95]
C-9	Medium I[b]	Batch	Photoautotrophic	2.64	[96]
CC-124	TAP[c]	Continuous	Mixotrophic	2.3	[97]

Abbreviation: GFP, green fluorescent protein.

[a]HSM: High salt medium, described by Sueoka.[99]

[b]Medium described by Sager and Granick.[100]

[c]TAP: Tris-acetate-phosphate medium, described by Gorman and Levine.[98]

(1.63 g L^{-1}), commonly used for *C. reinhardtii* growth.[47] In addition to increasing biomass production, the production of the heterologous protein cadaverine was also higher when compared to the standard condition.[46] Since photoautotrophic growth has several advantages, as previously mentioned at the topic "Growth regimes: advantages and applications," the development of this new culture medium may lead to an increase in research with this growth regime intending to a more sustainable cultivation.

Table 2 lists the highest biomass concentrations reported in the analyzed papers. As it shows, the highest concentration was 40 g L^{-1} in the heterotrophic culture of a transgenic *C. reinhardtii* strain. This value was achieved as a result of culture medium composition optimization.[48] The second highest biomass concentration was 25.44 g L^{-1} in the heterotrophic culture of *C. reinhardtii* CC-1690 wild strain. In this case, the authors optimized the culture temperature, increasing from 25°C (standard temperature) to 30°C and by using a 40:1 carbon and nitrogen ratio.[49] Although 25°C is the standard temperature for *C. reinhardtii* growth in the laboratory, a study using T1 transgenic *C. reinhardtii* strain also showed that 30°C temperature increased about 20% of growth rate in photoautotrophic growth.[50]

The third highest biomass production was 23.69 g L^{-1}, achieved with the CC-2937 transgenic strain expressing the heterologous protein green fluorescent protein (GFP), cultured in a 1 L bioreactor with 550 mL of working volume in modified TAP medium. The adopted strategy was to cultivate the *C. reinhardtii* transgenic strain with constant medium feeding, which resulted in biomass increase from 2.08 (batch mode) to 23.69 g L^{-1} (fed-batch mode).[51]

Table 2 shows that independently of the growth regime and cultivation process, it is possible to reach high biomass concentrations by optimizing the cultivation media. It is also important to note that, although the highest *C. reinhardtii* biomass production was reported in a heterotrophic culture, the papers that compared the three growth regimes in the same culture condition demonstrated that heterotrophic cultures usually do not provide the highest biomass production.[44,45,50,52,53] This fact indicates the need for conducting more studies comparing the three growth regimes while maintaining the same culture conditions. Although, the photoautotrophic growth was not the best regime for biomass accumulation, it was the most frequently investigated over the years, possibly due to its sustainable approach.

Even though biomass harvesting was not an explored objective in these papers, this is the final, essential, and challenging step of microalgae cultivation, in industrial processes.[54,55] Besides the traditional methods, such as centrifugation or filtration, bio-flocculation is an example of a harvesting technique that is gaining more attention lately. This technique can be applied for other organisms, such as fungi, or substances extracted from them, such as chitosan.[56,57] Likewise, self-flocculation is also a phenomenon studied in microalgae cultivation. In this case, the flocculating substances are produced by the microalgae themselves, which causes cell aggregation.[55] Though *C. reinhardtii* does not synthesize these molecules physiologically, they can be synthetized through genetic engineering.[58]

5.2 | Influence of growth regimes in lipids production

The second most investigated bioproduct in *C. reinhardtii* cultivation was lipid (25 papers) (Figure 2B). Lipids extracted from microalgae can be employed as a renewable

source for biofuel production. Third-generation biofuels are sustainable, renewable, nontoxic, and biodegradable fuel sources produced from vegetal biomass, that is, why this is a highly explored alternative to the global crescent demand for fuel. Nowadays, third-generation biofuels are mainly produced from higher plants; however, microalgal biomass is also a promising source for biofuel production. Compared to plants crop, microalgae can grow faster, in smaller spaces, with less water requirement, and without the need for arable land, in other words, with less environment impact.[59,60]

When lipids were the target compound in *C. reinhardtii* culture, mixotrophic growth was the most investigated (47% of the papers), followed by photoautotrophic (38% of the papers) and heterotrophic growth (15% of the papers). Among the papers that compared two or three growth regimes, results indicate that mixotrophic growth provided the best results to lipids and starch accumulation.[44,52,61]

Although photoautotrophic growth is a widely studied growth regime, for other microalga species this is not the most indicated growth regime for lipids accumulation. For instance, in *Chlorella protothecoides*, lipid accumulation was higher in the heterotrophic growth (55% of cell content) when compared to the photoautotrophic growth (15% of cell content).[62] On the other hand, a study performed with *Chlorella vulgaris* indicated that lipid accumulation was higher in mixotrophic growth when compared to heterotrophic and photoautotrophic growth.[63]

A strategy to increase lipids production in microalgae is to induce cellular stress in microalgae culture, such as by nutrient deprivation (mainly nitrogen), temperature, or light intensity. When facing a stress condition, growth is inhibited, and the available carbon is redirected to lipids accumulation in the form of triacylglycerol (TAG) and starch to store energy and prevent cellular damage. These molecules can be used industrially for bioethanol and biodiesel production.[53,59,64]

In Figueroa-Torres et al.[65] work starch and lipid yield in *C. reinhardtii* culture was successfully optimized for carbon, nitrogen, and phosphorus concentrations in TAP medium by constructing a multiparameter kinetic model in mixotrophic growth. Compared to the cultivation in standard TAP medium, the optimized one resulted in starch and lipid yield of 270% and 74% higher, respectively. *C. reinhardtii* UTEX 90 starch and lipid productivities were also increased by optimizing the cultures pH, temperature, and concentration of acetate and nitrogen. Results indicated an increase in starch productivity from 26.09 to 267.26 mg L^{-1} day^{-1}, and lipid productivity from 21.34 to 112.64 mg L^{-1} day^{-1} by setting the pH to 7.0, the temperature at 30°C, acetate concentration to 1.56 g L^{-1}, nitrogen (supplied in the form of ammonium chloride) to 100 mg L^{-1}, and adding 7% CO_2 air mixture to the cultivation.[66] For transgenic strain, Lee et al.[67] generated a *C. reinhardtii* overexpressing fructose-1,6-bisphosphate aldolase 1, an enzyme involved in the energy pathway. This transgenic strain exhibited a similar growth rate to wild strain in mixotrophic culture, as well as higher lipid and total fatty acids accumulation.

5.3 | Influence of growth regimes in heterologous proteins production

Heterologous proteins production was the main objective of 12 papers (Figure 2B). *C. reinhardtii* is an advantageous platform for heterologous protein production since it can perform posttranslational modifications necessary for the correct and efficient protein function, as well as easy and relatively cheap cultivation, and effective transformation techniques and vectors for accumulation and secretion of high rates of heterologous protein.[11] Table 3 lists the highest heterologous protein accumulation in the analyzed papers.

The highest heterologous protein yield was reported by Freudenberg et al.,[46] who cultivated *C. reinhardtii* in photoautotrophic growth. In this work, as mentioned before at biomass production topic, the new culture media 6xP successfully supported the optimization of both biomass and cadaverine production. The same culture media as used in Ref. [47] work, where the cadaverine production increased from 3.7 to 22.5 mg L^{-1} in photoautotrophic growth.

The second highest heterologous protein accumulation was reported by Torres-Tiji et al.[48] work, where the heterotrophic culture of a transgenic *C. reinhardtii* strain reached the maximum of 46.6 mg L^{-1} of heterologous ICAM-1 D2, a human protein that belongs to the immunoglobulin superfamily. The fourth paper on the list was developed with a wall-deficient transgenic strain expressing TPS4, an enzyme involved in the synthesis of *cis*-abienol. This was the first work that reported the pilot-scale cultivation of a cell wall-deficient strain of *C. reinhardtii*, and the results indicate a satisfactory biomass yield in a commercial-scale mixotrophic growth.[68]

The following highest heterologous protein accumulation was reported by Carrera Pacheco et al.[69] Their results indicate higher GFP production in mixotrophic growth (16 mg L^{-1}) when compared to photoautotrophic one (6 mg L^{-1}).[69] Since many chloroplast genes are regulated by light, they also investigated different light periods and intensities influence in recombinant protein expression. Their results indicated that these two variables have significant influence in both biomass and GFP production. Short light periods resulted in higher protein accumulation in both photoautotrophic and mixotrophic growth; however, this condition did not benefited growth.[69]

TABLE 3 Comparison of the highest results of heterologous protein accumulation expressed in *Chlamydomonas reinhardtii* reported in the analyzed papers.

Heterologous protein	Heterologous protein yield (mg L^{-1})	Growth regime	References
Cadaverine	240	Photoautotrophic	[46]
ICAM-1 D2	46.6	Heterotrophic	[48]
Cadaverine	22.5	Photoautotrophic	[47]
TPS4	22	Mixotrophic	[68]
Green fluorescent protein	16	Mixotrophic	[69]
(E)-α-Bisabolene	11	Mixotrophic	[101]

According to Kim et al.,[70] the fluorescent protein mCherry production yield in *C. reinhardtii* photoautotrophic regime was also low when compared to previous reports of heterologous protein yield in mixotrophic regime. The research studies also highlighted that the heterologous protein accumulation in the chloroplast in photoautotrophic growth is influenced by the CO_2 supply and light intensity.[70]

Benedetti et al.[71] work also reported high heterologous production of cellulase enzyme in mixotrophic growth, when compared to photoautotrophic and heterotrophic growths. The researchers also demonstrated phosphite dehydrogenase D production which allowed *C. reinhardtii* transgenic strain to grow in medium containing phosphite as the sole phosphorus source. Therefore, a cheaper growth medium was developed by replacing phosphate in TAP medium original composition by phosphite, which allowed microalgal growth in non-sterile condition.[71]

Braun-Galleani et al.[50] tested the effects of the three growth regimes in the production of the vivid Verde fluorescent protein (VFP). Their results indicated that the highest heterologous protein accumulation was achieved in mixotrophic growth (1.65 mg L^{-1}), followed by heterotrophic (0.66 mg L^{-1}) and photoautotrophic growth (0.63 mg L^{-1}). This work also highlighted the importance of temperature in *C. reinhardtii* growth, affecting both biomass and VFP accumulation. Results indicated a slightly higher VFP accumulation in 30°C mixotrophic growth when compared to the culture performed in the standard temperature of 25°C.[50]

It is important to highlight that beyond culture conditions, heterologous protein expression is dependent on several factors, such as the organelle in which the heterologous protein was expressed, the selected promotor, the chosen locus in the genome for heterologous gene insertion, the selected strain used for transformation, or the heterologous protein size.[11,50,69,72] For instance, the chloroplast usually presents higher heterologous protein expression than the nucleus[11] and different promoters can also influence in heterologous protein expression.[72] Thus, further studies must be developed to maximize the

heterologous protein production according and make it economically feasible.[73]

5.4 | Influence of growth regimes in hydrogen production

Hydrogen production was the fourth topic with more publications among the bioproducts production (11 papers). Hydrogen (H_2) is a renewable and clean energy carrier since its combustion does not emit polluting gases and generates high energy content, even higher than other gaseous fuels.[74] In microalgae, H_2 is produced during photosynthesis, when water molecules (H_2O) are converted into oxygen (O_2) and hydrogen ions (H^+), which are converted into H_2 by hydrogenase enzymes. This process stability depends on maintaining an anaerobic condition in the culture since oxygen presence cause hydrogenase inhibition.[60,75,76]

Among the papers, Jurado-Oller et al.[77] reported a very important achievement since they could establish H_2 production without cellular stress, by adding acetic acid in the medium on the fourth day of cultivation with O_2 aeration. Even though the cultivation resulted in low H_2 production (about 68 mL L^{-1}) in comparison to other productions reported in the literature, it is still an important result and a promising strategy when it comes to scaling-up for industrial application. They also reported that the mixotrophic growth accumulated more H_2 than the heterotrophic growth, indicating that illumination is important for H_2 production.[77] It is well known that acetate supply in *C. reinhardtii* growth enhances H_2 production, although acetate role in this process it is still not completely clear. Although acetate supply simultaneously with illumination enhance H_2 production, in the dark acetate they do not have the same effects.[77–79]

The highest H_2 production in *C. reinhardtii* reported in the analyzed papers was above 200 mL L^{-1} in a mixotrophic growth, using the mutant strain CC-4169 which is characterized by its higher photosynthetic efficiency in comparison to the wild strain. To optimize H_2 production, different nitrogen and carbon ratios were

tested in a modified TAP medium and the results indicated that the highest H_2 production was reached using 2 mM of nitrate as nitrogen source and 20 mM of acetate as carbon source.[80]

5.5 | Influence of growth regimes in other bioproducts production

The topic "other bioproducts" includes five papers. One of them, aimed at producing ketocarotenoids in *C. reinhardtii* by genetic engineering. The highest productivity of astaxanthin (3.1 mg L^{-1} day^{-1}) and total ketocarotenoids (4.3 mg L^{-1} day^{-1}) was achieved in mixotrophic growth using high light intensity (3000 μmol m^{-2} s^{-1}) and CO_2 supplementation.[81] Kang et al.[82] work aimed at accumulating glycolic acid in *C. reinhardtii*, produced through the photorespiration mechanism. After 20 days of cultivation, productions of 122.6 mg L^{-1} day^{-1} of glycolic acid and 800 mg L^{-1} of biomass were achieved.

The other three papers were published in 2022, and all of them aimed at using *C. reinhardtii* to produce terpenoids, organic chemicals of industrial interest. Among the terpenoids, those produced in *C. reinhardtii* were: limonene, reaching a maximum of 117 μg L^{-1} in mixotrophic growth[83]; patchoulol, reaching a maximum of 6.2 mg L^{-1} in photoautotrophic growth[84]; and sclareol, reaching a maximum of 656 mg L^{-1} in photoautotrophic growth.[85]

6 | CARBON AND NITROGEN SOURCES IN THE CULTIVATION MEDIUM

As well as growth regimes can influence this microalga growth and intracellular compound synthesis, nutrient availability is also essential for cellular growth and metabolism homeostasis. To find out which nutrients are important to cellular growth, one strategy is to verify the elemental cellular composition. Among the macronutrients that compose cellular structure, carbon, nitrogen, and phosphorus are the most abundant.[86]

Carbon is one of the most important nutrients to microalgae growth, it is present in carbohydrates, lipids, nucleic acids, pigments, and proteins composition, and constitutes about 50% of the dry biomass.[87,88] Through photosynthesis, *C. reinhardtii* is capable of consuming CO_2, and through respiration, cells metabolize organic carbon to obtain the energy necessary for cellular growth.

When analyzing the employed carbon source in *C. reinhardtii* cultivations most papers indicated acetate as the most common (48%). Acetate is the standard organic carbon source used for heterotrophic and mixotrophic growth.[18] The second most used carbon source was CO_2

gas (24% of experiments), which is the standard inorganic carbon source for photoautotrophic and mixotrophic growth. Glucose was used in 2% of the experiments; sodium bicarbonate, glycerol, potassium acetate, ammonium acetate, butyrate, sucrose, lactose, or volatile fatty acids were used in about 1% of the experiments.

Although acetate is a standard carbon source in these growth regimes, it is a relatively expensive synthetic reagent.[75] Therefore, aiming at reducing culture cost, other carbon sources are investigated in *C. reinhardtii* growth. For instance, Moon et al.[44] work intended to optimize cell growth and lipid production of the wild *C. reinhardtii* strain CC-124 by comparing the different organic carbon sources (acetate, glucose, glycerol, sucrose, and volatile fatty acids) in heterotrophic or mixotrophic growth. When comparing the three growth regimes, results indicated that mixotrophic growth with acetate (10 g L^{-1}) was the best condition for producing biomass (2.15 g L^{-1}). The use of 5 g L^{-1} of volatile fatty acids in *C. reinhardtii* increased fatty acid methyl esters production to 19.02%.

Banerjee et al.[66] demonstrated the importance of acetate in *C. reinhardtii* growth, in which UTEX 90 cultures were conducted in TAP medium with different acetate concentrations (0.53–4.2 g L^{-1}). The highest biomass productivity (235.7 mg L^{-1} day^{-1}) was achieved by using 2.63 g L^{-1} of acetate. Yet, higher acetate concentrations were unfavorable for *C. reinhardtii* growth. The authors also highlighted the biomass production improvement by growing the microalga mixotrophically with addition of 7% CO_2 air mixture. In this condition, biomass increased from 214.24 to 512 mg L^{-1} day^{-1}.[66]

Nitrogen is also an important nutrient for cellular essential molecules.[15,86] It is known that *C. reinhardtii* can metabolize different nitrogen sources, such as ammonium, amino acids, and nucleic acids.[89] Among the different organic and inorganic nitrogen sources, ammonium chloride was used in the majority of the experiments (73%), followed by those that did not mention or added a nitrogen source in the culture medium (8%), and papers that used urea (6%). Only 2% of the papers reported the use of sodium nitrate, nitrate, or ammonium sources, and 1% reported the use of ammonium nitrate, ammonium bicarbonate, ammonium sulfate, arginine, nitrite, peptone, or potassium nitrate.

Each nitrogen source has different forms of assimilation into the cell and metabolism; therefore, it can influence the biomass composition. For instance, Zhang et al.[49] investigated the influence of ammonium chloride, urea, and sodium nitrate in *C. reinhardtii* growth. Their assays were conducted with the wild strain CC-1690 in heterotrophic growth. The authors state that there was no significant difference in the growth rates and protein content among the culture conditions. On the other hand,

another work performed with the wild strain CS-51, also conducted in heterotrophic growth and testing ammonium chloride, urea, and nitrate, reported a significant difference between the results. Authors highlighted urea as the best nitrogen source for biomass production (610 mg L^{-1}), followed by nitrate (480 mg L^{-1}), and lastly ammonium chloride (440 mg L^{-1}).[90] These results indicate that other culture conditions can also influence the biomass production.

Munz et al.[91] reported higher cellular density when *C. reinhardtii* wild strain CC-1690 was cultured with arginine instead of ammonium as nitrogen source. Cell concentration increased from 1.88 to 6×10^7 cells mL^{-1} in photoautotrophic growth and from 1.24 to 1.97×10^7 cells mL^{-1} in mixotrophic growth. As well as the wild strain CC-125 when adding arginine in a photoautotrophic growth (from 0.51 to 3.53×10^7 cells mL^{-1}). The authors also demonstrated that arginine culture with the strains CC-1690 and *sta6* presented higher amounts of total lipids and TAG when compared to ammonium culture, although nitrogen-free culture had a higher yield than the arginine one.[91] Contrary to other amino acids, arginine can enter *C. reinhardtii* through a specific transport system that is induced by nitrogen starvation without the need of previous deamination or acetate supply.[92,93]

Nitrogen deprivation is a common condition used in *C. reinhardtii* growth to increase lipid accumulation. However, stress conditions usually cause disorders in cellular metabolism, harming cellular growth. Given the analyses and results present in this work, it can be observed that this area has more to be explored to balance biomass and lipids production. Adjustments in cultivation conditions are necessary to direct cell metabolism to our target, leading to plenty of opportunities yet to be explored to optimize the *C. reinhardtii* growth and its bioproducts production.

7 | RECOMMENDATIONS FOR *C. REINHARDTII* CULTIVATION

As demonstrated in the analyzed papers, culture conditions have a crucial role in *C. reinhardtii* growth and composition. According to the reports, the mixotrophic growth resulted in a higher production of biomass, lipids, and heterologous protein, when compared to the other two growth regimes. This growth regime is in advantage due to its metabolism flexibility, in contrast to photoautotrophic one that resulted in a low production of bioproducts. Photoautotrophic growth, instead, presents advantages in cultivation cost, by requiring cheaper input and easier to prepare culture medium, besides by its sustainable approach, which justifies this growth regime continuous improvement.

It is important to consider that other parameters were relevant to improve *C. reinhardtii* growth. One of the most relevant is the culture medium optimization. Also, as observed, the temperature has a notable impact on cultivation, and 30°C is more indicated for biomass production than the standard 25°C. As for cultivation processes, according to studies, fed-batch can lead to a higher biomass production than the batch mode, commonly used in laboratories. Finally, we point out that genomic studies are a great tool for selecting strains with the potential for higher production of biomass and other bioproducts for industrial application.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.