EXAMINING MICROALGAE SPECIES AS A BIOFUEL ENERGY CROP: A REVIEW

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One of the most important issues facing our society right now is the development of CO_2 neutral fuels. This truth has come to light during the previous eighteen months thanks to the "Climate Change Economics" [170] in the Stern Report and the "AR4 Synthesis Report" [84] in the National Security Council. The causes and effects of climate change are assessed in greater detail than has been done before in these publications. We conclude that we have crossed the threshold (now 455 ppm CO_2-e) 10 years sooner than the prior estimate. Essentially, they characterize atmospheric CO_2 levels as over 450 ppm CO_2-e (e=equivalent of all greenhouse gases) are currently in the dangerous range. These new discoveries have led the government to set a target (such as the European Union) to reduce CO_2, usually in the range of 10-20% by 2020. Germany has recently pledged to cut emissions by 30% by 2020 if other countries do the same.

However, these modest reductions are not sufficient to keep CO_2 levels within the accepted "optimal" range (i.e. below 450 ppm CO_2-e; IPCC [84]). In fact, they can stay stable above 550 ppm. Damage will be greater at this stage [84]. Therefore, although various CO_2 reduction scenarios have been developed to offset emissions, the IPCC concluded that emissions should increase by 2015 and that total CO_2 emission reductions should be achieved by 50-85% by 2050. More importantly, the IPCC is an understatement because current climate change models lack carbon feedback. As a result, lower emissions reductions (60% by 2020) are seen as overkill, and this concern prompted IPCC Chairman Rajendra Pachauri to say: "What we do in the next 2 to 3 years will determine our future [126]. However, achieving a 60% reduction in CO_2 emissions by 2020 or even a 50-85% reduction by 2050 is a major global challenge that requires a lot of improvement in terms of renewable energy. While biodiesel and bioethanol are considered the closest commercial options, consolidation efforts are underway to produce new biofuels. The sectors of biomass-to-liquid (BTL) diesel, biomethane, and biohydrogen are also expanding quickly. With an emphasis on biodiesel, which has the potential to be integrated with the production of renewable, CO_2-neutral gas through the power plant chimney and atmospheric CO_2 sequestration, this paper gives a brief summary of the history and current advancements in secondary microalgae processes. It specifically seeks to analyze the state of affairs in the biodiesel industry and pinpoint significant prospects for upcoming innovation. The use of biofuels and biodiesel worldwide The oil and power industries make up the two segments of the global energy market. Emissions reductions in both industries are required to fulfill global regulatory targets. About 33% of the world's energy is currently produced by energy, which is changing in a number of

ways to produce energy with minimal CO_2 emissions (eg nuclear, solar, wind, geothermal, hydroelectric, clean coal technologies).In contrast, in 2005, oil held a larger market share, accounting for approximately 67% of global electricity consumption, totaling around 15.5 terawatts (489 exajoules per year), as reported by the US Energy Information Administration. Despite the significance of fossil fuels, the establishment of CO_2-neutral (such as biodiesel, bioethanol, biomethane, and BTL diesel) and CO_2-free fuels for electricity generation has not yet been realized. The availability of biofuels highlights the convenience and potential of the biofuel industry. However, first-generation biofuel systems have been unable to fully harness this potential due to significant economic and environmental constraints (refer to "Challenges in Biofuel Production" and "Economic Viability of Microalgal Biodiesel"). In contrast, second-generation biofuel systems, including lignocellulosic and microalgae biofuel systems, have the capacity to overcome many of these limitations, aiming to reach the ambitious clean energy market target of $500 billion by 2050 or beyond [170]. The majority of biofuels produced to date originate from higher plants that utilize photosynthesis to convert solar energy into chemical energy, stored in various molecules such as lignin, cellulose, starch, and oil (see Figure 1).

Lignocellulose stands as the primary constituent of plant biomass and can serve as a raw material for ethanol production. This conversion can be achieved through processes like gasification or cellulolysis, which encompass chemical or biological enzymatic hydrolysis. Presently, this method is under development for secondary biofuel systems [41, 155] and is commonly referred to as "lignocellulosic processes."

Similarly, starches (e.g., from maize) and sugars (e.g., from sugarcane) are transformed into bioethanol through fermentation [23, 74], while oils (e.g., from sugarcane) are converted into bioethanol. Raw materials like rapeseed, soybean, and palm oil are employed in the production of biodiesel [80, 156]. Microalgae exhibit a remarkable capacity to synthesize cellulose, starch, and oil efficiently [16, 162]. Furthermore, some microalgae and cyanobacteria, which produce more glycogen than starch, can generate biohydrogen under anaerobic conditions [20, 35, 59, 76, 111], and their fermentation processes can be utilized for methane production.

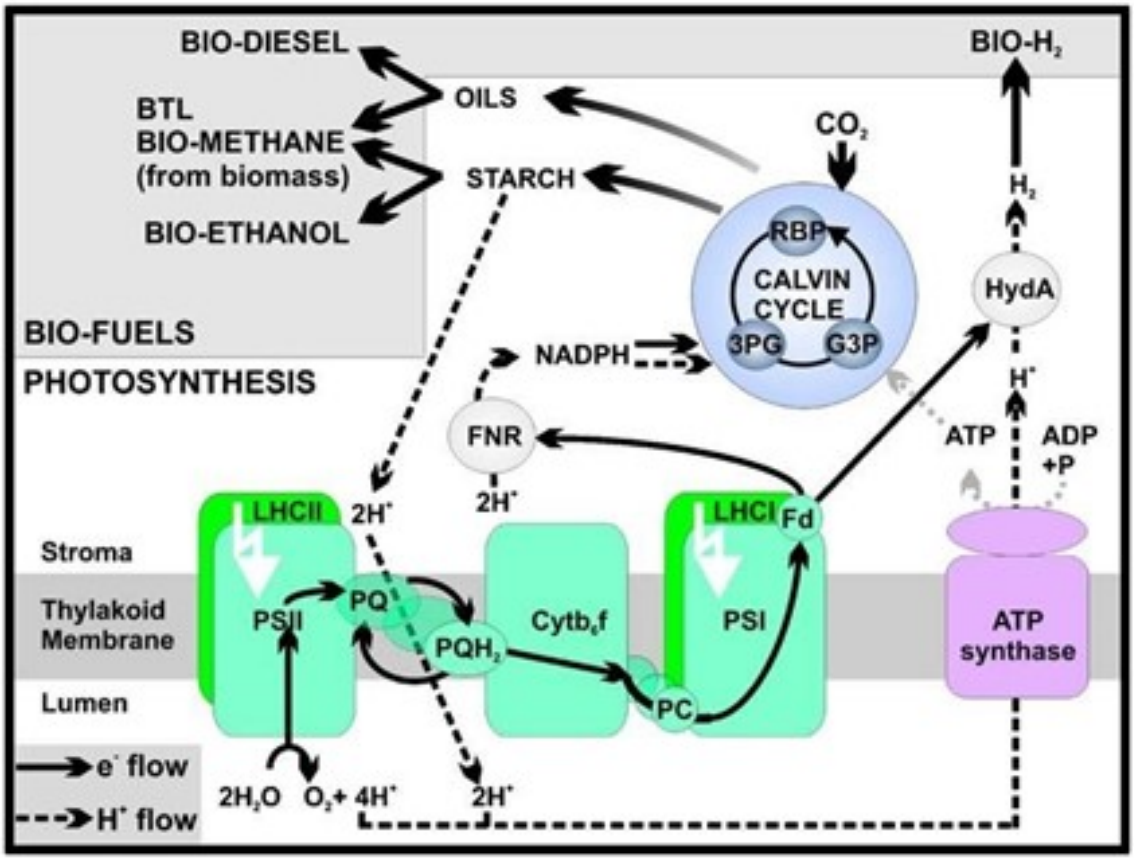

Figure 1: The process of photosynthesis, which converts solar energy into chemical energy, is essential for all biofuel production in plants. (Schenk et al., 2008)

I. FLEXIBILITY OF BIOFUEL PRODUCTION SYSTEMS

At the core of all light-driven biofuel production processes lies photosynthesis, which serves as the initial stage in converting light energy into chemical energy. It plays a pivotal role in generating the essential raw materials required for the synthesis of various fuels, including protons and electrons (for biohydrogen), sugars and starches (for bioethanol), fuel (biodiesel), and biomass (for bioethanol).

In higher plants and green algae, light is captured by specialized light-capturing proteins known as LHCI and LHCII (as shown in Figure 1). These proteins are encoded by extensive gene families with significant homology [51], and their expression is influenced by environmental factors, such as light intensity. They bind most of the chlorophyll and carotenoids within plants, playing a crucial role in light collection and dissipating excess energy to regulate the photosynthetic process, particularly in photosystem II (PSII; [81]). The excitation energy, essential for driving the photosynthetic reactions, is transferred to the photosynthetic reaction centers of photosystem I (PSI) and PSII via a highly coordinated network of pigments connected by LHC, PSII, and PSI subunits.

In an initial step, PSII harnesses this energy to initiate the photosynthetic water-splitting reaction, converting water into protons, electrons, and oxygen. Electrons are subsequently transferred to NADPH through the photosynthetic electron transport chain

involving plastoquinone (PQ), cytochrome b6f (Cyt b6f), photosystem I (PSI), and ferredoxin (Fd) (as depicted in Figure 1). Simultaneously, protons are released into the thylakoid lumen through the PSII and PQ/PQH2 cycles, creating a proton gradient that drives ATP production via ATP synthesis. Protons and electrons then combine with ferredoxin-NADP + oxidoreductase (FNR) to form NADPH. Both NADPH and ATP are utilized in the Calvin cycle and other biochemical pathways to produce sugars, starches, fats, and various biomolecules, collectively forming biomass. These biomass components serve as the basis for generating bioethanol, biodiesel, biomethane, and BTL biofuels.

Alternatively, in certain photosynthetic organisms, such as the green alga *Chlamydomonas reinhardtii*, protons and electrons extracted from water (or starch) can be transferred to hydrogenase (HydA) via the electron transport chain (ETC). The Calvin Cycle represents a pivotal component of the photosynthetic process responsible for carbon dioxide fixation in various organisms, ranging from primitive algae to higher plants. This process relies on ATP and NAD(P)H generated through light-driven mechanisms. In C4 and CAM plants, the Calvin Cycle is integrated with continuous processes leading to CO2 fixation, but the fundamental photosynthetic steps remain unaltered [175].

The Calvin Cycle can be segmented into three key stages, encompassing carboxylation, reduction, and substrate regeneration, with ribulose 1,5-bisphosphate (RuBP) serving as the substrate. The initial phase, involving the conversion of CO2 by reacting it with RuBP, is catalyzed by ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco). The significance of Rubisco cannot be overstated, as it single-handedly fixes all the carbon monoxide in the world from atmospheric carbon dioxide in a singular process. Furthermore, Rubisco stands as the most abundant protein globally, constituting approximately 30% of the total protein content in most leaves [129]. This abundance is partly attributed to its crucial role in photosynthesis, despite its relatively low catalytic carboxylase activity, processing only 2-3 RuBPs per second [104].

As the name suggests, rubisco performs two catalytic functions: it functions as a carboxylase, integral to the reduction phase of photosynthesis, and under aerobic conditions, it acts as an oxygenase, contributing to photorespiration. O_2 and CO_2 compete for the same catalytic sites, potentially affecting the efficiency of CO_2 fixation in certain aerobic environments. For example, although the enzyme is highly specific for CO_2 (Tobacco plants) 82 times, *Griffithsia monilis* (red algae) 167 times, *Rhodospirillum rubrum* (sulphur-free blood bacteria) 12 times [7], O2/CO2 molecular ratio in atmosphere 540:1, 24:125° C Air to water is saturated. In the first step of the Calvin cycle, rubisco catalyzes the formation of two molecules of 3-phosphoglycerate from RuBP, CO2 and H2O. Negative changes in the weak process leads to positive effects. In the second step, in the ATP/NADPH-dependent reduction step, carboxylic acids are reduced by phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase to produce two molecules of glyceraldehyde-3-phosphate. The third step consists of a reaction in which some of the glyceraldehyde-3-phosphate is converted to RuBP, which is necessary to maintain the reduction of photosynthesis [175].

II. ADDRESSING PROBLEMS IN BIOFUEL PRODUCTION

Although the biofuel production process holds significant promise as a means to generate carbon-neutral fuel, the primary production methods are burdened by numerous economic and environmental constraints, making biofuels a prominent subject of debate.

While some proponents underscore the advantages of biofuels [157, 167], others raise concerns about their commercialization and diminishing impact [19, 128, 131, 147, 190].

The most common problem with existing biofuel systems is that when there is a large production capacity, they compete with agriculture for arable land used for food production. For example, current biodiesel production from vegetable oil supplemented with small amounts of animal fat and waste cooking oil is estimated to account for only 0.3% of global fuel consumption (about 12 million tonnes in 2007) [24, 125] available and based on future transportation demands. Currently, about 8% of vegetable oil production is used to make biodiesel [125], which causes the price of vegetable oil to increase over the years.

Area Required for Biofuel Production: Earth's surface (510,072,000 km2) is estimated to receive an average of ~170 Wm−2 solar energy [31, 196]. This is equivalent to 2,735 YJ of energy per year, equivalent to 5,600 times the world's primary energy consumption in 2005 [196]. Therefore, the solar energy required to produce biofuels is plentiful. But even now oil crops will be planted on all arable land. If 2% of the world is land, 13% is suitable for agriculture, energy conversion efficiency from sunlight to biomass is 1% and oil production is 20%, these will meet half of our energy needs today. Using these figures, biofuel critics often conclude that biofuel production cannot contribute significantly to global oil demand. However, as will be mentioned here, higher photosynthetic efficiency and fuel production have already been achieved, thus paving the way for second generation biofuel technologies with great potential. The increase in arable land has created serious problems and bad practices around the world, giving rise to the term "hill land". For example, rainforests in Brazil and Southeast Asia are being cleared at an unprecedented rate to make room for soybean and palm oil plantations for biodiesel production.

Current biodiesel growth rates in Indonesia, Malaysia and Thailand range from 70% to 250% per year [181]. The increase in the amount of land that can now be used for food production can lead to food insecurity, particularly in countries where more than 800 million people already face hunger and malnutrition (figures exclude China; [53]). Also, the use of land extensively with fertilizers and pesticides can cause serious environmental problems.

Net energy balance when evaluating the cost and sustainability of the biofuel production process, it is necessary to establish the net energy balance (NEB). Agriculture, harvesting, processing, shipping etc. Considering the energy required for first-generation biofuels, the NEB is estimated to be about 25% corn ethanol and +□93% soybean biodiesel [80], but the number depends on the amount of production. While this report rejects the claim that the energy cost required to grow crops, machinery and facilities has led to negative NEB values for both biofuels, it has yet to take into account the Intergovernmental Panel on Climate Change's prediction that traditional crops may decline. There will be an increase of up to 50% by 2020 [84]. The carbon balance of the Carbon monoxide calculation process is equally important.

Biofuel crops are generally considered a nearly carbon neutral process because almost all of the carbon in carbon dioxide comes from the carbon dioxide released when burned in situ. But in reality, the overall CO2 balance of biofuel production needs to be evaluated by manufacturer, including energy consumption, plant use and refining, and the process of transporting the fuel from the CO2 that already emits fossil fuels. In addition, oil production

from palm oil fields established before the Kyoto emissions target policy is expected to reduce emissions compared to natural gas. Conversely, if the rainforest area has to be cleared first to make room for plantations, the CO2 balance will be negative [14]. CO2 Capture The integration of biofuel production with CO2 separation systems is a significant development that will not only greatly improve the net CO2 balance of the biofuel process, but will also help reduce atmospheric CO2. These usually involve the production of Agrichar via the pyrolysis process.

A number of second-generation biofuel production systems are currently under development that will have more NEBs, use more water and require less arable land. Lignocellulosic technologies and microalgae are of interest ([29,76,96,155]; Table 1). It has been reported that in one region, microalgae produce 15-300 times more biodiesel than crops (Table 1; [29]). In addition, microalgae often have a short cycle (~1-10 days depending on method) compared to traditional crops harvested once or twice a year, allowing them to be grown multiple or sequentially with increased results (Table 1).

Enhanced light capture and conversion rates ultimately lead to reduced fertilizer and food wastage, consequently lowering waste and pollution levels. Utilizing wastewater for algae cultivation represents a viable alternative [75, 103, 120]. Additionally, the cultivation of microalgae for biofuel production on marginal or soilless lands can alleviate land competition concerns and open up new markets in arid, dry, or saline regions. Furthermore, significant freshwater conservation can be achieved when conducting algae cultivation within low evaporation closed bioreactor systems, especially utilizing marine and halophilic microalgae species. The microalgal biomass generated in these bioreactors can also undergo gasification or pyrolysis to yield various biofuels and charcoal, serving as part of CO2 capture strategies. This approach facilitates the disposal of bio-GMO wastes in an environmentally sensitive manner. Carbon-rich biomass pellets can also be stored as part of a carbon sequestration strategy that utilizes CO2 from power plants as a feedstock for biomass production. Another noteworthy aspect of secondary microalgae systems is their suitability for biotechnological processes that can swiftly regenerate algae populations. These attributes hold promise for enhancing "Photosynthetic Efficiency."

Table 1 Comparison of crop-dependent biodiesel production efficiencies from plant oils

Plant source	Biodiesel (L/ha/year)	Area to produce global oil demand (hectares × 10^6)	Area required as percent global land mass	Area as percent global arable land
Cotton	325	15,002	100.7	756.9
Soybean	446	10,932	73.4	551.6
Mustard seed	572	8,524	57.2	430.1
Sunflower	952	5,121	34.4	258.4
Rapeseed/canola	1,190	4,097	27.5	206.7
Jatropha	1,892	2,577	17.3	130 (0[a])
Oil palm	5,950	819	5.5	41.3
Algae (10 g m^{-2} day^{-1} at 30% TAG)	12,000	406	2.7	20.5 (0[a])
Algae (50 g m^{-2} day^{-1} at 50% TAG)	98,500	49	0.3	2.5 (0[a])

Presented yields are for peak performing crops [16, 29, 162], although for example, Malaysia's average oil palm yield is actually about 4 tons/ha [119]. Algae yield scenarios are based on existing production systems and their potential [16, 162]. Current algal production systems fall between these ranges: Seambiotic Israel (currently at 20 g m^{-2} day^{-1} at 8–40% Triacylglycerides (TAG), HR BioPetroleum Inc Hawaii (aims to achieve 50 g m^{-2} day^{-1} at 30% TAG)

[a] If algal ponds and bioreactors are situated on non-arable land; jatropha is mainly grown on marginal land.

Numerous sectors have seen recent advancements, and it is being identified what constitutes better photosynthetic output and growth tolerance in saltwater or wastewater. The effectiveness of CO2 production and the harvesting process are two areas where secondary microalgae systems have been shown to have certain limits.

III. OTHER BIOFUELS FROM MICROALGAE

In addition to its use in oil production for biodiesel, microalgae also serves as a valuable feedstock for biofuel generation. The advancement of technologies for algal biodiesel production is closely linked with biomass liquefaction (BTL) methods, encompassing biohydrogen, biogas, bioethanol, and the cultivation of fast-growing algae. Below, we delve into the BTL, biohydrogen, and biomethane processes, as they are particularly pertinent to microalgae systems. The discussion of bioethanol is omitted here, as the process of producing bioethanol from microalgae closely resembles the initial technologies that employed food-derived products from sources like corn and sugar.

Microalgae-based biohydrogen production, which involves the photobiological generation of hydrogen from water, has emerged as a promising and potentially emission-free fuel source. It can be integrated with atmospheric CO_2 capture efforts. The concept of biohydrogen production by microalgae has been established for over six decades, with initial discoveries in green algae like *Scenedesmus obliques* [59] and subsequently in numerous other photosynthetic species, including cyanobacteria [20, 35]. Most investigations into algal hydrogen production have centered around the green alga *Chlamydomonas reinhardtii*, which serves as a model organism for studying photosynthesis, especially in the context of the aerobic-anaerobic cycle pioneered by Melis and colleagues in 2000 [78, 148]. This species was also prominently featured in studies dating back to 2000 [63, 111].

The biological hydrogen process holds appeal because it harnesses sunlight to convert water into hydrogen and oxygen through a two-step reaction. The initial reaction is common to all aerobic photosynthetic organisms, while the subsequent step involves specific iron-containing chloroplast hydrogenases and is limited to a more selective group of microalgae [57, 77]. Cyanobacteria also have the capability to produce hydrogen from water but employ a distinct biochemical method. Under standard light and aerobic conditions, H+ and e- generated by photosynthetic hydrolysis reactions are utilized to form ATP and NADPH. The second reaction takes place under anaerobic conditions, where the presence of O2 inhibits both ATP production through oxidative phosphorylation and NADH/NADPH formation [67]. In such conditions, certain microalgae, such as Rheinella, redirect the energy stored in carbohydrates like starch toward chloroplast hydrogenases [63, 111]. This redirection facilitates ATP generation via photophosphorylation and prevents an overload in the transport chain [96, 151]. Consequently, hydrogenases, in conjunction with various fermentation processes [93], serve as proton/electron release valves, producing hydrogen gas, which is expelled from the cell by combining protons and electrons and neutralizing ferredoxin [111].

Chlamydomonas reinhardtii is a fundamental organism in solar-powered biohydrogen production from water, and alternative fermentation processes can also be employed. An important advantage of hydrogen production is its rapid release into the gas phase, in contrast to other fermentation byproducts that can accumulate and prove toxic to cells. Recent advancements have led to improved efficiency in algal biohydrogen production. For instance, strains with elevated starch content, reduced cyclic electron flow around PSI (such as Stm6), and increased external storage of glucose (like Stm6glc4) have demonstrated enhancements [42, 95].

To create a profitable commercial algal hydrogen production system, the metabolic flow of hydrogen must be optimized through bioengineering and optimization of related processes in the bioreactor system [76]. Bioengineering includes methods to increase photon conversion from the current ~1% to ~7% commercially available [76]. The "Closed Bioreactor Design" section provides details on actual bioreactor design and ways to reduce costs. Combining hydrogen production with seawater desalination Biohydrogen production from algae can be combined with seawater desalination, even with limited production. Seawater can be converted by marine and halophilic algae into hydrogen (as protons and electrons) and oxygen, which are then burned to create fresh water.

Hence, there is a viable opportunity to integrate electricity generation with desalination by utilizing a fuel that employs hydrogen and oxygen to generate electricity within the nation. Despite the relatively modest freshwater yield, it offers advantages that traditional crops cannot provide. The water yield is directly correlated with the production of H2. With a light-to-hydrogen conversion efficiency of approximately 1% (which aligns with the current state of external light utilization through a photodilution generator), research indicates that 1 million liters of photobioreactor reactor space has the potential to yield up to 610 cubic meters (equivalent to 610,000 liters of hydrogen) of freshwater annually after processing is complete.

1. **Algal Biomethane Production:** The significance of biogas production from biomass has grown on a global scale. A recent investigation conducted by the Leipzig Energy and Environment Institute suggests that a substantial portion of Europe's methane requirements can potentially be fulfilled through biogas [18]. Nonetheless, our current comprehension of the biological mechanisms within biogas production facilities remains limited. Consequently, research in this domain is imperative and holds significant relevance for enhancing the biomethane production process. Biomethane has the capacity to be generated from diverse biomass sources and various plant species. An important constraint for the prospective advancement of biomethane-based facilities lies in the accessibility of photosynthetically cultivated biomass.

   Presently, a 500 kW biomethane facility necessitates approximately 10,000-12,000 tons of biomass feedstock each year, with corn serving as the primary crop of choice. Typical biomethane yields, ranging from 2,000 to 4,500 m3 per hectare per year, have been documented when utilizing cereals and sunflowers [5, 192]. Corn, characterized by its high yields, exhibits variability dependent on the variety and harvest timing, generating between 5,700 to 12,400 m3 of biomethane per hectare per year [5, 136, 192]. Some grass species, such as ryegrass, have been reported to achieve biomethane production levels of up to 4,000 m3 per hectare per year [192].

   Since the production of biomass per hectare by microalgae is five to thirty times that of crops, microalgae are crucial for monitoring [162]. Microalgae have high lipid, starch, and protein contents and low lignin content, which makes them perfect for fermentation-based biomethane production in biogas plants. Lipids are significant because, like biodiesel, they can convert into biomethane at a higher rate (790 L biogas, 72% CH_4, 28% CO_2) per kg of organic dry matter) than proteins (800 L biogas, 60% CH_4, 40% CO_2) and carbohydrates (746 L biogas, 50% CH_4, 50% CO_2) per kg of organic dry matter) [184]. About half a century ago, one of the earliest investigations into the viability of producing biomethane from microalgae was published, with the conclusion that the method is feasible and may be enhanced in the future [65]. Nowadays, it is possible to cultivate microalgae in huge volumes (150–300 tonnes per hectare annually) in indoor algae bioreactors that are helpful for producing biomethane and biomass feedstock. 29 and 138]

   Methane production from this quantity of biomass can reach 200,000–400,000 m3 per hectare annually. It should be highlighted, nevertheless, that because biomass is expensive to produce (the commercial value of green algae in Germany, where most biogas producers are located), biomethane produced by microalgae cannot yet compete with biomethane produced from corn or other crops. For instance, Chlorella vulgaris is 100 times more common than maize, but at the moment, its productivity is insufficient to meet the demands of biogas plants. These days, methane from biogas facilities is combined with carbon dioxide (typically between 50 and 75 percent methane), which makes it unusable due to its high impurity level. Since biogas is now mostly burned in combined heat and power plants, fuel storage is no longer popular. Nonetheless, the creation of the first pilot plants for biomethane separation was made possible by the development of effective purification systems, which began in Austria in 2005 (patent no. AT411332B (2003)) and Germany in 2006 (patent no. 304 26 097- BCM-method).

2. **Microalgae Cultivation:** Traditional outdoor pond algae production and even some indoor algae bioreactors have been commercially successful in producing valuable products such as astaxanthin and nutraceuticals. Biofuel production systems are less economically viable as they have a lower market price. Therefore, optimized biomass production forms the basis of commercial biofuel production [73,76,100,145,166], which requires careful optimization of crop growing systems.

IV. OPTIMIZING CULTURE CONDITIONS

Limitations may arise from the complexity and wide range of elements involved in optimizing culture conditions particular to a strain. These include water quality, pH, salinity [1,30,140,141], mineral and carbon regulation/bioavailability, cell fragility [70], cell density and growth inhibition [16], temperature [30], mixing [12], hydrodynamic and hydrodynamic stress [13], bubble size and distribution [14, 135], gas exchange [52], mass transfer [68]. While controlled agitation of the culture can provide important preliminary information, appropriate models must be properly constructed in laboratory bioreactors [150] to ensure mixing and mass transfer. Traditionally, most research on the relationship between algae growth and nutrition has been devoted to algae in the natural environment and the role of algae growth in ecosystems.

In contrast, algae production systems require maximal biomass growth to attain high cell densities. A more profound understanding of the underlying physical principles and advancements in bioreactor design [66] have resulted in increased achievable cell densities. The formulation of an optimal growth medium also assumes crucial significance as it ensures the provision of sufficient and consistent nutrients essential for maximizing and expediting cell growth, thereby enhancing overall biofuel production efficiency [36, 37]. Algae production processes are versatile and can involve multiple steps, allowing for considerable flexibility in each stage. For instance, techniques such as nitrogen limitation for oil production [165] or sulfur limitation for hydrogen production [111] can be employed. Employing fed-batch feeding for heterotrophic algae culture [100] and enriching photoautotrophic algae culture with CO2 can augment biomass yield, while fine-tuning mineral nutrient levels can enhance the productivity of insect cultures. Nitrogen and phosphorus typically emerge as the primary targets for improving the mineral environment [28, 86, 165, 203], although other minerals also play critical roles in supporting cellular processes and metabolic biochemistry. Mineral ions additionally exert significant influence in areas like osmoregulation, osmotic adaptation [89, 90], and the molecular configuration of photosynthetic complexes [112].

Certain algae varieties exhibit a twofold increase in growth rate and cell count when cultivated in the presence of yeast extract. Evidently, proliferating bacteria can effectively incorporate dissolved organic compounds, suggesting that wastewater can be viewed as a potential resource in specific scenarios. The scarcity of freshwater resources in numerous countries underscores the importance of wastewater recycling, with ongoing research exploring various wastewater utilization methods [10, 153, 154, 163, 201]. Notably, Zaslavskaia et al. [202] illustrated the transformation of Phaeodactylum tricornutum, a functional photoautotroph, into a heterotroph through genetic modification targeting the same gene responsible for encoding the glucose transporter.

The discovery, characterization, and manipulation of carriers hold substantial promise for future applications in algae cultivation, particularly in the context of biofuel production. Maintaining precise pH levels throughout the culture is of paramount importance, as it exerts a profound influence on all aspects of the medium's biochemistry. The ion uptake within the medium and the metabolic biochemistry of the cells can exert significant stress on pH levels, and in cultures with high activity, their impact can override the potential absence of exogenous buffers. Presently, the best practices and strategies for pH control encompass techniques such as microinjection of potent acids and bases, stabilization in heterotrophic cultures, and the management of CO2 dissolution in both photoautotrophic and heterotrophic cultures [52, 173].

V. OUTDOOR POND SYSTEMS

Most of the microalgae grown today are grown in open ponds. The construction and operation of open ponds is very efficient and therefore more efficient as long as the culture is preserved [193]. Outdoor pools can come in many shapes and sizes, but the most common design is a racetrack pool. Create a grid of rectangles in the region, with an elliptical channel in each. The impeller keeps the water flowing through the circuit continuously. They typically operate in water between 15 and 20 cm deep because productivity can reach 60–100 mg L-1 day-1 (i.e., 10–25 g m−2 days−1) and biomass concentration can reach 1 g dry weight per litre at this depth [137]. On the other hand, this efficiency is not consistently accessible and cannot be maintained annually. Reservoirs in Asia and the Ukraine also have comparable architecture [15]. Wastewater treatment plants can construct their algae pools to best fit the site; these are typically gravity-flow-guided pools rather than mixed pools. The 11,000-hectare Werribee wastewater treatment plant in Melbourne is one of the biggest of its kind.

The foundation of an algal wastewater treatment pond is made out of excavated trenches or retaining walls. Because more mechanisms (vanes) are needed to ensure the stability of the lake, building a dam costs more. Higher flow rates also necessitate the use of more sturdy structures. However, since transparent materials are not needed to create an open-air pool, various materials can be used for construction. Outdoor pools are also easier to maintain because they have wide open channels that remove surface biofilm. The biggest disadvantage of the open system is that they lose water by evaporation at the same rate as the products in the soil when they are open to the air, and they are also susceptible to harmful diseases. Newly opened ponds are often inoculated with the desired algae culture to encourage algae growth and preserve the pond's flora. However, undesirable species will occasionally appear and can significantly reduce yields even beyond inoculated species. When a big competitor enters the pool, it is very difficult to get rid of it. Of the more than 3,000 photosynthetic organisms collected by the Aquatic Species Program, none were found to dominate the open ponds and have the necessary biofuel and high lipid content [162]. In practice, it has been reported that open ponds are usually of two to six types and have many advantages: rapid growth, protection against animals, tolerance to high oxygen levels, etc. In open ponds, consistent and dependable reproduction of the same species

Breeding extremophiles, which avoid and outperform other species in certain settings (e.g., high/low pH or salinity), might, nevertheless, complement system development efforts. For instance, *Spirulina* is frequently the dominating species in soda lakes because it can grow and live at high pH levels (9 to 11.5) [17]. Because of its spiral design, collecting it is also simple. The world's largest producer of *Dunaliella* is Australia. Because of its high

intracellular glycerol concentration, which guards against osmotic stress, this unicellular species of green algae develops effectively in high salinity water. In shallow waters, *Dunaliella salina* is the best source of protective carotenoids that shield it from intense light [21].

VI. CLOSED BIOREACTOR DESIGN

Enclosed bioreactors offer numerous advantages beyond conserving water, energy, and chemicals, and they are increasingly becoming the preferred choice for biofuel production as costs decline (see Fig. 3). A pivotal aspect of these systems is their ability to support production volumes up to five times larger in terms of reactor capacity, resulting in a reduced production "footprint" [13]. A key consideration is efficiency, aiming to harness the maximum amount of solar energy from a given land area. Recent studies have indicated that the increased costs associated with bioreactors can be offset by enhanced production efficiency [29]. Most enclosed photobioreactors are designed in the form of tube reactors, plate reactors, or bubble column reactors ([137, 193]; refer to Figures 3 and 4). Additionally, less intricate structures, such as semi-hollow spheres, have demonstrated effectiveness in certain cases [152].

However, there is still a gap between the design of high-end reactors that meet all the needs of algae cells on the one hand, and the design of inexpensive reactors, on the other hand, to improve process economy [193]. Based on current electricity prices and production costs, the cost per square meter of the reactor should not exceed $15. The following paragraphs discuss some aspects of reactor design and current development from a business perspective. Most microalgae exhibit growth/photodynamics where light saturation occurs at medium light intensities. Therefore, they have low activity, photoinhibition, and even photobleaching under direct sunlight for *Chlamydomonas*; [110, 134]. To increase the efficiency of the process, photobioreactors must be designed to scatter light over a large area to provide moderate light intensity to cells. This is usually achieved by arranging tubular reactors in a fence-like structure (Figs. 3) The fence faces north/south to avoid blindness from the field. This elegance is way sunlight is diluted both horizontally and vertically. Dry weight up to 47 g m-2 day-1 can be obtained using this system [27].

The distribution of light around the circle of the tube is very interesting considering the reflections. However, compared to other biochemical engineering processes, this is often not achieved by precise measurements and kinetic methods. The bioreactor surface might be 10 times greater than the matching covering area to increase lighting. Photobioreactors that have bubble columns or plates positioned at an angle to the sun can also be explained in similar ways. Both kinds of structures promote better cell growth, but they need materials that are more transparent, like plastic or glass. However, "make the ratio of surface area to volume as large as possible" is the design philosophy. Since these structures are constructed along a short optical path, they may support larger biomass concentrations. The 400 m2/m3 value is state of the art. Therefore, small culture volume and less mixing power are required for a given biomass yield. Stirring is necessary in all photobioreactors because it inhibits cell aggregation and promotes CO_2 and O_2 distribution [117]. The partial pressure for CO2 is at least 0. To avoid limitation of kinetic CO_2 uptake, 15 kPa must be maintained and meet the stoichiometric requirement of 1.7 g of CO_2 per gram of biomass. This highlights the practicality of supplying CO_2 that has been purified from external flue gas, such as that from a power plant [45]. While the mixture does not affect light

attenuation in the reactor, there exists a relationship between the culture mixture and light attenuation, as each algal cell undergoes varying levels of interaction between darkness and light within the reactor [13]. Dark areas emerge due to mutual cell shadowing on the side of the reactor farthest from the light source. Consequently, it becomes essential to reevaluate algae physiology within the context of the "flashing light effect" [69].

Although high light intensity stimulates the photosystem, it can also lead to photoinhibition. Therefore, microalgae have developed photoprotection mechanisms to dissipate excess energy through fluorescence and heat. This energy wastage is circumvented when algae cells are subjected to low/high light cycles, as low light enables the transfer of energy within the photosystem to metabolic processes. These cycles should occur at a frequency of 10 Hz or faster, with the dark cycle being ten times longer than the light cycle [85]. Under such conditions, algal cells exhibit behavior similar to that under constant light [199]. Photobioreactors can effectively capture the flashing light effect through intelligent mixing to optimize the transition of cells between darkness and light. This concept has already been put into practice, as depicted in Figure 4 [109]. The reactor takes the form of a plastic plate air-lift reactor equipped with integrated baffles, inducing a consistent horizontal liquid vortex. However, the drawback lies in the energy consumption associated with the mixing technology. Nevertheless, such reactors have proven successful. The first CO_2 separation demonstration plant was scheduled for construction in Hamburg, Germany, in 2008. In various reactor types, combined energy consumption is estimated to account for 10-30% of the light energy problem. This level is evidently excessive, surpassing acceptable energy consumption thresholds.

Plastic bags made of polyethylene are utilized either as circular reactors (to prevent dark areas within the cylinder) or as plate reactors, which are available for purchase [146, 179]. It has been determined that a simple horizontal plastic pipe laid on the ground lacks the required strength for this purpose. One of the latest advancements in this field is the triangular reactor (as illustrated in Fig. 5). This innovation combines the bubble column principle with mixing through integrated static mixers within an external 'downcomer'. As per MIT's press release and external evaluation [139], even under optimal lighting conditions, this "3DMS-Reactor" achieves a dry air mass of 98 g m-2 per day over a 19-day period. Consequently, this stands as one of the most productive cultures ever developed, with a theoretical maximum average of approximately 100 g m−2 per day. Future improvements should focus on employing thin layers with a high internal area to enhance light distribution, thus increasing biomass concentration while reducing mixing energy requirements [150]. Moreover, gas transport should solely rely on conduction to avoid the formation of negative energy foam.

A different idea for a bioreactor gathers light using plastic Fresnel lenses and uses optical fibers to direct it into a lump reactor. By separating the sun's infrared rays, overheating issues during periods of high solar irradiance can be minimized and the expelled radiation can be utilized to produce energy. This can be used to mix cultures in the currently most energy-intensive bioreactors. This principle eliminates many of the problems associated with large outdoor reactors, but it is very expensive to use and the technology needs to be further developed to reduce its cost and ensure it is successful. Figure 6 presents a schematic of four commonly used bioreactor designs.

In summary, the design of photobioreactors, particularly those used to produce biofuel, is a fast-growing topic that is critical to the quick development of second-generation microalgal biofuel systems. The best and most profitable outcomes can only be achieved by combining innovative designs with theory and understanding of microalgae dynamics and growth efficiency.

Figure 3: A high-end closed bioreactor system. The world's largest closed photo-bioreactor in Klötze, near Wolfsburg, Germany (Bioprodukte Prof. Steinberg; www.algomed.de); the 700m3 are distributed in 500 km of tubes and produce up to 100 t algae biomass per year

Figure 4: Flat panel airlift from Subitec [38, 102] with usage of the flashing light effect, here for astaxanthin production Triangle Airlift Reactor, or 3D Matrix Algae Growth Engineering Scale Unit, GreenFuel,

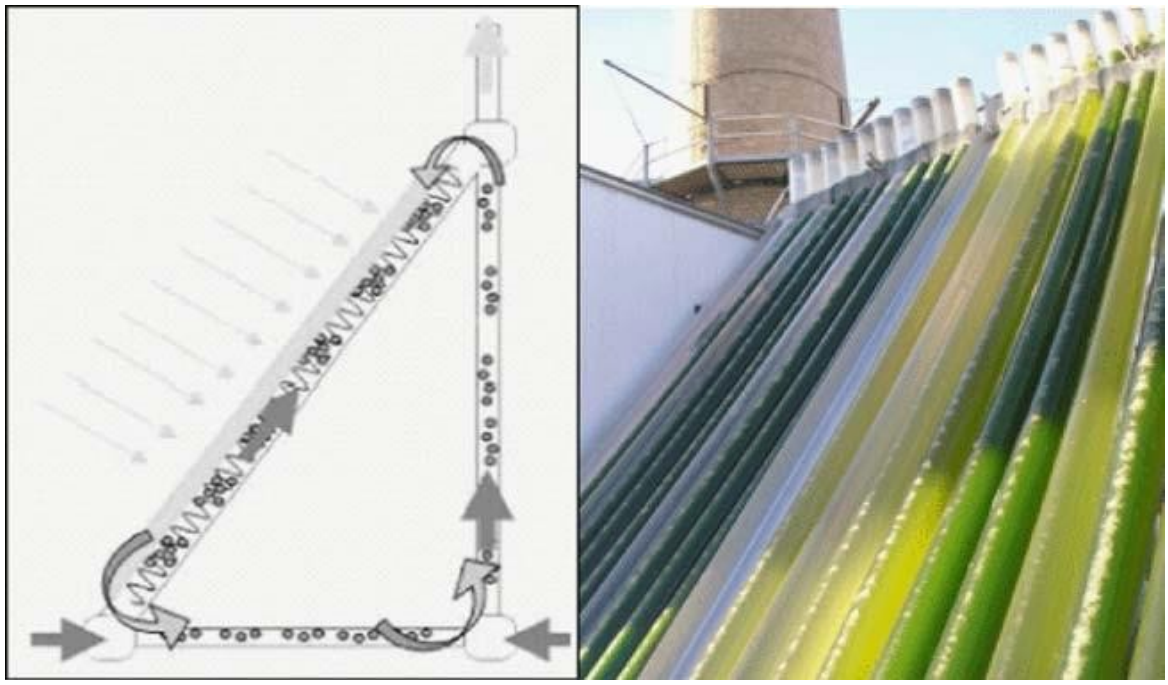

Figure 5: The patent drawing (US 20050260553) is on the left, while the demonstration plant at Red Hawk Power Plant in Arizona, USA, is on the right. 50]

Hybrid Systems: Outdoor ponds represent a cost-effective method for cultivating algae; however, they are susceptible to rapid contamination. In contrast, photobioreactors are highly effective at maintaining sterile cultures but are accompanied by setup costs that are often ten times higher than those of outdoor ponds. Combining these two systems emerges as the most efficient approach for cultivating crops valuable in the production of biofuels. In the practice of algae cultivation, inoculation has long played a crucial role. Open ponds are typically inoculated with the desired strain, which has invariably been cultivated in a bioreactor, ranging from something as simple as a plastic bag to a cutting-edge fiber optic bioreactor. It is worth noting that the size of the inoculum must be sufficiently large to allow the desired species to establish itself in the open system before any unwanted species take hold. However, contamination inevitably becomes predominant over time, necessitating system cleaning and re-inoculation.

Therefore, cleaning or washing the pools should be a part of the aquaculture process to reduce the problem. Aquasearch (Hawaii, USA) demonstrated this process using *Haematococcus pluvialis* to produce astaxanthin. Half of the Aquasearch area is dedicated to photobioreactors and the other half to open ponds. To promote the formation of astaxanthin, *Haematococcus pluvialis* was continually maintained in a nutrient-rich photobioreactor and then part of the culture was moved to an outdoor pond with limited nutrient availability. When the astaxanthin level peaks after three days, the open ponds are harvested, cleaned, and then re-inoculated with sufficient nutrients transferred with the inoculum [83]. This method works particularly well for producing biofuel because, in low-nutrient environments, algae quickly begin to transform solar energy into chemical energy that is stored as lipids for survival (see the section on "Lipid production by microalgae in nature"). There would need to be a succession of photobioreactors of escalating sizes, from beginning culture to the ultimate inoculum, for the large-scale generation of microalgae biofuel. To keep the cost per square

meter to a minimum, the complexity level should decrease as the bioreactors get larger. Smaller bioreactors must be maintained under strict axenic conditions; however, as the bioreactor size increases, the level of containment can be loosened if inoculum is continuously supplied to flush each stage of the scale-up, as long as procedures are in place to remove contamination should it arise early in the chain. It is crucial to use an algae species that is both quickly growing during the inoculum scale-up stage and extremely productive during the final open pond stage for such a strategy to be successful. Through this approach, fresh inoculum can be continuously added to open ponds with low nutrient levels that are good for the environment, preventing the domination of invasive species and promoting the ongoing production of algae biofuels.

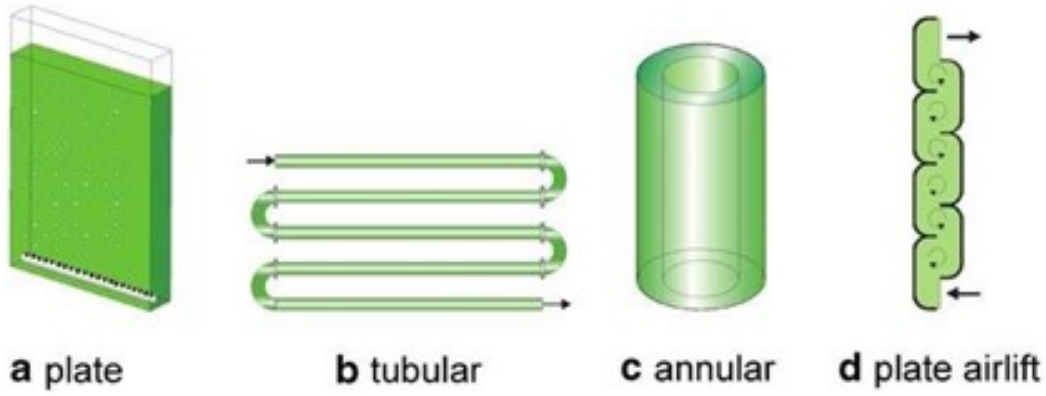

Figure 6: Various closed photobioreactor designs that are frequently used to produce important compounds The design of a plate reactor follows a classical approach. A tubular reactor is the largest closed photobioreactor. An annular reactor functions as a bubble column and has an empty inner cylinder to increase surface/volume ratio and prevent dark areas. A plate airlift reactor with baffles supports the flashing light effect through controlled fluid barrels. While all of these designs are thought to be generally appropriate, their cost makes them unsuitable for the generation of biodiesel.

VII. DOWNSTREAM PROCESSING HARVESTING METHODS

Because of their high water content, algae must be removed for harvesting and processing later on. Harvesting microalgae and lowering their water content cannot be done using a single optimal technique. The three most used harvesting methods in algal aquaculture nowadays are centrifugation, flocculation, and microscreening. The development of more inexpensive and energy-efficient harvesting techniques is required to increase the efficiency of the biofuel manufacturing process. Since certain species are far easier to harvest than others, strain selection is crucial in this regard. For instance, the long spiral form of the cyanobacterium. *Spirulina* naturally lends itself to the comparatively energy- and cost-efficient microscreen harvesting technique [11, 16]. Filtration that is both economical and efficient can only be used to filamentous or big colonial microalgae.

Although filtration is frequently used in laboratories, it has drawbacks in large-scale settings, including membrane clogging, the development of compressible filter cakes, and, most significantly, high maintenance costs. Stokes' law, which states that the sedimentation

velocity is proportional to the square of the cell radius (the Stokes radius) and the difference in density between the cell and medium, can be used to explain both sedimentation and centrifugation. Gravitational force-based techniques are not easily applicable to bacteria, but they are possible for yeast and microalgae with diameters more than 5 μm and rather thick cell walls. Some algae farms use pure sedimentation, but it takes a lot of time and space, therefore it's not a good option for producing biodiesel. Decanting centrifuges have also been effectively used, and separation is improved by commercial centrifuges accelerating to at least 10,000 × g (e.g. [195]). Centrifugation is now thought to be too expensive and energy-intensive for the first microalgae harvesting process.

An estimate of 3,000 kWh/ton has been made for the energy intake alone [16]. Nonetheless, centrifugation is a very helpful secondary harvesting technique that can be combined with oil extraction to concentrate an initial slurry (10–20 g/L) into an algal paste (100–200 g/L). Another widely used primary harvesting technique for concentrating algae is flocculation, which is the aggregation and sedimentation (or flotation) of algal biomass [118]. Adjacent settling ponds are utilized for flocculation, settling, and harvesting in raceway or mixing ponds. Although they are very powerful flocculants, inorganic compounds like lime, alum, and ferric chloride are thought to be too costly for large-scale operations. As they are more necessary and the algae can be utilized in downstream applications like animal feed or anaerobic digestion, organic cationic polyelectrolyte flocculants, such as the cationic polymer chitosan, are typically chosen [118]. Because flocculation enlarges the particle size, sedimentation proceeds more quickly (see Stokes' law) or, more effectively, interacts with floating bubbles. However, adding flocculants isn't the preferred approach for low-cost, environmentally friendly manufacturing at the moment. Current advancements entail promoting the self-flocculation of the cells, which may happen in the event of a pH change or carbon constraint. Moreover, polyelectrolytes can be utilized to promote bioflocculation, or spontaneous flocculation. Most likely, the least expensive harvesting method is bioflocculation.

While some species flocculate spontaneously, others do so in response to environmental cues, nitrogen stress, pH, and dissolved oxygen levels [16]. However, the unreliability of the time it takes for spontaneous flocculation to occur is an issue. Co-bio-flocculation is an intriguing variant of this that Ami Ben-Amotz recently introduced [3]. In this instance, flocs containing high lipid Nannochloropsis variants were formed using the naturally flocculating alga Skeletonema. Feeding the fish tilapia (*O. mosambicus*) algae is another intriguing technique that Mike Massingill recently shared. A conveyor belt with 10–14% solids is then used to collect the algal biomass from the sedimented droppings, and it is then air-dried ([3]; Kent Seatech: United States Patent 6447681). Transesterification and Lipid Extraction Nowadays, most biodiesel is made from plant or animal oils by transesterification, which can occur after oil extraction and may or may not disturb cells. As an alternative, methyl esterification combined with the application of immobilized lipases can speed up the process [100]. Basically, a typical extraction procedure is mechanically crushing and then squeezing. For the extraction of oils and other microalgal products, chemical solvents can be chosen in one- or two-step extraction approaches. While high-pressure homogenization ("French press") can be used to disrupt cells, a more modern method is electroporation, where a high electrical field is applied to the biomass leading to perforation of the cell wall and to better extraction. This can even be used directly on living algae [79] or in conjunction with transesterification [58], which yields glycerol and biodiesel when methanol and a catalyst like sodium methoxide are used.

VIII. CO_2 SEQUESTRATION

The CO_2 levels in the atmosphere are now considered to be "dangerously high" since they have surpassed 450 parts per million CO_2-e [84, 170]. Therefore, while the development of CO_2-neutral biofuel production systems is vital, the main purpose of their production is to stabilize atmospheric CO_2 levels at a level that is considered "dangerously high," rather than bringing it down to a level that is acceptable. Since separating CO_2 from other atmospheric gases is a technically tough task, physical sequestration of atmospheric CO_2 is sometimes seen as challenging. But over millions of years, photosynthetic organisms have perfected this process, and as a result, they are naturally suited to absorbing CO_2 and storing it as biomass. The generation of CO_2-neutral biofuels, such as biodiesel, and atmospheric CO_2 sequestration would be greatly enhanced if the captured CO_2 could be transformed into a more stable form for long-term storage (about 100 years or more). Large pond-style systems for capturing carbon dioxide were examined by Weissman and Tillett [194]. It has been demonstrated that the capture efficiency can reach 99% when operating in ideal circumstances [194, 204]. One gram of glucose requires 1.57 g of CO_2 to create, according to the following equation: Light · chlorophyll ¼ C6H12O6 ÷ 6 O_2 ÷ 6H_2O ÷ 6CO_2 Kurano et al. [97] found a ratio of 1.6 to 1 for the fixation of 4 g CO2 L−1 day−1 at growth rates of 2.5 g algae L−1 day-1.

Considering that, under some circumstances, glucose can be converted into other substances like lipids or starch, the consumption of CO_2 can reach up to 2 g CO_2 for every 1 g of algae. One hectare of algal ponds can sequester up to one ton of CO2 per day, assuming a growth rate of 50 g m-2 day−1. Following the extraction of oil (about 30% of the dry weight), 70% of the biomass can be fed into the biodiesel process mechanisms for sequestering carbon downstream. Particularly, the "slow burning" method of pyrolysis can transform the sequestered carbon into hard C-chips (Agri-char) [25]. This also has the added benefit that Agrichar, as its name implies, can be sold to the agriculture industry because it significantly raises the soil's fertility and carbon content. Through an impact on soil and crop gas exchange, this can further mitigate the effects of climate change [98, 105]. Additionally, pyrolysis serves as a sterilizing process for biomass waste, offering a waste disposal method that is sensitive to the environment and raising public acceptance of the use of genetically modified microalgae for the production of biofuel.

IX. MOLECULAR IMPROVEMENTS OF MICROALGAE FOR INCREASED BIODIESEL YIELDS

The following can be kept in mind so as to obtain the best performing microalgae strains for maximal biofuel production:

1. screening a wide range of natural isolates,
2. improve them by metabolic (genetic) engineering or
3. improve them by selection and adaptation.

Thousands of distinct algal strains are available for access in algae collections across the globe. For instance, the Algi-Net Database [4] provides information on the cultures of algae strains that are now cultivated in Europe. Notably, 3000 algal strains were gathered by the US Aquatic Species Program, which evaluated them for their ability to produce biofuel [162]. The Aquatic Species Program's global algae collections and species, along with current

developments in genetic engineering and material sciences, offer a solid foundation for the development of microalgal biodiesel production systems. In the future, techniques such as lipidomics, genomics, proteomics, and metabolomics may be employed to identify and cultivate novel strains exhibiting robust growth, elevated lipid biosynthesis rates, extensive resistance to diverse environmental conditions, and the production of valuable byproducts. Approaches from Systems Biology and Metabolic Engineering Transcriptomics, proteomics, and metabolomics techniques can be used to identify bottlenecks in the cell that affect the generation of algal biodiesel. When these techniques are combined, as opposed to when they are used in isolation, biological processes can be better understood. The measurement of metabolic flow can be used to confirm the identification of genes, proteins, or metabolites that exhibit variable expression. These signals point to the rate-limiting mechanisms within the cell. Through genetic or metabolic engineering, algal traits can be precisely tuned thanks to this systems biology method.

The goal of metabolomics is to identify metabolic profiles that characterize an organism's metabolome [56], in this example, lipid-rich algae. To determine the variations in metabolite levels brought on by either genetic or environmental alterations, a statistical technique is employed. Common methods for evaluating this include chemometric analysis after NMR spectroscopy or mass spectrometry (in conjunction with different chromatography techniques) [101, 123, 124]. The buildup may be caused by an enzyme that is downregulated upstream in the metabolic pathway or an enzyme that is upregulated downstream, making the interpretation of these data alone difficult. Consequently, the results of transcriptomics and proteomics should be considered when interpreting this data [121]. The metabolic flux can be investigated using a variety of methods to resolve the metabolic dynamics of microalgae, such as isotope labeling of important metabolite precursors or intermediates and time-dependent monitoring of these isotopes [55] or the monitoring of consumption and production of key compounds [44, 197].

The metabolic flux of an isotope from a given starting compound to a metabolic end product can be visualized time- and sometimes spatially-resolved using radioactive isotopes [34, 158, 198] or stable isotopes for NMR spectroscopy [159, 169] and mass spectrometry [158, 161, 185, 198]. The added benefit of using transcriptomics and proteomics is the ability to find differentially expressed genes and proteins that are either cooperatively regulated or directly engaged in the biosynthesis and breakdown of lipids. For instance, the over- or under-expression of certain regulatory genes and associated proteins in transgenic cells, including as kinases, phosphatases, and transcription factors, can effectively change entire physiological pathways [6, 43, 107]. By employing transgenes encoding for various enzymatic stages in fatty acid biosynthesis/modification pathways, metabolic engineering has been used to modify fatty acid production and composition in a number of plants, most notably canola [39, 49].

Genetic engineering may be able to increase algal production after the important enzymes and pathways are identified. Only a few chosen algal model species, such as C. reinhardtii, are currently routinely transformed; nevertheless, the area of transgenic microalgae is expanding and has a lot of potential [99, 189]. The "biolistic" procedure is the most widely used of the various transformation methods available for delivering DNA into the algal genome [189]. This method, which involves bombarding algae with microprojectiles coated in DNA, has been successfully used to a range of algae, including diatoms and green algae [8, 94]. It is also the preferred technique for transforming the genomes of mitochondria

or chloroplasts [143]. Other techniques for producing transgenic algae include electroporation [164, 174], agitation using silicon-carbide whiskers [46, 47], and cell agitation in the presence of glass beads and DNA [88]. More recently, vectors with nuclear matrix attachment regions (MARs) have been developed to boost transgenic over-expression by raising the degree of foreign gene expression. *Dunaliella salina*, a halotolerant algae, has been used for this [191]. Doebbe et al. [42] successfully inserted the HUP1 (hexose uptake protein) hexose symporter from *Chlorella kessleri* into the mutant strain *C. reinhardtii* Stm6 using Kindle's glass bead approach [88]. As a result, a modified mutant was created that can produce hydrogen using glucose that is given externally. Engineering the photosynthetic light capture apparatus to increase solar energy to biomass conversion is another way to begin the metabolic and genetic engineering of microalgae [122]. Recently, the genome of *C. reinhardtii* was sequenced, revealing hitherto unidentified genes linked to photosynthetic processes among other things [113]. By genetically altering specific PQ pathway components to reduce photodamage and boost photosynthetic efficiency in high-light environments, electron pathway optimization can also be explored as a means of raising PQ levels and, consequently, the rate of biomass production [122]. Acetyl-CoA is the precursor needed for the production of algal lipids. To increase oil production, acetyl CoA carboxylase and other lipid biosynthesis pathway enzymes have been targeted [142, 162]. In the context of optimization for the generation of biodiesel, lipid metabolism and the manufacture of fatty acids, glycerolipids, sterols, hydrocarbons, and ether lipids in eukaryotic algae have recently been reviewed [72, 115]. Even though C. *reinhardtii* is used as a model organism to research lipid biosynthesis in green algae [205], certain odd hydrocarbons and ether lipids from Botryococcus braunii have been reported, such as n- Alkadienes, trienes, methylated squalenes, tetraterpenoids, triterpenoid botryococcenes, lycopadiene; [2, 115].

The quality of the fuel product is significantly influenced by the type of oil used as a feedstock for biodiesel. A possible avenue for increasing lipid quantity and quality (chain length and saturation grade) is the genetic engineering of important enzymes involved in particular fatty acid manufacturing pathways within lipid biosynthesis. Since the alkyl ester content determines the fuel's durability and performance, lipid quality is crucial to the manufacture of biodiesel and ultimately plays a significant role in achieving international fuel requirements. Biodiesel's volumetric energy density is approximately 33 MJ/L, making it roughly 92% equivalent to petro-diesel. On the other hand, biodiesel burns hotter and burns longer than regular diesel fuel because its average hydrocarbon length is longer. The gasoline burns more thoroughly as a result. Considering this, the overall efficiency of biodiesel is almost 97% that of petro-diesel [92].

Nowadays, a variety of oilseed crops are used to make biodiesel, the most popular ones being soybean, rapeseed, and palm oil. Because a given biofuel crop's triacyl glyceride (TAG) profile is largely constant, the characteristics of the biodiesel made from that crop are predictable. When creating a new source of TAGs for biodiesel, several things need to be taken into account. Because they are mostly polyunsaturated, microalgal lipids are more vulnerable to oxidation. There is a significant problem with biodiesel while it is being stored. However, this disadvantage can be overcome by partially hydrogenating the oil by catalysis [29]. According to Gunstone and Hilditch's [71] measurements, the methyl esters of oleic (18:1), linoleic (18:2), and linolenic (18:3) acids had a relative rate of oxidation of 1:12:25. Thus, it is better to keep the amount of polyunsaturated fatty acids in biodiesel to a minimum. Higher polyunsaturated fat content, on the other hand, lowers the cold filter plugging point

(CFPP), which is the temperature at which engine fuel begins to crystallize or solidify and clog the filters. The melting points of the main fatty acids are shown in Table 2. It has been noted that the more unsaturated an oil is, the lower its melting point. Therefore, for fuel to function at low temperatures in colder climes, a larger unsaturated lipid concentration is needed.

Another metric for characterizing the quality of diesel fuel combustion during compression ignition is the cetane number (Table 2). Higher cetane fuels have been found to have shorter igniting delay times than lower cetane fuels in some diesel engines. As a result, it's critical to confirm that biodiesel's cetane number matches the engine's cetane rating [91]. In light of these factors, the "ideal mix" of fatty acids has been proposed to be 16:1, 18:1, and 14:0 in a 5:4:1 ratio. This kind of biodiesel has extremely low oxidative potential while maintaining a high cetane number and CFPP rating.

Algae possess remarkable potential for genetically modifying their lipid pathways, such as through up- or down-regulating fatty acid production or β-oxidation. It should be able to significantly raise the fraction of monounsaturated lipids by deleting or altering the enzymes that the cell uses to synthesize polyunsaturated lipids. Furthermore, it's likely that the homeostasis mechanism of the algal cells would need to change the lipid ratio's amounts of saturated lipids in order for it to stay fluid at low temperatures. As long as an algae species is cultivated in same conditions, its lipid profile will not change. Every algal species, however, will have a unique lipid profile, so it's critical to use species whose lipid profiles are appropriate for producing biodiesel.

X. PHOTOSYNTHETIC EFFICIENCY

Two other examples that may be helpful for large-scale cultivation, aside from metabolic engineering methods to boost lipid production, are briefly discussed below. These include improving photosynthetic efficiency and choosing and enhancing strains for the best growth, survival, and oil production using wastewater and saltwater resources. Any improvement in photosynthetic efficiency will boost the production of biofuels later on.

The initial step in the creation of all biofuels is driven by photosynthesis (Fig. 1). In particular, it absorbs solar radiation and transforms it into chemical energy (such as starch and oil). As a result, improving light capture efficiency is a crucial innovation in the creation of all systems for producing second-generation biofuels. In order to build massive light-harvesting antenna complexes that absorb sunlight and transport the resultant energy to PSI and PSII to power the photosynthetic processes, the majority of wild-type microalgae have evolved genetic methods (Fig. 1). This approach has an advantage in nature since it maximizes light collection in low light. The drawback is that higher plants and algae have to adopt photo-protective systems since too much light harms the photosynthetic apparatus [82, 130]. These usually release most of the captured energy as heat and fluorescence, or "waste" in the context of producing biofuels [134]. The majority of this energy dissipation occurs in the PSII-related light harvesting complexes (LHCII in Figure 1). Reducing the amount of chlorophyll-binding LHC proteins in each cell has been shown to significantly increase the total light conversion efficiency of bioreactors [122, 134]. With this approach, the light capture efficiency of the antenna systems designed specifically for oil production may be carefully adjusted and optimized. The culture is dark green because wild-type algae have extensive LHCII systems

that bind chlorophyll. At the same cell density, cultures produced by cell lines with tiny LHCII antenna systems have a much lighter green color (Fig. 7a). When exposed to high light levels, algal cells on the bioreactor's illuminated surface in the wild-type scenario absorb the majority of the light but can squander up to 90% of it as heat and fluorescence [122, 134].

The amount of light that the wild-type cells are exposed to decreases with distance from the lit surface. It is impossible for these darkened cells to absorb enough sun energy to support effective photosynthesis. This leads to a significant reduction in the culture's overall efficiency. Alternatively, the advantage of using smaller antenna cell lines with lower LHCII levels is that they allow more light to enter the bioreactor (Fig. 7a) and better match the light's energy needs for each photosynthesizing cell. Therefore, only the light required by the "small antenna" cells at the bioreactor surface is absorbed, thereby removing superfluous energy fluorescence. Consequently, this permits an increased amount of light—that is, the light lost in the wild-type as heat and fluorescence—to enter the bioreactor, ensuring that even the cells located further inside the culture receive almost ideal light exposure [122]. Small antenna cultures have a better overall photosynthetic efficiency as a result (Fig. 7b). Finally, the following are the main benefits of small antenna mutants for the generation of biofuel: (1) Lower heat losses as determined by fluorescence and LHCII Enhanced light penetration characteristics, less photo damage, increased yield, and enhanced bioreactor efficiency are the other four factors. These efficiency advantages could be further increased by combining enhanced mixing techniques with genetically modified strains.

Table 2 Profiles of fatty acids [92]

Fatty acid	Fatty acid	Cetane N°	Melting point (°C)	Ester m.p.
8:00	Caprylic	33.6	16.7	Ethyl, −43°C
10:00	Capric	47.7	31.6	Ethyl, −20°C
12:00	Lauric	61.4	44.2	Ethyl, −1.8°C
14:00	Myristic	66.2	54.4	Ethyl, 12.3°C
16:00	Palmitic	74.5	62.9	Ethyl, 24°C
16:1ω7	Palmitoleic	45	−0.1	NA
18:00	Stearic	86.9	69.6	Methyl, 39°C
18:1ω9	Oleic	55	14	Methyl, −20°C
18:2ω6	Linoleic	36	−5	Methyl, −35°C
18:3ω3	Linolenic	28	−11	Methyl, −57°C
20:1ω9	Gadoleic	82	23	NA
20:4ω6	Arachidonic	NA	−50	NA

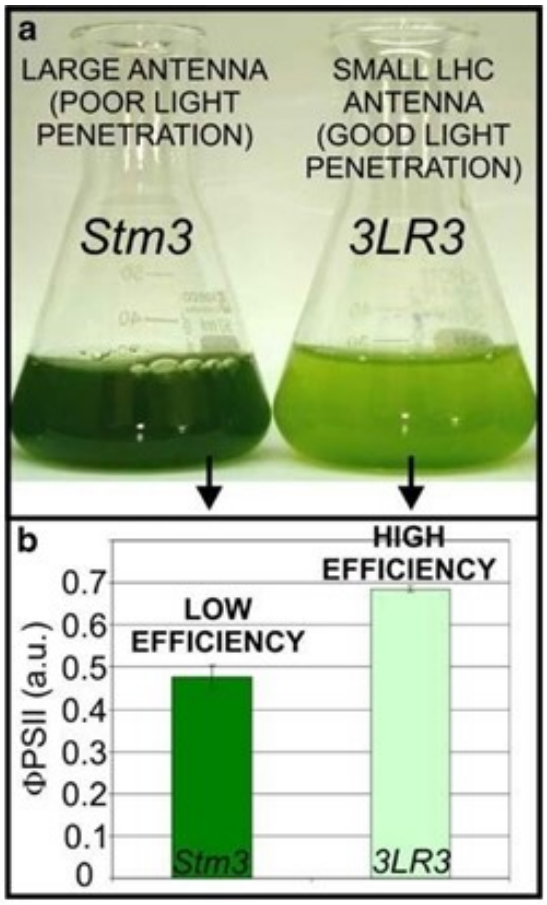

Figure 7: (a) Comparison of cultures of *Chlamydomonas reinhardtii* with parent strain (Stm3) and reduced antenna size (3LR3) at equal cell densities. a Cultures at densities of 6 × 106 cells/mL; (b) Photosynthetic quantum yield (8PSII); adapted from Mussgnug et al. [122]

XI. USING WASTEWATER AND SEAWATER RESOURCES

Using wastewater and seawater has obvious benefits over using more freshwater resources, which is becoming more and more of a concern due to population expansion and climate change-related water scarcity. But the water quality of both can differ, with wastewater having significant regional variations as well as long-term variations. Important nutrients like phosphorus and nitrogen can be found in wastewater [10, 163], but it can also contain pollutants, including heavy metals and excessive trace metals, are all quite concerning, especially when it comes to the manufacturing of biodiesel. Cadmium, for example, has been shown to inhibit lipid biosynthesis among many other cellular functions [64]. The same pollutants can also be found in seawater, though not always in the same amounts. Additionally, while using less expensive fertilizers of agricultural grade is preferable from an economic standpoint, it also adds another potential source of heavy metal pollution that may hinder sensitive types of algae.

Although it is preferable to conserve freshwater supplies, strains that have the ability to withstand elevated concentrations of specific toxins can be advantageous in open-pond settings and could mitigate worries about contamination by other opportunistic organisms. The use of algae in phytoremediation and toxicological investigations has led to significant advancements

in the fight against chemical contamination, however its usage has also generated significant controversy [15, 207]. Many contamination problems connected to metals are thought to have their root cause in the induction of oxidative stress [133, 171]. In addition to heavy metals [62, 186–188, 197], various non-metal organo- compounds [54] and trace metals like copper, which are often needed for adequate nutrition, can also be fatal or inhibitive at high concentrations [22, 180]. Resistance mechanisms differ not just on the type of algae species but also on strains within certain species and, of course, on the specific type of toxin. Although the resistance mechanisms, such as decreased accumulation, sequestration, and precipitation, were not fully explained by early studies [32, 33, 108], more recent research has made significant strides in identifying putative protein targets [64] and gene targets [40] associated with heavy metal resistance in algae. There is room for more research on structural aspects such cell wall composition [61] and concurrent studies on resistance mechanisms in plant models [116].

Some algae strains, such *Botryococcus braunii*, are freshwater strains, however many of the strains designated for oil production are marine strains. Thus, osmotic issues beyond those covered above may arise from using seawater for algae cultivation. Numerous reviews have been written about osmoadaptation in microorganisms [60, 90]. Despite the fact that salt stress has been linked to the production of oil [140, 176, 182, 183], freshwater species can experience a stress response that can be fatal or very inhibitory at osmolarities far lower than those of seawater. Recent developments in this area have also been made possible by genes in microalgae that show anti-salt action [177]. These genes are frequently general anti-stress genes that most likely work by reducing oxidative stress. One such protein is a glutathione peroxidase-like protein from *Chlamydomonas*, which, when cloned into tobacco, enhanced stress tolerance, particularly salt tolerance. Additionally, glutathione peroxidase has been linked in the past to stress reactions brought on by heavy metal exposure [133]. It is obvious that there is a lot of opportunity here for strain characterization and selection as well as for breeding, designing, and adapting strains with desired phenotypes that permit the utilization of water resources with varying water quality.

XII. RECENT ADVANCES IN MICROALGAL PRODUCTION

Recent developments were showcased at the 1st International Algae Biomass Summit in San Francisco in November [3]. This gathering brought together leaders of newly-established microalgae biofuel companies, existing aquaculture firms, prominent researchers from the Aquatic Species Program, and microalgae scientists worldwide [3]. The event served as a platform to encourage collaboration among world-renowned experts and to explore substantial funding opportunities in 2008 and beyond. The primary objective was to create a highly efficient, cost-effective system for algal oil production and optimize its conversion to JP-8 fuel. The economic benchmarks were set with algae hydrocarbon costs at US $0.48 per liter ($2 per gallon), with a minimum order volume of 210 million liters (50 million gallons) to ensure viability. To achieve this price point, consideration of algae by-products in the economic equation was imperative. Distinguished speakers at the event shared insights from historical experiences, current technologies, and innovative concepts. Key findings and conclusions can be summarized as follows:

1. Joseph C. Weissman calculated the theoretical maximum yield for algal production to be 100 g m−2 day−1 or 365 tons of dry biomass per hectare per year. Existing limitations

include the cost of obtaining CO2 at the required high concentrations for optimal algal growth and the challenge posed by algal grazers, a significant but often overlooked issue.

2. Cost-effective harvesting methods have been and continue to be a major limiting factor in the industry.

3. Biofuel production demands biomass at a cost of less than $300 US per ton of dry weight.

4. Ami Ben-Amotz presented findings of open pond yields averaging 20 g m−2 day−1 and suggested that overall production costs of $0.34 US per kilogram are feasible when the lipid content is sufficiently high. Effective co-bioflocculation using Skeletonema to enhance lipid-rich Nannochloropsis was highlighted as a cost-effective approach.

5. Tryg Lundquist pointed out the immense production potential of wastewater treatment ponds due to their high nutrient content and existing infrastructure. Limiting factors in this context include CO2 utilization and harvesting methods.

6. Mike Massingill demonstrated the cost-effectiveness of algae harvesting with the assistance of fish.

7. Mark Huntley presented hybrid systems achieving growth rates of *Tetraselmis suecica* at 62 g m−2 day−1 with 30% lipid content, although this was not maintained as a yearly average.

8. Bryan Willson introduced low-cost bioreactor designs utilizing disposable plastic materials, while Ben Cloud presented setups with standard farm-style configurations at an approximate cost of $15 US per square meter.

9. These insights and innovations collectively contribute to advancing the field of algal biofuel production.

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