Science of the Total Environment 794 (2021) 148636

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Review

A review on cyanobacteria cultivation for carbohydrate-based biofuels: Cultivation aspects, polysaccharides accumulation strategies, and biofuels production scenarios

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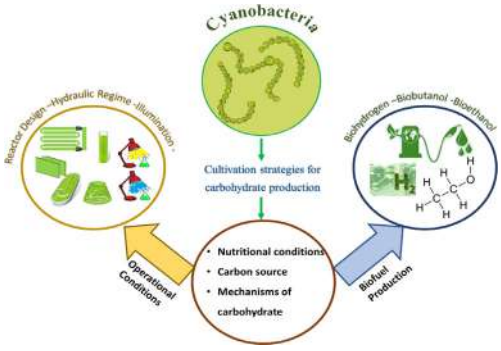
HIGHLIGHTS

- Carbohydrate-based biofuels from cyanobacterial biomass are reviewed.
- Feast and famine of carbon promote higher carbohydrate intracellular content.
- The economic feasibility of biofuels depends on high biomass productivities.
- Genetically engineered cyanobacteria biofuels promise the lowest GHGe.
- Nutrients and water recycling are crucial for low GHGe and net energy demand.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 6 April 2021
Received in revised form 3 June 2021
Accepted 19 June 2021
Available online 24 June 2021

Editor: Daniel CW Tsang

Keywords:
Bioenergy
Blue-green algae
Fermentation
Microalgae
Third-generation biofuels

ABSTRACT

Cyanobacterial biomass has constituted a crucial third and fourth-generation biofuel material, with great potential to synthesize a wide range of metabolites, mainly carbohydrates. Lately, carbohydrate-based biofuels from cyanobacteria, such as bioethanol, biohydrogen, and biobutanol, have attracted attention as a sustainable alternative to petroleum-based products. Cyanobacteria can perform a simple process of saccharification, and extracted carbohydrates can be converted into biofuels with two alternatives; the first one consists of a fermentative process based on bacteria or yeasts, while the second alternative consists of an internal metabolic process of their own in intracellular carbohydrate content, either by the natural or genetic engineered process. This study reviewed carbohydrate-enriched cyanobacterial biomass as feedstock for biofuels. Detailed insights on technical strategies and limitations of cultivation, polysaccharide accumulation strategies for further fermentation process were provided. Advances and challenges in bioethanol, biohydrogen, and biobutanol production by cyanobacteria synthesis and an independent fermentative process are presented. Critical outlook on life-cycle assessment and techno-economical aspects for large-scale application of these technologies were discussed.

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https://doi.org/10.1016/j.scitotenv.2021.148636
0048-9697/

Contents

1. Introduction

The increasing population growth and industrialization have stimulated a high demand and energy consumption from petroleum-based fuels (Okoye et al., 2017; Okoye and Hameed, 2016). The overexploitation of fossil-based sources, such as petrol, diesel, coal, and natural gas is unsustainable because of resource depletion, resulting in an impending energy crisis. Fossil fuels significantly impact the environment and disrupt the ecological structure due to their associated noxious greenhouse gases emission (Lelieveld et al., 2019; Perera, 2018). The greenhouse gases include toxic air such as CH_4, N_2O, fluorinated gases, and carbon dioxide (CO_2). The CO_2 emission is a critical human-produced climate-altering greenhouse gas, contributing to 80% of the total gas emissions (US Environmental Protection Agency, 2021). In the year 2020, the global CO_2 emission was estimated at 31.5 Gt, which was mainly generated from the combustion of fossil fuel (coal, diesel fuel, gasoline, oil, and natural gas) for electricity production (IEA, 2021). These environmental threats have attracted global attention to search for alternative energy sources with minimal environmental impact. Hence, the search for climate-neutral fuel technology as part of an energy security strategy in different parts of the world has become a crucial researchable area.

To date, there are three alternatives to reduce CO_2 emissions. First, by improving the current fossil fuel engine technologies, by CO_2 capture, and using biofuels from renewable energy technologies (Kumar et al., 2018; Thakur et al., 2018). Among the different mitigation strategies, the utilization of biofuels with CO_2 capture and storage provides a new alternative to achieve a significant reduction in greenhouse gases (Mishra et al., 2020). Cyanobacteria are diverging microbes with an important evolutionary history constituting the largest groups of gram-negative prokaryotes (Phélippé et al., 2019). Increasing research interest has been paid to these microorganisms because of their robust CO_2 consumption and their versatility as feedstock with several biotechnological and biorefinery applications. As a third-generation feedstock, cyanobacteria offer several advantages over first- and second-generation feedstocks. Cyanobacteria do not compete with land or agricultural resources, have a high biomass production rate, high productivity, and an ability to grow in hindering conditions such as saline or brackish water and in waste streams (Arias et al., 2020a). Moreover, the harvesting cycles are shorter (~1–10 days), than other materials, i.e., maize harvested once or twice a year (Harun et al., 2010; Varshney et al., 2015).

The advantages of cyanobacteria stimulated research in biotechnology to synthesize and accumulate a wide range of metabolites, mainly carbohydrates, lipids, proteins, and pigments (Ashokkumar et al., 2019). Based on their biochemical composition, different cyanobacteria species can be exploited for a variety of biofuels including biodiesel, bioethanol, colorants, and food supplements. In the case of biodiesel, several studies have reported the use of cyanobacteria and green algae intracellular lipids (Kumar et al., 2019, 2020; Nie et al., 2020). Nevertheless, large-scale biodiesel production from microalgae has not yet met economic feasibility because of several factors such as low biomass productivity, the high capital cost of reactors, low lipidic content, high energy requirements from biomass processing (harvesting, dewatering, drying), and lipid extraction/transesterification (Kumar et al., 2019; Zhu et al., 2017a). Consequently, more current studies focused on carbohydrate-based biofuels, such as bioethanol (Aikawa et al., 2018), biohydrogen (Bolatkhan et al., 2019), and biobutanol (Nilsson et al., 2020).

Fermentation is a biotechnological process in which carbohydrate-containing substrates are simultaneously treated while obtaining biofuels. This process has several advantages, including high production rates and low energy inputs. Additionally, fermentation processes have great versatility in various substrates, such as waste streams from agricultural, food, domestic, and industry sources (Sağır and Hallenbeck, 2019). Although biofuels obtained from the fermentation of carbohydrate-enriched cyanobacteria have the potential to replace petroleum-based products, there are still techno-economic challenges that need to be achieved to scale up the technology. Some of them are related to cost-effective cyanobacterial cultivation, high intracellular carbohydrate

content, improved downstream processes, efficient process of hydrolysis/saccharification, ensuring maximum fermentation yield, and life cycle analysis (LCA) and economic assessments.

In this review, biofuel production processes using carbohydrate-enriched cyanobacterial biomass as feedstock is critically elucidated. The following sections include a detailed technical analysis of key factors and limitations, and novel approaches in carbohydrate-enriched cyanobacterial biomass are elucidated, including their approaches in wastewater effluents. A detailed discussion is provided regarding the methods involved in converting cyanobacterial biomass to biofuels (bioethanol, biohydrogen, and biobutanol production) and co-products, research needs, and a techno-economical analysis and life cycle assessment of the several production alternatives.

2. Overview of cyanobacteria as a valuable third-generation biomass

Cyanobacteria are photosynthetic autotrophic microorganisms that perform essential ecological tasks in aquatic and terrestrial environments promoting phosphorus and carbon cycling and nitrogen fixation, contributing to renewable energy resources, environmental remediation, and energy conservation (Do Nascimento et al., 2019; Muñoz-Rojas et al., 2018). They can perform oxygenic photosynthesis to produce *chlorophyll a* and phycobiliproteins as light-harvesting pigments. Also, they contribute to CO_2 capture by incorporating carbon dioxide into their molecular structure in form of proteins, carbohydrates, and lipids providing carbon mitigation, and also by biomineralizing and store it as $CaCO_3$ (Mishra et al., 2020). There are vast cyanobacteria strains recorded in literature, and depending on the application channels, the strains are task-specific. Their intercellular compounds, nutrients, and varieties of protein in cyanobacteria have been exploited for wide-ranging applications, such as potential pharmaceutical additives (Arashiro et al., 2020a; Bhuvana et al., 2019), bioplastics (Kamravamanesh et al., 2018), fertilizers (Arashiro et al., 2020b), enzymes (Brandenburg and Klähn, 2020), aquaculture (Lin et al., 2019), and feed surrogates (Galetovic et al., 2020; Manzoni Maroneze et al., 2019).

Notably, cyanobacterial species have high resilience to several environmental conditions under extreme temperatures, high salinities, and resistance to variations in pH (Paliwal et al., 2017). Also, cyanobacteria can adapt to different radiances and could grow alone or in symbiosis with other organisms. Essentially, it is established that they perform a symbiotic relationship with heterotrophic bacteria and can efficiently be used to clean up wastewaters from different sources (Arias et al., 2017). In the case of cyanobacteria-based wastewater treatment, the nutrients and carbon contained in wastewater are a cheap source for cyanobacterial growth, while contaminants are removed from the wastewater (Uggetti et al., 2018). Noteworthy to mention is that cultivation of cyanobacteria or green algae in waste streams has been identified as the only cost-effective source of nutrients for biomass cultivation as long as cyanobacterial products are for non-food purposes (Park et al., 2011).

In the last decade, cyanobacterial production of carbohydrates has become an important research topic, generating about 1070 articles. These articles are published mainly in the areas of agricultural and biological sciences, biochemistry, genetics, and molecular biology. One of the promising carbohydrate applications is directed towards converting cyanobacterial biomass into biofuels, mainly bioethanol, biobutanol, and hydrogen. Some review articles are mainly focused on the metabolic engineering advances for production of α-polyglucans and glycogen production (Aikawa et al., 2015), mathematical modeling to understand the metabolism pathway of cyanobacteria (Baroukh et al., 2015), elucidating the contributions of small proteins to cyanobacteria metabolism (Brandenburg and Klähn, 2020), gene editing and multi-omics in cyanobacteria for biorefinery (Lin et al., 2019) and production of biofuels (Oliver et al., 2016), and biohydrogen (Bolatkhan et al., 2019). Also, some studies reported general applications of cyanobacteria (Khanra et al., 2018; Mathimani and Pugazhendhi, 2019), potential cost-effective cultivation alternatives (Arias et al., 2020a; Paliwal et al., 2017; Saha and Murray, 2018), and policies (Trentacoste et al., 2015). However, there is no detailed review encompassing research advances in cyanobacteria cultivation, integrated systems with wastewater processes, and strategies to produce carbohydrate-based biofuels, including LCA and economic evaluations of the different processes to the best of our knowledge. On this basis, the following section critically analyzes the most relevant and related studies about carbohydrate-based biofuels from cyanobacterial biomass.

3. Recent advances in carbohydrate accumulation by cyanobacteria

The primary metabolites accumulated by cyanobacteria are proteins, lipids, and carbohydrates. They contain mainly carbohydrates in the cell wall and as intracellular carbon storage. The carbohydrates present in cell walls provide structural support, while accumulated polysaccharides provide energy sources to the cell or act as protectors for survival under environmental repressions (Singh et al., 2019). Cyanobacterial biomass has aroused research interest in producing carbohydrate-based biofuels due to the high content of fermentable sugars and very low hemicellulosic and lignocellulosic content (Cheng et al., 2019). The feasibility of biofuel production from algal biomass is mainly governed by the carbohydrate content and composition. In this context, carbohydrate content and composition vary widely depending on several factors such as species type, cultivation conditions, and various hindering conditions.

3.1. Metabolic mechanisms of carbohydrate accumulation

All cyanobacteria species produce a variety of lipopolysaccharides and peptidoglycans in their cell walls (composed of outer and inner (plasma or cytoplasmic) membranes and a peptidoglycan layer in between). They store polysaccharides, including glycogen (α-1,6-branched α-1,4-glucan) as the major storage polymer like starch in algae or higher plants. They can accumulate amylopectin (α-1,4-glucan) (El Mannai et al., 2021), sucrose (α-D-glucopyranosyl β-D-fructofuranoside), glucosyl glycerol, trehalose, and some species produce extracellular cellulose (β-1,4-linked glucan), also by mixed-linkage glucans (MLG) (β-1,3-, β-1,4-linked glucan) (Maeda et al., 2018; Zhao et al., 2015).

Storage carbohydrates, especially glycogen, are accumulated as energy storage in chloroplasts' response to unfavorable environments, i.e., disproportionate C: N or C:P ratios, N and P limitation, or salt stress. The most accepted metabolic pathways for glycogen and other polysaccharides accumulation are presented in Fig. 1. Cyanobacteria synthesize glycogen during light periods from assimilated CO_2 through the following process: firstly, cyanobacteria fix CO_2 via the Calvin-Benson cycle; adding CO_2 to ribulose bisphosphate (Ribulose-bis-P, and subsequently converted into molecules of 3-phosphoglycerate (Glycerate-P), a product of the carboxylation reaction of ribulose-1,5-bisphosphate carboxylase or oxygenase (Aikawa et al., 2015). It must be noticed that Glycerate-P acts as an activator of ADP-glucose pyrophosphorylase (AGPase), the enzyme that catalyzes the ADP-glucose synthesis from glucose-1-phosphate, and this process is inhibited by Pi (Aikawa et al., 2015). The final step for glycogen synthesis consists of forming an α-1,6-glycosidic bond (Quintana et al., 2011).

Another important carbohydrate produced by cyanobacteria is the exopolysaccharides. This compound creates microenvironments within the soil, which help them to survive to hinder conditions i.e., light intensity (Phélippé et al., 2019), nutrients starvation (Marchus et al., 2018), temperature (Wang et al., 2014a), moisture (Mager and Thomas, 2011), and humidity changes (Adessi et al., 2018). Exopolysaccharides are mainly composed of soluble and insoluble fractions of monosaccharides, depending on the species. Glucose is the most abundant compound, but xylose, arabinose, fucose, galactose, mannose, rhamnose, and glucosamine are found in several species (De Philippis and

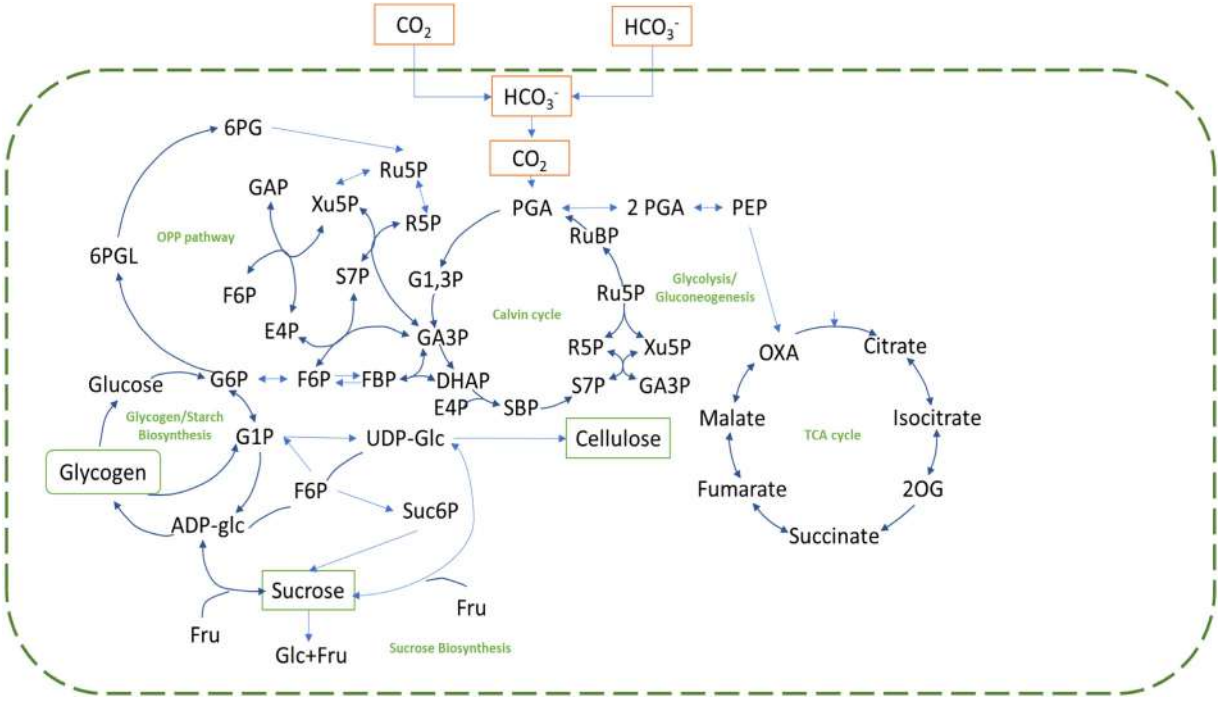

Fig. 1. Metabolic pathway in cyanobacteria related to polysaccharides metabolism. The pathways are reproduced from (Aikawa et al., 2015; Kamravamanesh et al., 2018; Quintana et al., 2011) with some modifications. All copyrights permission obtained. Abbreviations: AcCoA, acetyl-CoA; ADP-glc, ADP-glucose; DHAP, dihydroxyacetone phosphate; E4P, erythrose-4-phosphate; EtOH, ethanol; Fru, fructose; FBP, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; Glc, glucosa; GAP, glyceraldehyde-3-phosphate; G1P, glucose-1- phosphate; G1,3P, 1,3-bisphosphoglycerate; G6P, glucose-6-phosphate; 2OG, 2-oxoglutarate; OXA, oxaloacetate; PEP, phosphoenolpyruvate; 2PGA, 2-phosphoglycerate; 3PGA, 3-phosphoglycerate; 6PG, 6-phosphogluconate; 6PGL, 6-phosphogluconolactone; PYR, pyruvate; R5P, ribose-5-phosphate; RuBP, ribulose-1,5-bisphosphate; Ru5P, ribulose-5-phosphate; SBP, sedoheptulose-1,7-bisphosphate; S7P, sedoheptulose-7-phosphate; Suc6P, sucrose 6-phosphate; UDP-Glc Uridine diphosphate glucosa; Xu5P, xylulose-5- phosphate.

Vincenzini, 1998; Tiwari et al., 2020). Table 1 presents the accumulation of carbohydrates by different cyanobacteria and some green algae species under different growth conditions.

3.2. Cyanobacteria cultivation strategies for carbohydrate production

Cyanobacterial carbohydrates, compared to other higher plants or green algae, present certain advantages. For instance, cyanobacteria lack a cellulose cell wall but have cell walls composed of peptidoglycan, which can easily be degraded by fermentative bacteria or yeasts (Aikawa et al., 2015; Klanchui et al., 2018). Furthermore, as storage carbohydrates, glycogen is an excellent feedstock over starch, especially in the dark fermentation process. Carbohydrates production in cyanobacteria and other microalgae species occurs in response to several environmental factors. In controlled cultivations, many factors can cause stress in the cells and lead to cyanobacteria accumulating higher percentages of carbohydrates in dry cell weight. Cultivation of cyanobacteria with carbohydrate accumulation has been presented at the lab, pilot, and large scale. The most relevant strategies concerning cyanobacterial cultivation conditions are presented in the following sections.

3.2.1. Nutritional conditions

As observed in Table 1, the main strategies to optimize carbohydrate accumulation in cyanobacteria include nutritional factors such as nutrients limitation/depletion for nitrogen, phosphorus, sulfur, iron, and continuous addition of carbon. Other factors concern environmental conditions such as light intensity, pH, temperature, and salinity (Chen et al., 2013). All these strategies are used in green algae species as well. The primary approach for this purpose is mainly based on nitrogen and phosphorus depletion using inorganic carbon (Arias et al., 2020a). Relevant results on nitrogen limitation include the highest carbohydrate content (65–70%) (Arias et al., 2018b; Sassano et al., 2010), while phosphorus limitation also could result in high values up to 70% (Arias et al., 2018b). However, the maximum carbohydrate accumulation and periods of obtainment are species-dependent. For example, a reported study by Monshupanee and Incharoensakdi (2014) revealed that subjecting *Synechocystis* sp. PCC 6803 to 12 days of N and P starvation improved the carbohydrate accumulation by only 39% and 27%, respectively. Another nutrient such as sulfur has resulted in carbohydrate content of >50% in green algae species *Tetraselmis subcordiformis* and *Chlorella* sp. (Yao et al., 2012; Yuan et al., 2018).

The reason why N limitation/depletion in the culture is a good strategy is that it can propagate the conversion of fixed carbon in the Calvin cycle into storage molecules like lipids and carbohydrates instead of amino acids synthesis, hence, contributing to the enhancement of the NADH pool (Fig. 1). In the case of P, its limitation or depletion declines the ATP generation by degrading polyphosphate, creating an imbalance in the NADH:ATP ratio. Besides, P presence inhibits the process of activation of AGPase, the enzyme that catalyzes the ADP-glucose synthesis from glucose-1-phosphate, as previously mentioned (Rueda et al., 2020a).

3.2.2. Carbon source

Another crucial factor involved in carbohydrate accumulation is the availability and type of carbon source. The most common carbon sources are CO_2 or $NaHCO_3$, or NaH_2CO_3 addition. CO_2 is injected into the culture by gas bubbling. Once the gas is dissolved in the water, it is available in different forms in the function of pH: carbonic acid (H_2CO_3), bicarbonate (HCO_3^-), and carbonate (CO_3^{2-}) ions (De Farias Silva et al., 2017). In this sense, a pH ranging from 7 to 9 is necessary to ensure maximum dissociation. The availability of dissolved carbon is crucial for carbon conversion, and high concentrations of dissolved carbon can enhance storage carbon. Otherwise, the carbon stored in cells is the first component to be consumed (Arias et al., 2018a). Commonly, using an injection of 1%–5% is ideal for increasing the carbohydrate content (Möllers et al., 2014b; Rueda et al., 2020b). While the addition of $NaHCO_3$ or NaH_2CO_3 has resulted in up to 60%–70% carbohydrate accumulation (Arias et al., 2018b; De Farias Silva et al., 2016; Rueda et al., 2020b).

Table 1
Cyanobacteria and green algae growth conditions and carbohydrate accumulation.

Microorganism	Growth conditions	Stages	Carbon source	Hydraulic regime (cultivation/carbohydrate optimization)	Carbohydrate accumulation strategy	Dry cell weight ($g\ L^{-1}$)	Carbohydrates accumulation (%)	Ref.
Wastewater-borne cyanobacteria	N:P > 7, and synthetic	Two-stage	CO_2	Continuous/batch	N-depletion P-depletion	NA	69[b]	(Rueda et al., 2020b)
Synechocystis sp. and Synechococcus sp.	feast, and famine of inorganic carbon	Two-stage	CO_2-$NaHCO_3$	Batch/batch	Feast and famine	1	68.9[b]	(Rueda et al., 2020a)
Wastewater-borne cyanobacteria	220 μmolm^{-2} s^{-1}, 24 °C	Two-stage	$NaHCO_3$	Semi-continuous/ Batch	N- limitation/24:0 light: dark photoperiods N-limitation/12:12 h light:dark photoperiods P- limitation/24:0 light: dark photoperiods P-limitation/12:12 h light:dark photoperiods	0.97 0.99 1.62 1.35	62 75 46 36[b]	(Arias et al., 2018b)
Arthrospira platensis	SOT, 60–700 μmol m^{-2} s^{-1}	Two-stage	$NaHCO_3$	-/Batch	N- depletion	NA	65	(Aikawa et al., 2012)
Synechocystis sp. PCC 6803	BG11, $NaHCO_3$, 60 μmol m^{-2} s^{-1}	One-stage	$NaHCO_3$	-/Batch	N-depletion	NA	39	(Monshupanee and Incharoensakdi, 2014)
Dunaliella tertiolecta	60 μmol m^{-2} s^{-1} and 20–25 °C[a]	One-stage	Commercial cellulase and amyloglucosidase	Continuous	Pyrolysis temperatures	NA	40.5	(Kim et al., 2015)
Synechococcus elongatus PCC 7942	200 μmol m^{-2} s^{-1}, 28 °C, and 5% CO_2	Two-stage	Antibiotics	Batch	Overexpression of genes	NA	28	(Chow et al., 2015)
Scenedesmus bijugatus	Outdoor	Two-stage	Native freshwater	Semi-continuous	Two-step combined harvesting	NA	26	(Ashokkumar et al., 2015)
Chlorella sorokoniana	NA	One-stage	NA	Batch	Various pretreatment methods	NA	40.3	(Lorente et al., 2015)
Tribonema sp.	NA	One stage	NA	Batch	NA	NA	31.2	(Wang et al., 2014b)
Chlorella vulgaris KMMCC-9 UTEX26	150 μmol m^{-2} s^{-1}, 20–22 °C, Bubbling air[a]	One-stage	Na_2CO_3 (BBM)	Batch	N-limitation	NA	22.4	(Kim et al., 2014)
Synechococcus sp.	250 μmol m^{-2} s^{-1} and 1% CO_2	Two-stage	CO_2	-/Batch	N-limitation	3	59	(Möllers et al., 2014b)
Arthrospira platensis	150 μmol m^{-2} s^{-1}, 30 °C, Bubbling air[a]	Two-stage	Zarrouk medium	Semicontinuous	P- limitation	2–2.2	58	(Markou et al., 2013)
Chlorella vulgaris FSP-E	60 μmol m^{-2} s^{-1} and 2% CO_2	Two-stage	CO_2	Batch	N-limitation	7.5	52	(Ho et al., 2013a, 2013b)
Chlorella variabilis NC64A	150 μmol m^{-2} s^{-1}, 25 °C, and 2% CO_2	Two-stage	CO_2	Batch	N-limitation	0.43	37.8	(Cheng et al., 2013)
Scenedesmus obliquus CNW-N	210–230 m^{-2} s^{-1}, 28 °C, 300 rpm, and 2.5% CO_2	Two-stage	CO_2	Batch	N-limitation	4.5	51.8	(Ho et al., 2013b)
Dunaliella tertiolecta LB999	60 μmol m^{-2} s^{-1}, 20–25 °C, and 2% CO_2	One-stage	CO_2	Batch	Enzymatic action	NA	37.8	(Lee et al., 2013)
Chlorella sp. KR-1	80 μmol m^{-2} s^{-1},30 °C and 10% CO_2	NA	CO_2	NA	NA	NA	49.7	(Lee et al., 2013)
Scenedesmus dimorphus	50–1200, 25 °C and 2% CO_2	Two-stage	CO_2	Batch	N-limitation	5	45–50	(Wang et al., 2013)
Tetraselmis subcordiformis FACHB-1751	150 μmol m^{-2} s^{-1}, 25 °C and 3% CO_2	Two-stage	Artificial sea water	Semi- continuous	P-deprivation	4.5	40	(Yao et al., 2013)
Tetraselmis subcordiformis	200 μmol m^{-2} s^{-1}, 25 °C and 3% CO_2	Two-stage	Artificial sea water	Batch	N-deprivation S-deprivation	6	45–50	(Yao et al., 2012)
Scenedesmus obliquus	150 μmol m^{-2} s^{-11}, 25 °C, Bubbling air[a]	Two-stage	Bristol medium	Batch	Pre-treatment methods	NA	30	(Miranda et al., 2012)
Chlamydomonas fasciata Ettl 437	3000 Lux, 25 °C — 0.4 vvm CO_2	One-stage	CO_2	Batch	Ultrasonic treatment	NA	43.5	(Asada et al., 2012)
Leptolyngbya sp.	200 μmol m^{-2} s^{-1}, 28 °C,	Two-stage	Wastewater	Batch	Pre-treatment method	NA	40	(Tsolcha et al., 2021)
Chlorella sp.	1000 μmol photons m^{-2} s^{-1}, 28 ± 0.05 °C, 10% CO_2	Two-stage	CO_2	Semi-continuous	N-deprivation P-deprivation S-deprivation	NA	57–67	(Yuan et al., 2018)
Synechococcus PCC 7002	100 ± 5 μmol photons m-2 s — 1, 28 °C	Two-stage	$NaHCO_3$	Batch/Batch		6 g L^{-1}	25	(De Farias Silva et al., 2016)
Synechococcus PCC 7002	100 μE *m* $^{-2}$ *s* $^{-1,}$ 28 °C	One stage	CO_2	Batch/Batch	Urban wastewater	NA	60	(de Farias Silva et al., 2020)
Wastewater-borne cyanobacteria	343 W/m^2	Two-stage	$NaHCO_3$	Sequencing batch reactor/ batch	Previous feast and famine, N limitation	NA	48	(Arias et al., 2018a)

NA: not applicable.
[a] CO_2 from the air.
[b] VSS = volatile suspended solids.

Regarding the addition regime for carbon addition, in most studies, either CO_2 or bicarbonate is added continuously (CO_2) or in high concentration ($NaHCO_3$) to the culture to ensure carbon availability. In this context, maximum carbohydrate accumulation is obtained after several days, which indicates a low carbon uptake efficiency. Very few studies have been directed towards enhancing the carbon uptake in cyanobacteria. The most exceptional strategy is the one based on unbalanced growth, also called feast and famine (Fig. 2). This process consists of carbon addition during a short period (feast) and followed by a period without any carbon source (famine). Both processes are carried out during the light phase. The feast and famine strategy was firstly applied for wastewater-borne cyanobacteria in the study of Arias et al. (2018a), where cyanobacteria were submitted to an intermittent carbon addition (as $NaHCO_3$) in the cultivation phase (Fig. 2a). This operational condition improved carbohydrate accumulation to 48% in only 48 h of cultivation in a subsequent batch process. In consideration of these results, the feast and famine strategy was later applied in cyanobacteria monocultures (Rueda et al., 2020a) and full-scale photobioreactors (Rueda et al., 2020b), achieving carbohydrate contents up to 69% (Fig. 2b and c).

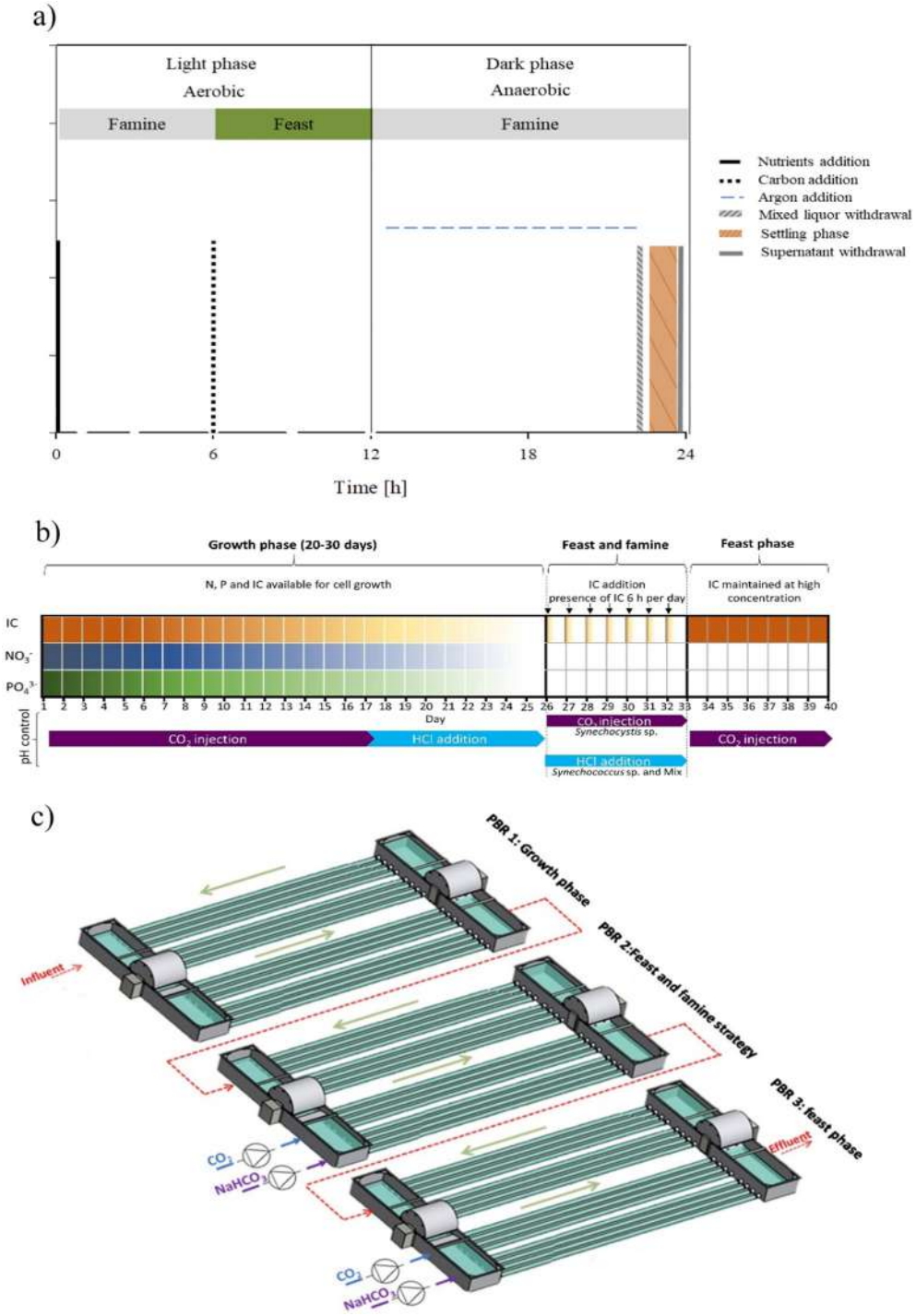

Fig. 2. Feast and famine strategies employed in the studies of a) (Arias et al., 2018a), b) (Rueda et al., 2020a), and c) (Rueda et al., 2020b). Copyrights permission obtained.

3.3. Operational conditions influencing carbohydrate-rich cyanobacterial biomass

While the limitation of nutrients is the most promising approach to achieve high carbohydrate content, the lack of nutrients, especially N or P, affects biomass productivity. The optimization of cyanobacterial biomass for carbohydrate production is frequently carried out in two different methods or stages to maintain the maximum biomass production. In the first stage, cyanobacteria are cultivated in a nutrient-rich growth medium to promote the highest cell concentration. Subsequently, the biomass is submitted to a second stage, with a nutrient-deprived medium, to increase the carbohydrate content. Successful biomass cultivation and carbohydrate accumulation depend on nutritional conditions and several factors concerning operational conditions, such as photobioreactor type, illumination, hydraulic regimes, and cultivation type.

3.3.1. Photobioreactor design

In the last decade, several configurations of open and closed reactors for microalgal cultivation were designed to increase biomass production (Posadas et al., 2014). Open reactors (i.e., raceways) are widely used for large-scale processes of bioactive materials, such as pharmaceuticals, oils, cosmetics, and functional foods (Mimouni et al., 2012). This type of system consists of shallow depth tanks, agitated mechanically by paddlewheels. Raceways present several advantages in terms of operation and costs; for instance, they require small amounts of energy because of mechanical agitation. The low depth and mechanical agitation allow light to penetrate uniformly throughout the tank, keeping suspended the culture, preventing sedimentation, and ensuring a well-mixed culture. The carbon requirement for cyanobacterial growth is usually provided by sparging with air and, in some cases, with CO_2 to avoid carbon limitation.

However, this system also presents several limitations, including a poor mass transfer, resulting in low biomass production. Notably, evaporative losses, CO_2 exchange to the atmosphere, the occurrence of other microorganisms as predators, and other fast-growing heterotrophic bacteria restrict this system's faster commercialization (Mendoza et al., 2013). The closed reactors, such as flat-plate, bubble columns, airlift reactors, stirred–tank, tubular, and internally illuminated reactors, have increased interest in the last years because it presents several advantages that overcome raceways' limitations. These systems have shown better mixing efficiency, higher CO_2, and light transfer rates, with improved biomass productivity (Ketheesan and Nirmalakhandan, 2012). So far, several photobioreactors have been developed, but only a few of them can be used for large-scale cultivation. Some reviews have reported the advances in the literature (Johnson et al., 2018; Lindblad et al., 2019; Saha and Murray, 2018). To the best of our knowledge, there are no studies related to the influence of the photobioreactor type on the accumulation of carbohydrates. Also, the accumulation of polymers or other compounds is more related to operational issues such as the hydraulic regime as discussed in the next section.

3.3.2. Hydraulic regimes

So far, the batch regime is the most used strategy to accumulate carbohydrates regardless of the type of growth medium. This hydraulic regime consists of adding a substrate to the photobioreactor, and the product remains in the reactor during a specific period. Although this cultivation regime has the advantage of high conversion rates, it has several disadvantages related to high costs and low production areas, which is difficult for large-scale implementation (de Farias Silva and Sforza, 2016). The second most employed hydraulic regimes are the semi-continuous operation, often called semi-batch. This operation consists of a single feeding and effluent removal per day, which helps select microorganisms with the highest affinity for nutrients and pressure the microorganisms to rapidly uptake the nutrients or carbon. This operation has been successful in carbohydrate accumulation, achieving similar percentages to batch cultures (Yao et al., 2013; Yuan et al., 2018). However, this process is also considered unpractical and is barely observed in full-scale processes. Conversely, the continuous regime is so far the most convenient operation applied in full-scale closed and open systems to cultivate several cyanobacterial or microalgal species. Nonetheless, very few studies have applied this hydraulic regime for carbohydrate accumulation, primarily for green algae species *Chlorella vulgaris* (de Farias Silva and Sforza, 2016), *Tetradesmus obliquus* (de Farias Silva et al., 2018), and *Dunaliella tertiolecta* (Kim et al., 2015), achieving a carbohydrate content higher than 40%. Despite being recognized that continuous cultivation can maximize microalgal biomass productivity under complete nutrients availability, very low biomass productivity is achieved when operated under N-depleted growth culture. In this sense, de Farias Silva et al. (2018) recommend optimizing the inlet nitrogen concentration to find an equilibrium between carbohydrate accumulation and biomass growth, which can be translated into higher yields.

Another less conventional regime used in green algae/cyanobacteria cultivation is the sequencing batch operation (Arias et al., 2019; Van Den Hende et al., 2016a). Like occurring in semi-continuous, this regime consists of a single feeding and effluent removal per day but with uncoupled SRT and HRT, which involves a settling period between removing the mixed liquor and supernatant and the feeding. This operation provides several advantages related to nutrients and species control and enhances easy-settling aggregates (Arias et al., 2018a). It must be noticed that this operation has been applied in successful pilot case studies (Van Den Hende et al., 2016a, 2016b) and used in conventional wastewater treatment plants.

3.3.3. Illumination

Light conditions as the light/dark photoperiods and light intensities significantly influence all physiological processes and cell cycle progression in all photosynthetic microorganisms (Aikawa et al., 2012; Yuan et al., 2018). Specifically, when light is limiting in the culture, cyanobacteria up-regulate their OPP pathway to consume more glucose and reduce its net CO_2 fixation rate (Wan et al., 2015). Light intensities requirements likely are species-dependent, observing the light conditions in all the studies shown in Table 1. A few studies have investigated the correlation of high intensities with higher carbohydrate accumulation. For instance, Aikawa et al., 2012 investigated *Arthrospira (Spirulina) platensis*, evaluating carbohydrate accumulation in different light intensities (20–700 μmol photons m^{-2} s^{-1}). In this study, *A. platensis* achieved the maximum glycogen of 65% at the highest light intensity 700 μmol photons m^{-2} s^{-1}. In the case of photoperiods, investigations by (Arias et al., 2018b) and (de Farias Silva et al., 2018) evaluated the effect of photoperiods in cyanobacteria and green algae, respectively. They concluded that biomass and carbohydrate production decreases significantly during the dark phase, likely because of the respiration processes. In the case of cyanobacteria, the carbohydrate content achieved a maximum of 74% of carbohydrate under 12 h permanent illumination after 12 days of incubation, whereas a maximum content of 63% was reached under light/dark periods in only eight days of incubation, this pattern was also observed in the biomass production (Arias et al., 2018b). It must establish that obtaining the maximum carbohydrate content under circadian cycles is crucial for the further up-scale implementation of the process under outdoor conditions.

3.4. Carbohydrate production in cyanobacteria-based wastewater-treatment

Wastewater is considered the cheapest substrate for algal biomass cultivation. From the first studies in the '50s, wastewater from different sources, especially from urban effluents, has been successfully used to grow microalgae in HRAP and photobioreactors (Emparan et al.,

2020b; Hernández-García et al., 2019). Although the balance of nutrients in the wastewater allows high biomass productivity, the carbohydrate content is usually low (Arcila and Buitrón, 2016; Arias et al., 2018c). One strategy until now is to use the two-stage process. In the first stage, cyanobacteria are cultivated in wastewater, and an optimization process with a growth medium follows this process in a batch reactor. This same strategy has been used in the lab (Arias et al., 2018b), pilot (Phélippé et al., 2019), and even large-scale studies (Rueda et al., 2020b).

Some studies have been focused on optimizing the carbohydrate content in one-stage wastewater (Table 2). Relevant studies include the cultivation of *Synechococcus* PCC 7002 in urban effluents with CO_2 addition, achieving 60% of carbohydrate content while reaching >80% of the removal rate of COD, N, and P in a batch study (de Farias Silva et al., 2020). Another interesting study was performed in a semi-continuous operation, in which soil cyanobacteria cultures were also cultivated in urban wastewater without CO_2 addition, evaluating the impact of different carbon and nutrient loadings. It was reported that low loadings and long hydraulic retention times (HRT) improved carbohydrate accumulation up to 46% (Arias et al., 2020b). The study of Sánchez-Contreras et al. (2021), revealed that industrial effluents could be a promising alternative to operate in high loads of wastewater. In this sense, high C:N of industrial wastewater can provide an unbalance in the nutrients to maintain high carbohydrate production without decreasing biomass productivity.

4. Biofuels from cyanobacterial carbohydrates

Genetic modifications propagated the generation of several kinds of biofuels in the last two decades because of the structural simplicity of cyanobacteria that allows easy genetic engineering. A myriad of research has targeted the utilization of mainly *Synechococcus* sp. and *Synechocystis* sp. as model cyanobacteria to produce several biofuels, such as free fatty acids, isoprene (Lindberg et al., 2010), isoprene hydrocarbons, ethanol (Miao et al., 2017), isobutyraldehyde, hydrogen (Srirangan et al., 2011), isobutanol (Nozzi et al., 2013), and 1-butanol (Gao et al., 2017). Although there is a notable metabolic pathways modification advancement in many research programs, however, low product yields are generally obtained (Ducat et al., 2011; McEwen and Atsumi, 2012). Thus, in the last few years, research attention was focused on biofuels' indirect production like bioethanol, butanol, and biohydrogen using cyanobacterial carbohydrates as a substrate for yeasts or fermentative bacteria. Advances in biofuels through metabolic routes of yeasts or fermentative bacteria and cyanobacterial metabolism are presented in the following sections.

4.1. Bioethanol production

Bioethanol, which is an alcohol, is mostly produced as a microbial metabolite, mainly from yeasts or some bacteria, such as *Saccharomyces cerevisiae*, and *Escherichia coli*, respectively, according to the following metabolic route (Fig. 3). So far, bioethanol is typically produced by fermenting crops such as sugarcane or lignocellulosic materials like paddy straw and thatch grass (Ho et al., 2013a, 2013b). However, using these crops competes with human food supplement production, thus contributing to food prices increase and generally increases the cost of production. Notably, bioethanol production from cyanobacteria can be broadly categorized into three-stage bioethanol production and single-stage production by genetic engineering.

4.1.1. Three-stage bioethanol production

At present, cyanobacteria conversion into bioethanol is increasing attention as a future biofuel feedstock to replace first- and second-generation biofuels (El-gamal and Tohamy, 2019). The green algae and cyanobacteria have simple cell wall structures compared to lignocellulosic land plants, as discussed in the previous section. However, cyanobacterial carbohydrates must be firstly hydrolyzed or saccharified to simple sugars to be available for yeasts. For this reason, cyanobacterial ethanol followed three unit operations similar to lignocellulosic bioethanol production, including the pre-treatment, fermentative stage, and distillation (El-gamal and Tohamy, 2019) (Fig. 3). In the case of pre-treatment, several methods have been tested for cyanobacterial carbohydrate hydrolysis or saccharification. Notably, enzymatic and chemical hydrolysis is the most common approach using mainly *Saccharomyces cerevisiae* (Table 3) (Markou et al., 2013; Rempel et al., 2019). In chemical hydrolysis, Markou et al. (2013) conducted a study on *Arthrospira platensis* carbohydrate hydrolysis employing four acids (H_2SO_4, HNO_3, HCl, and H_3PO_4), testing different conditions. Hydrolysis, based on chemical addition, although represents a cost-effective option, however, these chemicals can often lead to inhibition in the fermentative process without an optimized treatment (Castro et al., 2015). On the other hand, enzymatic saccharification is also recently recommended to cyanobacterial biomass as a straightforward method. Taking advantage of cyanobacteria's cell wall, that is

Table 2
Investigations of wastewater treatment with cyanobacteria and green algae for coupled biomass and carbohydrate production.

Cyanobacteria/green algae species dominating the culture	Wastewater	Carbohydrate production stages	Carbohydrate optimization treatment	Hydraulic regime	HRT (days)	Carbohydrates (% dcw)	Maximum biomass Concentration ($g\ L\ d^{-1}$)	Ref.
Gleiterinema sp.				semi-continuous	10	54	27	Sánchez-Contreras et al. (2021)
	Industrial	One-stage	NA	semi-continuous	8	57	33	
				semi-continuous	6	40	54	
Tetraselmis suecica				semi-continuous	10	9.13	0.65	Andreotti et al. (2020)
	Aquaculture	One-stage	NA	semi-continuous	7	4.47	0.49	Andreotti et al. (2020)
Parachlorella kessleri QWY28	Synthetic swine wastewater	One-stage	CO_2 injection	Batch	NA	54	6	Qu et al. (2019)
Desmodesmus spp.	Landfill leachate	One-stage	NA	Batch	NA	41	NA	Hernández-García et al. (2019)
Cyanobacteria dominated culture	Agricultural runoff	Two-stage	Remaining nutrients from cultivation phase with $NaHCO_3$ and CO_2 addition	semi-continuous	NA	69	0.3	Rueda et al. (2020b)
Soil cyanobacteria	Urban wastewater	One-stage	NA	semi-continuous	10	48	0.75	Arias et al. (2020b)
					8		0.54	
					6		0.32	
Synechococcus PCC 7002	Urban effluents	One-stage	CO_2 injection	Continuous	NA	60	NA	de Farias Silva et al. (2020)

NA: Not applicable.

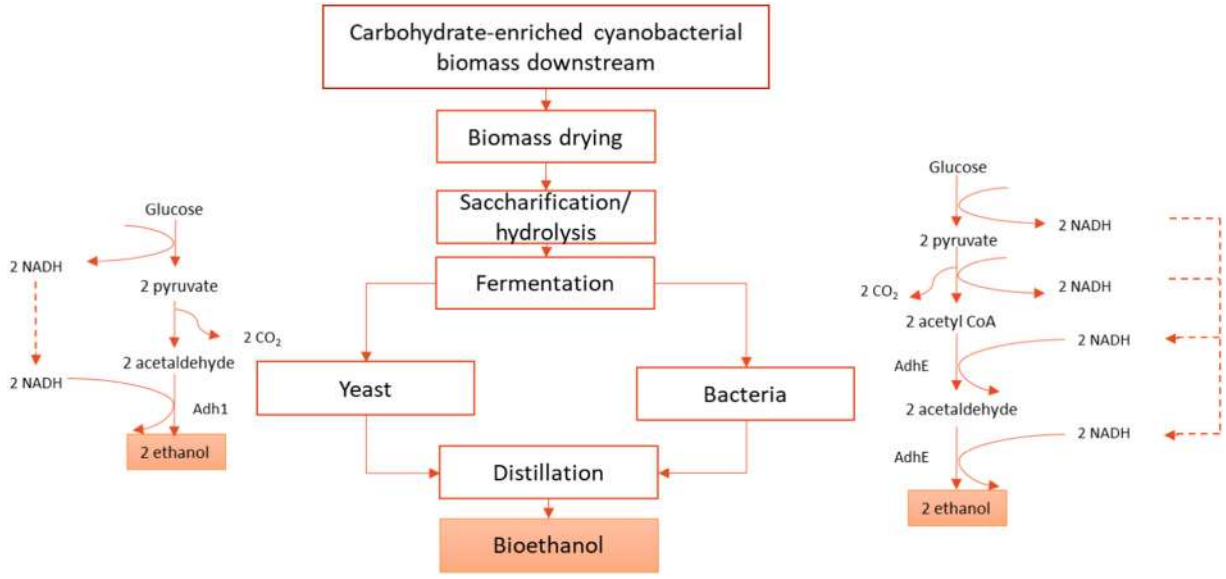

Fig. 3. Scheme of the production of bioethanol through fermentative processes with metabolic pathways in yeast and bacteria. Abbreviations: Adh1, alcoholdehydrogenase 1; AdhE, bifunctional CoA-dependent ethanol/aldehyde dehydrogenase.

less complex and less diverse than green algae, and have lack of lignin, hemicellulose, and even cellulose is a noteworthy endeavor. Enzymatic hydrolysis has shown different behaviors depending on the species, and the high cost of the process still limits their use.

4.1.2. One-stage bioethanol production by genetic engineering

Other studies also tried to make this process simultaneous using genetic biotechnology. In the studies of Aikawa et al. (2013, 2018), they used a recombinant yeast *S. cerevisiae* strain, which was able to keep α-amylase from the bacteria *Streptococcus bovis* and also exhibit glucoamylase from *Rhizopus oryzae*. They tested it on *A. platensis* surface to eliminate the need for biomass pre-treatment and amylase hydrolysis, with or without $CaCl_2$. This way, they directly converted *A. platensis* biomass to ethanol in a yield of 32% (gEtOH g^{-1} Biomass).

Other biotechnological efforts include ethanol production from genetically modified cyanobacteria strains, thus avoiding the two processes, hydrolysis and yeast fermentation (Dexter et al., 2015). Dienst et al. (2014) proposed the insertion of genes from *Zymomonas mobilis*

Table 3
Reported bioethanol production from different cyanobacteria species with remarks.

Cyanobacteria	Pre-treatment (hydrolysis)	Temp (°C)	Time (h)	Fermenter	Bioethanol yield (%)	Remarks	Ref.
Arthrospira (Spirulina) platensis	NA	30	96	*Saccharomyces cerevisiae* MT8-1dGS	86	Lysozyme was used to promote cell disruption to release glucose faster than it is consumed.	(Aikawa et al., 2013)
Synechococcus elongatus PCC7942	Acid (H_2SO_4)	30	48	*Zymomonas mobilis* ATCC 29191	91	The bacteria were engineered using co-expressing RNA of ictB, ecaA, and acsAB to increase the carbohydrate yield.	(Chow et al., 2015)
Synechococcus sp. PCC 7002	Enzymatic (lysozyme and two alpha-glucanases)	34	48	*Saccharomyces cerevisiae*	90	About 60% carbohydrate per dry weight basis accumulated under nitrogen-limited culture.	(Möllers et al., 2014b)
Synechocystis sp. PCC6803 genetically engineered	NA	NA	432	–	0.6084 (v/v) ethanol	Ethanol production delayed carbohydrate accumulation by 40%, however, microarray analysis revealed three dominant mRNAs (cpcB, adhA, and rps8) that strongly modified accumulation level.	(Dienst et al., 2014)
Synechocystis sp. PCC 6803	NA	27–29	144	*Zymomonas mobilis*	5.2 mmol OD_{730} $unit^{-1}$ L^{-1} day^{-1}	Cyanobacteria can autotrophically convert CO_2 into ethanol. It was performed using a double homologous recombination system to integrate the pyruvate decarboxylase (pdc) and alcohol dehydrogenase II (adh) genes under a light-driven psbAII promoter.	(Dexter and Fu, 2009)
Synechocystis strain Syn-HZ24	NA	30	240	–	0.9 g L^{-1}	The pH increasing strategy was used to suppress the effect of *Pannonibacter phragmitetus* that inhibited ethanol production. A Bicarbonate-based Integrated Carbon Capture System (BICCS) was designed and 180 mM $NaHCO_3$ was used to maintain the pH around 11.	(Zhu et al., 2017b)
Anabaena variabilis	Acid (2 N H_2SO_4)	30	80	*Saccharomyces cerevisiae*	28.2	It is a nitrogen fixer cyanobacteria and the carbohydrate was promoted via a biphasic phosphate-starved strategy reaching 63.4% carbohydrate yield.	(Deb et al., 2019)
Microcystis aeruginosa	Acid (2 N H_2SO_4)	30	80	*Saccharomyces cerevisiae*	23.9	The carbohydrate yield increased from 23.4 to 55.1% when the biphasic phosphate-starved strategy was implemented.	(Deb et al., 2019)
Arthrospira platensis	Amylolytic enzymes	38	168	*Saccharomyces cerevisiae*	93	In the presence of lysozyme, a recombinant yeast expressing α-amylase and glucoamylase successfully converted *A. platensis* directly to ethanol. However, ethanol productivity was increased by $CaCl_2$, which helped to delaminate the polysaccharide layer on the cell surface of *Arthrospira platensis*.	(Aikawa et al., 2018)

Note: The % yield was calculated based on the theoretical yield of ethanol. NA: Not applicable.

to codify for pyruvate decarboxylase and alcohol dehydrogenase to *Synechocystis* sp. PCC6803 strain. However, the application of these approaches is under continuous development, and further advances are necessary to reach better performances and upscale production.

4.2. Biobutanol

Another type of fuel that can be obtained from cyanobacteria is biobutanol. This type of fuel can be produced from *Clostridial* species via fermentative processing using the three-staged acetone-butanol-ethanol (ABE) process (Kushwaha et al., 2020b; Wang et al., 2014c) and also by single-stage genetically engineered cyanobacteria (Hendry et al., 2020).

4.2.1. Three-stage biobutanol production

ABE fermentation using *Clostridium* sp., especially *Clostridium acetobutylicum* and *Clostridium beijerinckii*, has been used to ferment simple and complex sugars, as well as gases, such as CO_2, H_2, and CO into butanol, producing also acetone and ethanol in the process (Wang et al., 2014c). Two different CoA-dependent pathways can produce butanol in *Clostridia* sp., according to Lan and Liao (2011). One of the pathways is called a synthetic 2-ketoacid mechanism using intermediates from amino acid biosynthesis routes. It briefly consists of the decarboxylation of a non-natural intermediate, 2-ketovalerate, and then its further reduction to 1-butanol. The other path, also known as the CoA-dependent pathway, consists of the synthesis of butyryl-CoA from acetyl-CoA and the subsequent reduction of butyryl-CoA to 1-butanol (Fig. 4). The viability of the microorganism's performance is mainly through an efficient enzymatic activity that could easily break carbohydrate polymers into monomers (Pugazhendhi et al., 2019). In general, cyanobacterial carbohydrates as a substrate for ABE fermentation present several advantages over second-generation feedstocks (Wang et al., 2014c). For instance, microalgae as a substrate without lignin and lower amounts of hemicelluloses help the current method for ABE fermentation with little or no modification. This way, the techniques used for saccharification processes, based on energy-intensive pretreatment and hydrolysis treatments, may not be necessary for cyanobacteria (Kucharska et al., 2018).

Similarly, like ethanol, ABE's downstream consists of three steps: drying of biomass, further hydrolysis, and fermentation (Arabi et al., 2019). So far, most of the studies regarding this fuel were carried out with carbohydrate-enriched green algae *Chlorella* sp. (Wang et al., 2014a) and *Neochloris aquatica* (Wang et al., 2017), reaching butanol concentrations, butanol yields, and butanol productivities up to 12 g L^{-1}, 0.6 mol mol^{-1} sugar (0.25 g g^{-1} sugar), and 0.89 g L^{-1} h^{-1}, respectively. Kushwaha et al. (2020), recently used cyanobacterial hydrolysates of *Oscillatoria obscura* and macroalgae *Lyngbya limnetica* with *Clostridium beijerinckii* ATCC 35702 as the fermenting microorganisms for biobutanol production. They obtained maximum biobutanol productivity of 1.565 g L^{-1} d and a butanol yield of 0.421 g butanol g^{-1} sugar, whereas, fermenting *Oscillatoria obscura* reached productivity of 0.826 g L^{-1} d^{-1} and a yield of 35% g of butanol g^{-1} sugar.

4.2.2. One-stage biobutanol production by genetic engineering

Advances in biotechnology have exploited genetic modification of cyanobacteria species by inserting gene expressions to perform CoA-dependent pathways similar to *Clostridia* species for biobutanol production (Hendry et al., 2020; Lan and Liao, 2011) (Fig. 5). This genetic modification has been challenging, because the anaerobic nature of these pathways expressed in oxygenic phototrophs microorganisms as cyanobacteria, negatively affects the enzymes involved. Hence, cyanobacteria can be subjected to anoxic conditions, and accumulate butanol from stored sugars (Wagner et al., 2019). Most of the investigations have been performed in two cyanobacteria model strains: *Synechococcus elongatus* PCC 7942 and *Synechocystis* PCC 6803. In the study of Lan and Liao (2011), *S. elongatus* PCC 7942 accumulated 14.5 mg L^{-1} butanol under anoxic conditions after seven days of culture. Also, Anfelt et al. (2015), tested *Synechocystis* sp. PCC 6803, obtaining n-butanol yield of 35 mg per g of biomass and productivity of 2.7 mg/g biomass d^{-1}.

4.3. Biohydrogen

Among the various biohydrogen production methods, the direct or indirect use of cyanobacterial carbohydrates through dark fermentation or indirect biophotolysis to synthesize biohydrogen is a noteworthy endeavor (Bandyopadhyay et al., 2010). Both processes are carried out by several heterotrophic bacteria or cyanobacterial species that possess nitrogenase or hydrogenase enzymes (Mishra et al., 2018). Dark fermentation is performed by anaerobic fermentative bacteria, such as

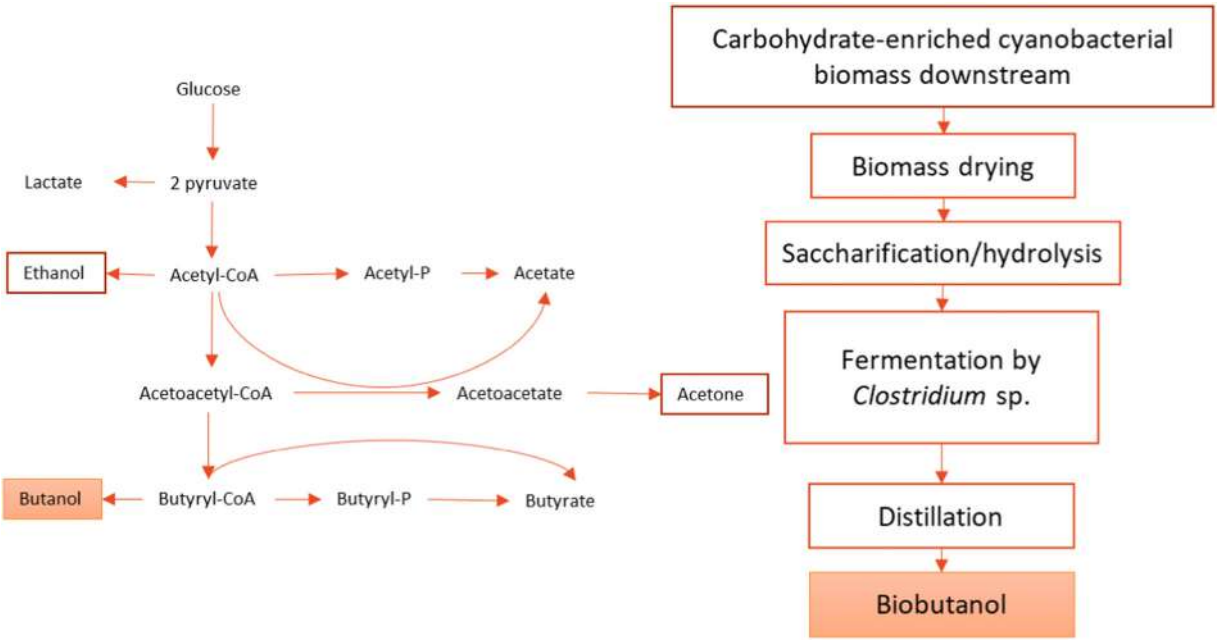

Fig. 4. Scheme of the production of biobutanol through ABE fermentative processes with simplified metabolic pathways of *Clostridium acetobutylicum* (Buehler and Mesbah, 2016).

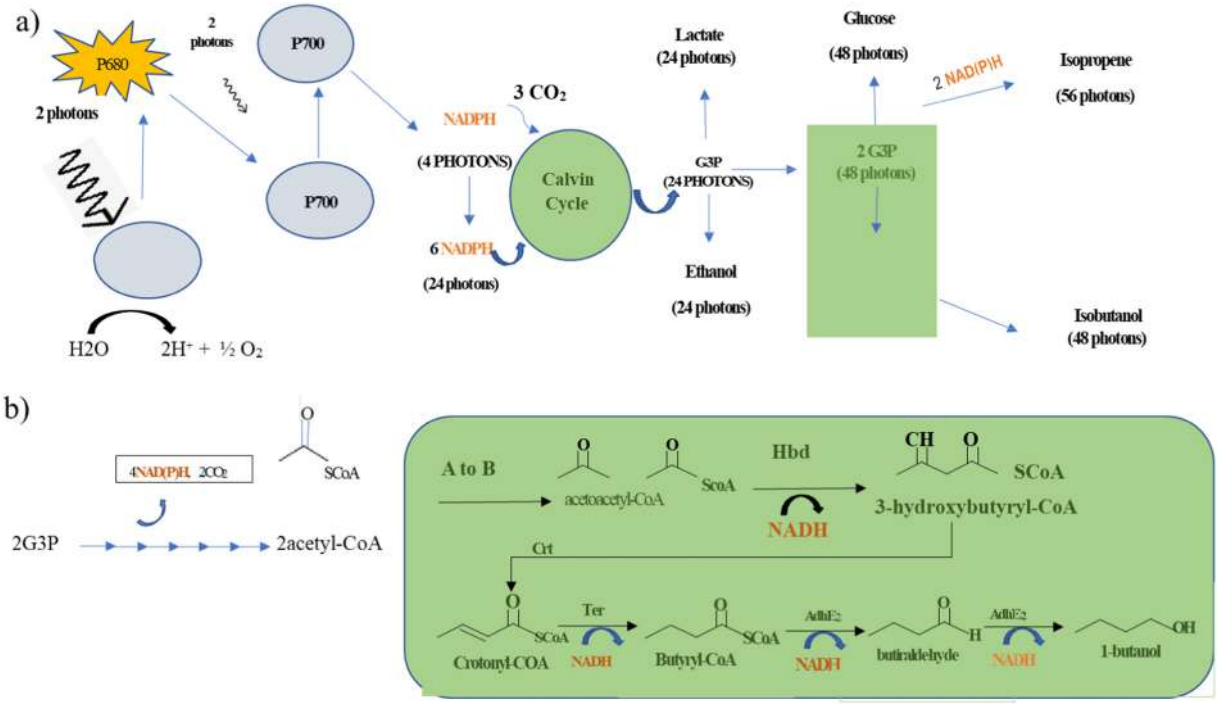

Fig. 5. 1-butanol production in engineered *S. elongatus* PCC 7942. a) light reaction provides NADPH as the reductant for carbon fixation into bioproducts, b) the engineered *S. elongatus* 7942 contains a heterologous expression of five enzymes for the conversion of acetyl-CoA to 1-butanol. Abbreviations: AtoB, acetyl-CoA acetyltransferase; Hbd, 3-hydroxybutyryl-CoA dehydrogenase; Crt, crotonase; Ter, trans-2-enoyl-CoA reductase; and AdhE2, bifunctional aldehyde/alcohol dehydrogenase. Figure recreated from Lan and Liao (2011). Copyrights permission obtained.

Clostridium sp. and *Enterobacter* sp., involving the light-independent anaerobic fermentation of carbohydrates or other organic substrates (Srirangan et al., 2011). In this process, proton-accepting electrons generated from carbohydrate oxidation to form hydrogen and fermentable sugars content are considered a crucial indicator for effective hydrogen production via the fermentative process (Xia and Murphy, 2016). Theoretically, the maximal amount of molecular hydrogen per mole of glucose is 12 mol as shown in Eqs. (1), (2), and (3):

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 12H_2 \quad (1)$$

$$C_6H_{12}O_6 + 6H_2O \rightarrow 2CO_2 + 2CH_3COOH + 4H_2 \quad (2)$$

$$C_6H_{12}O_6 + 6H_2O \rightarrow 2\,CO_2 + CH_3CH_2CH_2COOH + 2H_2 \quad (3)$$

Like cyanobacteria's benefits on bioethanol and biobutanol production, the lack of lignocellulosic material in cyanobacteria would benefit fermentation efficiency. Most conducted studies in this field have been performed with green algae (Batista et al., 2015; Nobre et al., 2013), but few studies using cyanobacteria species to produce biohydrogen has been reported with hydrothermal/steam acid pretreatment to improve the yield (Table 4). Notably, although the carbohydrate content is very low in some cases, the H_2 yields obtained by other biomasses or wastes are comparable (Łukajtis et al., 2018).

Conversely, cyanobacteria species can perform indirect biophotolysis, in which they auto-ferment their own stored carbohydrates through metabolic pathways (Aikawa et al., 2013). Table 5 shows hydrogen production from cyanobacteria through indirect biophotolysis. In this case, H_2 is produced and takes up/oxidize through the action of up to two different enzymes namely nitrogenases (only present in N-fixing cyanobacteria) and hydrogenases (Carrieri et al., 2010; Nyberg et al., 2015). Thus, the highest hydrogen yield depends on cyanobacteria species and environmental conditions (Khetkorn et al., 2010).

In H_2 production by nitrogenase activity, oxygen generation and hydrogen evolution are separated by the light intensity fluctuation. Then, nitrogen fixation occurs in cyanobacteria by heterocyst cells, under depleted oxygen conditions with reducing agents derived from the gluconeogenesis pathway, and the enzyme nitrogenase produces H_2 as a byproduct (Baebprasert et al., 2010). The enzyme nitrogenase is activated when heterocysts are subjected to an N_2-depleted medium

Table 4
Hydrogen production by dark fermentation of carbohydrate-enriched cyanobacterial biomass.

Cyanobacteria/green algae species dominating the culture	Carbohydrate content	Pre-treatment	Process	Hydrogen yield	Bacteria in the dark fermentation process	Ref.
Microcystis wesenbergii and *Microcystis aeruginosaare*	12	Microwave heating with H_2SO_4	Dark fermentation + Photo fermentation	256.74 (mL/gTVS)	Hydrogen producing bacteria and Photosynthetic bacteria	(Cheng et al., 2014)
	13	Steam heating with dilute H_2SO_4	Dark fermentation	18.63 (mL/gTVS)	*Clostridium butyricum*	(Cheng et al., 2019)
Microcystis aeruginosaare	13	Hydrothermal with dilute H_2SO_4	Dark fermentation	24.96 (mL/gTVS)	*Clostridium butyricum*	(Cheng et al., 2019)
Arthrospira platensis	19	2.5% dilute H_2SO_4 at 135 °C for 15 min,	Dark fermentation	85.0 mL/g VS	mixed anaerobic fermentative bacteria	(Xia et al., 2016)
Anabaena sp.	NA	NA	Dark fermentation	0.0114 kgH_2/kgbiomass	*Enterobacter aerogenes*	(Ferreira et al., 2012)

NA: Not applicable.

Table 5
Reported evolution of biohydrogen produced from different cyanobacteria strains through indirect biophotolysis.

Organisms	Description	Maximum hydrogen yield	Growth conditions	H_2 production conditions	Ref.
Arthrospira (Spirulina) maxima	Filamentous cyanobacteria	1.54 mmol H_2 g dry w^{-1} h^{-1}	Air, 40 μE m^{-2} s^{-1}, 30 °C,	Ar, (66 mM) Na_2CO_3, 6.5 g (77.4 mM) $NaHCO_3$, with a final Na (210 mM), 30 °C, 40 μE m^{-2} s^{-1}	(Carrieri et al., 2010).
Anabaena siamensis TISTR8012	Heterocyst filamentous cyanobacteria	8.68 mmol H_2 g dry w^{-1} d^{-1}	Air, 40 μE m^{-2} s^{-1}, 30 °C.	Ar, 30 °C, 40 μE m^{-2} s^{-1}	(Khetkorn et al., 2010).
Nostoc punctiforme ATCC 29133	Heterocyst filamentous cyanobacteria	20.7 mmol/g dry wt/d	Air, 40 μE m^{-2} s^{-1}, 30 °C.	Ar, 30 °C, 40 μE m^{-2} s^{-1}	(Khetkorn et al., 2010).
Synechocystis PCC 6803	Coccal cyanobacteria	0.02 mmol H_2 g dry w^{-1} d^{-1}	Air, 40 μE m^{-2} s^{-1}, 30 °C.	Ar, 30 °C, 40 μE m^{-2} s^{-1}	(Khetkorn et al., 2010).
Synechocystis PCC 6803	Coccal cyanobacteria	8.1 mmol H_2 mg chl a^{-1} h^{-1}	Air, 30 μE m^{-2} s^{-1}	Ar, 70 °C; 14 μE m^{-2} s^{-1}	(Baebprasert et al., 2010)
Nostoc PCC 7120	Heterocyst filamentous cyanobacteria	0.85 mmol H_2 mg chl a^{-1} h^{-1}	Air, 44 mmol photons m^{-2} s^{-1}	20% Ar in nitrogen (20Ar/80N_2) alternating, 44 mmol photons m^{-2} s^{-1}	(Nyberg et al., 2015)
Anabaena sp. CH3	Heterocyst filamentous cyanobacteria	1.6 mmol H_2	Air, 4% CO_2, 182 mmol photons m^{-2} s^{-1}, 25 °C	Ar, 4 °C, fructose, 130 mmol photons m^{-2} s^{-1}, 25 °C	(Chen et al., 2008)
Synechocystis sp. PCC 6803	Coccal cyanobacteria	0.81 mmol H_2 mg chl a^{-1} h^{-1}	Air. 30 °C, 50 μE m^{-2} s^{-1}	Ar, 50 μE $m^{2-1}s^{-1}$, 50 μE m^{-2} s^{-1}	(Burrows et al., 2008)
A. maxima sp. CS-328	Filamentous cyanobacteria	280 mL H_2/g DW	Air, 30 °C, 30–70 μE m^{-2} s^{-1}	Ar, dark, 30 °C,	(Ananyev et al., 2012)
ΔhupL mutant of Anabaena sp. *strain PCC 7120*	Heterocyst filamentous cyanobacteria	30 μmol H_2 mg chl a^{-1} h^{-1}	Air, 30 μmol photons m^{-2} s^{-1}, 25 °C	Alginate films, Ar, 6% and 3% CO_2, 13 to ~209 μmol photons m^{-2} s^{-1}	(Kosourov et al., 2017)
Calothrix sp.	Heterocyst filamentous cyanobacteria	7 μmol H_2 mg chl a^{-1} h^{-1}	Air, 30 μmol photons m^{-2} s^{-1}, 25 °C	Alginate films, Ar, 6% and 3% CO_2, 13 to ~209 μmol photons m^{-2} s^{-1}	(Kosourov et al., 2017)
Anabaena sp. (UTEX 1448)	Heterocyst filamentous cyanobacteria	67.07 μmol H_2 L^{-1} h^{-1}	Air, 4440 lx, 24 °C	Air, 4440 lx, 24 °C	(Vargas et al., 2018)
Calothrix 336/3	Heterocyst filamentous cyanobacteria	8.96 μmol H_2 L^{-1} h^{-1}	Air, 7 μmol photons $m^{2-1}s^{-1}$, 22 °C	Ar, 70 μmol photons m^{-2} s^{-1}, 23 °C	(Allahverdiyeva et al., 2010)

under anaerobic conditions with or without light. A conducted study by Shah et al. (2001), tested nitrogenase activity in *Anabaena variabilis* SPU 003 and produced 3.15 μmol H_2 h^{-1} mg^{-1} of biomass when subjected to dark anaerobic conditions. Other studies have also tested the activity of nitrogenase in light conditions; for instance, Chen et al. (2008) reported that when *Anabaena* sp. strain CH3 was subjected to 130 μE m^{-2} s^{-1} light intensity, about 0.8 mmol of H_2 was evolved. Conversely, the use of hydrogenase in non-N_2-fixing cyanobacteria has been enhanced by a sulfur-depleted medium. For instance, a reported study revealed that the growth of cyanobacterium *Synechocystis* PCC 680 in sulfur depleted medium and anaerobic conditions could enhance H_2 evolution, reaching 8.10 mmol H_2 mg chl a^{-1} min^{-1} (Baebprasert et al., 2010). Similarly, Burrows et al. (2008), produced H_2 of 0.81 μmol H_2 mg chl a^{-1} h^{-1} when their culture was subjected to an S-deprived medium.

Interestingly, some studies reported that N_2-fixing species are found with the highest H_2 yields. Hence, a comparison of H_2 evolution rates between N_2-fixing and non-N_2-fixing species of *Anabaena siamensis, Anabaena* sp. and *Nostoc punctiforme*, respectively was conducted by Khetkorn et al. (2010). Recently, genetic engineering has been explored as a strategy to improve H_2 yields. Nyberg et al. (2015) genetically modified N_2-fixing *Nostoc* PCC 7120, by improving hydrogenase performance. The culture was subjected to anaerobic conditions and obtained the highest H_2 volumetric production rate of 1.7 mL L^{-1} h^{-1} in irradiance of 44 μmol photons m^{-2} s^{-1}. It is worth mentioning that all the previous investigations are still performed at the laboratory scale. The study by Lindblad et al. (2002) up-scaled *Anabaena* PCC 7120 cultivation, achieving the highest H_2 production of 14.9 mL H_2 h^{-1} L^{-1} in outdoor conditions. However, generating hydrogen using indirect biophotolysis is still limited because of the low rates of the process and the high cost of photobioreactors.

5. Perspectives and recommendations

Cyanobacteria are promising microorganisms with great potential to become green factories to produce a wide variety of chemicals and valuable byproducts from natural resources. Their capacities in CO_2 fixation, growth rates, and resilience to harsh conditions make them an essential raw material for biorefinery. The use of carbohydrate-enriched cyanobacterial biomass as a substrate for third-generation biofuels production such as biohydrogen, bioethanol, or biobutanol, represents an attractive alternative to diminish the rapid depletion of fossil fuels reserves and minimize the effects on climate change. However, obtaining these biofuels from cyanobacteria is not yet economically feasible, mainly because of the high costs of the energy demand of the entire process. This limitation seriously affects the attempts to commercialize some biofuels, indicating that the technology in its current form is not economically viable (Nilsson et al., 2020). Several technical difficulties hinder the commercialization of large-scale cyanobacterial biofuels. The most critical aspects are cultivation, carbohydrates-rich biomass production, effective hydrolysis, and maximum carbohydrate conversion to biofuels, including the process energy and resources management efficiency. This section presents the technical, economic, and environmental challenges that need to be surmounted to realize carbohydrate-based biofuels production from cyanobacteria.

5.1. Technical challenges and research opportunities

Given all the operational aspects reviewed in this work, it is evident that several factors can influence carbohydrate content under N or P limitations. Hence, more studies must correlate illumination, hydraulic regimes, and carbon addition by theoretical and numerical optimization methods. The correlation of all those factors is needed because all the factors conditions could influence a large-scale outdoor implementation and could have pacifying or antagonizing effects on growth. The challenges related to cheap and high growth carbohydrate optimization are still an object of discussion and continued research gap. A possible alternative for the cost-effective cultivation of cyanobacteria is recycling nutrients from wastewater for growing the culture. This alternative could reduce the cost associated with fresh water and nutrient resources. Also, the objective is a promising alternative to urban/industrial wastewater treatment while at the same time valuable substrates can be achieved. Another promising strategy is the two-stage systems consisting of cyanobacteria cultivation in wastewater and further optimizing the carbohydrate accumulation culture to reduce growth medium and freshwater resources. The study of Rueda et al. (2020b),

showed the possibility of successfully performing this strategy at a large scale, achieving up to 70% of the carbohydrate content in an 11.7 m^3 semi-closed photobioreactor under outdoor conditions. Future studies are recommended to study the improvement of operational conditions by optimizing the accumulation of carbohydrates and growth of the biomass in a simultaneous process.

After successfully achieving the carbohydrate-enriched biomass, two processes can be performed to convert carbohydrates into biofuels. The first step consists of using carbohydrate-enriched biomass as a feedstock for fermentative bacteria or yeasts. In contrast, the second process exploits cyanobacteria's capacity to ferment their intracellular carbohydrate content, either for the natural or genetically engineered process. While fermentative methods are consolidated technologies because of the extensive research with first- and second-generation feedstocks, the effective biofuel production from cyanobacterial would depend on the previous carbohydrate-enriched process biomass optimization. This is because several lab-scale processes used low carbohydrate content to explore the fermentation process. Hence, the yields of ethanol, butanol, and hydrogen, per biomass or reducing sugars are often variable. In some cases, the process efficiency is a function of hydrolysis (i.e., butanol and ethanol production), reducing sugars content, and fermentative species (i.e., butanol and hydrogen).

Concerning cyanobacteria performing the production of biofuels, metabolic engineering offers the alternative to avoid manipulating other microorganisms and reducing the use of complex processes. However, this alternative would not allow the utilization of wastewater as a nutrient source. Instead, it would require controlled sterile conditions to maintain the genetically modified strain, increasing production costs. In general, more research about the optimization of the process is needed to improve the yields. Furthermore, more studies in the pilot and large scale are crucial to up-scale this technology, which will allow testing the effect of natural light and temperature. The hydrogen production by cyanobacteria performing indirect biophotolysis is a viable alternative to avoid other microorganisms. Although this technology is still limited by the low concentrations of H_2 obtained, further research can be directed to the optimization of the process through different culture conditions.

5.2. Techno-economic outlook of carbohydrate-based biofuels from cyanobacterial biomass

Previous studies of cyanobacterial cultivation agree that the primary goal of microalgae cultivation is to achieve more than 0.5 g L^{-1} (Fasahati et al., 2019; Nappa et al., 2020). Notably, the photobioreactor type influences the energy requirement and thus the final cost. For instance, the use of open-pond requires a specific electric energy consumption of 1.2 W m^{-3} to achieve a biomass content of about 0.5 g L^{-1}. However, this system has high CO_2 requirements (about 25%) due to gas leaks (Nappa et al., 2020). Conversely, closed-photobioreactors consume a higher specific electric energy of about 50 W m^{-3}, but they have several operational advantages including better light penetration, lower risk of contamination, higher biomass concentration (up to 2 g L^{-1}), and better use of CO_2 (about 10%) (Fasahati et al., 2019). Although open bond for biomass cultivation requires less energy than closed systems, however, the low biomass production of open ponds leads to an increase in the cost of harvesting and dewatering techniques. Dewatering techniques such as centrifugation, filtration, sedimentation, and flocculation are usually used. However, all these methods are costly and energy-intensive and are not applicable at a large scale, especially in low-cost harvesting to obtain low-value energy products (Zahra et al., 2020). The study of Fasaei et al. (2018) reported that the operational costs and energy consumption of mechanical and chemical options ranged from 0.5–2 € kg^{-1} and 0.2–5 kWh kg^{-1}, respectively for microalgae produced in open systems. Whereas, in closed cultivation systems the operational costs ranged from 0.1 € kg^{-1} to 0.6 € kg^{-1} and the energy consumption from 0.1–0.7 kWh kg^{-1}. Considering the lowest cost for harvesting/dewatering technologies, a recent report revealed a cost of 4.5 €/kg for biomass production in open ponds (Acién Fernández et al., 2019), while for closed systems between 4.15 €/kg and 5.96 €/kg is feasible (Norsker et al., 2011). The production costs can be enhanced by optimizing the cultivation area, solar irradiation, mixing, and changing the wastewater culture medium. In this way, the operational costs can be decreased by 1.4 €/kg (Acién Fernández et al., 2019), and the biomass could become an alternative feedstock for biofuel production.

5.3. Life cycle assessment

Besides the economic studies, the environmental benefits of biofuel production from cyanobacteria remain unclear. For this reason, the primary purpose of life cycle assessment (LCA) is to study cyanobacteria-based biofuels and their comparison with traditional biofuels. In addition, the results of the LCA provide a framework for evaluating the energy use, emissions, impacts of direct, indirect, and supply chain processes. Reported studies on cyanobacteria biofuel LCA focused on phases of biofuels synthesis by external fermentative processes (multiple stages) or auto-fermentation processes (one-stage) (discussed in previous sections). However, the large-scale production of carbohydrate-based biofuels from cyanobacteria is not yet clearly defined. No studies on LCA of cyanobacterial biofuels investigated commercial-size installations. All the studies focused on inputs and outputs from laboratory or pilot plant data with some assumptions of several variables of commercial production, which results in widely diverging results.

Most recent LCA studies have been directed towards one-stage bioethanol and butanol production by genetically modified cyanobacteria as shown in Fig. 6. Cyanobacteria cultivation (either performed in closed or open photobioreactors), including the downstream processing (harvesting, dewatering) and subsequent extraction of carbohydrate-based biofuels, require high energy inputs depending on the source. In the cultivation phase, several factors impact the performance, for example, the photobioreactor type. The flat-panel type reactors provide the lowest greenhouse gas emissions due to the higher productivity compared to other closed reactors. Other factors such as the energy demand for temperature regulation, mixing, or air sparging account for >60% of the emissions and induce a negative energy balance (Nilsson et al., 2020). Regarding the nutrients, sensitivity analysis has demonstrated that artificial nutrient sources led to a significant fraction of the total net energy requirements and greenhouse gas emissions (GHGe) due to the energy consumption embedded in industrial fertilizer production (Quiroz-Arita et al., 2017).

Concerning the subsequent process of biofuels processing, the study of Quiroz-Arita et al. (2017) produced ethanol from genetically modified *Synechocystis* sp. PCC6803, reaching biomass and ethanol productivities in the range of 0.12–0.76 g L d^{-1} and 0.04 g L d^{-1}- 0.24 g L^{-1} d^{-1}, respectively. They contemplated natural-gas-fueled combined heat and power system to provide process electricity and extra heat, and conservative assumptions around the ethanol separation process to achieve a net life cycle energy input of 0.55 MJ MJ^{-1}_{EtOH} - 0.20 MJ MJ^{-1}_{EtOH}, and greenhouse gas emissions (GHGe) reduction from 233.5 g CO_{2eq} MJ^{-1}_{EtOH} to 89.6 g CO_{2eq} MJ^{-1}_{EtOH}. Conversely, Luo et al. (2010) reported lower energy consumption and greenhouse gas emissions from 29.8 to 12.3 g CO_{2eq} MJ^{-1}_{EtOH} by employing higher efficiency heat exchangers in ethanol purification and/or with the use of solar thermal for some of the process heat. They considered lower mixing rates and the recycling of 90% of water. With these approaches, the emissions could meet the US standard for advanced renewable biofuels, targeted at 50% of gasoline emissions (45.6 g CO_{2eq} MJ^{-1}) (Arora et al., 2020). Another evaluated LCA of biobutanol production from genetically engineered *Synechocystis* (Nilsson et al., 2020). They produced the biobutanol in three types of closed reactors (G3, flat panels, and tubular), achieving productivities of 111–544 (mg_{BuOH} L^{-1} day^{-1}). They realized GHGe ranging from 16.9–58.6 gCO_{2eq} MJ^{-1}_{BuOH} and net life cycle energy from 3.8–13 MJ

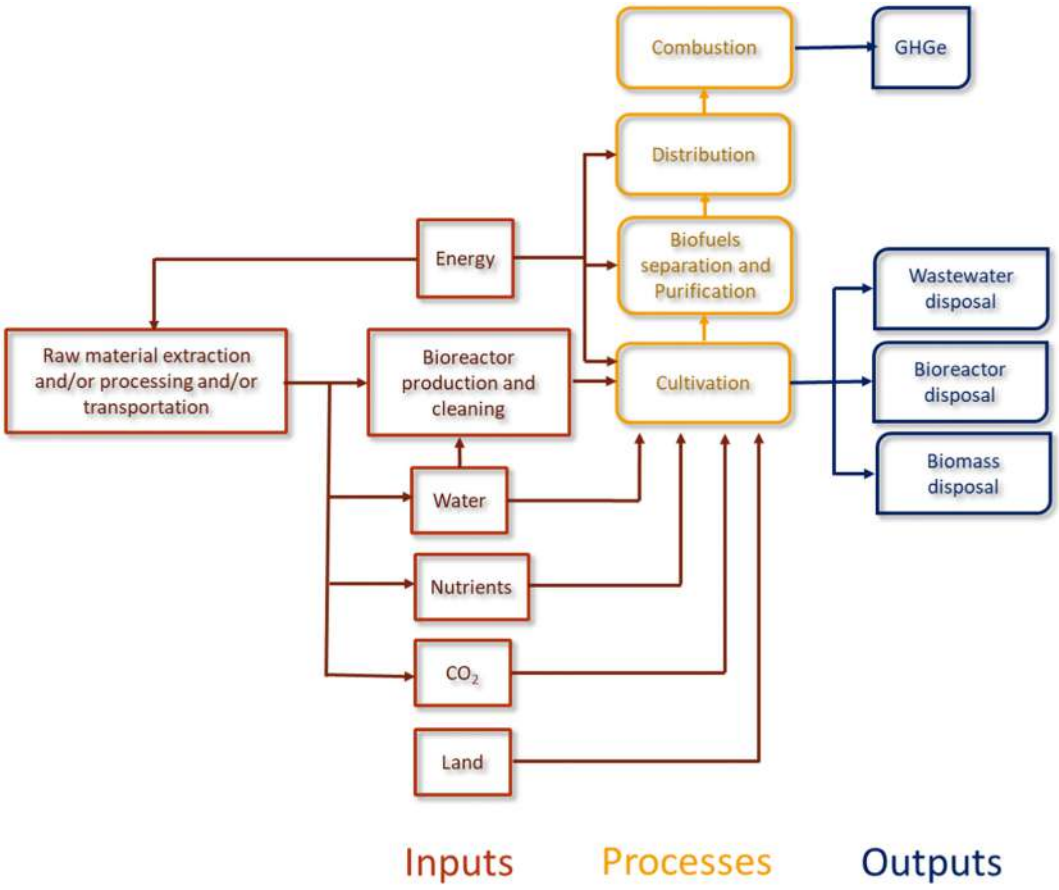

Fig. 6. Process schematic of the direct cyanobacteria-based biofuels production system.

MJ^{-1}_{BuOH}. Moreover, they found a higher emission reduction by 60% compared to fossil fuels, when the water was recycled.

A significant study evaluated LCA for the production of ethanol, isobutanol, and n-butanol production from cellulosic biomass by external fermentative processes. The conversion stage processes by fermentative routes exhibit higher direct CO_2, SO_2, and NO_2 emissions and high consumption of water. The results demonstrated that the fermentation of ethanol had the lowest net GHGe calculated on a gasoline gallon equivalent (GGE) basis (4300 g CO_{2eq} GGE^{-1}). The n-butanol via ABE fermentation requires minor fossil energy consumption (39 MJ GGE^{-1}) whereas, isobutanol exhibited modestly higher GHG emissions (5.0 kg CO_{2eq} GGE^{-1}) and fossil energy consumption of 51 MJ GGE^{-1} (Tao et al., 2014).

Mehmeti et al. (2018), estimated that the global warming potential of H_2 produced from the dark fermentation pathway with and without energy recovery are 9.8 and 19 kg CO_{2eq} kg^{-1} H_2, respectively. Notably, the impact achieved in H_2 by the fermentative process could be higher compared with other H_2 producing processes from microalgal biomass, such as tar-free catalytic reactive flash volatilization (8.24 kg CO_{2eq} kg^{-1} H_2) (Gholkar et al., 2021), wind energy-driven water electrolysis (0.97 kg CO_{2eq} kg^{-1} H_2) (Cetinkaya et al., 2012), and steam methane reforming (11.9 kg CO_{2eq} kg^{-1} H_2). To the best of our knowledge, there are no LCA studies on the production of cyanobacterial-biofuels by external fermentation processes. However, from conducted studies, it can be deduced that the different stages of the fermentative process increase the environmental impact and resource consumption. Hence, further research should be focused on these studies to test the assumptions.

One important thing to remark is that regardless of the biofuels production scenario, the cultivation stage plays an essential role in the total impacts of the whole process. Achieving the highest biomass productivity seems crucial for large-scale biofuel production and lower environmental impact. In addition, the energy demand and the impact of water and nutrients also represent an important issue. Recycling nutrients from wastewater for growing the culture could reduce the cost associated with fresh water and nutrient resources. Similarly, energy supply from renewable sources is the most sustainable option to consider in further studies. The LCA of these systems, including all the technical recommendations described, is an attractive and interesting research approach to decrease GHGe and improve resource management.

6. Conclusions

Cyanobacterial carbohydrates are a promising third and fourth-generation biofuels feedstock. The cultivation and high carbohydrate accumulation process present an attractive pathway for cost-effective large-scale implementation. The growth, accumulation of carbohydrates for the biorefinery approach is greatly influenced by the hydraulic regime, illumination, and type of reactor strategy implemented. Notably, the use of wastewater as a substrate for the cultivation of cyanobacteria could exploit nutrients in the wastewater for the accumulation of carbohydrates, achieve high removal of COD, and significantly reduce the cultivation cost and environmental impacts. However, the implementation of genetic modification cannot be realized in this medium because of the sterile conditions required for most genetic processes. Further conversion of these accumulated carbohydrates to biofuels, such as ethanol, hydrogen, and butanol production by independent or auto-fermentation is a noteworthy endeavor, however, studies are still limited to lab-scale. The most promising research seems now oriented towards mastering carbohydrate enriched biomass production, up-scaling efficacy, and cost-effectiveness. Future investigations should focus on the successful cultivation of these microorganisms

to find nutrient sources facilitating cost-effective exploitation, even in genetically modified cultures.

CRediT authorship contribution statement

ADM Conceptualization, Investigation, Writing - original draft; OSE and OPU Investigation, Writing - review & editing; RRH, BOA, LA, DER, and SPJ, Writing - review & editing, All the authors read, edited, and approved the manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: There is no financial or personal interest that is potential competing interest to declare.

Acknowledgment

The authors acknowledge the financial support provided by DGAPA (Dirección General de Asuntos del Personal Académico) under PAPIIT Project Nos.: IA203320, IN110118, IA102821, and IG100720 is acknowledged. Also, the authors thank the Consejo Nacional de Ciencia y Tecnología (CONACYT) for their financial support under the project No. 207450/P120.

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