

Chapter 11

Biofuels from Microalgae: Bioethanol

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Abstract The industrial potential of ethanol has been tested early in 1800 to be used as an engine fuel after the invention of an internal combustion engine. Currently, there are three generations of bioethanol that have been flourished based on different feedstocks. The first-generation bioethanol is derived from fermentation of glucose contained in starch and/or sugar crops. USA and Brazil are the main producers of bioethanol worldwide utilizing corn and sugarcane, while potato, wheat, and sugar beet are the common feedstocks for bioethanol in Europe. The term “second-generation bioethanol” emerged as a boon to overcome the “food versus fuel” that occurs by the first-generation bioethanol. The second generation also referred to as “advanced biofuels” is produced by innovative processes mainly using lignocellulosic feedstock and agricultural forest residues. The emergence of the third-generation bioethanol provides more benefits as compared to the first and second generations and is focused on the use of microalgae and cyanobacteria. These organisms represent as a promising alternative feedstock due to its high lipid and carbohydrate contents, easy cultivation in a wide variety of water environment, relatively low land usage and carbon dioxide absorption. This chapter will discuss the use of microalgae for the ethanol production and the main technological routes, i.e., enzymatic hydrolysis and yeast fermentation of microalgal biomass, metabolic pathways in dark conditions, and “photofermentation.”

Keywords Bioethanol · Microalgae · Carbohydrate accumulation
Hydrolysis–fermentation

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1 Introduction

Microalgae biomass is an interesting alternative to traditional bioethanol crops because it does not have the inherent disadvantages of bioethanol of the first or second generation. The cultivation of microalgae may occur in different culture media, without necessarily using potable water and can carry wastewater, salt water (seawater) and brackish water in its composition. Microalgae production does not compete for freshwater intended for irrigation of plantations or for human and animal consumption. In addition, microalgae cultivation can occur in small areas and in non-arable, semiarid, or desert lands, since the main factors that influence the development of microalgae are the availability of sunlight and water for cultivation (Brennan and Owende 2010). Thus, the cultivation of microalgae does not directly compete for arable land for food production nor does it increase the occurrence of burning and deforestation, the main methods for obtaining new arable land. Another advantage is that when using carbohydrates produced by certain species of microalgae, the productivity of bioethanol of the third generation (in liters per hectare per year) may be some orders of magnitude greater than the productivity of raw materials used in the production of bioethanol of the first and second generations, according to Table 1.

Historically, microalgal biomass has been largely employed in the production of several compounds for human consumption and industrial application, including sterols, amino acids, fatty acids, and carotenoids, despite being considered in the last few years for biofuel production. The interest in converting microalgae into biofuels relies on some points: productivities superior to those of conventional energy crops, after lipid and carbohydrate extraction; potentially possible to recover high-value coproducts from the debris, such as proteins and pigments; low water consumption in comparison with the irrigation of energy crops; possibility of cultivation in non-arable lands, using non-potable water, such as wastewaters and without the application of pesticides and herbicides; and improvement of air quality, due to CO₂ fixation for biomass growth.

Table 1 Bioethanol productivity from different feedstocks

Feedstocks	Bioethanol productivity (L/(ha year))
Corn straw	1050–1400
Wheat	2590
Cassava	3310
Sweet sorghum	3050–4070
Maize	3460–4020
Beet	5010–6680
Sugarcane	6190–7500
<i>Panicum virgatum</i> (switchgrass)	10,760
Microalgae	46,760–140,290

Adapted from Mussatto et al. (2010)

Because of their simpler structure than those of higher plants, microalgae can achieve much higher photosynthetic efficiencies than terrestrial plants. Thus, a larger share of the captured solar energy is stored through the accumulation of carbohydrates inside the cell. Similarly, microalgae biomass production occurs in relatively short times, much lower compared to terrestrial plants used in the production of the first- and second-generation bioethanol. The possibility of recovering the microalgal several times or continuously, depending on the type of bioreactor used for biomass production. Thus, there is an abundant and inexpensive source of biomass for the production of bioethanol. Considering the potential of microalgae use, the great diversity of species, and the different possible conditions of cultivation, the knowledge of the physiology and metabolism of these microorganisms becomes imperative for the development of new industrial processes.

The microalgae serve as raw material for different types of biofuels, among them methane, hydrogen, biodiesel, and bioethanol, which could be used together or substituting the gasoline in light vehicles (Mata et al. 2010). Since global consumption of light fossil fuels is greater than the consumption of diesel heavy vehicles, researches' efforts on microalgae bioethanol production should be increased, an economically interesting alternative.

The selection of the appropriate microalgae species for the production of biofuels is an important factor for the success of the productive process as a whole. The desirable characteristics for a microalgae to be potential organism to biofuels production are tolerate shear stresses found in the reactors (especially in closed photobioreactors), to be dominant in relation to contaminant microorganism strains, large CO₂ absorption capacity in photoautotrophic systems (high photosynthetic efficiency), tolerate large temperature variations resulting from daily and seasonal cycles, low nutrient requirement, potential of high value-added coproducts in addition to the desired product, present a short productive cycle and self-flocculation to facilitate the recovery stage of the microalgal biomass.

The use of microalgae and cyanobacteria for the production of the third-generation biofuels has many advantages over higher plants in view of producing the first- and second-generation biofuels, mainly due to their faster growth under several conditions, including in wastewater. The biochemical composition of microalgae grown under normal conditions, that is, without nutrient limitation, primarily encompasses proteins (30–50%), carbohydrates (20–40%), and lipids (8–15%). Microalgae present several compounds in their cells, such as lipids, carbohydrates, proteins, and pigments, in different concentrations. This chemical profile directly reflects the nature of the microorganism (as its species or lineage), the influence of the chosen culture conditions, and the stage of growth of the culture. In this way, the same microalgae species can present different compositions when handling the specified factors (Zepka et al. 2008). For the production of the third-generation bioethanol, one should select a microalgae species with the ability to produce high concentrations of carbohydrates instead of lipids as energy reserve compound (Mussatto et al. 2010).

Several studies have shown that limiting the amount of nitrogen in the culture medium is one of the main factors that leads to the accumulation of carbohydrates by microalgae (Dragone et al. 2011). According to Behrenset et al. (1989), microalgae in a nitrogen-deprived culture medium direct the flow of carbon to the synthesis of carbohydrates in detriment of the production of proteins. Thus, effort to increase yields of biofuels produced by microalgae is underway, including the optimization of light technologies to modify the carbon uptake pathways, aimed at a higher accumulation of biomass or specific compounds such as carbohydrates and lipids or, more recently, the use of genetic engineering for producing bioethanol, biohydrogen, and other special fermentation products (de Farias Silva and Bertucco 2016). Photosynthetic organisms are favorable for the production of biofuels, mainly because of their low cost of cultivation, but biofuel yields obtained under normal conditions are not satisfactory. In addition to the production of biodiesel, microalgae and cyanobacteria serve as attractive feedstock for the production of bioethanol, although the scientific and technological knowledge on this context is still scarce. On the contrary, studies have documented that the contents of oil and carbohydrates in microalgae cells can be increased under stress conditions, resulting, for instance, in a decrease of the protein content under nitrogen depletion (Ho et al. 2013; Wang et al. 2013). This approach could be applied to cultivate microalgae biomass richer in carbohydrates, thereby leveraging their use for the production of bioethanol, which is currently the most widely used biofuel in the world.

However, under or non-optimized growth conditions, some microalgae strains have been receiving special attention because they present the potential of industrial application for the production of bioethanol of the third generation. Hirano et al. (1997) found two with high starch: *Chlamydomonas reinhardtii* (UTEX 2247) with 45% starch (dry basis) and *C. vulgaris* (IAM C-534) with 37% starch. The microalgae yields were, respectively, 11 and 32 g dry mass/(m² day). Dragone et al. (2011) produced biomass of *C. vulgaris* with up to 41% starch (dry basis) under low nitrogen culture conditions. According to Doucha and Lívanský (2009), a mutant strain for the production of starch from *Chlorella* sp. can accumulate 70% starch (dry basis) under conditions of suppression of protein production.

Technologies for the first (sugar or starch feedstock) and second generations (lignocellulosic feedstock) of bioethanol basically involve two stages: the conversion of sunlight into chemical energy (such as carbohydrates and lipids) and the conversion of chemical energy into biofuel. These two stages are related to each other and result in increased production costs. As an improvement of this process, the use of a single-stage system that is capable of capturing sunlight directly and converting it into biofuel (bioethanol) would avoid one step, thereby reducing the cost of production and increasing the sustainability of the bioethanol production process. Three possible routes involving the use of microalgae and cyanobacteria biomass for bioethanol production are discussed in the literature, accordingly summarized in Fig. 1 (de Farias Silva and Bertucco 2016). The first one is the traditional process in which the biomass undergoes pretreatment steps, enzymatic hydrolysis, and yeast fermentation. The second route is the use of metabolic

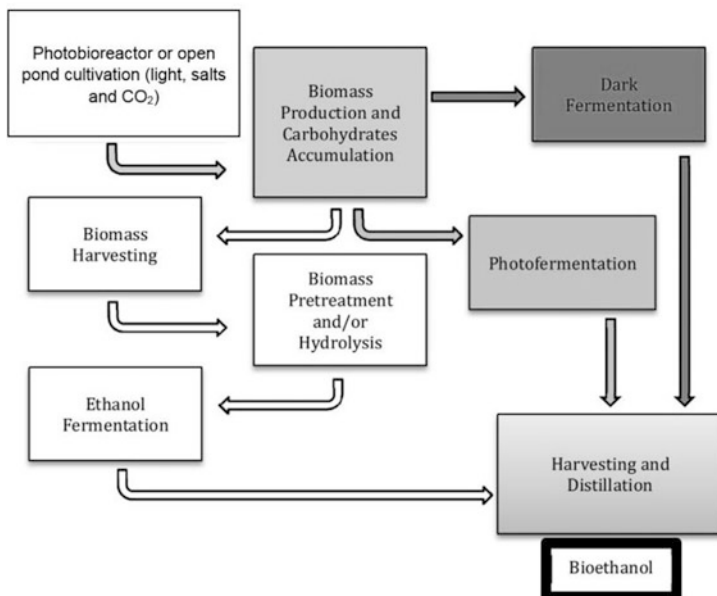


Fig. 1 Routes of bioethanol production from microalgae (adapted from de Farias Silva and Bertucco 2016)

pathways in dark conditions, redirecting photosynthesis to produce hydrogen, acids, and alcohols (such as ethanol). The third way is via “photofermentation,” which is impracticable in nature. The last route requires the use of genetic engineering to redirect the preexisting biochemical pathways of microalgae for a more subjective and efficient production of bioethanol.

Photosynthesis is a vital process that drives the synthesis of all biofuels, by converting light energy into biomass, carbon storage products (carbohydrates and lipids), and a small amount of H_2 . In green algae, the light-harvesting complex (LHC) (chlorophylls and carotenoids) absorbs photons from sunlight as chemical energy. This energy is used by the photosystem II (PS II) for the catalytic oxidation of water to form protons, electrons, and molecular oxygen. Low-potential electrons are transferred to the electron transport chain for the reduction of ferredoxin and then the formation of nicotinamide adenine dinucleotide phosphate (NADPH). An electrochemical gradient is formed, and the release occurs after oxidation of water in the thylakoid lumen, which is used to produce adenosine triphosphate (ATP) by ATP synthase. Photosynthetic products (NADPH and ATP) are substrates for the Calvin–Benson cycle, where CO_2 is fixed as C3 molecules that are assimilated to form sugars, lipids, and other biomolecules essential for cell growth.

Biofuels from microalgae have been the subject of intense research mainly focused on the production of biodiesel and biogas, although bioethanol and biohydrogen are also considered. The production pathways and operating conditions vary for each biofuel. Studies have already demonstrated the potential viability of

industrial processes for the production of biodiesel, according to the previous chapters. However, studies aimed at consolidating a suitable process for the production of bioethanol are still ongoing. On the contrary, cyanobacteria strains have been shown to produce relevant amount of bioethanol. Markou et al. (2013) evaluated the potential of bioethanol production using carbohydrate-enriched biomass of the cyanobacterium *Arthrospira platensis*. The biomass acid hydrolysates were used as substrate for ethanolic fermentation by a salt stress-adapted *Saccharomyces cerevisiae*, with highest bioethanol yields of $16.32\% \pm 0.9$ (gram ethanol per gram biomass) with HNO_3 0.5 N. The production of bioethanol from microalgae and cyanobacteria is a feasible technological development, as they showed higher productivity than certain crops such as sugarcane and corn (already consolidated as feedstocks for bioethanol production). Moreover, microalgae and cyanobacteria can reach 50% of their dry weight (DW) in carbohydrates, which can then be hydrolyzed and fermented with high yields.

2 Carbohydrate Accumulation by Microalgae

Microorganisms with potential for bioethanol production in this way are selected primarily in accordance with their ability to accumulate carbohydrates, which depends on environmental and nutritional conditions. The main environmental factors are light intensity, pH, salinity, and temperature, while the nutritional factors include availability and source type for nitrogen, carbon, phosphorus, sulfur, and iron (Chen et al. 2013; Markou et al. 2013).

Genera *Scenedesmus*, *Chlorella*, *Chlorococcum*, and *Tetraselmis* from *Chlorophyta* division and *Synechococcus* among other cyanobacteria have been extensively studied as feedstock for this type of bioethanol production. In general, the cultivation in a high light intensity ranged from 150 to 450 $\mu\text{mol}/(\text{m}^2\text{s})$ using a mix of CO_2 in air between 2 and 5% and mesophilic temperatures (20–30 °C) achieves around 50% of carbohydrate content under nutrient starvation, mainly nitrogen, according to Table 2 (de Farias Silva and Bertucco 2016). However, carbohydrate content could be extremely variable and the productivity depends on the cell growth too, that is, growing conditions that allow the simultaneous accumulation and growth. According to Rizza et al. (2017), generally microalgal strains that accumulated the highest levels of carbohydrates did not accumulate lipids under identical growth conditions.

The positive effect of increasing light intensity on the accumulation of starch and lipids is feasible only up to a point, usually equal to saturation of photosynthesis under given conditions in a particular species. Nutritional factors directly or indirectly influence the rate of photosynthesis and biochemical composition of microalgae. Macroelement (nitrogen, sulfur, or phosphorous) limitation is the most widely used and so far the most successful strategy for enhancing starch accumulation. For example, availability of nitrogen enhances the synthesis of proteins, pigments, and DNA, the amount of iron affects the photosynthetic electron

Table 2 Microalgae carbohydrate content in different growth conditions (adapted from Dragone et al. 2011; de Farias Silva and Bertucco 2016; Rizza et al. 2017)

Microalgae	Growth conditions	Carbohydrate (%)
<i>Arthrospira platensis</i>	150 $\mu\text{mol}/(\text{m}^2\text{s})$, 30 °C, bubbling air	58.0
<i>Chlamydomonas reinhardtii</i> UTEX 90	450 $\mu\text{mol}/(\text{m}^2\text{s})$, 23 °C, 4 days, and 130 rpm	59.7
<i>Chlorella vulgaris</i> KMMCC-9 UTEX26	150 $\mu\text{mol}/(\text{m}^2\text{s})$, 20–22 °C, bubbling air	22.4
<i>Chlorella</i> sp. KR-1	80 $\mu\text{mol}/(\text{m}^2\text{s})$, 30 °C, and 10% CO ₂	49.7
<i>Chlorella</i> sp. TISTR 8485	BG11 medium for 20 days	27.0
<i>Chlorococcum</i> sp. TISTR 8583	BG11 medium for 20 days	25.9
<i>Scenedesmus obliquus</i>	150 $\mu\text{mol}/(\text{m}^2\text{s})$, 25 °C, bubbling air	30.0
<i>Scenedesmus obliquus</i> CNW-N	210–230 $\mu\text{mol}/(\text{m}^2\text{s})$, 28 °C, 300 rpm, and 2.5% CO ₂	51.8
<i>Synechococcus elongatus</i> PCC 7942	200 $\mu\text{mol}/(\text{m}^2\text{s})$, 28 °C, and 5% CO ₂	28.0
<i>Synechococcus</i> sp. PCC 7002	250 $\mu\text{mol}/(\text{m}^2\text{s})$ and 1% CO ₂	59.0
<i>Tetraselmis subcordiformis</i> FACHB-1751	150 $\mu\text{mol}/(\text{m}^2\text{s})$, 25 °C, and 3% CO ₂	40.0
<i>Ankistrodesmus</i> sp. strain LP1	BG11 medium supplemented with 1 mM NaNO ₃	51.3
<i>Desmodesmus</i> sp. strain FG	BG11 medium with 1 mM NaNO ₃	53.5
<i>Pseudokirchneriella</i> sp. strain C1D	BG11 medium with 1 mM NaNO ₃	40.5
<i>Scenedesmus obliquus</i> strain C1S	BG11 medium with 1 mM NaNO ₃	29.9

transport, nitrite/nitrate and sulfate reduction, nitrogen fixation, and/or detoxification of reactive oxygen species (ROS). Sulfur involves the formation of sulfolipids, polysaccharides, and proteins, as well as in the electron transport chain. When sulfur is present at limiting concentrations, it inhibits cell division, whereas high concentrations inhibit the photosynthetic assimilation of carbon-rich compounds, such as carbohydrates. CO₂ is the most common source of carbon (autotrophic condition), and under nitrogen depletion conditions, the supplementation of CO₂ in conjunction with light intensity causes the carbon to be absorbed and converted into carbohydrates more efficiently.

According to Dragone et al. (2011), increasing microalgal starch content by nutrient limitation has been regarded as an affordable approach for the production of the third-generation bioethanol. Thus, these authors have evaluated starch accumulation in *C. vulgaris* P12 under different initial concentrations of nitrogen (0–2.2 g_{urea}/L) and iron (0–0.08 g_{FeNa-EDTA}/L) sources, using an experimental design. Starch accumulation occurred at nitrogen depletion conditions. Cell growth was much slower than that observed during nitrogen-supplemented cultivations. The authors proposed a two-stage cultivation process for high starch accumulation:

a first cultivation stage using nitrogen- and iron-supplemented medium, followed by a second cultivation stage in a nitrogen- and iron-free medium. The high starch content obtained (up to 41.0% of dry cell weight) suggests *C. vulgaris* P12 as a very promising feedstock for bioethanol production.

Carbohydrates are the major products derived from photosynthesis and the carbon fixation metabolism (Calvin cycle), which are either accumulated in the plastids as reserve materials (starch), or become the main component of cell walls (cellulose, pectin, and sulfated polysaccharides). However, the composition and metabolism of carbohydrates (mainly starch and cellulose) in microalgae may differ significantly from species to species. Microalgae that contain glucose-based carbohydrates are the most feasible feedstock for bioethanol production (Chen et al. 2013). The cell walls of microalgae primarily consist of an inner cell wall layer and an outer cell wall layer. The composition of the outer cell wall varies from species to species, but usually contains specific polysaccharides, such as pectin, agar, and alginate, while the inner cell wall layer is mainly composed of cellulose and other materials. Table 3 shows the compositions of the cell walls and the storage products. For some microalgae, the glucose polymers produced via cellulose/starch are the predominant component in the cell walls and stored products of microalgae. Starch and most cell wall polysaccharides can be converted into fermentable sugars for subsequent bioethanol production via microbial fermentation.

The accumulation of carbohydrates in microalgae is due to CO₂ fixation during the photosynthetic process (Fig. 2). Photosynthesis is a biological process utilizing ATP/NADPH to fix and convert CO₂ captured from the air to produce glucose and other sugars through a metabolic pathway known as the Calvin cycle. The metabolic pathways of energy-rich molecules are closely linked. Some studies demonstrated that there was a competition between lipid and starch synthesis because the major precursor for triacylglycerols synthesis is glycerol-3-phosphate (G3P), which is produced via catabolism of glucose (glycolysis). Thus, to enhance biofuels' production from microalgae-based carbohydrates, it is vital to understand and manipulate the related metabolisms to achieve higher microalgal carbohydrate

Table 3 Composition of microalgal cell wall and storage products (Chen et al. 2013)

Division	Cell wall	Storage products
Cyanophyta	Lipopolysaccharides, peptidoglycan	Cyanophycean starch
Chlorophyta	Cellulose, hemicellulose	Starch/lipid
Dinophyta	Absence or contain few cellulose	Starch
Cryptophyta	Periplast	Starch
Euglenophyta	Absence	Paramylum/lipid
Rhodophyta	Agar, carrageenan, cellulose, calcium carbonate	Floridean starch
Heterokontophyta	Naked or covered by scales or with large quantities of silica	Leucosin/lipid

accumulation via strategies like increasing glucan storage and decreasing starch degradation. The starch forms around a crystallizing nucleus and is present as an amorphous starch grain. When a chloroplast gathers enough starch, it may become an amyloplast. However, the detailed changes in enzymatic activity and metabolic flux of carbohydrate biosynthesis of microalgae are poorly understood. The manipulation of the carbohydrate metabolisms of microalgae by genetic engineering has also been proposed. With the development of genetic engineering of microalgae, and a better understanding of the biochemistry of microalgae carbohydrate metabolisms, superior strains for carbohydrate accumulation could be developed.

Except the starch in plastids, microalgal extracellular coverings (cell wall) are another carbohydrate-rich part, which could be transformed to biofuel. However, the compositions of microalgal extracellular coverings are diverse by species. Among them, cellulose is one of the main fermentable carbohydrates in most of green algae. Cellulose synthesis is a complicated process that includes many enzymatic reactions. The starting substrate for cellulose synthesis is UDP-glucose, which is formed from the reaction of UDP and fructose catalyzed by sucrose synthase. Despite the understanding of main carbohydrate metabolism in microalgae, in-depth knowledge on its regulation is still lacking. It is important to integrate updated information of genomic sequences, transcriptomes, proteomes, and metabolomes data at systems level to meet the challenges on economic biofuels production from microalgae.

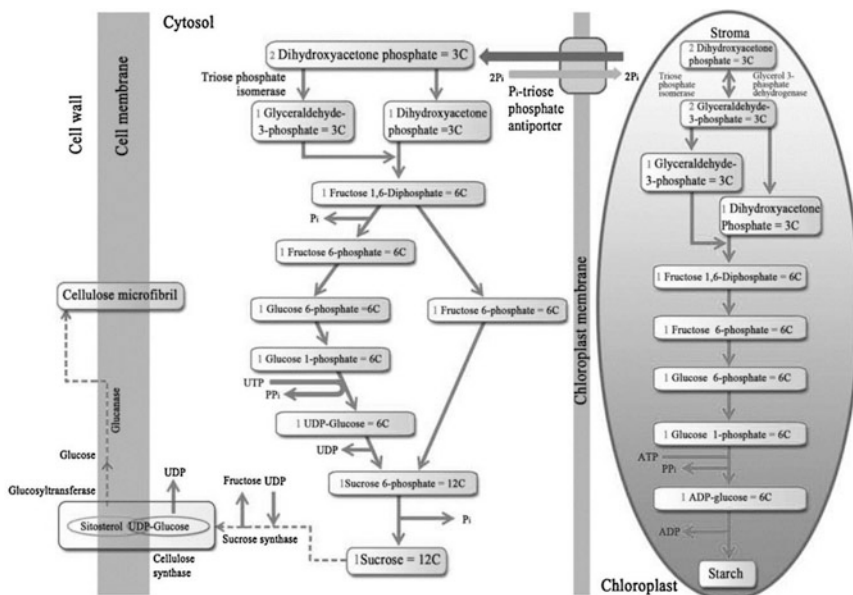


Fig. 2 Proposal of carbohydrate metabolism in green algae (adapted from Chen et al. 2013)

Although systems study of microalgae on carbohydrate metabolisms is currently in its infant stage, omics studies on microalgae have made significant progress. Such a strategy will open a door for efficient carbohydrate metabolic regulation and genetic engineering of microalgae for biofuels' production.

3 Technologies of Microalgal Carbohydrates to Bioethanol

The main technological routes for bioethanol production by microalgal biomass involve hydrolysis–yeast fermentation, the use of metabolic pathways in dark conditions, and “photofermentation.”

The hydrolysis of biomass is the most used method for the use of microalgal carbohydrates. Hydrolysis–fermentation of microalgal biomass is based on the production of microalgae biomass succeeded by pretreatment steps, involving breakdown of the cell structure and hydrolysis of the biomass, and frequently by the addition of enzymes. The treated biomass is then fermented with yeasts or bacteria to obtain ethanol. The main drawbacks of this route are the multistep processes required, which demands more energy, and the use of enzymes and yeasts, which accounts for a considerable proportion of the costs. On the contrary, the hydrolysis/fermentation process converts biomass at the highest rate, because of the well-known high efficiency of enzymes and yeasts in converting biomass into products.

Markou et al. (2013) studied the potential of bioethanol production using carbohydrate-enriched biomass of the cyanobacteria *A. platensis*. For the saccharification of the carbohydrate-enriched biomass, four acids (H_2SO_4 , HNO_3 , HCl , and H_3PO_4) were investigated. The hydrolysates then were used as substrate for ethanol fermentation by a salt stress-adapted *Saccharomyces cerevisiae* strain. According to the authors, the highest bioethanol yield was observed at acid concentration of 0.5 N. At this concentration, fermentation of hydrolysates with HCl as catalyst had the lowest bioethanol yield (13.41% gram of ethanol per gram dry biomass), while hydrolysates with H_2SO_4 and HNO_3 as catalysts had bioethanol yield of 16.27 and 16.32%, respectively. *Chlorella* biomass was hydrolyzed in the presence of 2% HCl and 2.5% MgCl_2 , a sugar concentration of nearly 12%, and a sugar recovery of about 83% was obtained. Fermentation experiments demonstrated that glucose in the *Chlorella* biomass hydrolysates was converted into ethanol by *S. cerevisiae* with a yield of 0.47 g/g, which is 91% of the theoretical yield (Zhou et al. 2011).

Rizza et al. (2017) researched *Desmodesmus* sp. strain for production of biomass fermentable. Hydrolyzed preparations were brought to pH 5.5–6.0 with $\text{Mg}(\text{OH})_2$ crystals and used directly or after concentration by freeze-drying for ethanol fermentation. A detailed time-course analysis of the increase in biomass and accumulation of total carbohydrates and proteins indicated that *Desmodesmus* sp. strain FG grew robustly, its reaching ratios of carbohydrates to protein over 2.

Microalgae biomass at 100 g/L was hydrolyzed according to the optimized conditions to yield soluble carbohydrates preparations. These preparations were inoculated with *S. cerevisiae* cells and accumulated about 23 g ethanol per liter, representing approximately 81% of the maximum theoretical. These results indicated that microalgae biomass could be converted into ethanol by baker's yeast efficiently as commercial grade dextrose and that other nutrients, usually used to improve fermentation, such as the N-source, were already present in the hydrolyzed microalgal biomass. Both almost complete exhaustion of carbohydrates from the fermentation broth and high conversion efficiency of carbohydrates into ethanol indicated very high enrichment of fermentable sugars in the biomass of the strains selected in this study and in their corresponding hydrolysates. It also indicated that sugar loss and/or generation of fermentation inhibitors from microalgal biomass remained at negligible levels after the optimized saccharification treatment. These results contribute to support the potential of microalgae biomass as an alternative feedstock for bioethanol and the value of bioprospecting programs to identified candidate strains among natural biodiversity.

Yuan et al. (2016) evaluated liquid hot water pretreatment prior to enzymatic hydrolysis of *Scenedesmus* sp. The concentration and recovery of total sugars and glucose at 100 °C were 0.85 and 0.26 g/L, respectively, while 13.4 and 0.16 g/L at 200 °C. These results indicated that the increase of temperature could accelerate the motions of solvent molecules (sulfuric acid) and improve the liberation of sugars. Thus, according to these authors, liquid hot water pretreatment could greatly enhance the enzymatic efficiency and could be regarded as an ideal method for glucose recovery from microalgae.

Mixed microalgae cultures could be considered as an attractive research area compared to traditional pure culture to dominate cultivation contamination risk and enhance economic feasibility of large-scale biofuel production. In this sense, Shokrkar et al. (2017) evaluate the effect of different pretreatment strategies including acidic, alkaline, and enzymatic hydrolysis on the sugar extraction from mixed microalgae. According to these authors, total carbohydrates content of microalgal biomass increased about 20.1% in the absence of nitrogen (about 36% in terms of volatile suspended solids amount). Dilute acids decompose cellulose, and starch in the biomass to release simple sugars. Hydrolysis kinetic depends on the type of substrate, temperature, acid concentration, and reaction time. Results showed that the mixture of dilute sulfuric acid and MgSO₄ exhibited a higher sugar yield than dilute acid. Among all pretreatments used, the enzymatic treatment with thermostable enzymes showed the highest recovery of 0.951 g of extracted glucose per gram of total sugar. Moreover, the enzymatic pretreatment of wet microalgae was compared with dried ones at identical operational conditions and dried biomass concentration of 50 g/L, and similar sugar yields were achieved which would be advantageous to reduce the need for drying of the microalgae biomass. Fermentation of the acidic and enzymatic treated samples to ethanol using *Saccharomyces cerevisiae* showed yield of 0.38 and 0.46 g/g glucose, corresponding to 76 and 92% of the theoretical values, respectively. These authors reported that bioethanol yield after enzymatic hydrolysis of mixed microalgae

culture is higher than that of acid hydrolysis. Carbohydrates in microalgae biomass are mainly cellulose and starch. Cellulose molecules are glucose polymers linked together by β -1,4 glucosidic bonds, as opposed to the α -1,4 and α -1,6 glucosidic bonds for starch. In the enzymatic pretreatment of algae, β -glucosidase/cellulase hydrolyzed β -1,4 glucosidic bonds of algal cellulose, whereas α -amylase liquefied algal starch to oligosaccharides through the hydrolysis of the α -1,4 glucosidic linkages, and then amyloglucosidase hydrolyzed α -1,4 and α -1,6 glucosidic bonds of oligosaccharides into glucose. Therefore, it is desirable to use three enzymes in the enzymatic pretreatment of microalgae, thus improving the hydrolysis yields even further.

Another process known as “dark fermentation” refers to the conversion of organic substrates into biohydrogen (de Farias Silva and Bertucco 2016). Fermentative and hydrolytic microorganisms hydrolyze complex organic polymers into monomers, which are subsequently converted into a mixture of organic acids of low molecular weight and alcohols, mainly acetic acid and ethanol. Various microalgae and cyanobacteria that are capable of expelling ethanol through the cell wall by means of intracellular process in the absence of light include *C. reinhardtii*, *Chlamydomonas moewusii*, *C. vulgaris*, *Oscillatoria limnetica*, *Oscillatoria limosa*, *Gleocapsa alpicola*, *Cyanothece* sp., *Chlorococcum littorale*, and *Spirulina* sp. and *Synechococcus* sp. However, dark fermentation is disadvantageous in terms of hydrogen productivity, because approximately 80–90% of the initial chemical oxygen demand (COD) remains in the form of acids and alcohols after the process. Even under optimal operating conditions, typical yields vary only between 1 and 2 mol H₂ per mol of glucose. The production of ethanol is favored by the accumulation of carbohydrates in the microalgae cells through photosynthesis, and then, the microalgae are forced to synthesize ethanol through fermentative metabolism directly from their carbohydrate and lipid reserves when switching the growth to dark conditions. However, it can be concluded that dark fermentation of microalgae is not an efficient process for the production of bioethanol.

“Photofermentation” is a process of growing interest principally after the announcement of the installation of industrial plants where modified cyanobacteria are used to produce bioethanol directly. The “photofermentative” route (simply, photanol) is a natural mechanism of converting sunlight into products of fermentation through a highly efficient metabolic pathway. Photanol is not only limited to ethanol production, but it is also used for a large number of naturally occurring products resulting from glycolysis-based fermentation (Rai and Singh 2016). Thus, several cyanobacteria species can be genetically modified by introducing specific fermentation cassettes through molecular engineering procedures, and then tested as a fermentative organism. *Synechococcus* sp. is a unicellular cyanobacterium living in freshwater that has been relatively well characterized. It is capable of tolerating insertion of foreign DNA to be transformed and replicated using shuttle vectors between *Escherichia coli* and cyanobacteria, or insertion of foreign DNA into the chromosome through homologous recombination at selected active sites. *Synechocystis* sp. PCC 6803 was the first photosynthetic organism that had its genome sequenced and one of the best characterized cyanobacteria.

Thermosynechococcus is also naturally transformable. The metabolic pathway of ethanol synthesis is briefly summarized: After fixation of inorganic carbon by Calvin cycle, it forms phosphoglycerate that is converted into pyruvate by two enzymes (pyruvate decarboxylase and alcohol dehydrogenase), and finally into ethanol. Therefore, the “photofermentation” process for obtaining ethanol includes two stages: photosynthesis and fermentation. Each stage has its key factors that determine the efficiency of the process and the metabolic needs of the cyanobacteria. In any case, this route requires the use of genetically modified microorganisms.

Figures 3 and 4 present the schematic diagram of the assumed fermentative pathways operating in dark-incubated wild type *Chlamydomonas reinhardtii* and mutant PFL1-deficient strain 48F5 (Philipps et al. 2011). In fermenting *C. reinhardtii* wild type cells (CC-124), pyruvate from glycolytic glucose oxidation, serves as substrate for several enzymes. Pyruvate formate lyase (PFL1) cleaves pyruvate into formate and acetyl CoA. Acetyl CoA is converted to acetate by the successive action of phosphotransacetylase (PTA) and acetate kinase (ACK), resulting in ATP production, or to ethanol by a bifunctional aldehyde/alcohol dehydrogenase (ADH1), resulting in oxidation of NAD(P)H. Pyruvate decarboxylase (PDC) decarboxylates pyruvate yielding acetaldehyde, which is further reduced to ethanol by alcohol dehydrogenase (ADH). Another pathway leads to D-lactate production by the action of D-lactate dehydrogenase (D-LDH). Pyruvate ferredoxin oxidoreductase (PFR1) oxidatively decarboxylates pyruvate, resulting in reduced ferredoxin (FDX_{red}), CO₂, and acetyl CoA. The latter can probably be metabolized by PTA and ACK or ADH1 (indicated by a dotted line). Reduced FDX could then function as an electron donor for the hydrogenase (HYD1), resulting in hydrogen evolution in the dark.

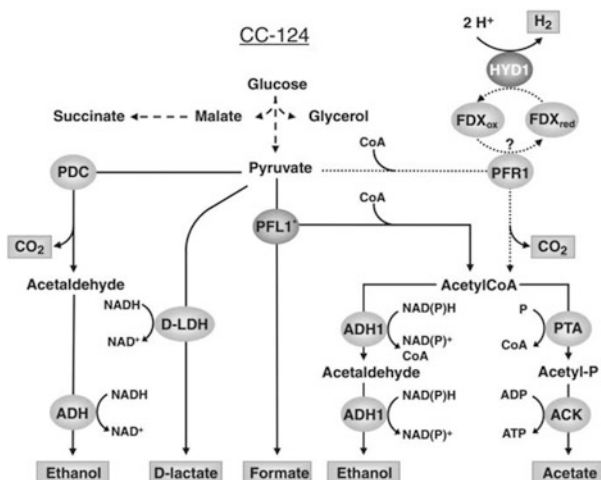


Fig. 3 Fermentative pathways of wild type *Chlamydomonas reinhardtii* and mutant PFL1-deficient strain 48F5

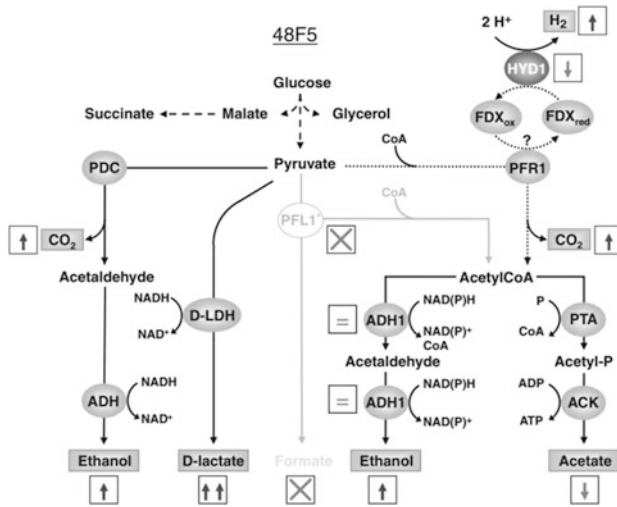


Fig. 4 Fermentative pathways of *C. reinhardtii* mutant PFL1-deficient strain 48F5

Pathways could be resulting in the other products as malate, succinate, and glycerol, which have been reported to be fermentative products of *C. reinhardtii*.

In contrast, the PFL1 pathway is not active in strain 48F5 because of disruption of the PFL1 gene (indicated by light gray lines and text, and red crosses near PFL1 and below formate in Fig. 4). Instead, the dark-incubated PFL1 mutant generated more H₂, CO₂, ethanol, and D-lactate than the wild type, while acetate secretion was reduced. Strain 48F5 also showed reduced *in vitro* hydrogenase activity and reduced HYD1 transcript and HYD1 protein levels. The amounts of ADH1 were almost identical in the wild type and the PFL1 mutant. Red downward arrows indicate a reduction, green upward arrows indicate an increase, and orange equal symbols indicate unchanged results. The double upward arrows for D-lactate indicate a more than twofold increase in this metabolite.

Costa et al. (2015) reported in their study the effect of inoculum concentration and carbon source to *C. reinhardtii*, as well as the influence of hybrid system and coculture (*C. reinhardtii* and *R. capsulatus*) on the photofermentative ethanol production. Maximum ethanol content (19.94 g/L) and productivity (0.17 g/(Lh)) were achieved by hybrid system in which the effluent of *C. reinhardtii* containing organic acids was used as substrate to *R. capsulatus*. The results from this work are beneficial to comprehend the potentiality of microalgae and photosynthetic bacteria to synthesize ethanol concerning several strategies such as media composition and different culture systems (hybrid and cocultivation).

4 Cases and Outlook for Commercial Production

It is broadly accepted that microalgal-based biofuels' economics would be largely improved if obtained in the frame of biomass biorefineries for the production of multiple commodities and higher value products. According to the US Energy Information Administration (EIA 2017), world biofuel production will increase from approximately 1.3 million barrels per day in 2010 to approximately 3.0 million barrels per day in 2040 (Kim et al. 2017). Fermentations run in study of (Rizza et al. 2017) yielded as coproducts 0.06 kg dry edible yeast *S. cerevisiae* per 1 kg dry *Desmodesmus* sp. biomass and the spent fermentation broth that would be used as animal feed supplements or other biotechnological applications. It is presumed that CO₂ produced as a fermentation product (at least 0.22 kg/kg of dry *Desmodesmus* biomass) could be recycled into microalgae to increase productivity and reduce the C footprint of bioethanol production, as previously reported in the literature (Stewart and Hessami 2005).

Moreover, sufficient carbohydrate content and efficient biomass harvest are required for economical bioethanol production from microalgae. Kim et al. (2017) studied the red algae *P. cruentum*, which is one of the most promising candidate organisms for producing fatty acids, lipids, carbohydrates, and pigments, from seawater and freshwater. In this, research was compared to the separate hydrolysis and fermentation, and simultaneous saccharification and fermentation methods. After optimizing each process, these authors designed an overall mass balance for bioethanol production: 100 g of seawater microalgae consists of 16.9 g glucose, 5.3 g of galactose, and 4.7 g of xylose, whereas 100 g of freshwater microalgae consists of 16.6 g glucose, 5.5 g galactose, and 6.4 g xylose. Saccharification and fermentation processing (5% substrate loading, w/v) of microalgae was conducted with pectinase (4.8 mg/g), cellulase (7.2 mg/g), and *S. cerevisiae* at 37 °C for 12 h, resulting in ethanol production of 5.58 and 5.90 g, respectively (Fig. 5). These results suggest that freshwater is a more efficient candidate for bioethanol production than seawater biomass.

Algenol is an American company owner of the first industrial plant for bioethanol production from engineered microorganisms. *Cyanobacterium* sp. with plasmids of a heterologous alcohol dehydrogenase gene (from *Synechocystis*) and pyruvate decarboxylase gene (from *Zymomonas*) (Piven et al. 2015). These high photosynthetic efficiency values can be ascribed not only to the species used but also to the geometrical characteristics of the photobioreactors (vertical bags) and to the cultivation under continuous conditions. The main limitations reported about this process are the fixed carbon/ethanol ratio, incidence of light, contaminants, and CO₂ supply time.

Other companies have been research of bioethanol production from microalgae, according to review (de Farias Silva and Bertucco 2016). In 2011, Joule Unlimited started a project to build an industrial plant using an engineered cyanobacterium from light, carbonic gas, water, and salts, with authorization of the Environmental Protection Agency (EPA) in 2014. This company claims to have an efficient system

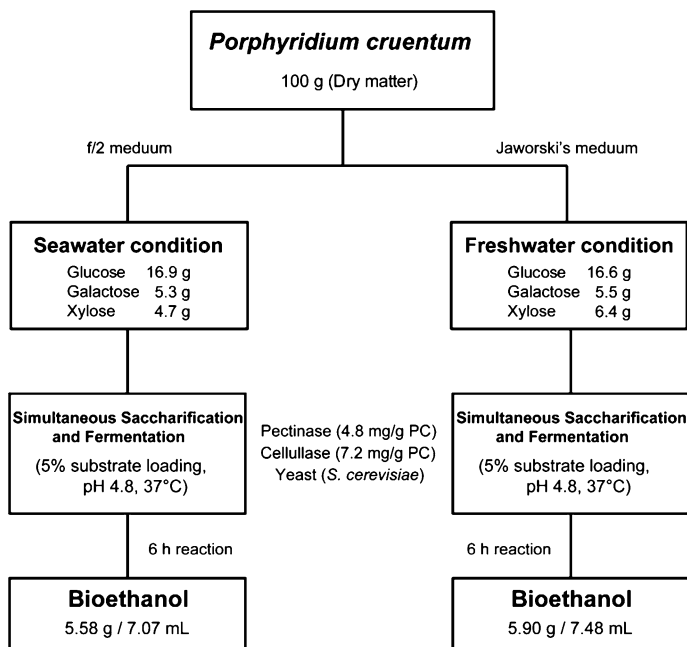


Fig. 5 Overall mass balance for bioethanol production from seawater and freshwater *Porphyridium cruentum* (Kim et al. 2017)

to directly produce biofuels such as alkanes and ethanol from CO₂. It was reported that a photosynthetic efficiency of 6–7% was achieved, in comparison with algal open-pond values of 1.5%, both in outdoor conditions. The system proposed is based on a reactor called SolarConverter® (a horizontal thin film plastic using CO₂ in a closed system and outdoor), where the mixing, culture density, and geometry (depth and surface area) have been studied to optimize the capture and conversion of CO₂ by an appropriate combination of the light and dark areas with the reactor. The company estimated ethanol productivity >230,000 L/(ha year) with a production cost of US\$ 0.16/L of ethanol with subsidies (US\$ 0.32/L without subsidies).

The costs of bioethanol production from sugarcane (Brazil, 0.16–0.22 US\$/L) are lower than those from corn (USA, 0.25–0.40 US\$/L), sugar beet (Europe, 0.43–0.73 US\$/L), and lignocellulosic materials (USA, 0.43–0.93 US\$/L) (Gupta and Verma 2015). It is quite difficult to estimate the economics of bioethanol from genetically engineered cyanobacteria. Algenol announced a production cost of approximately 0.79 US\$/L, and potential application of this method of bioethanol production will be increased with the continuous decrease.

5 Conclusions

According to available scientific literature and company initiatives, it is clear that the bioethanol production from microalgae should focus on not only the increase of the carbohydrate content but also the higher productivity of biomass. Technical and economic evaluations are necessary to verify the gains and losses of energy involved in the production of ethanol from microbiological biomass. The relevance of genetically engineered microorganisms with traditional processes must also be discussed, as it is well known that enzymes and yeasts can efficiently produce bioethanol with high productivity. The main technological bottlenecks of hydrolysis and fermentation seem to be being solved by several researches in this area, even helped by the production of the second-generation ethanol. Finally, more studies are necessary, particularly for better understanding of carbohydrate accumulation (hydrolysis and fermentation), as well as metabolic pathway of dark and photofermentation, which appears indeed as a highly promising technological application in the future.

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