

Review

Sustainable Microalgae and Cyanobacteria Biotechnology

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Abstract: Marine organisms are a valuable source of new compounds, many of which have remarkable biotechnological properties, such as microalgae and cyanobacteria, which have attracted special attention to develop new industrial production routes. These organisms are a source of many biologically active molecules in nature, including antioxidants, immunostimulants, antivirals, antibiotics, hemagglutinates, polyunsaturated fatty acids, peptides, proteins, biofuels, and pigments. The use of several technologies to improve biomass production, in the first step, industrial processes schemes have been addressed with different accomplishments. It is critical to consider all steps involved in producing a bioactive valuable compound, such as species and strain selection, nutrient supply required to support productivity, type of photobioreactor, downstream processes, namely extraction, recovery, and purification. In general, two product production schemes can be mentioned; one for large amounts of product, such as biodiesel or any other biofuel and the biomass for feeding purposes; the other for when the product will be used in the human health domain, such as antivirals, antibiotics, antioxidants, etc. Several applications for microalgae have been documented. In general, the usefulness of an application for each species of microalgae is determined by growth and product production. Furthermore, the use of OMICS technologies enabled the development of a new design for human therapeutic recombinant proteins, including strain selection based on previous proteomic profiles, gene cloning, and the development of expression networks. Microalgal expression systems have an advantage over traditional microbial, plant, and mammalian expression systems for new and sustainable microalga applications, for responsible production and consumption.



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1. Introduction

Growing concern about the production of sustainable chemicals has led to the investigation of alternative feedstock to produce chemicals and fuel using a green technology approach. There are numerous potential alternative feedstocks available today, including industrial wastes, agricultural residues, lignocellulose material, aquaculture wastes, etc. Such material can be transformed by microorganisms, which have been shown to have a high potential for partially replacing today's feedstocks for chemicals and fuel. Many fine biochemicals, such as pigments and exopolysaccharides from *Porphyridium cruentum* [1] and other value-added products such as tocopherols [2], are produced in various amounts by microorganisms such as microalgae and cyanobacteria. Microalgae are microscopic and unicellular eukaryotic species found individually or in chains or groups in the water column and sediments. These organisms are capable of performing photosynthesis,

important for life on the earth, producing close to half of the atmospheric oxygen by using carbon dioxide and sunlight. Microalgae, cyanobacteria, and bacteria are the base of the food web, providing energy for all the trophic levels above them. Cyanobacteria are microscopic organisms that perform photosynthesis as well, but they are prokaryotic organisms. The production of microalgae or cyanobacteria, on the other hand, is entirely dependent on the species, nutrients for mutation, cultivation conditions, particularly light intensity and quality, as well as the mode of process operation and configurations. Finally, all important factors affecting growth performance and productivity should adhere to the best strategy for improved compound productivity. Some microalgal metabolites have also been reported to act as antimicrobial agents, particularly in inhibiting the growth of various bacteria and fungi. Marine algae antibacterial substances have been found to be effective against some enterobacterial strains such as *Escherichia coli*, *Salmonella typhoid*, *Staphylococcus aureus*, and *Enterococcus faecalis* [3]. Because of the enormous diversity of marine organisms and the incalculable potential for discovering new antimicrobials, they are very promising candidates for antibiotic isolation. Furthermore, extracts from marine algae have been used to treat wounds, fever, and stomach aches, as well as harmful agents such as leishmaniasis, chagas, and trypanosomiasis. Another novel natural biochemical derived from algae has been used for cancer therapy (breast, cervical, and stomach cancer) with a satisfactory reduction in side effects. Antiviral agents produced by microalgae have also been discovered, which may represent other important biochemicals; these have different chemical compositions and apoptosis-focused action mechanisms [4,5].

Microalgae has also demonstrated a high potential as an alternative feedstock for third-generation biofuel production. Among the potential biofuels that can be produced from microalgal biomass are biodiesel, bioethanol, biobutanol, biogas, and biohydrogen [6]. Because microalgae do not contain lignocellulosic material, production of biofuel from microalgal biomass has been an alternative to terrestrial plant biomass, particularly carbohydrates and lipids from algae, which makes production of biofuel easier than terrestrial plants. Microalgal biomass can be used completely; however, the integration of production schemes would be essential for the sustainable production of microalgae from primary raw material, reused material, or waste material [7].

2. Applications of Biotechnology on Microalgae and Cyanobacteria Utilization

Table 1 summarizes the applications of microalgal biotechnology in various sectors: bioenergy, health care (bioactive phenolic compounds) [8], environmental applications (CO₂ capture, sustainable production from waste material) [7], aquaculture, raw material to elaborate balanced feed [9], cosmetics (the protein, lipids, ash, amino acids, carbohydrate composition of microalgal biomass give suitable functional properties to cosmetic formulation: moisture, brightness, firmness of skin, skin protection) [10], and food. Microalgal biotechnology can be described according to the sector of applicability by development stages: the first stage, which can be considered a short period of 2–5 years, the second stage with a medium period of time up to 5 years, and the third stage up to 7 years or longer, developments which applications are expected to have the technological appropriation at industrial level.

The period visualizing developments in the different sector of application is the time necessary for each stage to complete before the next step.

Table 1. Microalgal biotechnology developments visualized in terms of stages according to the application sector.

Sector	Stages		
	First Stage	Second Stage	Third Stage
Bioenergy	Bioprospecting of biofuels	Optimization of the production process	Use of biocatalysts, or new routes of biofuels production
Health care, therapeutics	Bioprospecting of health care compounds	Protein recombinant technology	Clinical assays for biomedical purposes
Environment	Biodegradation and biotransformation assays	Selection of phycoremediation or wastewater treatment process	On-site applications in which an integrated process has been developed
Aquaculture	Selection of strains and evaluation for specific fish or crustacean aquaculture	Balanced diets design	Feed additive production and implementation in aquaculture farms
Cosmetics	Bioprospecting of natural products from microalgae	Skin applications	Medical care supplements for skin applications
Foods	Natural products	Probiotics	Food supplements

2.1. High Valuable Compounds from Microalgae

2.1.1. Pigments

Pigments in microalgae are important because they are cytoplasmic compounds with a diverse chemical structural composition and three distinct activities, namely light energy capture, electron transfer reactions, and antioxidant activities. All these reactions are part of a complex photosynthetic chain mechanism system that performs functions such as light harvesting, energy transfer, photochemical redox reactions, and photoprotection. It is divided into three major pigment classes: chlorophylls, carotenoids, and phycobilins, all of which are light energy harvesting molecules. These pigments could absorb light quanta and then deliver photon energy to sites where the photosynthesis process began [11]. Table 2 compares the pigment components found in microalgae and cyanobacteria.

Table 2. Differences in pigment characteristics (chlorophyll, carotenoids, and phycobilins).

Characteristics	Chlorophyll	Carotenoids	Phycobilins
Common found place	Green plant and cyanobacteria	Brown algae (phaeophyta) and green algae (chlorophyta) red algae (rhodophyta)	Red algae (rhodophyta) and cryptomonads and cyanobacteria
Structural formulae description	Tetrapyrrole ring with a central magnesium atom	Polyene chain consisting of 9–11 double bonds and terminating in rings	Tetrapyrrole unit with open chain of four pyrrole rings (tetrapyrrole)
Absorption wavelength	450–475 nm (blue/blue–green); 615–675 nm (red)	400–550 nm (blue to green light)	500 nm to 650 nm (green–red)
Water solubility	Insoluble in water	Insoluble in water	Soluble in water
Examples	Five types of chlorophylls a, b, c and d	Xanthophylls (molecules containing oxygen) and carotenes (oxygen free molecule).	phycoerythrin, phycocyanin, allophycocyanin
Functions	Colour pigment used in photosynthesis as a photoreceptor	Biological abilities, including photosynthesis, photoprotection, plant coloration, and cell signaling.	Supplement the light-capturing ability especially red, orange, yellow, and greenlight.

Pigments are considered natural food colorants, and pigments extracted from microalgae and cyanobacteria have emerged as an appealing source to replace synthetic coloring. Apart from that, the sustainable and renewable properties of microalgal culture, combined with a diverse pigment profile, have made microalgae a popular choice for the above-mentioned applications. All these pigments have been shown to be capable of being produced on a large scale from cyanobacterial and microalgal cultures grown under optimal conditions [12,13].

Microalgae and cyanobacteria, as well as macroalgae, are a reliable source of natural bioactive compounds. Among pigments, carotenoids have shown significant commercial and industrial applications, with global demand increasing year after year. It was predicted that it would increase by 33 percent or reach 2.0 billion dollars in 2026 compared with 2019 [14]. This subsequently highlighted the promising market value with a variety of business opportunities. Along with these global market trends, green extraction technologies have emerged, which could provide new advantages in the sustainable production of these high-value products. Extraction technologies that have been widely reported in the extraction process for pigment carotenoids from algae samples include pressurized liquid extraction, supercritical fluid extraction, and subcritical fluid extraction [15].

Chlorophylls

Chlorophylls (chl) are the primary light-harvesting toward energy-transforming pigments in photosynthetic organisms and are structurally arranged by non-covalent bond to specific apoproteins [16].

Chlorophyll molecules, in general, have a tetrapyrrole ring with a central magnesium atom. Chlorophyll molecules are classified as chl a, b, c, and d, with structural differences in the side-group substituents on the tetrapyrrole ring. Apart from chl c, the chlorophylls a, b, and d are distinguished by the presence of long-chain terpenoid alcohol [17]. All chlorophylls have two major absorption bands, 450–475 nm for blue or blue–green and 615–675 nm for red, which results in the chlorophyll green color characteristic. Chl a is part of the core reaction center, a pigment–protein complex, located in the light-harvesting antennae component, accompanied by chl b or chl c. The accessory pigments, chl b, c, and d extend the broad range of light absorption. Thus, more energy can be captured from sunlight by the photosynthetic organism [18].

Chlorophyll derivatives (chl a, chl b, and chl c) in microalgae and cyanobacteria are promising for health-promoting activities such as anti-inflammatory properties, anti-colorectal cancer, human hygiene properties, anti-stomach disorder activities, and anti-bad breath protection [19]. A summary of previous research and development on the medical applications of chlorophyll and its derivatives has been published to provide a better understanding of science-based health claims [20].

Chlorophyll and its derivatives have structural functional properties and pharmaceutical properties. Photosynthesis is a process that converts solar energy into chemical energy by harvesting light energy along with water and carbon dioxide to produce oxygen and carbohydrates as byproducts. Based on the structural properties of chlorophyll, it contained a heme moiety part that was like the hemoglobin structure, which may serve to facilitate CO₂/O₂ exchange by conferring the functionality to be used in the treatment of ulcers, stimulation of cell growth, acceleration of tissue formation, and increasing the rate of healing. Other chlorophyll derivatives have antioxidant and antimutagenic properties by trapping mutagens in the gastrointestinal tract [21].

Carotenoids

Carotenoids are a large group of biological chromophores with an absorption range of 400 to 550 nm, at which wavelength the carotenoids have strong absorption, giving them their yellow–orange color [22]. Carotenoids play three major roles in the photosynthetic organism: (i) as accessory light-harvesting pigments by transferring electrons to chl a [23]; (ii) structural entities within the light-harvesting and reaction center pigment–protein

complexes [24], and (iii) molecules involved in the protection against oxygen reactive species [25].

Carotenoids have two profiles: a primary activity involved in photosynthetic processes and a secondary activity produced by microalgae under the following stress conditions [26]. There are over 400 known carotenoids, with β -carotene and astaxanthin being the most common carotenoid produced on a large scale. *Dunaliella salina*, which grows under high salinity and light intensity conditions, produced 14 percent of the dry biomass weight of carotenes [27]. In addition to their biological role, carotenoids have piqued the industry's interest in health applications due to their bioactive potential as antioxidants, anti-inflammatory agents, and anti-tumor agents when compared to others. Consumption of a diet supplemented with β -carotene has been linked to cancer prevention in some tissues [28]. The freshwater microalgae *Haematococcus pluvialis*, which contains more than 3% dry weight biomass, is the best source of astaxanthin production on a large scale [29]. Cyanobacteria and microalgae can grow in a variety of environments. The ability to modify the metabolism process in cyanobacteria or microalgae under different growth conditions has resulted in the improvement and optimization of a specific compound over two or three industrial bioprocess stages.

Cyanobacteria could also produce a wide range of terpenoids and carotenoids via the carotenogenesis biosynthetic pathway. *Anabaena* and *Nostoc* species, for example, converted phytoene to lycopene via a reaction catalyzed by a phytoene desaturase [30]. Lycopene is an important molecule in the synthesis of β -carotene from lycopene, which is catalyzed by lycopene cyclase [31], while γ -carotene is an intermediate in the synthesis of β -carotene in two steps catalyzed by lycopene cyclase [32]. The genes involved in carotenogenesis are conserved in photosynthetic aquatic organisms. However, carotenoids are only expressed and produced by a few cyanobacteria and microalgae species. In the biotechnological industry, the expression of certain genes during carotenogenesis is critical to increase carotenoids productivity. Genetic engineering was used in another study to improve carotenoids production in cyanobacteria [33]. The approach of genetic alteration has received a lot of attention in recent years, and a few examples have already been described in cyanobacteria. As a result, effective genetic engineering for other microalgae or cyanobacteria can be explored further by emphasizing the bioactivities of compounds they produce.

Phycobiliproteins

The major antennae component of cyanobacteria and red algae contain phycobilins (phycoerythrobilin, phycocyanobilin, and phycourobilin), which are packed into complex structures called phycobilisomes. These complexes are linked to photosynthetic membranes [34]. Phycobilisomes are linear tetrapyrroles with a magnesium atom attached. The wavelength absorption range of these accessory pigments is 500–650 nm, with colors ranging from blue–green to green, yellow, orange, and red. Phycobiliproteins, unlike chl proteins and carotenoid proteins, are water soluble, and the pigments are covalently bound to apoproteins. In general, cyanobacterial species have three important protein-pigment complexes that are covalently linked to chromophores: C-phycocyanin (PC, λ_{\max} 610–620 nm), allophycocyanin (APC, λ_{\max} 650–655 nm), and phycoerythrin (PE, λ_{\max} 540–570 nm) [35].

Phycocyanin is a blue pigment that has important applications as a food supplement and a fluorescent biomedical marker in a variety of pharmaceutical applications. Phycocyanin is made up of a protein moiety (and apoprotein subunits) and bilin-type chromophores. Phycocyanin accounts for approximately 0.439 g/g of cyanobacteria biomass [36], but the concentration of phycocyanin in the cell is completely dependent on environmental growth conditions. Phycocyanin is thought to be an accessory pigment that absorbs light energy and transfers it to chlorophyll-a [35]. Phycocyanin has been proposed by several authors as a carbon storage material [37] or as a nitrogen source during nitrogen deficiency [38]. Some cyanobacteria species, such as *Calothrix* sp. [39], *Oscillatoria* sp., [40],

Phormidium sp., [41], and *Synechocystis* sp., have previously been proposed as a source for industrial production of phycocyanin [42].

Previous research found that highly purified phycocyanin (PC) extracted from the thermophilic cyanobacterium *Thermosynechococcus elongatus* had anti-cancer activity against three cancer cell lines: colorectal adenocarcinoma, hepatocellular carcinoma, and mammary gland breast carcinoma. An in vitro assay demonstrated the cytotoxic effect against cancer cells, yielding these results [35]. In another study, the combination effect of PC and betaine also showed a good result on the growth of A549 lung cancer, with a decrease in cancer cell viability of up to 60% through in vivo studies [43]. Previous research revealed that this PC has two protein bands visible in SDS-PAGE analysis: α -phycocyanin (15 kDa) and β -phycocyanin (16.5 kDa) [35]. Another study found that using PC had an effective inhibitory effect on A53T α -synuclein and A β 40/42 fibril formation at low stoichiometric ratios [44]. Further research should be conducted to identify natural biomolecules that can be used to develop therapeutic aids for protein-related diseases characterized by an inadequacy in protein folding, such as cataracts, Alzheimer's disease, and Parkinson's disease [44].

2.1.2. Superoxide Dismutase

To maintain the balance of reactive oxygen species, microalgae and cyanobacteria have an effective protective mechanism against highly reactive species produced during the electron harvest–transport chain (ROS). A high accumulation of ROS causes an imbalance in ROS production, resulting in oxidative stress. Some enzyme activities involved in cyanobacteria photosynthesis are mentioned to share a protective mechanism against ROS but act in different ways [45].

Superoxide dismutase (SOD) is an enzyme found in plants, algae, microalgae (including the Antarctic marine microalgae *Chaetoceros brevis*), and cyanobacteria that catalyzes the disproportionation of the anion radical to molecular oxygen and hydrogen peroxide. This enzyme protects cells, at least in part, from reactive oxygen species formed during photosynthesis; under normal conditions, the enzymatic protection mechanism maintains the balance of reactive species. ROS are either free radicals, reactive anions containing oxygen atoms, or molecules containing oxygen atoms that can either produce or be chemically activated by free radicals. The main cause of cell damage is the alteration of macromolecules caused by ROS, such as polyunsaturated fatty acids in membrane lipids, essential proteins, and DNA (Figure 1).

SOD expression is highly regulated, with specific metalloforms acting as inducible protectors in specific cellular compartments. SOD works efficiently during photosynthesis, but little is known about its response to irradiance and oxygen accumulation stresses when these microorganisms grow in photobioreactors. When SOD has maximum accumulation into the cell, SOD accumulation during growth requires a stress event because SOD expression is highly regulated and inducible. Okamoto et al. [46] discovered a significant increase in SOD activity when the microalgae *Tetraselmis gracilis* was exposed to high cadmium levels. CCAP 19/18 was also reported in another study on the enhancement of SOD production from *Dunaliella salina*. The study found that cultivating these microalgae under high light intensity and nitrogen-depleted conditions resulted in a higher level of SOD than the control condition [47]. There are few reports on the production of SOD in photobioreactors, whether an environmental stress is required, such as increasing light irradiance to the culture, or whether oxygen accumulation in the PBR is required. Microalgae and cyanobacteria grown in photobioreactors under stress conditions could be a good alternative for producing this valuable fine product.

Extracts of cyanobacteria and eukaryotic algae (red, green, and brown algae, diatoms, *Euglena*, and *Charophyta*) have been found to contain SOD [48–50]. In general, these SOD were resistant to cyanide and antibodies against Cu, Zn, indicating the presence of Fe- and/or Mn-enzymes and the absence of Cu, Zn-SOD enzymes, although an aerial green alga lacks Cu, Zn-superoxide dismutase, aquatic angiosperms and ferns, such as other

land plants do. Thus, the distribution of Cu, Zn-superoxide dismutase reflects the organism's phylogeny.

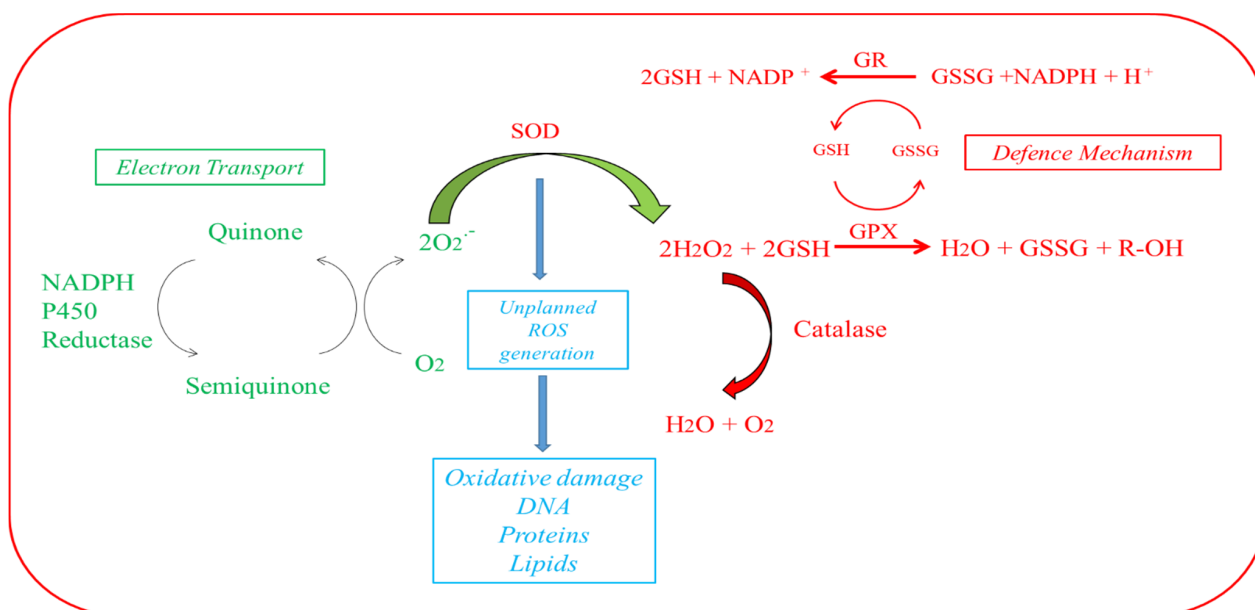


Figure 1. The schematic diagram of ROS action on DNA, protein, and lipid in the microalgae cell body.

Sport injuries, knee joint osteoarthritis, rheumatoid arthritis, and intestinal cystitis have all proven to be effective anti-inflammatory treatments. Other diseases, such as Alzheimer's disease, Parkinson's disease, cancer, and aging, have been reported or implicated because of oxidative stress and the release of an excessive amount of ROS. SOD can be used to treat these diseases; in fact, microalgae can be a good source of SOD.

2.1.3. Antibacterial, Antiviral, Anticancer, and Anti-Inflammatory Activities

Bioactive compounds derived from different microalgae have been identified as promising substances for use as antibacterial, antiviral, anti-inflammatory, and antifungal agents [51]. Several microalgae, particularly extremophile strains, have been shown to have a diverse distribution of metabolites that can be used as antimicrobials. The genetic diversity of cyanobacterial and microalgal organisms is linked to their biosynthetic pathway diversity. Cyclic peptides are secondary metabolites produced by cyanobacteria that construct libraries of their biosynthetic pathways to produce analog compounds. The antibacterial activity of pitipeptolide A is determined by post-translational n-methylation of phenylalanine. Some cyanobacterial genera produce alkaloids, hapalindole-type compounds composed of an indole moiety and a cyclized isoprene. Calothrixin A, another alkaloid, has potent inhibitory activity against tumor cell lines at nanomolar concentrations; their activity is related to DNA topoisomerase inhibition [52].

Streptococcus pneumoniae, *Escherichia coli*, *Staphylococcus aureus*, and other transmitted healthcare microbes such as *Acinetobacter baumannii*, *Klebsiella* sp., and *Pseudomonas aeruginosa* have all been shown to be inhibited by microalgal metabolites [53,54]. Several factors, including the solvent used during the extraction process, can influence the antimicrobial efficiency of microalgal metabolites. Mashhadinejad et al. [55] discovered that different solvents had varying inhibitory effects on Gram-positive and Gram-negative bacteria such as *Bacillus subtilis*, *Staphylococcus* sp., *E. coli*, and *P. aeruginosa*.

It has been reported that the presence of metabolites and lipid content accumulated in microalgal cells influences the efficiency of microbial inhibition by microalgal extract. Polysaturated and unsaturated fatty acids found in microalgae have been found to be antimicrobial, particularly against Gram-positive bacteria. Other compounds found in microalgae include hexadecanoic acid (16:0) and octadecadienoic acid (C18:2), which have

been shown to have antimicrobial activity [56,57]. Other metabolites found in microalgae include astaxanthin, violaxanthin, squalene, lutein, and sulphate polysaccharides, which have been shown to have high antioxidant and antimicrobial activity. Table 3 summarizes metabolites from different microalgal biomass.

Table 3. Metabolite extracted from different microalgal biomass.

Microalgae	Metabolite Compound	References
<i>Chlorella zofingiensis</i>	Astaxanthin	[58]
<i>Chlorella minutissima</i>	Phytol	[59]
<i>Spirulina</i>	Polysaccharides	[60]
<i>Spirulina platensis</i>	Phycocyanin, oleic acid, linolenic acid	[61,62]
<i>Dunaliella</i> sp.	Diacylglycerol	[63]
<i>Nannochloropsis</i>	Diacylglycerol	[64]
<i>Dunaliella salina</i>	Palmitic acid	[65]

2.2. Biofuels from Microalgae

Microalgal biomass contained several major biomolecules, such as lipid and carbohydrate, which could be converted into third-generation biofuels. The amount of lipids in microalgal biomass varies by species. Several studies have investigated the lipid content range of 20–50 percent in various microalgal species. Microalgal lipid extracted from biomass can then be converted into biodiesel via a catalytic chemical reaction using either a chemical or an enzyme as a catalyst [66]. Microalgal-based biodiesel is thought to be competitive with terrestrial crops due to the high growth rate, high lipid intracellular accumulation, and non-seasonal production. Furthermore, microalgal growth can be performed on bioremediation processes, wastewater treatment, or CO₂ sequestration [67], or in limited growth conditions for other microorganisms [68].

Other liquid biofuels, such as bioethanol and biobutanol, can also be produced by utilizing microalgal biomass. The production of these biofuels involves the fermentation of microalgal carbohydrate polymer using microorganisms such as yeast and bacteria as biocatalysts [69]. Prior to the fermentation process, the biomass must be pretreated and the carbohydrate extracted (Figure 2). Yeast, such as *Saccharomyces cerevisiae*, which is a common microorganism used in fermentation, will consume the carbohydrate and convert it into bioethanol via anaerobic fermentation during the fermentation process. Several studies have been conducted to investigate the potential of microalgal carbohydrate as a bioethanol feedstock using various pretreatment methods (Table 4). Clearly, the majority of the research indicated that different pretreatment processes could have a significant impact on the release of microalgal carbohydrates and bioethanol production.

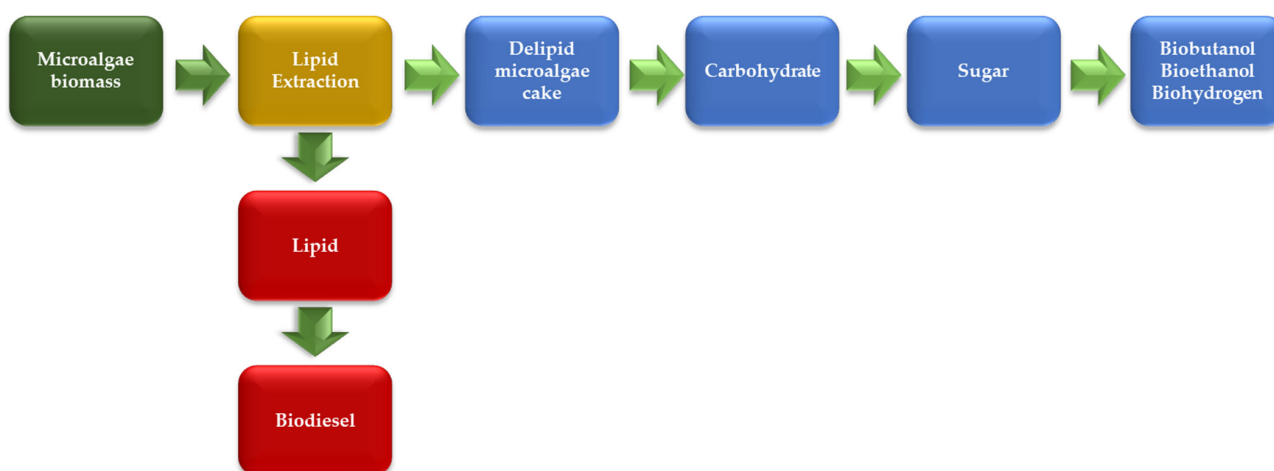


Figure 2. Flow diagram of microalgal biomass pretreatment process prior to carbohydrate polymer production and fermentation.

Table 4. Bioethanol production from different microalgal biomass feedstock.

Microalgae Strain	Pretreatment Condition	Bioethanol	References
<i>Chlorella</i>	Dilute acid 5% sulphuric acid	0.28 g/g biomass	[70]
Mixed microalgae	Dilute sulphuric acid 0.5 N H ₂ SO ₄ at 120 °C for 4 h	0.18 g/g biomass	[71]
<i>Chlorella</i> sp.	Dilute alkaline treatment at 2% NaOH for 2 h at 120 °C	0.081 g/g biomass	[72]
<i>Chlorococcum infusionum</i>	0.74% NaOH at 120 °C for 30 min	0.26 g/g biomass	[73]
<i>Nannochloropsis gaditana</i>	1 M NaOH, 120 °C for 30 min	0.094 g/g biomass	[74]
Defatted <i>Nannochloropsis oculata</i>	4% H ₂ SO ₄	0.062 g/g sugar	[75]
<i>Dunaliella tertiolecta</i>	Pretreatment using 1% (v/v) sulfuric acid at 120 °C for 15 min	0.62 g/g biomass	[76]

Another study on the fermentation of lipid-extracted *Chlorella* sp. KR-1 biomass found that when the fermentation was performed using *S. cerevisiae* KCTC 7931 under separate hydrolysis and fermentation (SHF) processes, approximately 0.4 g/g sugar of bioethanol corresponding to 0.16 g/g biomass was produced. Other studies on bioethanol production from microalgal species such as *Tetraselmis suecica*, *Dunaliella tertiolecta*, *Chlamydomonas* sp., and *Stigeoclonium* sp. have also been published [77–80].

Biobutanol, on the other hand, is produced through the conversion of microalgal carbohydrate by *Clostridium* sp. under strictly anaerobic fermentation conditions. Various products such as butyric acid, acetic acid, acetone, ethanol, and butanol are produced during the fermentation process, which involves two major metabolic pathways: acidogenesis and solventogenesis [81]. Acidogenesis is a biological conversion reaction by bacteria of carbohydrate or monomer sugar into volatile fatty acids such as acetic acid and butyric acid. As the second phase of ABE fermentation, the biological reaction is continuing to solventogenesis, which is a biochemical reaction by *Clostridium* species to produce acetone and butanol. There have been few studies on biobutanol production from microalgal biomass to date. For instance, Hong et al. [82] reported that approximately 3.48 g/L of biobutanol was produced from the fermentation of *Gelidium amansii* pretreated with 2% sulfuric acid. Another study on ABE fermentation of *Chlamydomonas reinhardtii* CCAP 11/32C produced 10.31 g butanol/g biomass under optimal conditions. The study also found that pretreatment processes could significantly influence biobutanol production [83]. Investigation on biobutanol from enzymatic hydrolyzed *Nannochloropsis* sp. also indicated that approximately 2.61% of butanol was produced from the ABE fermentation [84]. In addition, the production of butanol from different microalgae such as *Chlorella* sp. and *Borodiniopsis texensis* CCALA [85] has been reported. A study reported that microalgae cultivated using wastewater could accumulate high carbohydrate content, a procedure that can be useful for butanol production from *Borodiniopsis texensis* CCALA.

3. Implementation of an Efficient Production System: Mixotrophic Cultivation of Microalgae for Biodiesel Production

The main cultivation systems for microalgae are open (such as raceway) and closed photobioreactors. The selection of a cultivation system depends on several factors, which can be chosen in order of application and economic objectives. Combining the selection criteria of the cultivation system in order of importance the selection can be as: the algal species, the desired final product, water supply, availability of sunlight, the cost of facilities, availability of nutrients, climatic conditions, and CO₂ supply [86]. The amount of nutrients and certain metals (i.e., iron and magnesium) must be optimal because they are important for the growth of microalgae and CO₂ fixation efficiency. A well-designed cultivation system can improve the efficiency of CO₂ capture by microalgae [86]. Another important factor when selecting a cultivation system is the likelihood of contamination by other microorganisms. For example, algal species like *Chlorella*, *Dunaliella*, and *Spirulina* can grow only in specific environments, hence are not likely to be contaminated by other microalgae when

cultivated in an open-air system, and low possibility of bacterial contamination. In contrast, some marine microalgae such as *Tetraselmis*, *Skeletonem*, and *Isochrysis* are susceptible to foreign invasion and some additional manipulation should be considered [87].

Light as a source of energy for photosynthetic organisms is the main limiting factor in the development of a cultivation system for microalgae. The photosynthesis rate is directly proportional to the light intensity above the light compensation point (not photosynthesis inhibition and not overshadowed by other cells). Mostly in microalgae, the photosynthetic system becomes saturated to a radiation close to 30% of the total solar irradiance, i.e., between 1700–2000 $\mu\text{Em}^{-2}\text{s}^{-1}$. Some species of phytoplankton grow to optimal intensities of 50 $\mu\text{Em}^{-2}\text{s}^{-1}$ and are photoinhibited to 130 $\mu\text{Em}^{-2}\text{s}^{-1}$. The most significant issues for commercial cultivation of photosynthetic cells are photoinhibition and light limitation. One possible solution is to use photosynthetic cell heterotrophic metabolism to replace or supplement energy and carbon material from organic sources. According to some research, heterotrophic and autotrophic metabolic activities coexist, resulting in mixotrophic growth [88]. The relative contribution of photoautotrophic metabolism to biomass production increases with an increase in the coefficient of absorption of light or an increase in the supply of CO_2 and a decrease in the supply of the organic carbon source. Certain cellular components can accumulate during heterotrophic growth [89,90].

It was assumed that autotrophic and heterotrophic growth in *Euglena gracilis* [91] and *Spirulina platensis* [92] occur simultaneously and independently in cells growing in mixotrophic condition. In general, by mathematical terms, the mixotrophic growth is the sum of the growths in autotrophic and heterotrophic conditions, and can be expressed as:

$$\frac{dX_M}{dt} = \frac{dX_A}{dt} + \frac{dX_H}{dt} \quad (1)$$

where X_M are cells growing in mixotrophic conditions, X_A are cells growing in autotrophic conditions, and X_H are cells growing in heterotrophic conditions. However, it is difficult to know how many cells are growing in autotrophy or heterotrophy at any time during the cell growth. To simplify the conceit, cells growing in autotrophic mode can be defined as an α fraction of the total cell biomass and a β fraction of cells growing in heterotrophic metabolic conditions. In other words, Equation (1) may describe as.

$$\frac{dX_M}{dt} = \alpha \frac{dX_M}{dt} + \beta \frac{dX_M}{dt} \quad (2)$$

The values of α and β can be calculated on the basis of a ratio of the autotrophic growth rate dX_A/dt and heterotrophic growth rate dX_H/dt , both of which proceed, or are suggested to proceed, during mixotrophic cultivation dX_M/dt .

$$\alpha = \frac{dX_A/dt}{dX_M/dt} \quad (3)$$

$$\beta = \frac{dX_H/dt}{dX_M/dt} \quad (4)$$

The values of α or β are 0.0 when growth occurs in the absence of organic carbon and light energy, whereas, the summation of α and β are 1.0, signifying growth in mixotrophic conditions. In other words, the fractions α and β during mixotrophic growth vary as follows [93]:

(i) For the autotrophic fraction

The condition $\alpha \leq 1$ at initial time (t_0) signifying that light is unlimited and the organic carbon source consumption is negligible.

The condition $0 < \alpha < 1$ at any time (t_i).

The condition $\alpha \approx 0$ at final time (t_f) light irradiance is limiting and growth is almost entirely dependent on organic carbon source consumption. In other words:

$$Y_{X/S} = \frac{X_i - X_0}{S_0 - S_i}$$

$$\int_{X_{M_i}}^{X_{M_{i+1}}} \Delta X_M = \frac{Y_{X/S}(S_0 - S_i)}{1 - \alpha_i} \int_{t_i}^{t_{i+1}} \Delta t_i \tag{5}$$

where $Y_{X/S}$ is the yield of biomass (X) produced by substrate (S) consumed.

(ii) For the heterotrophic fraction

The condition $\beta = 0$ at time = t_0 that light is unlimited and organic carbon source consumption is negligible

The condition $0 < \beta < 1$ at any time = t_i

and with the condition $\beta \approx 1$ at final time = t_f signifying that light irradiance is limiting and growth depends on organic carbon source consumption. In other words:

the light saturation constant, $K = \frac{Y_{kj}A}{V}$, biomass produced per energy unit and illuminated area, this constant can be used as photobioreactor design criteria, because it involves the light energy absorbed by a specific microalgal specie and the amount of light irradiated in a specific photobioreactor geometry.

$$\int_{X_{M_i}}^{X_{M_{i+1}}} \Delta X_M = \frac{Kl_o}{1 - \beta_i} \int_{t_i}^{t_{i+1}} \Delta t_i \tag{6}$$

And then, β_i at any time can be calculated

$$\beta_i = 1 - \frac{Kl_o}{X_{M_{i+1}} - X_{M_i}} (t_{i+1} - t_i) \tag{7}$$

Kinetic of Biofuels Production by Microalgae

Microalgae can be grown in three ways: photoautotrophically, heterotrophically, and mixotrophically [94,95], as shown in Table 5. Due to the lower production costs, photoautotrophic cultivation using sunlight as an energy source is preferred over the other two types of cultivation [94]. In the course of mixotrophic cultivation, microalgal cells absorb both CO₂ and organic carbon [94,96]. Several reports on the cultivation of microalgal species in mixotrophic conditions with various organic carbon sources have been published. In general, the amount of biomass increases significantly under mixotrophy. The carbon mass balance can be summarized as follows: total carbon of microalgal biomass equals sum of inorganic carbon source (CO₂) plus carbon from organic source. However, due to further limitations on an important growth factor, such as nitrogen limitation or nitrogen starvation, some molecular components are stored in special compartments.

Biodiesel production kinetics can be described as follow:

Considering Equation (1), biodiesel is produced under mixotrophic condition, some amount is produced autotrophically and other parts heterotrophically; mathematically lipid production may be described by Equation (2).

However, lipid content is a part of biomass produced in both metabolic routes of growth, which depends essentially on the microalgal specie and the type of organic carbon source. Biodiesel production may be described in terms of α_b and β_b as the fraction of lipids in autotrophic and heterotrophic growth, respectively:

$$\frac{dB_M}{dt} = \alpha_b \alpha \frac{dX_M}{dt} + \beta_b \beta \frac{dX_M}{dt} \tag{8}$$

The fractions α_b and β_b are the fractions of biomass that represent total lipid content in microalgae grown under mixotrophic conditions.

Table 5. Biomass and lipid productivity under different production strategies.

Microalgal Specie	Biomass Productivity mg/L/d	Carbon Source	Metabolic Cultivation	Lipid Content %	PFA Content mg/g	Reference
<i>C. vulgaris</i>	137.43	molasses	Mixotrophic	39	59.7	[94]
<i>C. vulgaris</i>	91.57	CO ₂	Autotrophic	19	36	[94]
<i>Scenedesmus dimorphus</i>	119.25	Sugar cane Bagasse	Mixotrophic		40.02	[97]
<i>Scenedesmus dimorphus</i>	140.37	Apple-pomace hydrolysate	Mixotrophic	41	64	[95,96]
<i>Scenedesmus dimorphus</i>	96.4–96.55	CO ₂	Autotrophic	28	37.65	[95]
<i>Chlorella vulgaris</i>		Sorghum bagasse	Mixotrophic	34.4		[96]
<i>Arthrospira platensis</i>	1330	Sucrose	Mixotrophic	3.68 mg/g cell		[98]
<i>Arthrospira platensis</i>	153	CO ₂	Autotrophic	3.12 mg/g cell		[98]
<i>Graesiella</i> sp.	170	CO ₂ + Glucose	Mixotrophic	45.8	1.47	[88]
<i>Graesiella</i> sp.	120	CO ₂	Autotrophic	19.4	16.66	[88]
<i>Dictyosphaerium</i> sp.	230	CO ₂ + Glucose 10 g/L	Mixotrophic	32		[99]
<i>Dictyosphaerium</i> sp.	230	CO ₂ + Glucose 20 g/L	Mixotrophic	42		[99]

4. Other Important Applications of Microalgal Biotechnology

Microalgae are a diverse group of photosynthetic microorganisms that grow in a variety of metabolic modes (autotrophic, heterotrophic, and mixotrophic). These cells are represented by dozens of families, both eukaryotes and prokaryotes, all over the world. Their classification has been based on biochemical profiles, pigments, microstructures, and sizes ranging from 2 to 200 microns, among other factors [100–102]. Microalgae play a critical role in the fixation of carbon and nitrogen, making them the primary producers of excellence by maintaining the homeostasis of biological systems and trophic chains. They may also act as regulators of rising carbon emissions, allowing climate change to be mitigated [103,104].

Humans have increased their use of microalgae in recent years; they are among the three most abundant groups (diatoms, green algae, and cyanobacteria), and they are the most widely used in biotechnological processes [105]. Three applications of microalgae have had an impact due to their importance in economic activities: (1) animal feed additives, (2) phycoremediation, and (3) waste management (Table 1).

4.1. Additives for Animal Feeding

The use of microalgae as nutritional additives for animal feed and human consumption, for instance, is recognized by the production of omega-3 polyunsaturated fatty acids [106]. Of all the species studied, the most widely used are the genera *Chlorella*, *Tetraselmis*, *Isochrysis*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*, *Nannochloropsis*, *Skeletonema*, and *Thalassiosira* for aquaculture activities, mainly because of their high nutritional content, presence of vitamins, minerals, pigments, and antioxidant compounds [107,108]. Several microalgae such as *Aphanizomenon*, *Chlorella*, and *Arthrospira*, are high protein-rich species, making them essential in a world where population density continues to increase [109,110]. Instead, lipids and highly polyunsaturated fatty acids such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (ARA) have been found in microalgae; similarly, a high concentration of linoleic and linolenic acids has been found in several species of microalgae such as *Spirulina* [111,112]. Other essential compounds obtained from microalgae are β -carotene, lutein, astaxanthin, chlorophyll, phycobiliprotein, among others, such as vitamin precursors, antioxidants, immune system promoters, and anti-inflammatory agents

have been obtained from several microalgal genera such as *Anabaena*, *Nonstock*, *Botryococcus*, *Synechococcus*, *Chlamydomonas*, *Scenedesmus*, *Perietochloris*, and *Porphyridium* [113,114]. *Nannochloropsis* biomass has shown functional properties in the inclusion to balanced diets of the tropical edible fish *Atractosteus tropicus*, Gill 1863 [115].

4.2. Phycoremediation

The phycoremediation of wastewater using microalgae is a highly efficient and economically viable process that allows the removal of chemical pollutants and nutrients by using them as energy sources, as well as the use of CO₂ to produce biomass with multiple applications using biotechnology and bioprocesses such as biofuel production, feed additive extraction, and so on [116]. To achieve maximum efficiency in the phycoremediation process, it is critical to use the most efficient metabolic growth mode, which can be classified into three types that correspond to the use of a single source of carbohydrates, though in waste management or phytoremediation, these processes proceed with a mixture of carbohydrates or a non-common carbohydrate, respectively: (1) autotrophic, (2) heterotrophic, and (3) mixotrophic [117]. Photo-autotrophic microalgae require light, water, atmospheric CO₂, and nutrients to grow, resulting in high lipid, carbohydrate, and protein production. In the dark, heterotrophic microalgae can produce carbohydrates and lipids by using organic and inorganic CO₂. This type of microalgae has the advantage of simple maintenance and operation, as well as low production costs; however, it has the disadvantages of a limited number of microalgal species that can be used, the addition of nutrients, contamination with opportunistic bacteria, and the inability to produce sunlight-induced metabolites [118]. Mixotrophic microalgae, on the other hand, combine the capabilities of autotrophic and heterotrophic microalgae at the same time and use a variety of organic carbon sources such as glycerol, carbohydrates (hexoses and pentoses), amino acids, organic acids, and low-cost substrates such as agro-industrial residues [117], making them the most interesting for use in industrial processes by reducing cultivation costs and achieving maximum production, as proven by species namely *Cryptocodinium cohnii*, *Chlorella vulgaris*, and *Chlorella sorokiniana* [87,118,119].

4.3. Waste Management

One of the most important applications of microalgae, such as *Chlorella vulgaris*, that has recently been developed is wastewater treatment [120]. The advancement of this technology allows microalgae to absorb various types of contaminants in water from municipal, industrial, agro-industrial, and livestock wastewaters, producing algal biomass and allowing the water to be safely disposed. It should be noted that the use of microalgal treatment plants has a positive effect because bioenergy is produced, allowing for a more effective, sustainable, simple, economical, and environmentally friendly remediation process [121,122].

The advantage of using microalgae for wastewater treatment is that it allows the process of bioenergy production during remediation to be channeled through the following processes [123]: (1) biohydrogenesis, which involves converting waste to carbohydrates and ethanol; (2) complete solventogenesis, which involves converting waste to butyrate and butanol; (3) methanogenesis, which involves converting waste to simple sugars and methane; (4) bioelectrogenesis, which involves converting waste to simple sugars and CO₂ (glycolysis, ATP production, and gluconeogenesis); (5) incomplete solventogenesis, in which wastes are converted into carbohydrates and ethanol; (6) anoxic respiration, in which wastes are converted into volatile fatty acids (VFA) and polyhydroxyalkanoates (PHA); and (7) lipogenesis, in which microalgal biomass and lipids are produced (fatty acids).

Of course, the efficiency of the remediation method will be determined by the species of microalgae (microorganism partners) that are handled, in addition to the pretreatment [124]. For example, in wastewater treatment, the use of *Clostridium* with acid pretreatments and a bacterial consortium (symbiotic processes) is highly efficient [125]. However, to achieve maximum metabolic efficiency, the remediation process with microalgae requires various

pretreatments such as thermal, chemical (acidic and alkaline), oxygen, and infrared light, among others [126]. To improve the efficiency of the remediation process, several types of bioreactors have been developed, including suspension, embedded bed, biofilm, fluidized bed, reverse flow, anaerobic sludge bed, extended bed, and granular sludge, immobilized systems, and membrane-based systems [127]. Another benefit of using microalgae in wastewater treatment is the removal of toxic minerals such as As, Br, Cd, Hg, Pb, Sc, and Sn ions, as well as the production of biomass (organic matter) that can be used in fermentation processes [128,129].

During the degradation process, wastewater (domestic or industrial) acts as an electron donor (anolyte fuel) to generate reduced equivalents through the action of microalgal and bacterial consortia (microbial fuel cells, MFC) at a very high metabolic rate for extended periods of time. However, environmental variables such as pH, temperature, salinity, light, water flow rate, microelements, C:N:P ratio, etc., must be controlled [130,131].

Biodiesel, on the other hand, can be produced from the production of microalgal biomass from wastewater treatment, where microalgae fix inorganic or organic CO₂ to produce fatty acids (biofixation) via the autotrophic/heterotrophic biosynthetic pathway of lipids, and it is converted into a biofuel via transesterification processes. *Scenedesmus* microalgae, for example, were cultured in wastewater fermented with swine feces, allowing for high lipid production and waste removal [132]. Furthermore, several microalgal species, including *Chlamydomonas reinhardtii* and *Chlorella vulgaris*, contain a high concentration of glycogen, starch, cellulose, agar, and other compounds that can be converted into bioethanol during fermentation [133,134]. However, tolerance to high CO₂ concentrations is a limitation for biofixation; as a result, many microalgal species have been studied, with the most efficient being *Chlorella* spp., *Arthrospira* (formerly *Spirulina*) spp., *Scenedesmus dimorphus*, *Botryococcus braunii*, and *Nannochloropsis oculata* [135–141].

5. New Tools to Improve Microalgal Applications: Recombinant Protein Production in Microalgae

The recombinant protein industry has developed more than 170 recombinant proteins used in medicine. Third generation has transformed the therapeutic recombinant protein, in which third generation focus on new routes of administration and increasing efficiency and safety [142,143].

In the biotechnological industry: medical biotechnology, enzyme technology, biopolymers, bioplastics, biofuels, bioremediation, and agricultural biotechnology have seen an intense productivity improvement by the selection of higher producing microbial strains with the application of recombinant DNA technology. New technologies should search new targets for strain improvement programs [144]. The eukaryotic systems used for pharmaceutical production include mammalian cell cultures, bacteria, insect cells, yeast, and plants, however with some limitations [145–148]. Some limitations such as complex nutritional, maintenance, and sterile conditions in mammalian expression systems, in plant expression systems have technical and regulatory concerns for full scale production [147,149–151].

In this sense, microalgae and cyanobacteria are diverse organisms with properties relevant for large-scale production systems, making them an attractive host for recombinant protein production [152,153]. Proteins, peptides, unsaturated fatty acids, vitamins, pigments, and other valuable compounds can be produced at lower producing costs [145,154].

Recently, a geminiviral vector has been used for the effective expression of therapeutic proteins in microalgae, with two advantages over other receptors: the innocuity of several strains and an excellent platform for the development of biological products [155]. For example, the SARS-CoV-2 receptor binding domain (RBD) and the basic fibroblast growth factor (bFGF) were subcloned in a geminiviral vector and used for nuclear transformation to temporarily express these proteins in *C. reinhardtii* and *C. vulgaris*, respectively [156]. Some advantages are important for the use of *C. reinhardtii* and *C. vulgaris*, they are safe to consume as dietary supplements, easy cultivation, lack of pathogens, simple culture media,

and growth in sterile conditions; these properties make an attractive heterologous protein production system [151,155,157–160].

Microalgal metabolism has been manipulated to produce high-valuable compounds according to photobioreactor engineering. In other strategies, transcriptional remodeling of lipid metabolism in *Chlamydomonas*, some regulators have been identified [161]. For example, Jia et al., [162] demonstrated that in *Chlamydomonas*, overexpression of a gene encoding a DofF transcription factor significantly increased intracellular lipid content. Some stress regulators have been identified, TF-encoding genes (an important strategy used by plants to adapt to environmental changes) [163], the Myb transcription factor in response to Pi starvation response [164], and lipid remodeling regulator 1 (LRL1) in response to P-depleted condition [165].

The green alga *Chlamydomonas reinhardtii* produces H₂ gas in anaerobic conditions, reactions catalyzed by hydrogenase enzymes, another important application possible in microalgae. The expression of the HYDA genes is influenced by anoxia condition. In the presence of O₂, transcriptional control of HYDA1 or HYDA2 genes is paralyzed, seems to be regulatory sequences involved in the hypoxia response [164]. It is necessary to isolate mutants that express hydrogenase in the presence of O₂ to dismiss the enzymes sensitive to oxygen [166]. By identifying mutant strains with constitutively expressed HYDA gene transcripts would be a very useful tool in assisting with the difficult task of producing O₂-tolerant hydrogenase enzymes [165]. These technologies generate a valuable platform to produce relevant industrialize biotechnology with significant yields and financial feasibility.

6. Concluding Remarks

Some microalgae have been cultivated successfully as large-scale or commercially available products: *Chlorella vulgaris* and *Chlamydomonas reinhardtii* for bioethanol production [167], β-carotene and coenzyme Q produced by *Isochrysis galbana* [168,169], *Tetraselmis* sp. produce skincare substances that reduce the size of melanocytes and the amount of hyperpigmentation [170], halotolerant marine microalgae, such as *Chaetoceros mulleri* and *Tisochrysis lutea*, produce sterols used in the pharmaceutical industry [171], the pigment Astaxanthin successfully commercialized produced by *Haematococcus pluvialis* [172], and *Chlorococcum* sp. produce carotenoids and phytoene [173]. Although there are some successful samples of microalgal cultures, some technologies can help to improve biomass are high-valuable compounds production, revalorize waste material by microalgal metabolism, and integrate a downstream process to obtain several products in one cultivation.

Microalgae and cyanobacteria have numerous biotechnological applications in a variety of industries (Figure 3). Microalgal processes can be divided into two main systems based on their technological application: closed and open. The closed system can be used for high valuable compounds, such as the production of food additives, nutraceuticals, pharmaceutical, and recombinant protein; these types of photobioreactors can be assembled with a full control equipment of pH, temperature, CO₂ supply, light supply, medium supply, broth recovery, oxygen control, and sterile conditions, and all are susceptible for high purity compound production. Close photobioreactors can be used to develop recombinant therapeutic protein production, which can be a cost-effective production system compared with mammalian cell culture. Open systems in the other part of applications are useful to produce large amounts of biomass without the need for sterility. The focus in the latter system is a biorefinery process, in which the main product can be biofuels—biodiesel, and biohydrogen—but other compounds such as glycerin, biosurfactants, health products, and feed protein can also be obtained. Microalgal growth has been used in wastewater treatment and phycoremediation processes, clean water recycling in aquaculture, and sustainable fish and vegetable production, such as aquaponics technology. For extremely valuable compounds, OMICS technologies in microalgae require the discovery of new genetically modified microalgae capable of producing pharmaceuticals for human health applications; these systems may have technical advantages as well as cost-effective processes.

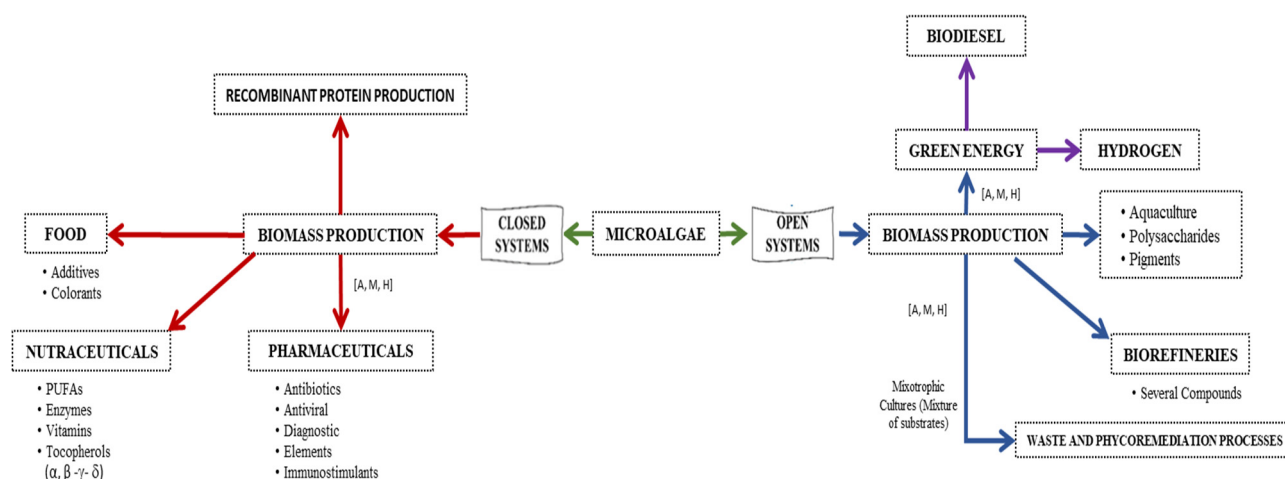


Figure 3. Biotechnology integration process for responsible production and consumption. Process integration in both open and closed systems.

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