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Carbon Dioxide Bio-fixation and Wastewater Treatment via Algae Photochemical Synthesis for Biofuels Production

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ABSTRACT

We are faced with the problem of energy/carbon dioxide (CO_2) in the coming decades. Microalgae has been considered as one of the most promising biomass feedstocks for biofuels production. Meanwhile, the productivity of these photosynthetic microorganisms in converting CO_2 into carbon-rich lipids, only a step or two away from biodiesel, greatly exceed that of agricultural crops, without competing for arable land. Worldwide, research and demonstration programs are being carried out to develop the technologies needed to expand algal lipid production from a craft to a major industrial process. This paper narrates the recent advances on microalgae used for biofuels (e.g., biohydrogen, biodiesel and bioethanol) production, including their cultivation, harvesting, and processing. The various aspects associated with the design of microalgae production units are described as well, providing an overview of the current state of development of algae cultivation systems (photobioreactors and open ponds). Algal cultivation systems integrated with the algae-based biorefineries could yield a diversity of bioresources, such as biodiesel, green gasoline, bio-jet fuel, isolated proteins, food starches, textiles, organic fertilizers), which mitigate the costs of biofuels production. Utilizing the energy, nutrients and CO_2 held within residual waste materials to provide all necessary inputs except for sunlight, the algae cultivation becomes a closed-loop engineered ecosystem. Consequently, developing this biotechnology is a tangible step towards a waste-free sustainable society.

Keywords: photosynthesis; algae; biodydrogen; biodiesel; hydrothermal processing

1. Introduction

Natural photosynthesis is the process, by which sunlight is captured and converted into the energy of chemical bonds of organic molecules that are the building blocks in all living organisms, oil, gas and coal. These fossil fuels, the products of photosynthetic activity millions of years ago, could provide the energy to power our technologies, heat our homes and produce the wide range of chemicals and materials that support our life. As a consequence of ever-growing utilization of fossil fuels, we are faced with a severe problem of increasing levels of CO_2 and other greenhouse gases in the atmosphere with implications for global climate change.

Photosynthesis as a successful energy generation and storage systems is derived from a fact that the raw materials and power needed for biomass synthesis are available in almost unlimited amounts; sunlight, water and CO_2. The core process of photosynthesis is the water splitting by sunlight into oxygen and hydrogen equivalents. The oxygen is released into the atmosphere, where it is available for living organisms to breathe and for burning fuels to drive our technologies. The hydrogen equivalents are used to reduce CO_2 to sugars and other types of organic molecules. When fossil fuels, biomass and other biofuels are burned to release energy, we are simply combining the 'hydrogen' stored in these organic molecules with atmospheric oxygen to form water. Similarly, energy is also released from the organic molecules constituting our food, when they are metabolized within our bodies by the respiration process. Thus, in the biological world, photosynthesis brings about the splitting of water into oxygen and hydrogen, whereas respiration is the reverse, combining oxygen and hydrogen in a carefully controlled and highly efficient way so as to create the metabolic energy. From an energetic view, the synthesis of organic molecules implies a way of storing hydrogen and storing solar energy in the form of chemical bonds[1,2].

This article comprehensively reviews the current progresses on green biofuels production from algae, mainly consisting of four parts. The first part states the energy utilization along with the CO_2 problem within the coming decades, and discusses the contributions that can be made from photosynthetic biofuels based on the successful principles of photosynthesis. The global energy situation, CO_2 and solar energy capture, and photosynthetic biofuels are presented as well. In particular, it emphasizes the potential of exploiting the vast amounts of solar energy available to produce biofuels via algae photosynthetic reaction combining the advanced technologies. The second part describes the current barriers and challenges of biofuels production from algal biomass, including the new technologies for cultivation, harvesting and processing. The third part discusses the production of main biofuels (i.e., biohydrogen, biodiesel and bioethanol) from algal biomass. In addition, the integration of biodiesel and bioethanol production in the biorefinery approaches have been presented to search for a better understanding of microalgae biofuel production and path forward for research and commercialization. Ultimately, the integrated algal systems for wastewater treatment and bioremediation to capture carbon (C), nitrogen (N) and phosphorus (P) from

specialty industrial, municipal and agriculture wastes are introduced. To bring more profits, the value added biofuels and chemicals can be developed by the sustainable and applicable ways.

1.1. Global Energy Consumption and Demands

Currently the global annual energy consumption rate is about in the region of 16.3 TW[3], with the USA and the extended EU each representing about 40% of this. In the future, this global value will rise due to industrialization in underdeveloped and developing countries coupled with the increase of world population. Based on the current projections, the global annual energy consumption rate will reach 20 TW or even more by 2030, doubled by 2050 and tripled by the end of the century[4-6]. About 85% of the total global energy consumed at present comes from burning fossil fuels with the proportion approaching 90% for the developed countries. Oil, gas and coal contribute approximately equally to this demand. The remaining sources of energy are hydroelectric, nuclear, biomass and renewable, such as solar, wind, tide and wave. At present, the utilization of biomass plays a dominated role in the underdeveloped regions such as Africa, where woody biomass and other organic matters are used as fuels.

The low level contribution of non-fossil fuels to present-day global energy demand reflects the readily available resources of oil, gas and coal. Even when oil reserves become limiting, there will remain large reservoirs of gas (including from shale) and, particularly, coal to exploit[7]. Therefore, in the global arena, the problem for the immediate future is not a limitation of fossil fuel reserves but the consequences of its combustion. If the total fossil fuel reserve is burnt, the CO_2 level would rise to values equivalent to those that existed on our planet long before humankind evolved[8]. Despite of this consideration, it is certain that fossil fuels will continue to be a major source of energy for some years to come but it is vital that they should be used in such a way as to minimize CO_2 release into the atmosphere. Technologies for CO_2 sequestration have been developed[9]. Hand in hand with this, there is an improvement in the efficiency of energy use and supplementation whenever possible from non-fossil fuel sources. Against this background, we must also strive to develop new technologies based on principles that have yet to be revealed from basic studies and in particular those that focus on using the enormous amount of energy available to us as solar radiation[10]. The sun provides solar energy to our planet on an annual basis at a rate of 1×10^5 TW. Therefore, the energy from 1 h of sunlight is equivalent to all the energy humankind currently uses in a year. We do have existing technologies to capture sunlight and produce electricity and the efficiency and robustness of these photovoltaic systems is improving daily[11-13]. Compared with the present-day price of fossil fuels, photovoltaic systems represent an expensive way to generate electricity because of high construction costs. In time, these costs will decrease relative to the cost of fossil fuel. Moreover, a combination of the principles of photovoltaic systems, especially those using cheap organic or inorganic materials, with concepts derived from natural photosynthetic systems may provide a long-term solution via artificial photosynthesis technology[6,10].

1.2. Carbon Dioxide (CO_2) and Solar Energy Bio-capture

Since 1850, the atmospheric CO_2 levels, which were stable between 200 and 280 ppm for the previous 4×10^5 years[14], have risen sharply to 370 ppm[15]. Although the increased atmospheric CO_2 level is now widely accepted as a major contributor to global warming, its potential effects are only beginning to be understood. Recent high profile reports for example indicate that atmospheric CO_2 levels of 450 ppm are likely to result in severe and probably irreversible coral reef damage[16]. At levels of 550 ppm, the melting of the West Antarctic ice sheet will cause 4-6 m rising in sea level[16] and the extinction of 24% of plant and animal species are predicted[17]. A level of 650 ppm has been predicted to result in disrupted thermohaline circulation (*e.g.*, switching off the *Gulf Stream*), major local climate changes[16] and the extinction of 35% of plant and animal species[17]. More recent global climate change models[18] suggest that the effects may be even more pronounced than previously predicted emphasizing the importance of stabilizing 2 levels as close to 450 ppm as possible and preferably below[15,16,19]. However, it appears highly unlikely that CO_2 levels will be kept below this target, due to the high CO_2 emission levels and the long residence time of CO_2 in the atmosphere. Hoffert and colleagues reported that about 11 TW CO_2-emission-free fuel by 2025 was required to achieve a stabilization of atmospheric CO_2 levels at a level of 450 ppm[3]. If Hoffert's predictions are correct, we are faced with the challenge of installing systems capable of producing energy free of CO_2 emissions at a level almost equivalent to the total current global energy demand in 2000 (13 TW) in the twenty years time. It means that an abundant zero-CO_2 emission fuels (e.g., biohydrogen) is needed urgently. Even biofuels such as biodiesel and bioethanol still produce CO_2, the difference will depend on the overall life cycle analysis, which takes carbon assimilation during feedstock production into account.

In the current society, the development of zero-CO_2 emission fuels is one of the greatest energy challenges because of two urgent reasons. The first one is the rapid depletion of oil reserves, which requires the development of replacement fuels and infrastructure on the decades to a century time horizon. Secondly, future fuels will increasingly have to be free of CO_2 emissions, as fossil fuel combustion causes anthropogenic CO_2 emissions that exacerbate global warming. The constraints of global warming clearly indicate that the implementation of clean fuel technologies must take place much more quickly. The non-CO_2 emitting energy options currently considered to be the most viable, including nuclear power, coal-fired power stations coupled to anticipated CO_2 sequestration systems, and renewable energy sources such as solar, geothermal, wind and hydroelectric. Of these, only renewable energy sources can sustain long term supplies and energy security (millennia) owing to their borderless distribution. The promise of clean energy by nuclear fusion remains inaccessible. Among the renewable resources, incident solar energy is by far the largest (1.78×10^5 TW per year)[20] and capable of supplying 1.35×10^4 times the total global energy demand (13 TW per year in 2000). However, solar energy capture is both expensive and inefficient.

Nearly all life on the earth needs to capture solar energy and converts it into chemical energy and biopolymers by photoautotrophic organisms. Many organisms have developed complex molecular machinery for converting efficiently sunlight into chemical energy over the past 3 billion years, but there is no any man-made technologies to match it up to now. Chlorophyll photochemistry within photosystem II (PSII) drives the water-splitting reaction efficiently at room temperature, in contrast with the thermal dissociation reaction that requires a temperature of *ca.* 1,550 K. The high-resolution structure of PSII, particularly the structure of its Mn_4Ca cluster[21-24] has successfully provided an invaluable blueprint for designing solar powered biotechnologies for the future. Combing this knowledge with new molecular genetic tools, fully sequenced genomes, and physiological processes of oxygenic phototrophs, researchers have been strongly inspired to develop new biotechnological strategies to produce renewable CO_2-neutral energy from sunlight[25].

An obvious target is manipulating photosynthesis to increase the initial capture of light energy, which at present is less than 2%. Recently, this approach has had some success using engineered genes from plants and photosynthetic bacteria. For example, ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO), the plant enzyme that converts CO_2 to organic carbon by carboxylation during photosynthesis, also conducts a competing, less efficient oxygenation reaction. When an inorganic carbon transporter gene from *Cyanobacteria* was expressed in plants, the more efficient carbon fixing photosynthetic reaction of RuBisCO was favored. In another approach, the *cyanobacterial* versions of two rate-limiting enzymes in the chloroplast's carbon-fixing 'dark reaction' were overexpressed in tobacco, resulting in an elevated rate of photosynthesis and increased plant dry weight[26]. Besides, the manipulation of genes involved in nitrogen metabolism has also been a successful approach to increasing biomass[27,28].

1.3. Photosynthetic Biofuels

Most life-cycle studies have found that replacing gasoline with ethanol modestly reduces greenhouse gas emissions if made from corn and substantially if made from cellulose or sugarcane[29-46]. These studies compare emissions from the separate steps of growing or mining the feedstocks (e.g., corn or crude oil) and processing them into the transportation fuels. Corn and cellulosic ethanol emissions exceed or match those from fossil fuels and therefore produce no greenhouse benefits. However, because growing biofuel feedstocks removes CO_2 from the atmosphere, biofuels can in theory reduce greenhouse gas emissions relative to fossil fuels. Studies assign biofuels a credit for this sequestration effect, which we call the feedstock carbon uptake credit. It is typically large enough that overall greenhouse gas emissions from biofuels are lower than those from fossil fuels, which do not receive such a credit because they take their carbon from the ground. It is our belief that the next generational change in the use of bioresources will come from a total integration of innovative plant resources, synthesis of biomaterials, and generation of biofuels and biopower. The premise of photosynthesis for the direct generation of fuels (*Photosynthetic Biofuels*) is that a single organism can serve both as a photo-catalyst and a

producer of ready-made fuel. This concept is exemplified in the schematic of Fig.1, where H_2O, sunlight and CO_2 are inputs and O_2, biomass, fuels (H_2, hydrocarbons) and chemicals are outputs. In this model, conversion of solar-to-chemical energy, and biohydrogen, hydrocarbons, or other chemicals take place within a single cell, possibly involving the photosynthetic apparatus and the adjacent cellular metabolism [47-49].

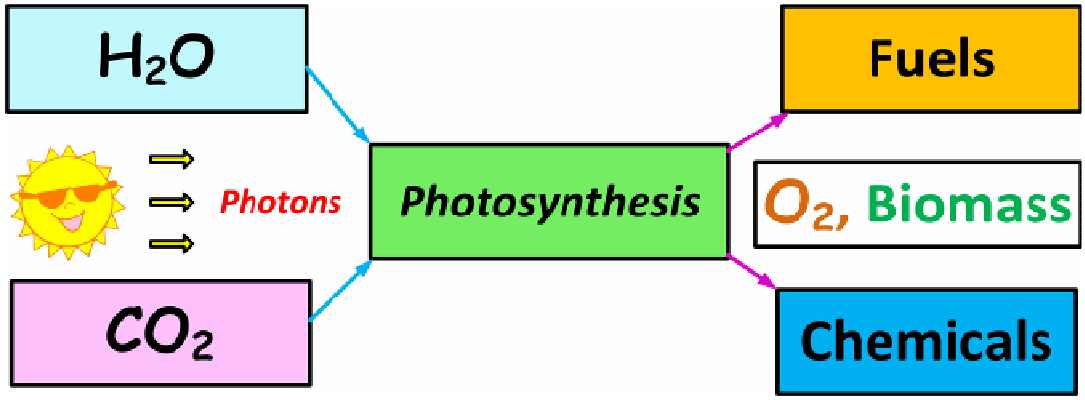

Figure 1. Schematic depicting the concept of '*Photosynthetic Biofuels*', where a single organism converts, via the process of oxygenic photosynthesis, H_2O and CO_2 into biomass and O_2. Alternatively, photosynthate can be directed toward the generation of fuels and chemicals. Oxygen is a by-product of photosynthesis[47].

So far, biofuels production from plants or algae photosynthesis has focused on closure of the carbon cycle, but not the nitrogen cycle. Either plant-based or algae-based biofuels require application of nitrogen fertilizer produced from the *Haber-Bosch* process. The reduced nitrogen is assimilated by the plant or algal species to make proteins and nucleic acids, which are not utilized for fuel production. Instead, the high-nitrogen containing residuals are used mainly as animal feed, and eventually result in dispersion of reduced nitrogen on earth, which increases the production of nitrous oxide (N_2O), a greenhouse gas 300 times worse than CO_2[50]. Feeding biofuel production residues to animals is currently economically attractive and may offset the energy and environmental cost of feed production, but is not a scalable solution if biofuels are to replace the majority of the liquid fuel used today. Recycling the ammonia from the protein-rich residuals as a fertilizer for photosynthetic feedstocks can close the nitrogen cycle. Corn ethanol, algal biodiesel, and other traditional feedstock (Fig.2A) do not utilize proteins and thus the reduced nitrogen is lost from the biofuel production cycle[51]. Only the utilization of protein in a controlled manner will allow for the recycling of ammonia. Fig.2B shows the conceptual scheme for closed carbon and nitrogen cycles to optimize the biofuel production. This idea could be implemented in both plant (Fig.3A) and algal (Fig.3B) biofuel production processes to recycle nitrogen fertilizer in practice[51].

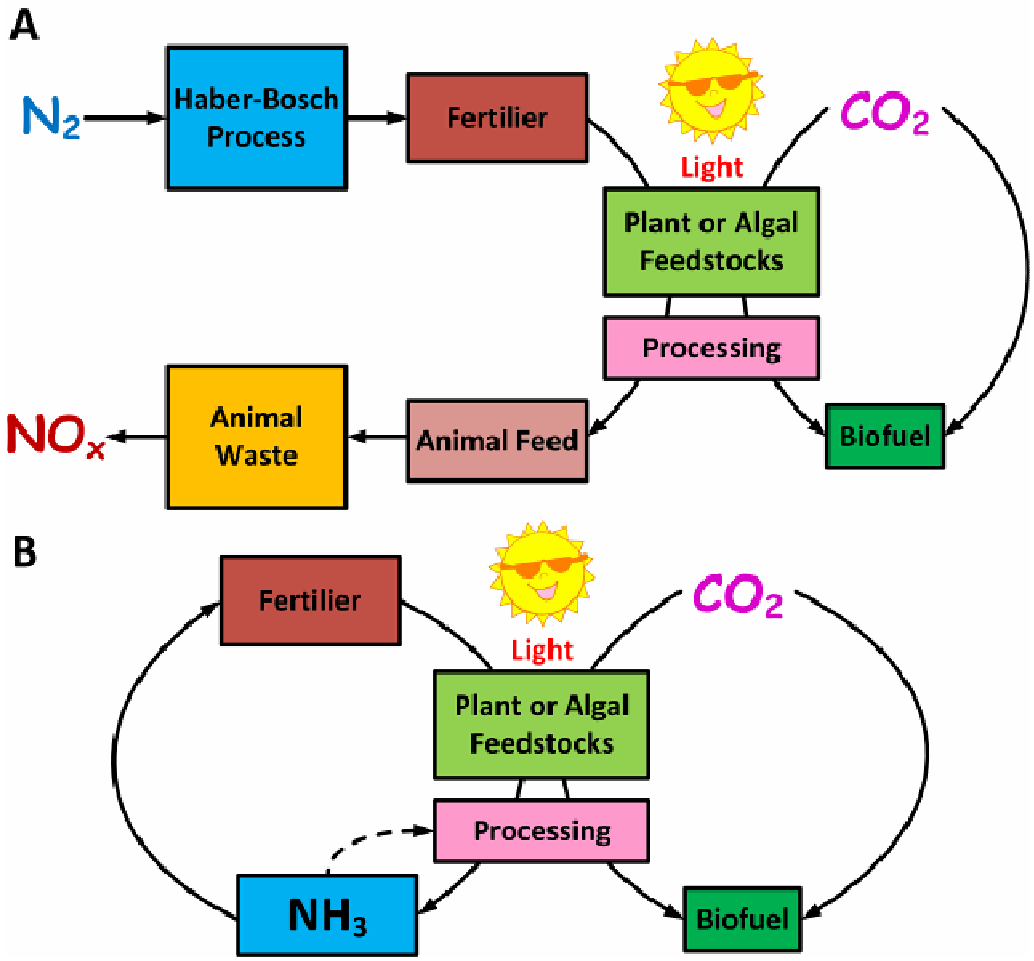

Figure 2. Carbon and nitrogen cycles in biofuel production. (a) Traditional biofuel production from plant or algal feedstocks closes the carbon cycle but imbalances global nitrogen flux. Nitrogen is fixed through the Habor-Bosch process to synthesize fertilizer, which is assimilated to proteins in biomass. The nitrogen-rich residual is commonly sold as an animal feed by-product and leads to NO_x emissions from animal wastes. (b) Utilization of proteins for fuel production can close both the carbon and nitrogen cycles. Protein conversion releases ammonia as a by-product. The ammonia may be reapplied as a fertilizer or nitrogen source for fermentation.

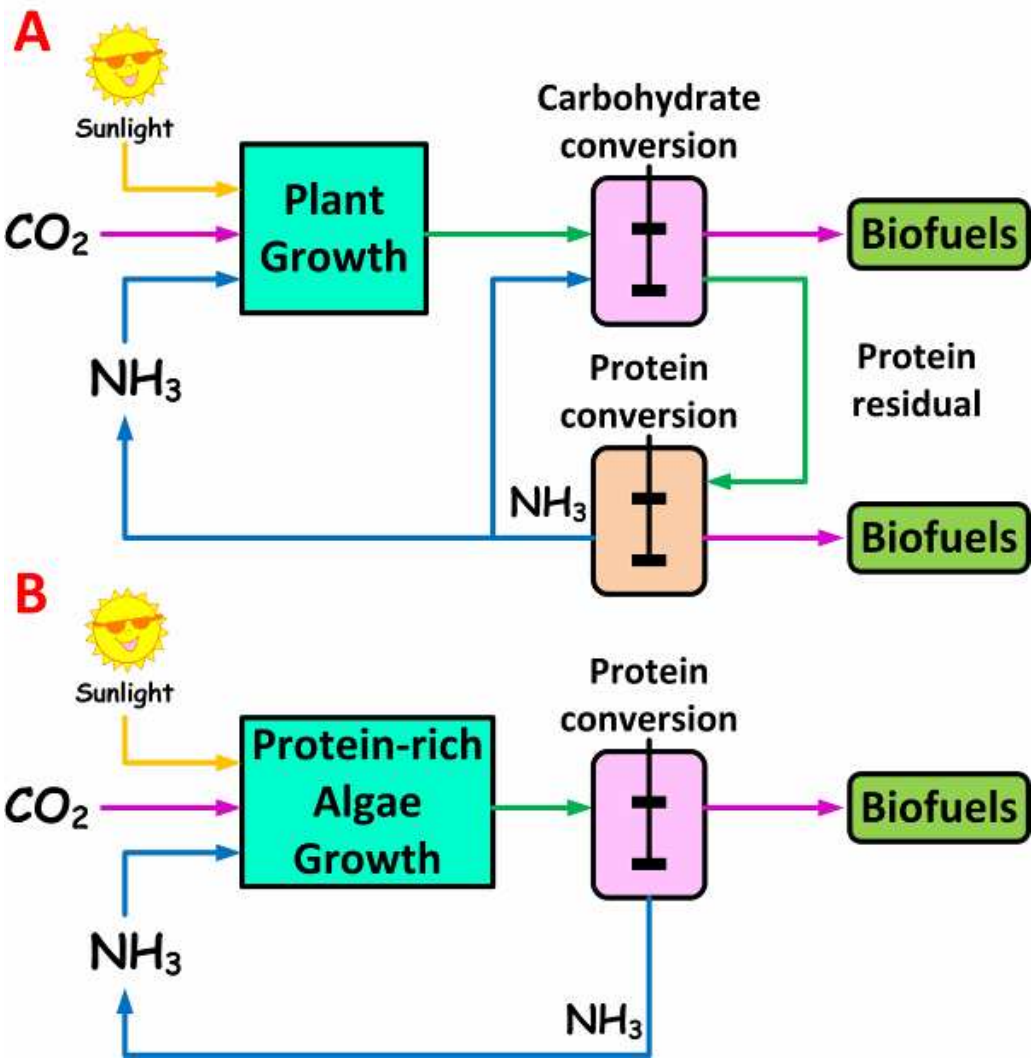

Figure 3. (A) A Conceptual process for biofuels from plant biomass can recycle fertilizer when protein residual is utilized for ammonia recycling and fuel production; (B) Biofuel production from protein-rich algae also releases ammonia to be directly reapplied for subsequent algae growth.

Because of the low photosynthetic efficiency and the competition of energy plants with food plants for agricultural land, some researchers suggested that it is unreasonable to grow plants for biofuel production[52-54]. The main reason is the growth of such energy plants will undoubtedly lead to an increase in food prices. Meanwhile, most prior studies have found that substituting biofuels for gasoline will reduce greenhouse gas emissions because biofuels sequester carbon through the growth of the feedstock. These analyses have failed to count the carbon emissions that occur as farmers worldwide respond to higher prices and convert forest and grassland to new cropland to replace the grain (or cropland) diverted to biofuels[53]. Converting biomass into the valuable building blocks for chemical syntheses

may be the best choice. Compared with biofuel production, available biomass, instead of fossil fuels, is more preferable to be used for heat to generate electricity. The saved fossil fuels could be used for transportation purposes. Clearing rainforests in the tropics and converting them into oil palm plantations is highly dangerous because the underlying layers of peat are oxidized and much more CO_2 is released by the oxidation of organic soil material than can be fixed by the oil palms. The rainforests plays an important role for the climate and constitute a valuable resource for novel compounds for drug discovery. With respect to the carbon footprint, it will be much better to reforest the land used for growing energy plants, because at a 1% photosynthetic efficiency, growing trees would fix around 2.7 kg/m^2 of CO_2, whereas biofuels produced with a net efficiency of 0.1% would only replace fossil fuels which release about 0.31 kg/m^2 CO_2 upon combustion[54].

2. Algae Photosynthesis for Biofuels

2.1. Photosynthesis

Photosynthesis is a process converting light energy into the organic molecules of biomass, which is mainly composed of carbohydrates symbolized as CH_2O. On a global basis, the photosynthetic efficiency is much lower than for agricultural and energy crops or algal cultures growing under the optimal conditions because of seasonal changes and the large portions of land and oceans, which do not sustain higher photosynthetic activity[55]. Thus, the rate of energy storage averaged over a year by photosynthesis is 100 TW, representing just 0.1% conversion given that solar energy arriving at our planet is at a rate of 1×10^5 TW over the same period of time. This energy is mainly stored in wood and fibers of terrestrial trees and plants. A similar amount of photosynthetic activity occurs in the oceans, but the fixed carbon is rapidly recycled into the food chain[56]. Therefore, a global photosynthetic efficiency is about 0.2% but with only half being stored in biomass (i.e., 0.1%). Absolutely, it was terrestrial biomass that was the major source of energy for humankind prior to the exploitation of fossil fuels. Therefore, it is not surprising that there is a growing interest in returning to the use of biofuels as an alternative to fossil fuels because of their CO_2 neutral characteristic. Nevertheless, the scale required for satisfying the global energy requirement is far from attainable because of competing with large-scale food production and general land use needed to sustain a global population of seven billion.

Although it is possible to engineer plants and other types of photosynthetic organisms (i.e., algae) as energy-converting ‘machines’ and ‘chemical factories’, the overall efficiency of solar energy conversion will rarely exceed 1% and will usually be much less, so that this approach can make only a minor contribution to our future energy requirements. However, the efficiencies of the early photochemical and chemical reactions of photosynthesis, which are not directly involved in biomass production, are significantly higher. As a result, there are alternative and complementary approaches for using solar energy. It may develop a highly efficient, artificial, molecular-based, solar-energy-converting technology that

exploits the principles of the 'front-end' of natural photosynthesis. Indeed, our knowledge of the natural process is to provide a blueprint for the design and assembly of such 'artificial photosynthetic' devices as described as follows. The process is based on the light-driven water-splitting reaction that occurs in PSII of plants, algae and cyanobacteria (Fig.4). Firstly, solar energy is absorbed by chlorophyll and other pigments. And then, it is transferred efficiently to the PSII reaction center where charge separation takes place. This initial conversion of light energy into electrochemical potential occurs in the PSII reaction center with a maximum thermodynamic efficiency of 70%, and generates a radical pair state $P680^{\cdot+}Pheo^{\cdot}$, where P680 is a chlorophyll a molecule, and Pheo is a pheophytin a molecule. The redox potential of $P680^{\cdot+}$ is highly oxidized (about +1.2 V), while that of $Pheo^{\cdot}$ is about 20.5 V. The latter is sufficiently negative because it could drive the hydrogen formation. Instead, the reducing equivalent is passed along an electron transport chain to PSI, where it is excited by the energy of a second 'red' photon absorbed by a chlorophyll molecule, known as P700, to lift it to a reducing potential of 21 V or even more. By this way, sufficient energy is accumulated to drive the CO_2 fixation, which not only requires the generation of the reduced hydrogen carrier, i.e., nicotinamide adenine dinucleotide phosphate (NADPH), but the energy-rich molecule adenosine triphosphate (ATP) formed by some energy during electron transfer releasing from PSII to PSI in the form of an electrochemical potential gradient of protons.

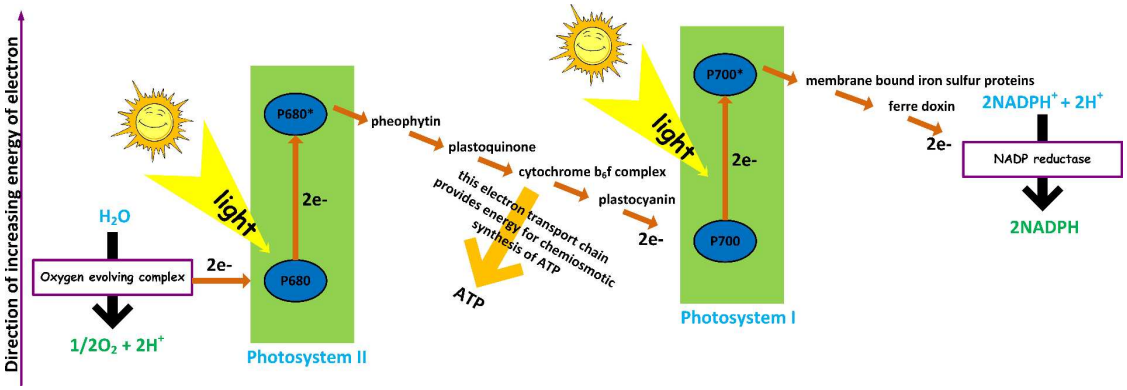

Figure 4. A simplified scheme of the light reactions of photosynthesis.

2.2. Algae Photosynthesis and CO_2 Biomitigation

Algae are recognized as one of the oldest life-forms[57] and are present in all existing earth ecosystems, representing a big variety of species living in a wide range of environmental conditions[58]. They are primitive plants (thallophytes), i.e., lacking roots, stems and leaves, have no sterile covering of cells around the reproductive cells and have chlorophyll *a* as their primary photosynthetic pigment[59]. Under natural growth conditions, phototrophic algae absorb sunlight, and assimilate CO_2 from the air and nutrients from the aquatic habitats[60]. The term 'microalgae' is not a biological, but rather a practical, description, and its scope may differ depending on the context and the author. In its widest definition, microalgae are unicellular, photosynthetic microorganisms from several related branches of the tree of life, comprising, for example, prokaryotic *cyanobacteria*, eukaryotic green algae,

red algae and heterokonts (e.g., brown algae and diatoms)[58,61]. Microalgae can produce lipids, proteins and carbohydrates in large amounts over short periods of time. These products can be processed into both biofuels and valuable co-products[60]. However, the production of lipids, proteins and carbohydrates may be limited by available sunlight due to diurnal cycles and the seasonal variations; thereby limiting the viability of commercial production to areas with high solar radiation[62]. Microalgae can fix CO_2 from three different sources, viz. atmosphere, discharge gases and soluble carbonates[63]. Under natural growth conditions, microalgae can assimilate CO_2 from the air, tolerating and utilizing substantially higher levels of CO_2 (up to 1.5×10^5 ppmv)[64]. Therefore, in common production units, CO_2 is fed into the algae growth media either from external sources such as power plants[65,66] or in the form of soluble carbonates such as Na_2CO_3 and $NaHCO_3$[67,68]. Other required inorganic nutrients for algae production include nitrogen, phosphorus and silicon[69]. Algal cells are veritable miniature biochemical factories, and appear more photo-synthetically efficient than terrestrial plants as these are very efficient CO_2 fixers. The ability of algae to fix CO_2 has been proposed as a method of removing CO_2 from flue gases to reduce emission of greenhouse gas emissions from power plants. Many algal cells have been found exceedingly enriched with oil globules, which could be converted into biodiesel[70]. Three distinct algae production mechanisms, photoautotrophic, heterotrophic and mixotrophic are in use, all of which follow the natural growth processes. Photoautotrophic production is autotrophic photosynthesis, and heterotrophic production requires organic substances (i.e., glucose) to stimulate growth, while some algae strains can combine autotrophic photosynthesis and heterotrophic assimilation of organic compounds in a mixotrophic process[60]. Many microalgae strains have high lipid content (20-50% dry weight), which can be enhanced by optimizing the growth determining factors[71,72].

Most of the current research and development efforts have focused on microalgae due to their high growth rate and oil content. Algae contain oils, sugars, and functional bioactive compounds that can be used for commercial products. Recently, special attention has been given to cultivate microalgae as an energy crop with the aim of replacing traditional oil crops for biodiesel and bio-oil production. Algae have the potential to produce up to ten times more oil per acre than traditional biofuel crops such as oil palm. They can survive where agricultural crops can't, such as in salt water and on marginal land. They thrive on a diet of waste CO_2 and the nutrients in agricultural run-off and municipal wastewater. And in addition to fuels, valuable co-products, such as biopolymers, proteins and animal feed can be made during the process. The concept of using algae to make fuel was first discussed more than 50 years ago but a concerted effort began with the oil crisis in the 1970s[69]. The US Department of Energy (DOE) from 1978 to 1996 devoted $25 million to algal fuels research in its aquatic species program at the National Renewable Energy Lab (NREL) in Golden, Colorado. The program yielded important advances that set the stage for algal biofuel research today[73].

In the 1980s and 1990s, researchers tried various approaches. They grew algae in outdoor open ponds and enclosed photo-bioreactor tanks, experimented with breeding, fed algae smokestack CO_2 emissions to boost their growth, and tested species that can tolerate extreme salt and pH environments. The first genetic transformation of microalgae came in 1994. And a few years later, scientists successfully isolated and characterized the first algal genes that express enzymes thought to enhance oil production. From 1990 to 2000, the Japanese government funded algae research through an initiative at the *Research Institute of Innovative Technology for the Earth* (Kyoto). The program focused on CO_2 fixation and improved algal growth with concentrated mirrors that collect light. These approaches yielded some successes and many are still the focus of scientists today, but none have proven economical on a large scale. The DOE program closed partly in 1996, because algal systems could not compete with the cheap crude oil of the late 1990s. The NREL-Chevron partnership started in 2007 and concluded in 2011, many efforts have been ongoing for the revived algae research program. Like all photosynthetic organisms, with a little water, a few nutrients and CO_2, microalgae-pond scum use energy from the sun to grow. With just these inputs, they can easily double their population in a day. Faced with stresses such as nutrient deprivation, algae put their energy into storage often in the form of natural oils such as neutral lipids or triglycerides and growth slows. Similar to the oils from crops such as soybeans, jatropha and oil palm, algal oil can be extracted from the organisms and refined into biodiesel by transesterification with short-chain alcohols (i.e., methanol) or by esterification of fatty acids[73]. Algae can also be synthesized into other fuel products, such as hydrogen, ethanol and long-chain hydrocarbons that resemble as crude-like oil. Microalgal H_2 is the direct product of the light reactions of photosynthesis. To bypass the H_2 storage problems, an alternative approach would be to enable and harvest biofuel products from the carbon reactions of photosynthesis. Of particular interest is the process of generating and accumulating hydrocarbons via the fatty acid or terpenoid biosynthetic pathways[74-77]. Hydrocarbons can be viewed as a biological way of storing hydrogen (Fig.5).

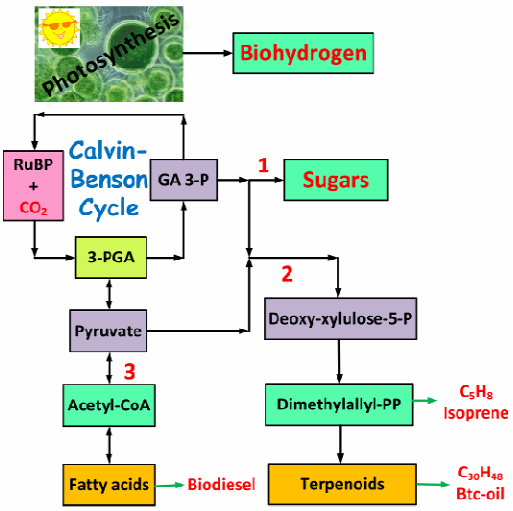

Figure 5. Photosynthetic products generation from the light and carbon reactions of photosynthesis

Vegetable and animal oils have long served as important raw materials for a number of applications, including surfactants, lubricants, polymers and foodstuffs[78]. The primary precursors for these products are mono-, di- and poly-functional linear alkyl alcohols, aldehydes and acids are derived from the oxidative or reductive functionalization of acyl lipids and fatty acids[79-81]. These modifications generally occur at either the carboxyl or olefinic moieties on the lipid, and the resulting products thus depend on both the tail length and the degree of unsaturation of the lipid precursor[82]. Algal lipids are very similar to many plant lipids, with the notable exception that algal lipids are more likely to contain fatty acid components having higher degrees of unsaturation[69,83]. Fig.6 presents the values of both tail length and unsaturation for several representative algae and plant crops[78,84-87]. It can be observed that many plants and algal crops have an average tail length in the 17/18 carbon range. And highly unsaturated lipids in algae occur more frequently in polar lipid fractions, specifically phospholipids[88]. Depending on species and growth conditions, phospholipids can compose anywhere from 8-47% of the total fraction of algal oil[89]. In contrast, soy oil contains only 2-3% phospholipids[90]. Owing to the presence of the phosphate moiety, these lipids complicate many transesterification, reduction and combustion processes[91,92], and are therefore not desirable for biodiesel production without pre-treatment.

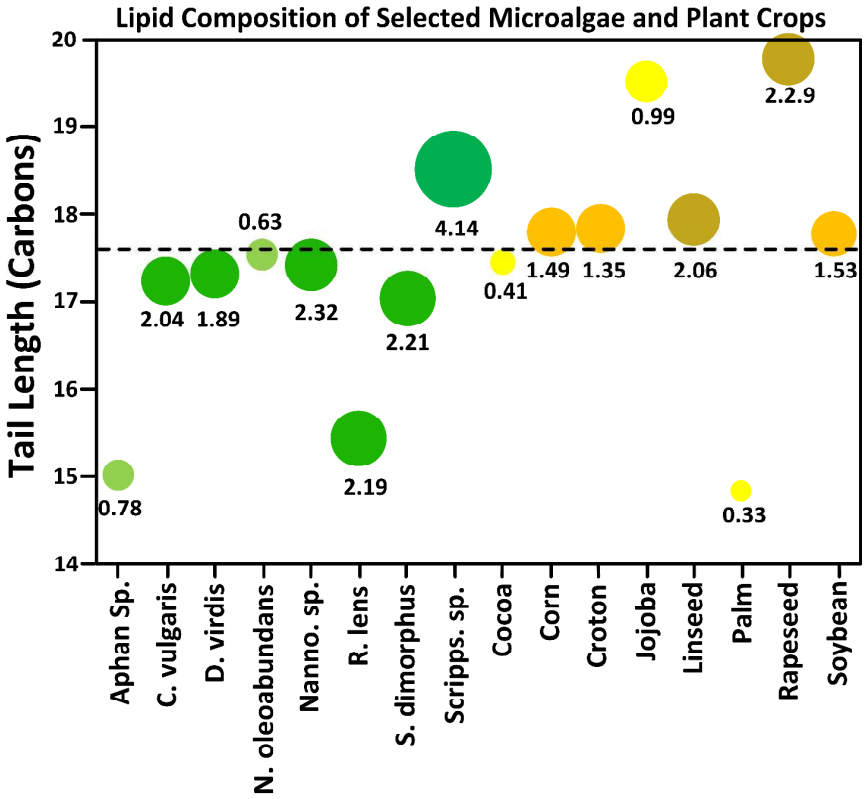

Figure 6. Lipid compositions of selected algae and plant crops; the circle size corresponds to the average degree of unsaturation per lipid tail [78, 84-87]

Most of the current biofuel production is from the fermentation of sugar produced from grains by conventional yeast strains, or on transesterification by acid/alkali or enzyme based catalysts. It is the first generation of biofuel production which is thought to have negative impacts on food security and controversial energy balance[93]. Second generation biofuels involve biological processing of lignocellulosic biomass to overcome the fuel vs. food dilemma[94]. Both 3rd and 4th generation biofuels use photosynthetic microorganisms to create renewable fuels: the former is basically processing of algae biomass for biofuel production, while the latter is about metabolic engineering of algae for producing biofuels from oxygenic photosynthetic organisms (Fig.7). Algae metabolic engineering forms the basis for 4th generation biofuel production. It uses recombinant DNA and other biological and bioengineering techniques for directed modification of cellular metabolism and properties through the introduction, deletion, or modification of algal metabolic networks to create or enhance biofuel production[95,96].

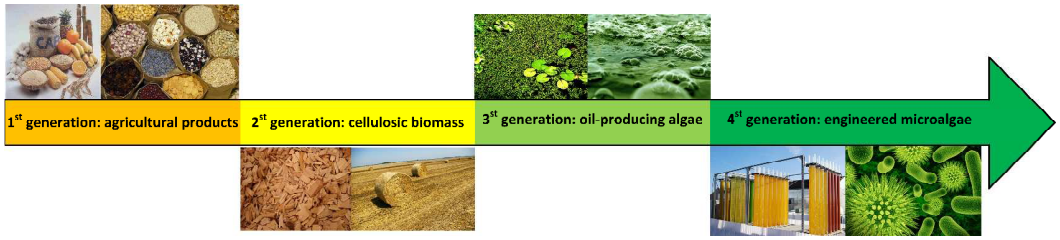

Figure 7. Four generations of biofuel production: from agricultural products to algae

Additionally, algae are more productive than plants. Under suitable culture conditions, the oil lipid productivity of microalgae can greatly exceed that of vascular plants[97,98]. For example, the median value of the maximum specific growth rate of microalgal species is approximately 1 per day whereas for higher plants it is 0.1 per day or less[99]. Each algal cell is photosynthetically active whereas only a fraction of plant biomass photosynthesizes. Each algal cell can absorb nutrients directly from its surroundings, so algae do not have to rely on energy-consuming, long-distance transport of nutrients via roots and stem. In addition to light, photosynthesis requires CO_2. In plants, photosynthetic tissue can access CO_2 only through pores known as stomata. These pores are not always open and CO_2 must move through them against a flow of water vapor. The CO_2 diffusion pathway from the surface of the photosynthetic tissue to a photosynthesizing cell is much longer in plants than in microalgae and increases with increasing the thickness of the photosynthetic structure[99,100]. Therefore, algae can access CO_2 more easily than vascular plants and this contributes to the relatively rapid growth. Owing to their high solubility in water, the equilibrium concentration of CO_2 in an algal suspension is greater than in the atmosphere above the suspension. Effectively, water enriches CO_2 that is essential for photosynthesis. This also improves algal productivity relative to plants. Furthermore, because of a short life-cycle, algal biomass can be harvested daily or hourly, whereas plant biomass typically remains in the field for much longer. Unfortunately, owing to the low productivity of plants, existing plant-derived biofuels cannot displace petroleum-based transport fuels to any significant extent. This severe limitation can only be overcome with a new generation of

biofuels such as algae-based fuels. Unlike the existing crop-derived biofuels, algal fuels can be produced without encroaching on cropland and without further deforestation. Production of algal biofuels need not reduce the supply of food, feed, other agricultural products and freshwater[97,98].

Production of some existing biofuels demands unsustainable inputs of nitrogenous fertilizers, which are generated from fossil fuels and require huge inputs of energy to produce[100,101]. Plant-symbiotic bacteria, algae and other photosynthetic microorganisms can naturally convert the atmospheric nitrogen to a form that can be used by life-forms, but most crop plants and microalgae being considered for producing biofuels do not do this. Therefore, engineering plants and algae for nitrogen fixation capability is important for sustainable production of biofuels. Production of all kinds of biofuels can be improved substantially by genetic and metabolic engineering[97,102-112], bioprocess engineering[113-115], the use of extremophilic species[116], and in other ways[117]. The future of biofuels is intertwined with genetic and metabolic engineering. No form of renewable energy can fuel infinite growth and, therefore, society will have to learn to live within limits, including limits on population. Increasing the efficiency of energy use will be essential and will need to be achieved without changes to the lifestyle that we are accustomed to in the developed world. Within the constraints of sustainability, all humanity must attain an equitable quality of life. Algal biofuels have a clear potential for contributing to environmental, social and economic sustainability[118].

Photosynthesis is the fundamental system required for all potential bioenergy surrogates production from photosynthetic microorganisms. However, it is a relative low-efficiency process in terms of energy conversion when compared to the downstream synthesis of targeted products. More than 90% of the photon energy delivered to a given photosynthetic footprint can be dissipated as heat or fluorescence, and current estimates for realistic photosynthetic conversion efficiency fall around 6% of total incident light energy[119-121]. Maximization of photosynthetic potential is one of the most important and complex challenges in current efforts to exploit primary productivity for bioenergy applications (Fig.8). It is reported by Doan et al.[122] that some researchers tried to directly exploit the abundant algae or plants from the marine or lakes for biofuels production. However, it should be noted that utilizing excessively the algal biomass (i.e., marine algae) in existence for biofuels production may destroy the earth's aquatic ecosystem and change the global climate. However, according to the mechanisms of microalgae photosynthesis, the algae could be rapidly grown and harvested in small-scale aquatic artificial systems under the optimum conditions as well. Crucial components for the photosynthetic process are antenna proteins, which absorb light and transmit the resultant excitation energy between molecules to a reaction center. The efficiency of these electronic energy transfers has inspired much work on antenna proteins isolated from photosynthetic organisms to uncover the basic mechanisms at play[123-127]. Intriguingly, recent works have documented[128-130] that light-absorbing molecules in some photosynthetic proteins capture

and transfer energy according to quantum-mechanical probability laws instead of classical laws at temperatures up to 180 K. This contrasts with the long-held view that long-range quantum coherence between molecules cannot be sustained in complex biological systems, even at low temperatures[131]. Collini et al.[132] and Richards et al.[133] used two-dimensional photon echo spectroscopy measurements[134-137] to study coherently wired and vibronic coupling, respectively, light-harvesting in photosynthetic marine algae at ambient temperature. These observations provide compelling evidence for quantum coherent sharing of electronic excitation across the 5-nm-wide proteins under biologically relevant conditions, suggesting that distant molecules within the photosynthetic proteins are 'wired' together by quantum coherence for more efficient light-harvesting in cryptophyte marine algae[132].

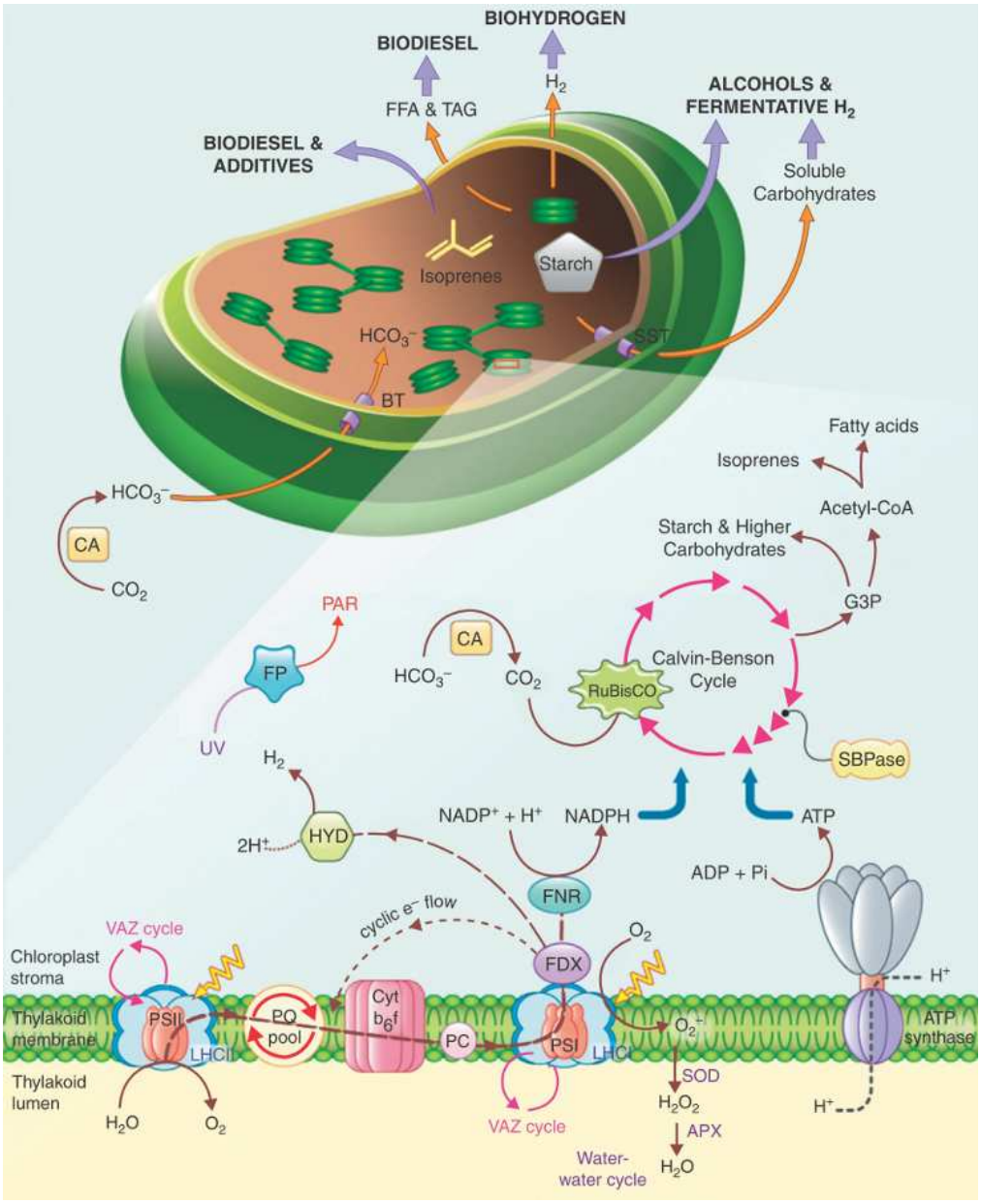

Figure 8. Generic chloroplast of a green alga showing placement of fuel-relevant primary metabolites and their integration into bioenergy production. Also depicted are the major components of photosynthesis and carbon fixation, including elements with the potential to be engineered for optimization of these pathways, as described in the text (specifically BT, CA, FP, HYD, LHC, RuBisCO, SBPase, VAZ, water-water cycle). APX: ascorbate peroxidase, BT: bicarbonate transporter, CA: carbonic anhydrase, Cyt b_6f: cytochrome b_6f, FDX: ferredoxin, FFA: free fatty acids, FNR: ferredoxin-NADP+ reductase, FP: fluorescent protein, G3P: glyceraldehyde 3-phosphate, HCO_3^-: bicarbonate, HYD: hydrogenase, LHC: light-harvesting complex, PAR: photosynthetically active radiation, PC: plastocyanin, PS: photosystem, PQ pool: plastoquinone pool, SBPase: sedoheptulose-1,7-bisphosphatase, SOD: superoxide dismutase, SST: soluble sugar transporter, TAG: triacylglycerol, UV: ultraviolet light, VAZ: xanthophyll cycle [122].

During the photosynthetic process, microalgae utilized CO_2 from atmosphere as carbon source to grow and reproduce. Microalgae cells contain approximately 50% carbon, in which 1.8 kg CO_2 are fixed by producing 1 kg microalgal biomass[97]. Hence, this method is recognized to be more environmental friendly and technologically feasible to bio-mitigate CO_2 compared to physicochemical adsorption or direct inject into deep ocean. However, the low concentration of CO_2 in the atmosphere (0.04%) with poor mass transfer rate in water have resulted to the use of expensive air pump to deliver CO_2 efficiently to microalgae rather than relying on natural diffusion from atmosphere[138]. On the other hand, flue gases from industry usually contain more than 15% (v/v) of CO_2[139] and therefore, could be a prospective carbon source for microalgae. This is a win-win strategy in which air pollution from industry can be controlled through microalgae cultivation while the microalgae biomass can be used to produce biofuels.

Currently, extensive research has been focused to identify suitable microalgae strains that can grow under high concentration of CO_2 while producing lipid for subsequent biodiesel production. The desired microalgae strains should have the following characteristics: (1) high growth rate and biomass productivity; (2) high tolerance to trace the amount of acidic components from flue gases such as NO_x and SO_x; (3) able to sustain their growth even under extreme culture conditions (e.g., high temperature of water due to direct introduction of flue gases). A few recent studies have reported that *Chlorella* sp., *Scenedesmus* sp., and *Botryococcus braunii* are among the microalgae strains that have shown promising result to mitigate CO_2 emission with typical CO_2 consumption rate of 200-1300 mg/L/day[140-144]. Besides, a pilot-scale system has been successfully developed to culture microalgae using industrial flue gases and *Scenedesmus obliquus* was able to tolerate a high concentration of CO_2 up to 12% (v/v) with optimal removal efficiency of 67%[145]. Moreover, supplying a high concentration of CO_2 to microalgae can enhance the accumulation of polyunsaturated fatty acid in the microalgae cells[146]. This is an encouraging observation as higher content of polyunsaturated acid tends to reduce the pour point of biodiesel produced and making it feasible to be used in cold climate countries.

2.3. Algae Cultivation and Photobioreactors (PBRs)

One of the most understudied methods for CO_2 mitigation is using biological processes (*via microalgae*) in a direct CO_2 to biomass conversion from point source emissions of CO_2 in engineered systems such as PBRs. Microalgal biofixation in PBRs has recently gained renewed interest as a promising strategy for CO_2 mitigation. PBRs utilized for microalgal CO_2 sequestration offers the principal advantages of increasing microalgae productivity, owing to controlled environmental conditions, optimized space/volume utilization, and more efficient use of costly land. Actually, the photosynthetic solution when scaled up would present a far superior and sustainable solution under both environmental and economic considerations[147]. Fig.9 shows the integrated diagram of the PBRs applications on waste CO_2 capture and wastewater treatment by microalgae. The produced microalgal biomass can be used for biofuel production (e.g., biodiesel and methane) and other by-products, such as animal feeds and polymers[148,149].

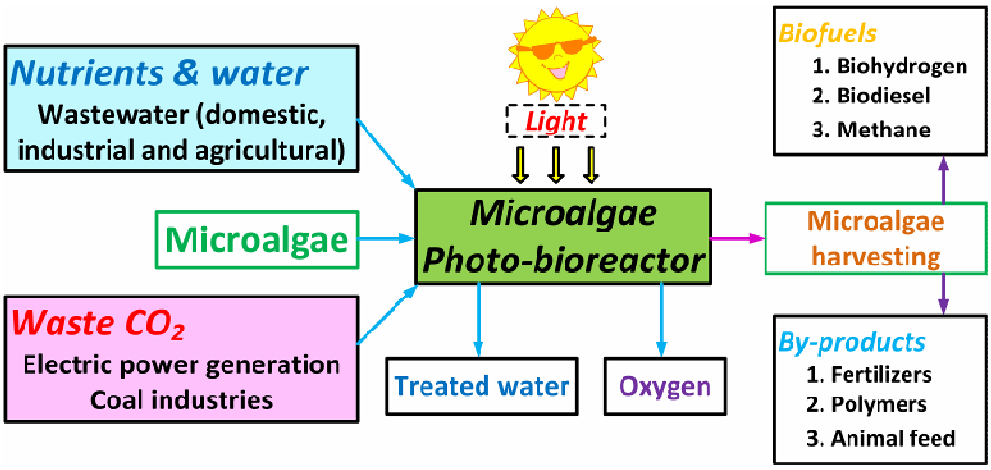

Figure 9. Schematic diagram of microalgae photo-bioreactors applications on CO_2 capture and biofuels production

In general, microalgae could be cultivated in open (pond) systems or closed systems. Considering all the limitations and shortcomings of the pond systems, most researchers had oriented their research works towards the development of an unconventional way for microalgae culture, which should be fully closed and compact with high surface-to-volume ratio and all the growth factors be optimized. Closed reactors could be tubes, plates or bags made of plastics, glass or other transparent materials, in which the algae are supplied with light, nutrients and CO_2[150,151]. However, only a few of these designs can be practically used for mass production of algae[152,153]. For energy production, algal biomass is too much expensive up to now. On one hand, this price is governed by the perceived nutritional value of algal biomass that is mostly produced for animal feed and not for energetic usage. On the other hand, it is caused by the low productivities of open ponds, the high demands of auxiliary energy and high costs of classical PBRs designs. But the problems are being addressed by engineering and science. Encouraging results have been obtained using new reactor geometries, optimized aeration and mixing strategies[154-157].

An experimental helical-tubular PBR has been designed by Briassoulis et al.[158] for controlled, continuous production of *Nanochloropsis* sp.. Its main advantages includes: combination of large ratio of culture volume to surface area along with the optimized light penetration depth, easy control of temperature and contaminants, effective spatial distribution of fresh air and CO_2, better CO_2 transfer through extensive interface surface between fresh air and culture-liquid medium and novel automated flow-through sensor providing continuous cell concentration monitoring. Henrard et al.[159] evaluated the potential of semi-continuous cultivation of *Cyanobium* sp. in closed tubular bioreactor, combining factors such as blend concentration, renewal rate, and sodium bicarbonate concentration. Cultivation was carried out in vertical tubular PBR for 2 L, in 57 d, at 30 ^{o}C, 3200 Lux, and 12 h light/dark photoperiod. The maximum specific growth rate was observed as 0.127 per day, when the culture had blend concentration of 1.0 g/L, renewal rate of 50%, and sodium bicarbonate concentration of 1.0 g/L. The maximum values of productivity 0.071 g/L/d and number of cycles (10) were observed in blend concentration of 1.0 g/L, renewal rate of 30%, and bicarbonate concentration of 1.0 g/L. The results showed the potential of semi-continuous cultivation of *Cyanobium* sp. in closed tubular bioreactor, combining factors such as blend concentration, renewal rate, and sodium bicarbonate concentration.

The hydrodynamic and mass transfer characteristics of a flat-panel airlift PBR with high light-path are more efficient than those reported elsewhere for tubular and other flat-plate PBR, which opens the possibility of using PBRs with higher light paths than yet proposed[160]. Janssen et al.[152] studied light regime, photosynthetic efficiency, scale-up, and future prospects of enclosed outdoor PBR. In this study it is shown that productivity of PBRs is determined by the light regime inside the bioreactor. In addition to light regime, oxygen accumulation and shear stress limit productivity in certain designs. In short light-path systems, high efficiencies, 10-20% based on photosynthetic activate radiation (PAR 400-700 nm), can be reached at high biomass concentrations [>5 kg/m^3 (dry weight)]. However, it is demonstrated that these and other PBR designs are poorly scalable (maximal unit size 0.1-10 m^3) and applicable for cultivation of monocultures. This is why a new PBR design is proposed in which light capture is physically separated from photoautotrophic cultivation. This system can possibly be scaled to larger unit sizes, 10 to >100 m^3, and the reactor liquid as a whole is mixed and aerated. It is deduced that high photosynthetic efficiencies, 15% on a PAR-basis, can be achieved. Future designs from optical engineers should be used to collect, concentrate, and transport sunlight, followed by redistribution in a large-scale PBR. The research co-operation project between *The Norwegain Institute for Agricultural and Environmental* research in Norway, Uppsala University in Sweden and IIT Kharagpur in India, the BioCO_2 project (2008-2011), has designed, constructed and tested a flat panel, rocking PBR for algae cultivation (non-rocking mode) and hydrogen production (rocking mode). It consists of two glass plates fixed between an inner frame made of stainless steel and outer frames made of aluminum, an air bubbling tube and a tube designed for temperature regulation[161].

2.4. Algae Genetic and Metabolic Engineering

In recent years, new biotechnological approaches relating to genome perturbation of microalgal cells to endow them with different properties are dramatically increasing. However, the full potential of genetic engineering of some microalgal species, particularly diploid diatoms, can be fully realized only if conventional breeding methods become firmly established, thereby allowing useful mutations to be easily combined[112,162]. Significant advances in microalgal genomics have been achieved during the last decade[112,162-165]. Expressed sequence tag databases have been established; nuclear, mitochondrial, and chloroplast genomes of several microalgae strains have been sequenced. Historically, the green algae *Chlamydomonas reinhardtii* has been the focus of molecular and genetic phycological research. Therefore, most of the tools developed for the expression of transgenes and gene knockdown are specific for this kind of species. Current genetic engineering pursuits are towards microalgae that are of greater interest in industrial applications and environmental conservation[162]. To improve microalgal biomass or lipid production and CO_2 capturing efficiency, several approaches have been developed.

Up to now, efforts to increase the lipid content of microalgae have been mainly focused on the optimization of growth and induction conditions, such as temperature, light, salinity and nutrient content/depletion, for instance[165-167], reported genetic modifications of microalgae to alter either lipid quantity or quality (i.e., composition) are still sparse. The main reason is probably lack of a generally applicable transformation protocol for microalgae. Since microalgae are such a diverse group or organisms, it is not guaranteed that a method that works for one species can be applied to another one. For example, some species, such as *D. Salina* without a rigid cell wall, whereas diatoms often have a very rigid silicate shell. This directly affects the method of gene transfer into the cell[168]. Another problem is the limited range of available markers. Although auxotrophy markers are available for some species such as *C. reinhardtii*, stable transformation of other species still has to rely on co-transformed genes conferring resistance to antibiotics. However, some substances routinely used in the transformation of plants, such as kanamycin and hygromycin, are sensitive to increased NaCl concentrations and cannot be used for strains requiring sea water. Also, heterologous gene expression (e.g., the expression of genes not originating from the organisms) in microalgae suffers from the lack of available promoter sequences to control expression, and the possibility of codon usage bias. In summary, any protocol for the genetic transformation of a new microalgal strain (not necessarily a new species) has to be carefully modified to meet and overcome its specific requirements and limitations. Despite the obstacles described above, genetic modification is already one of the main tools to study metabolic pathways in microalgae, and is strongly contributing to our knowledge about their biology. Metabolic engineering by genetic modification is expected to be one of the main steps that will lead to versatile, sustainable and economically viable biofuels from algae[96,111,166-174]. As shown in Fig.10, unicellular algae are capable of synthesizing a range of biofuels. Lipids and carbohydrates represent the main energy storage molecules in algae, and a broad understanding of primary metabolism

is necessary to manipulate electron flux toward these products or H_2 for bioenergy applications. Complicating these efforts are the distinct metabolic processes that occur within algal organelles and the numerous enzyme isoforms present in a cell[111,175-177].

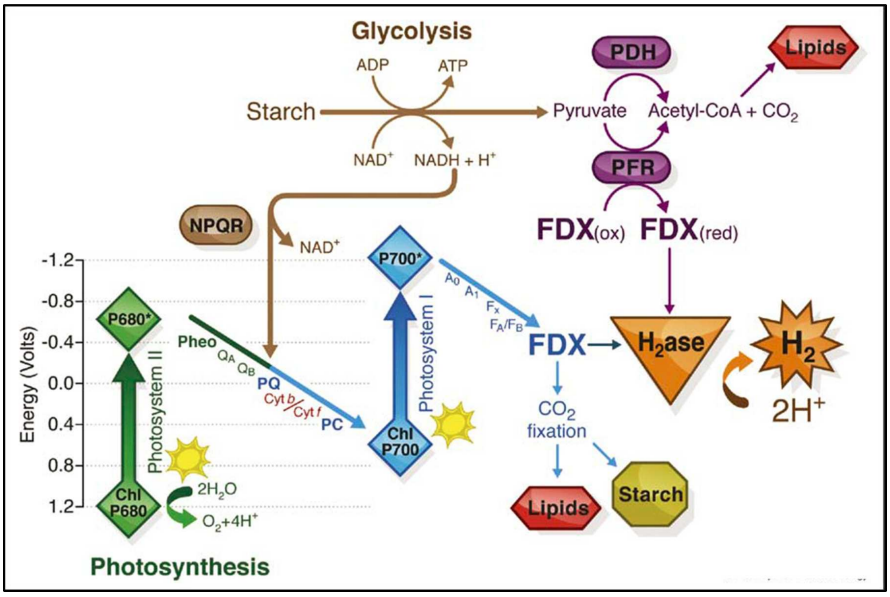

Figure 10. Photosynthetic and glycolytic pathways in green algae related to biofuel and biohydrogen production. Simplified illustration of the pathways used for lipid, starch, and H_2 production in *Chlamydomonas reinhardtii*. [111]

Research interest into microalgal lipid production for biofuels is at an all time high, with a whole range of studies from growth optimizations[178-180] to induced mutagenesis of microalgae to improve lipid yield[169-172]. It can be envisaged that careful strain selection and improvements of microalgae for a variety of useful traits hold a lot of promise, and can be compared with efforts in conventional agricultural crop breeding. Current bottlenecks for large-scale cultivation appear to be in harvesting/extraction processes as well as cheap and energy efficient cultivation systems. Commercial production of biodiesel from algae depends on lipid productivity in industrial scale cultivation systems, production costs, and the energy ratio of production[181]. Against each of three aspects, microalgae lipid production presents a mixed picture. A positive energy balance will require technological advances and highly optimized production systems. The mitigation of environmental impacts, and in particular water management, presents both challenges and opportunities, many of which can only be resolved at the local level. Existing cost estimates need to be improved and this will require empirical data on the performance of systems designed specifically to produce biofuels. At the current time it appears that the sustainable production of biofuels from microalgae requires a leap of faith, but there are nonetheless grounds for optimism. The diversity of algae species is such that it is highly likely that new applications and products will be found. As experience with algal cultivation increases, it may also be found that biofuels have a role to play[182-184].

3. Algae Harvesting and Processing for Biofuels

3.1. Algae Harvesting

A major challenge in downstream processing of microalgae is separating the microalgae from their growth medium, that is, the harvesting process. A high biomass concentration leads to mutual shading of the microalgal cells and thus a reduction in productivity, therefore, biomass concentrations in microalgal cultures are usually low: from 0.5 g/L in open pond reactors to about 5 g/L in PBRs. This means that a large volume of water has to be removed to harvest the biomass. As a result of the small size of the microalgal cells (2-20 μm) and their colloidal stability in suspension, harvesting by means of sedimentation or simple screening is not feasible, except perhaps for larger species such as *Arthrospira*. When microalgae are produced for high-value added products, harvesting is done by centrifugation. Besides, flocculation, electro-coagulation-flocculation and membrane filtration are suggested because of economical reason. However, centrifugation is too expensive and energy-intensive if biomass is to be used for low-value products such as biofuels due to the large volumes of culture medium that need to be processed. Finding an alternative technology that is capable of processing large volumes of culture medium at a minimal cost is essential to reduce the cost and increase the scale of microalgal biomass production[60,185-187].

To realize large-scale production of microalgal biomass for low-value applications, new low-cost technologies are needed to produce and process microalgae requiring the separation of a low amount of culture medium. Flocculation is considered as one of promising low-cost harvesting methods[188]. Methods available for harvesting algae from broth include centrifugation, filtration, flocculation, and gravity sedimentation. The method chosen, to a great extent, depends on the final product and the processes subsequently used: some processes require the algae to be completely dewatered, and others do not[189,190]. The cost and energy demand for harvesting microalgae could be significantly reduced if the cells could be pre-concentrated by flocculation[191,192]. During flocculation, single cells form larger aggregates that can be separated from the medium by simple gravity sedimentation. When flocculation is used for harvesting microalgae, it is part of a two-step harvesting process. Flocculation is used during the first step to concentrate a dilute suspension of 0.5 g/L dry matter 20-100 times to slurry of 10-50 g/L. Further dewatering using a mechanical method such as centrifugation is then required to obtain an algal paste with 25% dry matter content[193]. The energy requirements for this final mechanical dewatering step are acceptable because the particles are relatively large and the volumes of water to be processed small[187]. The economics are very different when flocculation is used for harvesting microalgal biomass than when it is used for removing impurities from a liquid. Also, contamination is a major issue because any chemicals added to induce flocculation end up in the harvested biomass. These chemicals can interfere with the final applications of the biomass (i.e., food or feed) or with further processing of the biomass (e.g., lipid extraction)[186]. Flocculation could be achieved in several ways, which

have been widely explored for microalgae harvesting in recent years. These approaches range from traditional flocculation methods that are widely used in other fields of industry (e.g., chemical flocculation) to novel ideas based on the biology of microalgae (e.g., bioflocculation) and the utilization of emerging technologies (e.g., magnetic nanoparticles utilization)[194].

Chemical flocculation

Metal salts (i.e., alum and ferric chloride) are widely used for flocculation in industries such as water treatment and mining. Metal salts are being utilized for harvesting microalgae (i.e., *Dunaliella*[195]) resulting in high concentrations of metals in the harvested biomass. Then, these metals remain in the biomass residue after extraction of lipids or carotenoids[196]. Furthermore, the metals may interfere with the use of the protein fraction in this residue as animal feed. The valorization of the protein fraction as animal feed is said to be important for making microalgal biofuels economically viable[197]. Despite this shortcoming, metal coagulants provide a good model system to study the interaction between flocculants and microalgal cells because their properties are well understood[198,199]. Other commonly used chemical flocculants in other industries are synthetic polyacrylamide polymers, which may contain traces of toxic acrylamide and also contaminate the microalgae[200]. Therefore, flocculants based on natural biopolymers are a safer alternative. To be able to interact with the negative surface charge of microalgal cells, these biopolymers should be positively charged, which is rare in nature. A well-known positively charged biopolymer is chitosan, which is derived from chitin, a waste product from shellfish production. Chitosan is a very efficient flocculant but it works only at low pH, but pH in microalgal cultures is relatively high[201]. An alternative to chitosan is cationic starch, which is prepared from starch by addition of quaternary ammonium groups. The charge of those quaternary ammonium groups is independent of pH and therefore cationic starch works over a broader pH range than chitosan[202]. Other examples of biopolymers that can be used to flocculate microalgae are poly-γ glutamic acid (an extracellular polymer produced by *Bacillus subtilis*)[203] or polymers present in flour from *Moringa oleifera* seeds[204]. A general problem of polymer flocculants is that they undergo coiling at high ionic strengths and become ineffective. Therefore, they are less suitable for harvesting microalgae cultivated in seawater.

Recently, Rashid et al.[205,206] used chitosan as a flocculant to harvest freshwater microalgae *Chlorella vulgaris*. In chitosan-based microalgae harvesting process, bridging was the primary mechanism of flocculation. Chitosan is one promising choice due to its high molecular weight and charge density. It contains positively charged amino groups (NH^{3+} and NH^{2+}), which have a tendency to adsorb with negatively charged microorganisms, including microalgae[207].When chitosan co-exists with negatively charged algal cells in asolution, electrostatic repulsion between the cells decreases. The decrease in electrostatic repulsion reduces zeta-potential and promotes flocculation[208]. If the chitosan binds partly with microalgae cells, the empty cell surface attaches to another cell, forming a chain like

structure called bridging. At high flocculant concentration, microalgae cells are covered by cationic polymer leaving insufficient empty sites, generating a net positive charge[208]. This positive charge also attaches with surrounding negatively charged cells to make flocs. This phenomenon is called patching. Chitosan holds tremendous potential for high biomass recovery from microalgae culture. Low dose requirement and short settling time are the distinct advantages of chitosan over common flocculants. Microalgal culture can be concentrated up to 10 times at optimal pH (6.0) and flocculant dose (120 mg/L chitosan). Further studies should be carried out to explore the possible ways to reduce the chitosan dose for cost-effective microalgae harvesting. Then, Farid et al.[209] studied nano-chitosan for harvesting microalga *Nannochloropsis* sp. Nano-chitosan showed better biomass recovery. Dosage of chitosan consumption was decreased from 100 to 60 mg/L and biomass recovery increased about 10% by using nano-chitosan. The best initial cell density was 665×10^6 cells/mL for minimum flocculant dosage consumption and minimum cost process. The presence of acetic acid in recycled water from harvesting showed an increase in microalgae growth. Using recycled water increases biomass concentration and at the same time has no treatment cost.

Lee et al.[210-212] also utilized the aminoclays having high density amino sites ($–NH_2$) and water-soluble, transparent, and less ecotoxic effects in aqueous solution[213] for rapid harvesting of freshwater and marine microalgae. The aminoclays placed in the metal (i.e., Fe^{3+}) center were synthesized by sol-gel reaction with 3-amino-propyltriethoxysilane as a precursor, producing $–(CH_2)_3NH_2$ organo-functional pendants, which are covalent-bonding onto cationic metals. The protonated amine groups in aqueous solution lead the efficient sedimentation (harvesting) of microalgal biomass within approximately 5 min and 120 min for fresh and marine species, respectively[210]. Significantly, the aminoclays did not depend on microalgae species or media for microalgae harvesting. In particular, the harvesting efficiency (%) was not decreased in a wide pH region. The harvesting mechanism can be explained by the sweep flocculation of microalgae, which is confirmed by measurement of zeta potential of aminoclay in aqueous solution where aminoclay shows a positively charged surface in a wide pH region. To reduce the cost of aminoclays and simplify the harvesting procedures, the membrane process using aminoclay-coated cotton filter was employed for the treatment of 1 L-scale microalgae stocks. It was successfully performed with three recycles using the same aminoclay-coated cotton filter after removing the harvested microalgae. In conclusion, the aminoclay-based microalgae harvesting systems are a promising means of reducing the cost of downstream processes in microalgae-based biorefinery[210].

Autoflocculation

Flocculation often occurs spontaneously in microalgal cultures when pH increases above 9[214]. This flocculation type is usually referred to as autoflocculation, because it occurs spontaneously in microalgal cultures as a result of a pH increase due to photosynthetic CO_2 depletion. Autoflocculation is associated with the formation of calcium or magnesium

precipitates. Depending on the conditions, these precipitates carry positive surface charges and can induce flocculation through charge neutralization and/or sweeping flocculation. Calcium phosphate precipitates are positively charged when calcium ions are in excess of phosphate ions and interact with the negative surface charge of microalgal cells[215,216]. High phosphate concentrations are required for this type of flocculation to occur. As a result of the declining phosphate reserves and increasing prices of phosphate, flocculation by calcium phosphate precipitation is unsustainable, except perhaps in applications where microalgae are used for wastewater treatment and excess phosphate needs to be removed[217]. Magnesium hydroxide or brucite also precipitates at high pH. These precipitates are positively charged up to pH 12, consequently interacting with the microalgal cell surface to cause flocculation[218,219]. Most waters contain sufficiently high background concentrations of magnesium for this process to occur. Calcium carbonate or calcite also precipitates at high pH, but whether it can induce microalgae flocculation remains to be demonstrated. Flocculation at high pH is caused by formation of inorganic precipitates and not by pH as such, so the harvested biomass contains high concentrations of minerals[220]. Although these have a low toxicity, it is nevertheless preferable to remove them from the algal biomass.

Physical flocculation methods

Biomass contamination would be avoided if it were possible to induce flocculation by applying only physical forces. For instance, microalgae flocculation can be accomplished by applying a field of standing ultrasound waves. Although this method works well in the laboratory, it is difficult to apply on larger scales[221]. In electrocoagulation flocculation, flocculation is induced through electrolytic release of metal ions from a sacrificial anode[222]. The efficiency of this method might be improved by changing the polarity of the electrodes[223]. Similar to flocculation by metal salts, electrocoagulation flocculation results in contamination of the biomass with metals, albeit to a lesser extent than when metal coagulants are directly used. OriginOil claims to have developed a solution for this problem by using only electromagnetic pulses to neutralize the surface charge of microalgal cells and induce flocculation[224].

Recently, several studies have explored the use of magnetic nanoparticles to harvest microalgae. Magnetite (Fe_2O_3) nanoparticles may adsorb directly on the microalgal cells, upon which the cells can be separated from the medium by applying a magnetic field. Thus, this method combines flocculation and separation in a single process step[225,226]. Magnetite nanoparticles seem to adsorb more easily on some microalgal species than on others[227]. Adsorption can be improved by coating the nanoparticles with cationic polymers[228,229]. An advantage of using magnetite nanoparticles for harvesting microalgae is that the nanoparticles can be recovered after harvesting and subsequently reused[225]. Bejor et al.[230] investigated the low cost harvesting of microalgal biomass from water using physical method. Four fabric filters (stretch-cotton, polyester-linen, satin-polyester and silk) were used for microalgae harvesting by filtration method. For the three algae communities with

cell size of 2-20 μm, stretch-cotton filter showed a harvesting efficiency of 66-93%, followed by polyester-linen (54-90%), while satin-polyester and silk fabrics achieved harvesting efficiencies of 43-71% and 27-75%, respectively. The research revealed that for wastewater generation of 1500 m^3/day and algae concentration of 200 mg/L, microalgae harvesting cost per m^2 per kg of algae per m^3 would be ≤ £0.15 using stretch cotton filter. Thus, fabric filters utilized for algae harvesting have been proven to be a cheap and reliable harvesting technique especially in areas where skilled labor is rarely feasible.

Bioflocculation

In natural blooms of microalgae occurring in lakes or rivers, flocculation sometimes occurs spontaneously. This spontaneous flocculation is assumed to be caused by extracellular polymer substances in the medium and is called bioflocculation[231]. Bioflocculation is often successfully used for harvesting microalgae in facilities where microalgae are used in wastewater treatment[232]. The underlying mechanism, however, is poorly understood and deserves further research because it may lead to a chemical-free method for flocculating microalgae. Some microalgal species flocculate more readily than others and such naturally bioflocculating microalgae can be mixed with other species to induce flocculation[233,234]. There are indications that bioflocculation may be initiated by infochemicals[235]. Recently, an infochemical isolated from a senescent and flocculating culture of a *Skeletonema* sp. was found to be capable of inducing flocculation in a culture of another species of microalgae[236].

Bacteria or fungi can also induce bioflocculation of microalgae. Some fungi, for instance, have positively charged hyphae that can interact with the negatively charged microalgal cell surface and cause flocculation[237,238]. Specific consortia of bacteria can also induce flocculation of microalgae[239,240]. These flocculating fungi or bacteria can be cultivated separately or in combination with the microalgae. Cultivating bacteria or fungi in combination with microalgae requires a carbon source in the medium. In wastewater, a carbon source is usually present and this allows cocultivation of microalgae and bacteria. This results in a culture of mixed algal-bacterial flocs that can be easily harvested[241,242]. The use of bacteria or fungi as a flocculating agent avoids chemical contamination of the biomass but results in microbiological contamination, which may also interfere with food or feed applications of the microalgal biomass[243].

The energy intensive of harvesting tiny microalgae cells (1-70 μm) from culture broth can account for at least 20-30% of total costs of algal biomass production. Recently, Zhou et al.[244] developed an alternative fungus pelletization assisted bioflocculation method for harvesting microalgae (*Chlorella vulgaris UMN235*) using pellet-forming fungal strain (*Aspergillus oryzae*) isolated from municipal wastewater sludge. Under heterotrophic growth condition, the key factors including spore inoculums, organic carbon concentration in medium as well as pH variation had significantly positive effects on fungus-algae pellet

formation. The process parameters of 1.2-104 spores/mL, 20 g/L glucose, and pH ranged from 4.0 to 5.0 were found optimal for efficient fungus-algae pellet formation. For autotrophic growth, when pH of culture broth was adjusted to 4.0-5.0 with organic carbon addition (10 g/L glucose), almost 100% harvesting efficiency of microalgae was obtained. Moreover, it was observed that diameter and the concentration of fungus-algae pellets were affected by the shaker rotation. The novel harvesting technology might reduce the microalgae harvesting cost and will have potential to be applied to all types of microalgae species as alternative to other traditional harvesting methods. In addition, Lee et al.[245] proved that bacteria play a profound role in flocculating by increasing the floc size resulting in sedimentation of microalgae. And the collective presence of certain bacteria was the determining factor in flocculation of *C. vulgaris*.

Electro-coagulation-flocculation

Electroflocculation is a process that uses electric currents to dissolve sacrificial metal to supply the ions required for the flocculation. In comparison with auto-, bio- or microbial flocculation, electroflocculation is a physical/chemical process that has the advantages of being non-species specific, simpler to operate and results are more predictable. Unlike chemical flocculation, electroflocculation does not introduce unnecessary anions such as SO_4^{2-} or Cl^- which can result in the lowering of pH[246]. The construction of the electroflocculation cell is also relatively simple; it consists of a container with electrode plates and a direct current power supply, and hence involves modest capital investment. For these reasons, electroflocculation has been selected as a potential harvesting technique for microalgae. Lee *et al*.[247] studied the electroflocculation for marine microalgae harvesting. By combining electroflocculation with mixing and settling, an overall energy consumption of 0.33 MJ/m^3 has been achieved. On a large scale, the mixing can be made energy efficient by the use of a baffled hydraulic mixer. The total cost for the harvesting, including electrical energy, electrode metal dissolution and capital depreciation, is estimated to be \$0.19 kg^{-1} of the ash-free dry mass. Therefore, electroflocculation is more economical than other harvesting techniques for marine microalgae.

Membrane Filtration

Membrane technologies have been used for the removal of bacteria, viruses and other microorganisms[248]. As manufacturing techniques improve and the range of applications expands, the cost of membranes and membrane systems have steadily decreased, which may make it possible to use membrane technology for microalgae harvesting. Most significantly, membrane filtration can achieve complete removal of algae from the culture media[248]. Different membrane filtration technologies have been used for the removal or concentration of microalgae. Zhang et al.[249] evaluated the feasibility of using a cross-flow membrane ultrafiltration (UF) process to harvest and dewater algae suspension, and the microalgae was concentrated 150 times and final algae concentration reached 154.85 g/L. Hung et al.[250] studied how operating parameters affect microfiltration (MF) and examined the effect of preozonation on flux behavior when using hydrophobic and hydrophilic membranes. Zou et al.[251] investigated the effect of physical and chemical parameters on

forward osmosis (FO) fouling during algae separation. In addition, the effect of solute reverse diffusion on FO fouling was systematically studied. Pressure-driven MF and UF membrane processes are prone to fouling and are relatively energy intensive, while the FO membrane process showed a very low permeate flux[252]. Chow et al.[253] compared MF and UF methods and found both techniques attractive for removal of cyanobacterial cells. Rossignol[254] compared MF and UF technologies for continuous filtration of microalgae.It showed that although the pure water fluxes of MF membrane were higher, during separation of microorganisms, fluxes of the UF membrane became higher than MF membrane.

The membrane separation efficiency is greatly affected by fouling. It can be further explained that the microorganisms accumulation on membrane surface or in membrane pores causes decline in permeate flux[254]. Many efforts have been made to understand and reduce fouling, including membrane surface modification and new membrane material development[255]. Conventional polymeric materials membranes have been widely used in filtration and concentration of microalgae[249,256-258]. Rossignol et al.[259] evaluated the performances of inorganic filtration membranes. Liu[260] utilized a thin, porous metal sheet membrane to harvest microalgae, which exhibited high properties of membrane area packing density, chemical/thermal stability, mechanical strength, high permeability and low cost. Sun et al.[261] evaluated several commercial MF and UF membranes for filtration and concentration of *Chlorella* from dilute culture media. The results showed that permeate fluxes increased with the increase in feed solution temperature, and the fluxes were probably limited by released extracellular polymeric substances (EPS) at higher temperatures. Moreover, MF membranes and UF membranes showed similar flux in this work, indicating that pore size and porosity are not important for this application. This suggested that the permeate flux of different membranes is controlled by the fouling layer that acts as the membrane selective layer. The work also demonstrated that a membrane with hydrophilic surface shows very little fouling for algae harvesting.

To reduce fouling formation, Hwang et al.[262] proposed a fatal problem of membrane technology by means of surface-coating with a functional coating material, i.e., hydrophilic polyvinyl alcohol (PVA) polymer. The PVA coating caused the membrane surface to become more hydrophilic and it was confirmed by decreased contact angles up to 64% compared to the unmodified membranes. The surface-coated membrane found to exhibit substantially enhanced performance: a maximum flux increase of 36% and almost 100% recovery rate. It showed that the membrane performance can be improved simply by applying a surface-active coating, even to the level of economic feasibility.

The enhancement of membrane shear-rates has long been recognized as one of the most efficient factors for fouling control. It is implemented either by moving the fluid or the membrane. The membrane can be moved in a circular rotation, a torsional vibration or in

vertical and horizontal oscillation systems[263,264]. Application of a rotating disk system for algal harvesting showed that it almost doubled the membrane productivity compared to a reference cross-flow system, ascribed to the high shear-rates at the liquid-membrane interface[265,266]. However, Ladner et al.[267] found a very significant impact of enhanced shear on the microalgal cells. The algal organic matter released from sheared microalgal cells caused increased membrane pore blocking. This phenomenon was not observed in the other studies[265,266], probably due to different types of microalgae (cell wall), type of pumps, filtration experimental designs (shorter time-frame), etc. Therefore, a process that would maintain a high shear-rate only at the liquid-membrane interface, and not in the whole bulk, would be beneficial to achieve an efficient filtration process. Bilad et al.[268] investigated the effectiveness of submerged microfiltration to harvest both a marine diatom *Phaeodactylum tricornutum* and a *Chlorella vulgaris* in a magnetically induced membrane vibrating (MMV) system. They assessed the filtration performance by conducting the improved flux step method (IFM), fed-batch concentration filtrations and membrane fouling autopsy using two lab-made membranes with different porosity (Fig.11). The full-scale energy consumption was also estimated. Overall results suggested that the MMV offered a good fouling control and the process was proven to be economically attractive. By combining the membrane filtration (15×concentration) with centrifugation to reach a final concentration of 25% w/v, the energy consumption to harvest *P. tricornutum* and *C. vulgaris* was, as low as 0.84 and 0.77 kW·h/m^3, respectively, corresponding to 1.46 and 1.39 kW·h/kg of the harvested biomass.

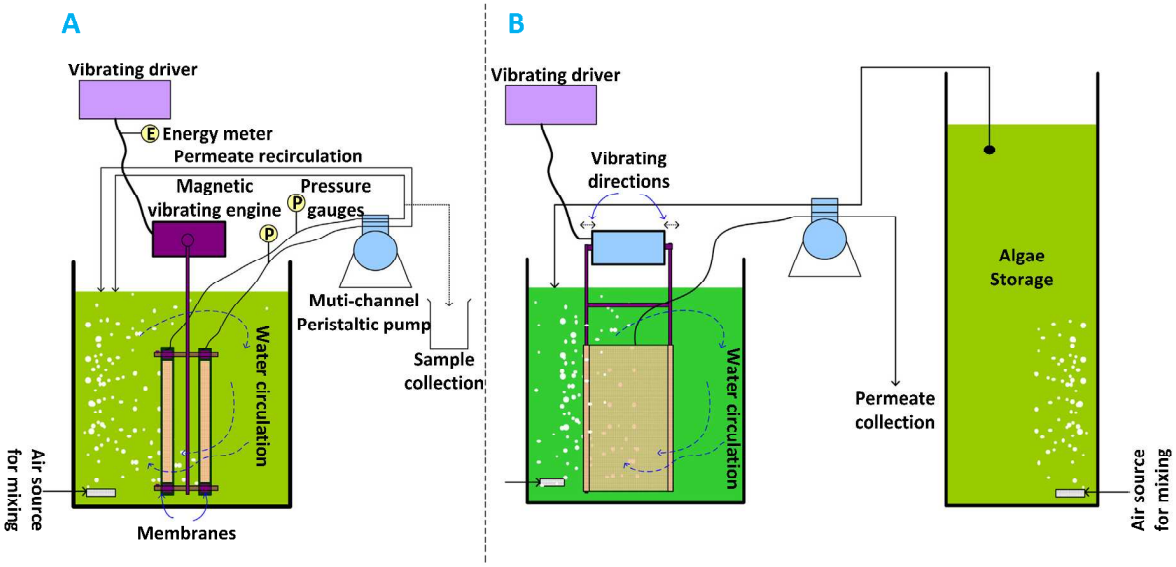

Figure 11. Experimental set-up for (A) the improved flux stepping filtration method (IFM) test in a total permeate recycle filtration mode, also showing the parallel view of the narrow edges of the two vibrating membranes, and (B) the fed-batch concentration filtration showing the set-up in a full surface view of the vibrating membranes [268].

Development of an efficient flocculation technology for microalgae may yield major cost and energy savings in large-scale production. Generally, chemical flocculation could result in contamination of the microalgal biomass, as the use of natural polymers may minimize this problem. Alkaline flocculation promises to be a low-cost flocculation method, but result in contamination of the biomass, albeit with mineral precipitates with low toxicity. Bioflocculation by fungi or bacteria holds a potential feasibility when microalgae production is combined with wastewater treatment, for wastewater can provide the necessary carbon source for the flocculating microorganisms. Physical flocculation has the advantage that it may avoid biomass contamination due to chemicals or microorganisms. Fundamental researches into infochemicals that induce flocculation in microalgae are necessary, because this may contribute to a highly controllable method for inducing flocculation that avoids contamination. The same holds true for approaches to induce flocculation through genetic modification. Further studies should examine the flocculation efficiency under specific conditions, and investigate how flocculation is affected by properties of the microalgal cells or culture conditions, particularly interfered by organic matters in the culture medium. Cost evaluation should not only take the cost of flocculation step itself into account, but also the influence on the entire production process.

3.2. Algae Hydrothermal (HT) Processing

Several processing approaches for biofuels from land-based biomass have been developed and partly commercialized up to now. Nevertheless, algae also contain carbohydrates that could be converted by similar processes. In regards to biomass, not every process is suitable for application in an efficient and economic manner. Therefore, well-known processes have to be checked for algal biomass. Additional to this, microalgae are offering novel pathways of producing biofuels, which have to be taken into account[269]. One of the economic and energetic drawbacks in the processing of microalgae is the dewatering stage, as microalgae typically grow to a solid concentration of 1-5 g/L[60]. The challenges of concentrating and drying result in the energy intensive. Macroalgae can be harvested more easily due to their large size, but the moisture content is still very high compared with terrestrial biomass[270]. Microalgae biofuel is usually produced by the extraction of lipids and subsequent transesterification to biodiesel. Most common lipid extraction techniques require a dry feedstock before transesterification, as do conversion to thermal energy or syngas by combustion or gasification. This can account for as much as 25% of the energy contained in algae[271].

Hydrothermal (HT) processing avoids the step of drying, as algae is treated as slurry in hot-compressed water. Operating conditions depend on the desired product: at low temperatures, less than 200 °C, the process is referred to as HT carbonization (HTC) and predominantly produces a char; at intermediate temperatures of 200-375 °C, the process is known as HT liquefaction (HTL), primarily producing an oil; at the higher end of the temperature range, greater than 375 °C, the process is called HT gasification (HTG), predominantly producing a syngas. These HT processing routes is to generate a product

with higher energy density. The char produced from HTC can be co-fired with coal or used as biochar for soil amendment[272], the biocrude from HTL can be upgraded into a variety of fuels and chemicals, while the syngas from HTG can be used for combustion or converted into hydrocarbons by either biological or catalytic processing, e.g., *Fisher-Tropsch* synthesis. Other than the above mentioned HT processes, there are some additional wet processing methods that have been used for algal biomass, as wet extraction techniques offer a distinct energy requirement advantage. For example, Levine et al. proposed the *in situ* lipid hydrolysis of wet algae followed by the supercritical transesterification with ethanol[273]. Alternatively, Patil et al. have suggested the wet transesterification to fatty acid methyl esters in supercritical methanol[274]. There have also been limited studies on the co-liquefaction of algal biomass with coal or organic solvents to improve the yields and quality of biocrude[275,276]. During the carbonization stage, the carbon content is enhanced and the oxygen and mineral matter contents are decreased, the gaseous product is low and a biochar is produced by carbonization reactions. During liquefaction, biomass is decomposed to smaller molecules, which are reactive and can repolymerize into oily compounds[277-280].

The products from HTL consist of a biocrude fraction, a water fraction containing some polar organic compounds, a gaseous fraction and a solid residue fraction. At the more severe conditions in HTG, the desired product is a syngas, consists of varying amounts of H_2, CO, CO_2, CH_4 and light hydrocarbons. The initial reaction steps are the same as during liquefaction, but the more severe conditions lead to the small fragments decomposing even further to low-molecular weight gaseous compounds. At high temperatures (>500 °C) H_2 production is favored, while CH_4 production is favored at 350-500 °C, although all these conversion pathways can be influenced with the use of catalysts[281-286]. The high ionic product supports acid- or base-catalyzed reactions and can act as an acid or base catalyst precursor due to the relative high concentrations of H_3O^+ and OH^- ions from the self-dissociation of water[283]. The advantage of this method is the additional acid or base catalysts can be avoided. The ions concentration can reach maximum at 275 °C, which is therefore the optimum temperature for acid- or base-catalyzed reactions. Above 350 °C, the ionic product decreases rapidly by five orders of magnitude or more above 500 °C[284]. Between 300 and 450 °C, the density at 30 MPa changes from a liquid-like 750 kg/m^3 to a gas-like 150 kg/m^3; however, there is no phase change occurring. The density change directly associates with the properties such as solvation power, degree of hydrogen bonding, polarity, dielectric strength, diffusivity and viscosity[287].

Chemical reactions in hydrothermal conditions and in supercritical fluids can provide new, potentially cheaper paths to renewable fuels from wet algal biomass[288]. The methods used to make large quantities of liquid fuels from algae involve extracting the oil with an organic solvent such as hexane, and converting the oil into either biodiesel by catalyzed transesterification with alcohol or to green diesel by catalytic hydrotreating. Drying algae prior to extracting takes time, consumes energy, and adds expense. Producing fuel directly

from wet algal biomass could improve the economics and environmental sustainability of algal biofuels. Thus, some alternative ways such as hydrothermal and solvothermal processes have been developed[289]. HT processing is an energy efficient approach favoring of the required reactions[290]. Hot compressed water (e.g., 300 °C, 8.6 MPa) could readily dissolve organic compounds, and its elevated ion product (10^{-11} *versus* 10^{-14} for ambient water) could accelerate acid-catalyzed, hydrolytic decomposing biomacromolecules[290,291]. Algal biomass contains amounts of macromolecular proteins, polysaccharides, and lipids, along with inorganic components. The lipid fraction is usually targeted for fuels, but the protein and polysaccharide fractions also have heating value. Thus, conversion of the whole biomass into fuels can lead to biocrude yields exceeding the lipid content of the algae, whilst a greater partition of the heating value originally resident in the biomass into the final fuel products (Fig.12). HT processing can also facilitate reuse of nitrogen (N) and phosphorus (P) needed for a sustainable processing [289,292]. Herein, Fig.13 illustrates a photobioreactor for microalgae cultivation where nutrients, water, light and CO_2 are the only required inputs. A similar concept could be described for open pond cultivation or for macroalgae, where the cultivation layout could include growth in either closed tanks or in marine environments. More importantly, some dewatering is still required, when the algal biomass is treated by the HT processing. Low-cost dewatering has more challenges for microalgae than macroalgae, but many processes are available, such as flocculation described above[289].

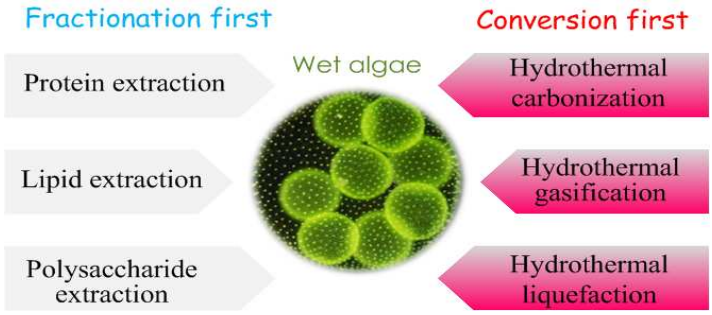

Figure 12. Hydrothermal and supercritical fluid processing approaches for transformation of wet algal biomass into fuels and other products fractionate the biomass first or process the entire biomass first.

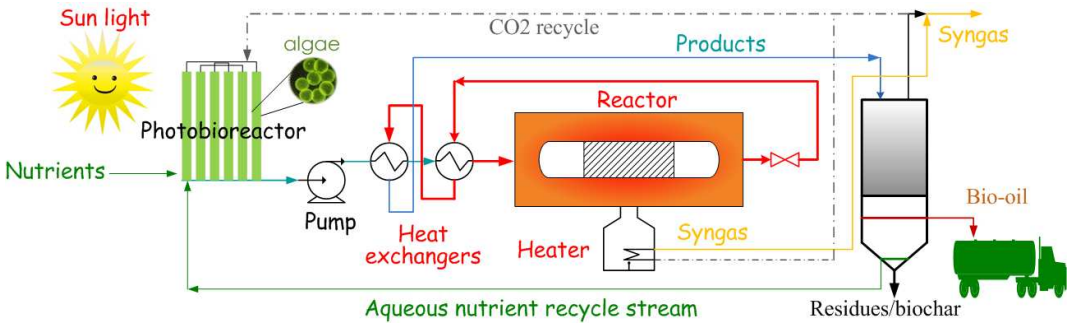

Figure 13. Integrated hydrothermal process with nutrient and CO_2 recycling for algae photosynthesis.

In summary, culturing microalgae for biofuels production could be combined with wastewater treatment to minimize heavy dependency on inorganic nutrients source. Apart from that, incorporation of baffled system in open pond and closed-photobioreactor is recommended to enhance mixing intensity between microalgae, nutrient sources and CO_2 while reducing the energy input. Also, effective harvesting and drying of microalgal biomass can be easily achieved through immobilization technology; however, extensive research is still required to strengthen this visionary strategy. For the downstream processing, lipid extraction from microalgae presents a complicated task as well. Physical extraction method which is suitable to extract oil from crops is not efficient in extracting lipid from microalgae, since the lipid is embedded within a layer of cell wall. Cell disruption method such as chemical or thermal extraction is necessary to recover the lipid effectively. However, some of the cell disruption methods require large quantity of energy input that could lead to negative energy balance. In addition, it is noteworthy that the chose of cell disruption methods, chemical solvents and extraction conditions are significantly relied on microalgae strains. In other words, no single method can give optimum lipid extraction for all types of microalgae strains. Several breakthrough technologies such as supercritical extraction/transesterification, in-situ transesterification, hydrothermal processing and transesterification assisted with ultrasonication or microwave are yet to be discovered to enhance microalgae biocrude production. Moreover, biodiesel derived from microalgae still would ideally be the main product. Additionally, diversified biofuels (i.e., biohydrogen, bioethanol) production from microalgae is necessary to improve the overall energy balance. For instance, the microalgal biomass after lipid extraction can be recycled for bioethanol production, since high concentration of carbohydrates remain in the biomass. Other potential biofuels derived from the microalgal biomass residue are, such as bio-oil from pyrolysis or hydrothermal process. This is a win-win strategy in recycling the waste to produce another source of energy which greatly amplifies the sustainability of microalgae biofuels. Nevertheless, bioethanol and bio-oil production from microalgae is still at the infancy stage and the real potential is yet to be completely discovered. In the next part, the main biofuels such as biohydrogen, biodiesel, and bioethanol derived from algal biomass will be presented in details..

3.3. Biohydrogen

Uniquely among organisms of oxygenic photosynthesis, many green microalgae encode for genes of hydrogen metabolism, including two [Fe-Fe] hydrogenases[293,294], and genes encoding proteins that are required for the [Fe-Fe] hydrogenase assembly[295,296]. Hydrogen metabolism-related proteins in green microalgae are localized and function in the chloroplast, such that the [Fe-Fe] hydrogenase can receive high potential energy electrons directly from reduced ferredoxin (*Fd*) at the end of the photosynthetic electron transport chain (Fig.14A). Since the green microalgal H_2 metabolism discovered by Hans Gaffron et al. in the early 1940s[297-299], it escaped no-one's attention that green microalgae can serve as the photosynthetic producers of H_2, essentially derived from sunlight and H_2O[299,300]. A measure of green microalgal hydrogen production is offered upon consideration of existing

approximately 3×10^6 photosynthetic electron transport chains per green algal cell[301-303], each capable of transporting 100 electrons per second. Theoretically, a 1 L culture containing 10×10^6 cells per mL could produce hydrogen 200 mL/h. In practice, anoxic conditions are a strict requirement for the expression and activity of the H_2 production machinery in the green microalgal cell. Oxygen, produced at the H_2O-oxidation site of the photosynthetic apparatus (Fig.14A), is a potent inhibitor of the [Fe-Fe] hydrogenase and a positive suppressor of H_2-related gene expression, blocking the transcription of all genes associated with hydrogen metabolism. This may be seen as nature's provision of a powerful and effective mechanism that prevents the co-production of hydrogen and oxygen from the photosynthetic apparatus. Thus, upon turning on illumination of a dark-adapted anoxic green microalgal culture, hydrogen production has been observed to last for as long as 90 seconds, before the oxygen fully inhibits hydrogen production[304].

The conundrum of O_2 inhibiting H_2 production could not be solved in 70 years of related research[305]. However, an experimental approach was designed and applied to bypass the O_2 problem in 2000. Continuous photosynthetic H_2 production was sustained for several days, achieved upon a regulated slow down of O_2 evolution in the green algae *Chlamydomonas reinhardtii*[306,307]. This breakthrough successfully employed the cell's own respiration to consume the photosynthetically generated O_2[298,308], in a process where internal starch reserves were used to sustain the cells' respiration[309]. Fig.14B depicting the coupling of the cellular chloroplast photosynthesis with mitochondrial respiration to explain how anoxic conditions can be maintained in the cell permitting expression of the HydA hydrogenase, and enabling sustained hydrogen metabolism in the chloroplast. Initially, balancing of photosynthesis and respiration was achieved upon sulfur-deprivation of the algae[307], a condition that lowered the level of photosynthesis to just below that of respiration, resulting in an anoxic environment that supported hydrogen production. Maintenance of anoxia by the cell's own respiration has already become the platform of green algal H_2 production in the field, and is currently employed by many labs in several countries, as a vehicle by which to further explore the properties and premise of green microalgal H_2 production[310-312]. Fig.14B also depicts in the mechanism of the process of H_2 production, which depends on the availability of starch or endogenous substrate to help sustain cellular respiration for the consumption of photosynthetic O_2. In wild type microalgae, starch reserves can suffice to sustain hydrogen production for about 4-5 days. When starch reserves are consumed, cells need to go back to normal photosynthesis, where biomass accumulation and O_2 evolution would take place. The latter is necessary and sufficient to replenish endogenous substrate and otherwise to rejuvenate the microalgae, so that the stage of H_2 production can be repeated. Experimental results from such cycling of the 'stages' are shown in Fig.14C, where alternating O_2 and H_2 production could be sustained ad infinitum[308]. Furthermore, the critical role of endogenous substrate in maintaining anoxia in the cells was demonstrated with mutants of *Chlamydomonas reinhardtii* that over-accumulated starch. These were able to sustain H_2 production for about twice as long, and reach yields about twice as high, compared to those measured with wild type strains[313]. In the laboratory, sequestration and quantification of hydrogen

can be achieved upon collection of H_2 in upside-down graduated cylinders or burettes by the method of water displacement (Fig.14D). The method of H_2 storage by the displacement of water in glass containers satisfies the requirement of easy H_2 sequestration and a subsequent easy retrieval for use. However, the method is not practical for large-scale commercial exploitation, where substantial amounts of hydrogen must be reversibly stored-and-retrieved without a significant energetic expenditure[314]. To date, there are no simple storage alternatives, especially when considering H_2 as a fuel for the transportation sector. A main barrier is the requirement of high capacity storage and on-demand retrieval, in a reversible process where the energetic requirements of storing-and-retrieving are low. Different approaches have been investigated, including hydrogen liquefaction[315,316], compression up to 5000 psi[317,318], storage in metal hydrides[319-323], boron-nitrogen (B-N) based hydrides[324-331] and adsorption (physisorption) in porous materials, notably carbon nanotubes[332,333]. Current problems associated with these approaches include a combination of low capacity, high cost, high energetic requirement, and safety. Alternative methods of storing H_2 in N_2 (conversion to NH_3), CO_2 (conversion to CH_4 or CH_3OH) have also been proposed. Energetic and economic feasibility of the latter has not been established as yet. Difficulties in hydrogen storage are an impediment in transportation, distribution, and on-board storage, all of which raise questions as to the present-day practicality of renewable hydrogen in industrial and automotive applications.

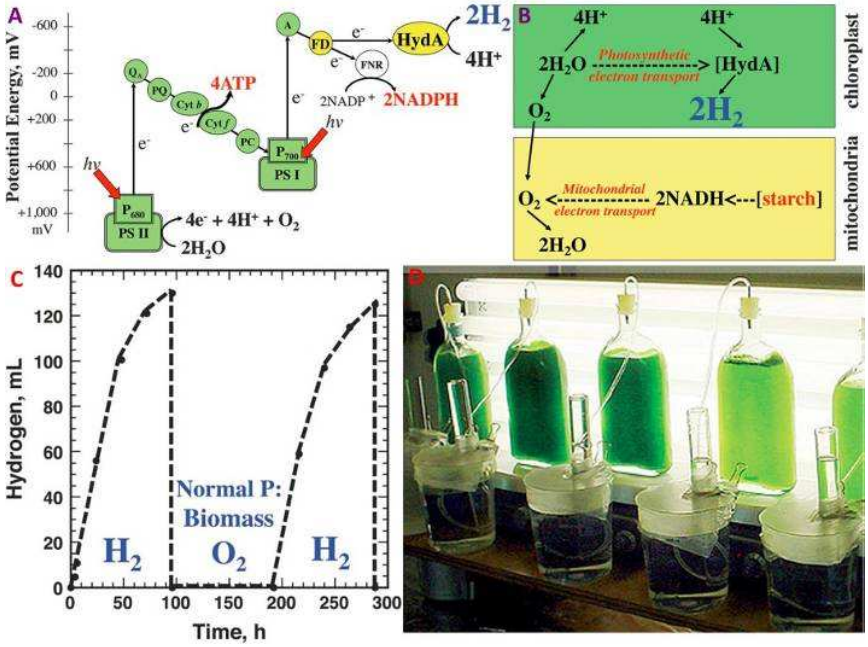

Figure 14. (A) Linked H_2O oxidation and H_2 production in the photosynthetic apparatus of green microalgae; (B) Coordinated photosynthetic and respiratory electron transport that leads to anoxia (absence of oxygen) and H_2 production in green microalgae; (C) Cycling of a green microalgal culture between the stages of H_2 production and normal photosynthesis (Normal P); (D) Light-driven green microalgal H_2 production, sequestration, and quantification measurements conducted in the laboratory [47].

Biohydrogen Production Pathways

While the pathways of biohydrogen production are noticeably different in algae and *cyanobacteria*, both organisms share a fundamental commonality that hydrogen is a secondary metabolite produced to balance the organisms' redox energetics. In general, in photosynthetic organisms, the hydrogen yield is appreciably higher when photosynthesis ceases and the stored sugar (or other carbohydrates) is catabolized. Under illuminated and anaerobic conditions, certain algal species also evolve hydrogen, but with a much lower yield, to facilitate a basal level of metabolism through the photosynthetic production of ATP; On the other hand, some *cyanobacterial* species evolve hydrogen as a byproduct of nitrogen fixation mediated by nitrogenase.

In green algae, there are three pathways including two light-dependent pathways, and possibly one light-independent fermentative pathway for hydrogen evolution mediated by either [Fe]- or [Fe-Fe]-hydrogenases, both of which are unidirectional[334,335]. In all three algal pathways, the reduced *Fd* acts as a key station to supply electrons to the hydrogenase via the irreversible reaction: $2H^+ + 2e^- \rightarrow H_2$. Given that the first two pathways are light-dependent, the electron transport chain is used to shuttle electrons (gained through the oxidation of various compounds) for the reduction of *Fd*. In the first pathway, water is the source of electrons and is photosynthetically oxidized via the catalytic activity of PSII. In the second pathway, however, electrons are gained through the catabolism of endogenous carbohydrate stores (i.e., the glycolysis pathway and citric acid cycle) or other organic macromolecules such as lipids. The catabolism of these compounds generates NAD(P)H molecules, which are subsequently oxidized by NADP-PQ oxidoreductase (NPQR) to liberate electrons (in addition to protons and $NAD(P)^+$). The electrons are fed to the electron transport chain medially at the level of plastoquinone (PQ). Finally, the analysis of algal cultures placed under dark anoxic conditions has revealed a putative third pathway for hydrogen evolution. Under dark anoxia, algae degrade its endogenous starch reservoirs to sustain a basal level of metabolism, generating fermentative end products such as formate, acetate, ethanol, and possibly hydrogen. Since the electron transport chain is inactive during dark periods, pyruvate provides the electrons to reduce *Fd*, a step mediated by pyruvate ferredoxin oxidoreductase (PFR1) (Fig.16)[335,336].

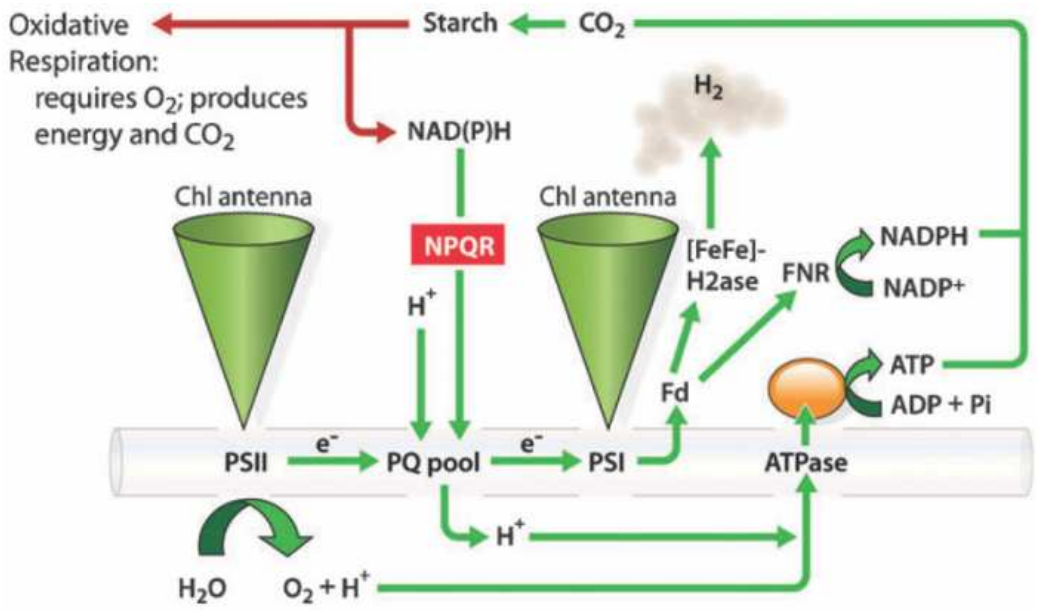

Figure 16.. Hydrogenase-catalyzed H_2-photoproduction pathways in green algae [335]

Since hydrogenases are the most active molecular catalysts for hydrogen production and uptake[337,338], and could therefore facilitate the development of new types of fuel cell[339-341]. In [Fe-Fe]-hydrogenases (i.e., HydA1), catalysis takes place at a unique di-iron centre (the [2Fe] subsite), which contains a bridging dithiolate ligand, three CO ligands and two CN^- ligands[342,343]. Through a complex multi-enzymatic biosynthetic process, this [2Fe] subsite is first assembled on a maturation enzyme (i.e., HydF), and then delivered to the apo-hydrogenase for activation[344]. Synthetic chemistry has been used to prepare remarkably similar mimics of that subsite1, but it has failed to reproduce the natural enzymatic activities thus far. Berggren et al.[345] proved that three synthetic mimics (containing different bridging dithiolate ligands) can be loaded onto bacterial HydF (*Thermotoga maritime)*, and then transferred to apo-HydA1 (one of the hydrogenases of *Chlamydomonas reinhardtii* algae). Full activation of HydA1 was achieved only when using the HydF hybrid protein containing the mimic with an aza dithiolate bridge, confirming the presence of this ligand in the active site of native [Fe-Fe]-hydrogenases [346,347]. This is an example of controlled metalloenzyme activation using the combination of a specific protein scaffold and active-site synthetic analogues. This simple methodology provided both new mechanistic and structural insight into hydrogenase maturation and a unique tool for producing recombinant wild-type and variant [Fe-Fe]-hydrogenases, with no requirement for the complete maturation machinery. Because this procedure has been shown to work with proteins (HydF from *Thermotoga maritima* and HydA1 from *Chlamydomonas reinhardtii*) from two completely different organisms, it is very likely that [Fe-Fe]-hydrogenases from other microorganisms, overexpressed in their apo formin *E. coli*, which lacks the maturation machinery, could also be activated through simple reaction with 2-HydF. Thus, this reaction could be used for exploring a large variety of [Fe-Fe]-hydrogenases, for instance, from different species or derived from directed

mutagenesis-with the aim of finding the most active and stable enzymes for exploitation in biotechnological processes of H_2 production[348] as well as in bioelectrodes in (photo)electrolysers or fuel cells[339-341].

The *cyanobacteria* also have three different hydrogen evolution pathways, which are different from algal pathways due to two different [Ni-Fe]-hydrogenases (bidirectional [Ni-Fe]-hydrogenases and uptake [Ni-Fe]-hydrogenases), and a [Mo-Fe]-nitrogenase found exclusively in nitrogen-fixing *cyanobacteria*[349]. Two of them use water and organic compounds, respectively, as the electron donor, releasing electrons that are supplied to bidirectional [Ni-Fe]-hydrogenase for hydrogen evolution. However, the organic compounds (i.e., glycogen) that are catabolized for hydrogen production are formed through CO_2 fixation with the electrons supplied by the splitting water. In this case, water is the indirect electron donor for hydrogen evolution. Owing to the bidirectional nature of the *cyanobacterial* [Ni-Fe]-hydrogenases, hydrogen can be either produced or consumed via the reversible reaction: $2H^+ + 2e^- \rightarrow H_2$. Bidirectional [Ni-Fe]-hydrogenases are thought to be associated with the cytoplasmic membrane and accept electrons from both NAD(P)H and H_2 (Fig.16).

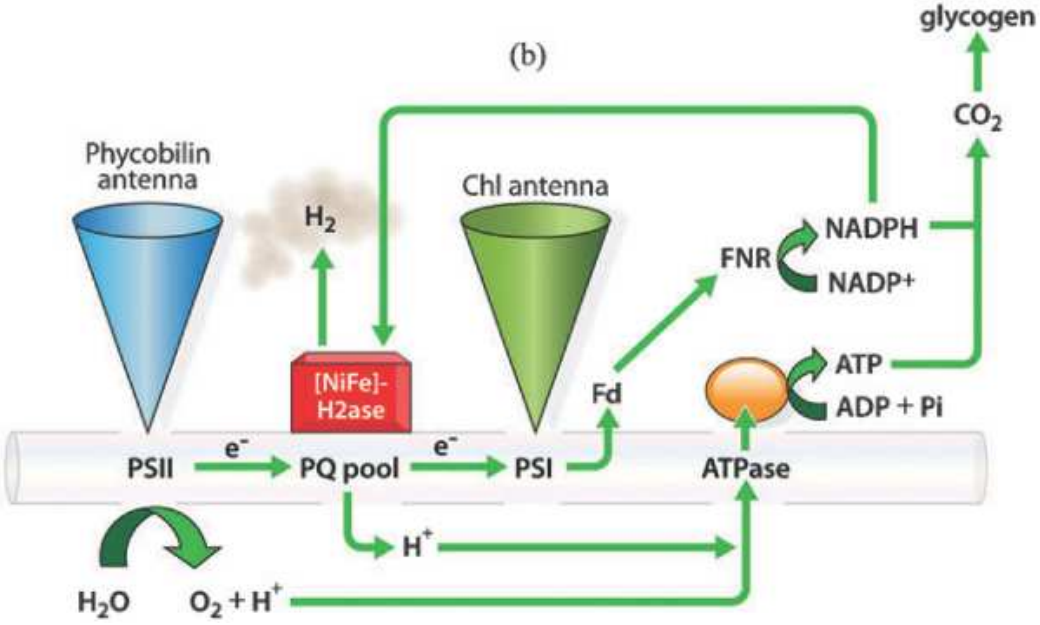

Figure 16. Hydrogenase-catalyzed H_2-photoproduction pathways in *cyanobacteria* [335]

Studies from a small number of *cyanobacterial* mutant stains suggests that the hydrogen evolution pathway mediated by bidirectional [Ni-Fe]-hydrogenase is possibly coupled to the photosynthetic electron transport chain. This putative pathway is different from the algal pathway, as it does not solely rely on reduced *Fd* as an electron donor. Electrons could be shuttled directly to bidirectional [Ni-Fe]-hydrogenase at the level of NPQR (located near the photosynthetic membrane, between PSII and PQ). And electrons that are

not diverted via NPQR continue along the electron transport chain through various electron acceptor intermediates (i.e., PQ, $Cytb_6f$, PC, and PSI) for the *Fd* reduction. While the majority of electrons gained by *Fd* are siphoned into other more essential assimilatory pathways (e.g., CO_2 fixation), a small number of them are relayed back to NPQR through cyclic electron flow. At the onset of dark anoxia when the electron transport chain is nonfunctional, the second hydrogen production pathway can become active. This pathway is the most widely accepted hydrogen production pathway for *cyanobacteria*, and it is analogous to the aforementioned putative fermentation pathway for hydrogen production in green algae; where NAD(P)H generated through the catabolism of endogenous glycogen stores is oxidized by NPQR to yield the electrons required for hydrogen evolution[350]. The third hydrogen production pathway is found only in nitrogen-fixing *cyanobacteria*, in which nitrogenase fixes atmospheric nitrogen to form ammonia and hydrogen: $N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$ [335,351]. However, this pathway is energetically expensive since 2 ATP molecules are required for every electron being transferred. The electrons and ATP molecules fed to nitrogenase are obtained from either the electron transport chain associated with photosynthesis or the catabolism of carbohydrates. The electrons gained from these oxidations are first relayed to NPQR or ferredoxin/NAD(P)H oxidoreductase (FNR). NPQR will donate these electrons to the electron transport chain at the level of PQ, whilst FNR can ferry directly the electrons to nitrogenase. Furthermore, the spent reducing power for hydrogen evolution during nitrogen fixation can be regained by hydrogen consumption via uptake [Ni-Fe]-hydrogenase. The electrons gained from hydrogen uptake are recycled back into the photosynthetic electron transport chain via the PQ pool and can be used by cytochrome c oxidase (cyt. c) for the reduction of O_2 to water (i.e., Mehler reaction) or transferred back to nitrogenase via PSI and a heterocyst-specific *Fd* [350,352].

Biohydrogen production by microalgae is considered as the most favorable pathway[353]. Microalgae split water into proton (H^+) and oxygen (O_2) in the presence of light. The process can converted H^+ into hydrogen via hydrognease, called direct-photolysis[354]. However, the hydrogen production in this process is low because of two main reasons that H_2 and O_2 are produced concomitantly, mixing and reacting into H_2O immediately, and hydrognease itself is sensitive to oxygen[355,356]. This inhibitory effect can be fixed by adopting indirect bio-photolysis, consisting of two stages of stage-I and stage-II called aerobic and anaerobic stage, respectively. In stage-I, the cells do photosynthesis to accumulate organic compounds (mostly glucose) and oxygen is evolved. In stage-II, the cells degrade stored organic compounds under anaerobic condition[357]. In two-stage process, oxygen (in stage-I) and hydrogen (in stage II) are evolved separately. Stage-II can be under light condition called, photo-fermentation, or without light named dark fermentation[358]. Fig.17 illustrates the concept of two-staged hydrogen production by microalgae. Several factors affect the hydrogen yield in stage-I and stage-II. Healthy grown cells in stage-I produce hydrogen efficiently. The microalgae growth in stage-I is controlled by different parameters like, light, nutrients, carbon source, temperature, pH, and bioreactor design. These parameters are equally important in stage-II also.

Immobilization and sulfur deprivation are the key intermediate steps of stage-I and stage-II. For immobilization, the cells are suspended in a solidifying material and cut into small pieces. Immobilized cells are easy to handle, have high stability and produce more hydrogen than free cells. Sulfur deprived (S-deprived) cells yield more hydrogen than sulfur-provided cells. In the presence of sulfur, the cell synthesizes protein which suppresses the hydrogen production[359].

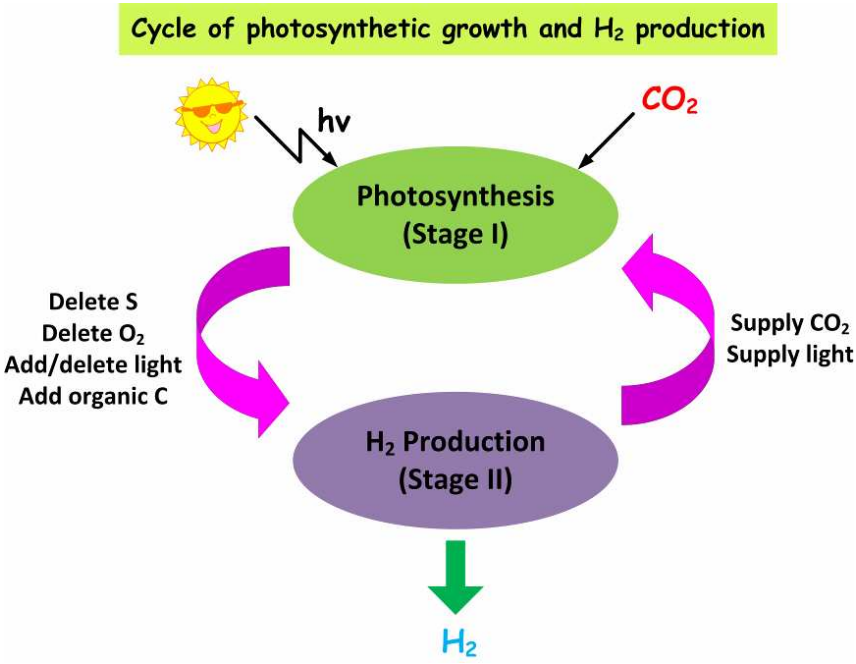

Figure 17. Concept of two-staged biohydrogen production by microalgae

Life-cycle Assessment of Biohydrogen

Although the outcomes of biohydrogen from photosynthetic microorganisms (i.e., microalgae) are still small, different studies are carried out to increase the production yield and optimize the process to lessen the negative impact on the environment and climate change. Biohydrogen production has been produced continuously at laboratory scale[360], while a commercial-scale production is expected in the very near future. Given the expected market penetration of hydrogen technologies and the fact that the relative environmental impacts of biohydrogen production systems have not been scientifically established to date, there is a need for a reliable life cycle assessment (LCA) of environmental impacts associated with biohydrogen production systems or technologies[361]. LCA can give the possibility to compare different biohydrogen production approaches using different photosynthesis methods and, at the same time, identify the environmental 'hot spots' of the whole process. Romagnoli et al.[362] provided a starting point for a quantitative LCA approach to assess the environmental impacts of a scale-up photobiological hydrogen production process[363,364]. In light of a cyclic hydrogen production process *Chlamydomonas reinhardtii* has been developed by researchers at the NREL and the University of California-Berkeley[365,366]. *C.* reinhardtii cells are grown in a

stirred tank reactor with light in a medium containing a low level of sulfur, then transferred into an anaerobic medium in a second stirred tank. The results of the analysis show that using biohydrogen to produce electricity offers more environmental benefits than using a fossil fuel based source. The analysis provided a quantification of the avoided CO_2 emissions from fossil based fuel if a cycling photobiological hydrogen production from green algae (i.e., *C. reinhardtii*) with forced sulfur deprivation is used instead. This amount can be attested on a maximum level around 25.5 tCO_2 per year if coal is the replaced energy source for electricity production. At this stage, the positive result of LCA can be clearly seen in term of the climate change and human health categories[362].

To determine the energy consumption and CO_2 emissions, Ferreira et al.[367] presented a life cycle inventory of biohydrogen production by *Clostridium butyricum* through the fermentation of the whole *Scenedesmus obliquus,* which was accomplished through the fermentation of the microalgal biomass cultivated in an outdoor raceway pond, and the preparation of the inoculum and culture media. The scale-up scenarios are discussed aiming for a potential application to a fuel cell hybrid taxi fleet. The H_2 yield obtained was 7.3 g H_2/kg of *S. obliquus* dried biomass. A total energy consumption of 88 (71-100) MJ/MJ_{H2} and 5776 (5119-6268) gCO_2/MJ_{H2} emissions was obtained, which is considerably high and unsustainable if pilot/industrial scale is envisaged. The stage of microalgae culture required the highest energy consumption (55 MJ/MJ_{H2}) and emitted the maximum CO_2 (3605 gCO_2/MJ_{H2}), respectively, and contributing with 62.4% of the energy consumption in the overall process. When CO_2 absorption is considered, the microalgae culture becomes responsible for 41.1% of the overall CO_2 emissions, with 1516 gCO_2/MJ_{H2}. Other studies and production technologies were taken into account to discuss an eventual process scale-up. Increased production rates of microalgal biomass and biohydrogen are necessary to become competitive with conventional production pathways.

Biohydrogen Production in a Bio-refinery Concept

To facilitate successful targeted mutagenesis in the future, bioengineering approaches will have to expand the identification of bottlenecks of the hydrogen production metabolism and the key factors controlling it. Consequently, both phylogenetic and system biological approaches are being established to model biochemical pathways of H_2 production in more detail and elucidate the essential regulatory networks involved in H_2 production[363-366,368-372]. In particular genomic, transcriptomic, proteomic, and metabolomic data are being combined to develop reliable metabolic flux models to identify energy, H^+ and e^- sources and sinks. It still has to become established whether the subsequent elimination of identified bottlenecks using targeted molecular engineering approaches will be successful[373,374]. From the current state of the art, however, it is likely that the best biohydrogen production capacities will be achieved with the application of genetic manipulation.

Recent comprehensive evaluation studies on the feasibility of algal biofuel production, performed by the *Solar Biofuels Consortium*, concluded that a diversification into various co-products is an important part for the development of a standalone microalgal biofuels industry [117]. Consequently, new biorefinery concepts are needed to combine hydrogen with the production of other biofuels such as biogas (methane), oils (i.e., biodiesel), and the separation of valuable co-products. Such biorefinery concepts can be designed with the aim of achieving CO_2 neutral systems in which CO_2 and nutrients are recycled (Fig.18). Since H_2 is a volatile product that can be readily collected from the culture, hydrogen can be considered an excellent component of such new bio-refinery concepts[311].

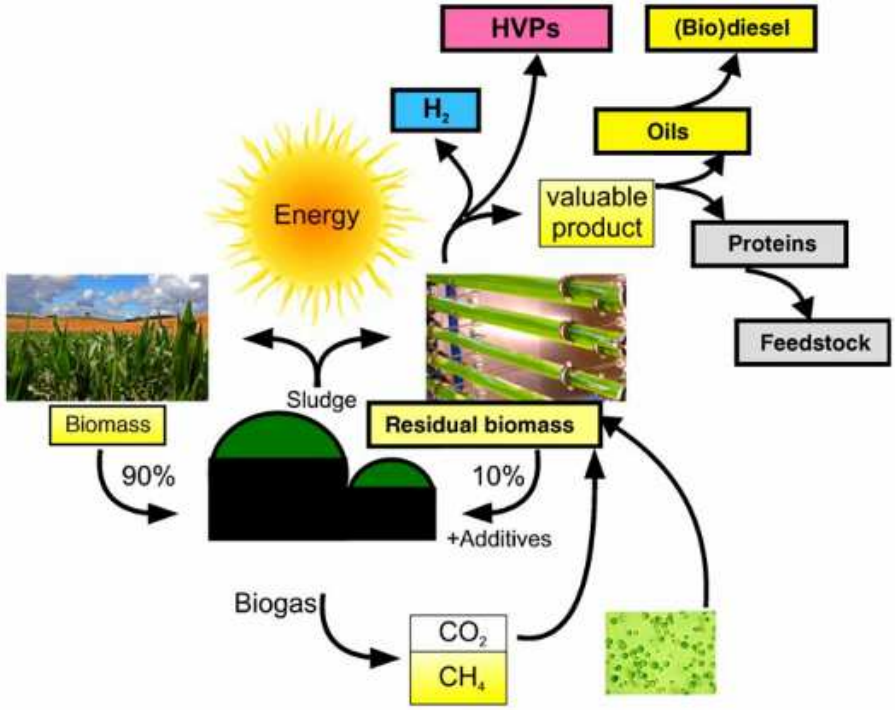

Figure 18. Concept of a biorefinery system for bio-energy and bio-products in algae. Bio-products include bio-energy products such as hydrogen, oils for bio-diesel, sugars for bio-ethanol and biomass for bio-methane, intermediate value products such as proteins for animal feedstocks, and high value products (HVPs) for example for pharmaceutical purposes. CO_2 and nutrients released during the fermentation of residual biomass during the production of bio-methane will be recycled. Biomass can also be pyrolyzed to produce 'sequestered carbon' in the form of biochar, which has value as a soil enhancer [311].

Biohydrogen Application on Fuel Cell

Hydrogenases are abundant enzymes that catalyze the reversible interconversion of H_2 into protons and electrons at high rates. Those hydrogenases maintaining their activity in the presence of O_2 are considered to be central to H_2-based technologies, such as enzymatic fuel cells and for light-driven H_2 production. Among three phylogenetically distinct types of hydrogenases, two enzyme classes prevail in nature. According to the metal content of

their active sites, they are classified as nickel-iron ([Ni-Fe]) and di-iron ([Fe-Fe]) hydrogenases. [Fe-Fe]-hydrogenases are highly productive in H_2 evolution, but are irreversibly inactivated during catalysis by even trace amounts of O_2. However, [Ni-Fe]-hydrogenases function usually in the direction of H_2 oxidation and are less sensitive to O_2. Oxygen tolerance implies that, upon approaching the catalytic center, O_2 has to be removed reductively through an immediate delivery of four electrons and protons for the complete reduction of O_2 to water. Because the oxidized active site is blocked and cannot bind H_2, electrons must be delivered by reverse electron flow.

High-yield biohydrogen production in combination with photosynthesis will require an oxygen-tolerant hydrogenase (i.e., [Fe-only]-hydrogenase). This could be achieved by the intelligent combination of random mutagenesis, site-directed mutagenesis and directed evolution, which has already been applied successfully to improve other enzymes[375]. For instance, the existing oxygen-tolerant hydrogenases of *Ralstonia eutropha* with its identified maturation apparatus[376] are a valuable starting point. And most recent strategies in this field are summarized[377]. If succeed, the future scenario for a designed organism with engineered biophotolytical hydrogen production might be similar to the model (Fig.19)[378]. Future energy balances for such systems should consider the following parameters: (1) the progress in energy transformation efficiency that can be obtained hopefully using designed organisms with improved hydrogenases; (2) the development of the high energy content algal biomass, the low-cost fermenters and media; (3) decreasing the doubling time of algal culture; (4) the option to use sunlight instead of artificial light (indoor systems would also be possible using fiber optics). The environmental benefits derived from zero-CO_2 emission and the increasing costs of gasoline and natural gas should eventually make the natural system, which still has potential for improvement, more competitive.

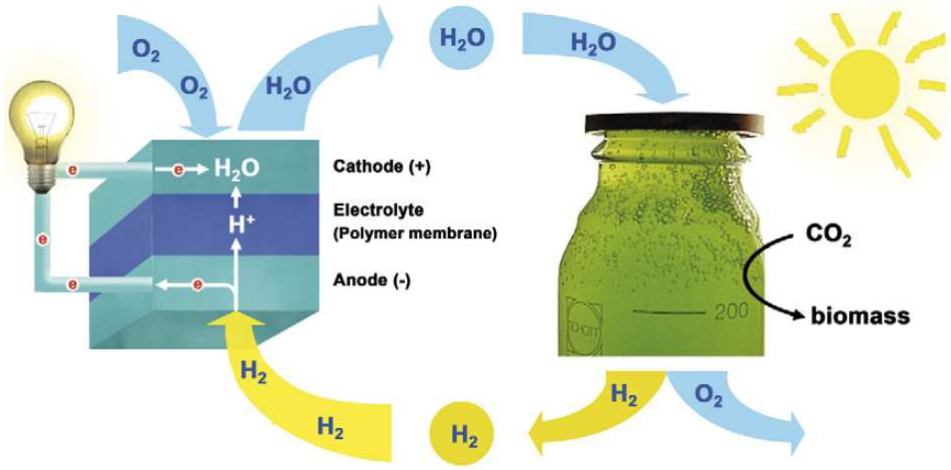

Figure 19. The circuit of water and hydrogen in a system consisting of hydrogen-producing microalgae and a fuel cell that transforms hydrogen into electrical energy [378].

As for biohydrogen applications, it is mentioned above that biohydrogen produced from microalgae could be widely used for hydrogen-oxygen fuel cells driving the fuel cell vehicles (FCVs). It is not only environmental friendly and highly energy-efficient, but can also be produced using a variety of readily available raw materials. Thanks to these characteristics, FCVs are ideal for achieving sustainable mobility. Therefore, many automobile manufactures have tried their best to make this vehicle technology widely available as soon as possible as shown in Fig.20. Some significant components, such as hydrogen, oxygen, catalysts, membrane, circuit, have attracted more attention and need to be developed for designing the superior performance FCVs.

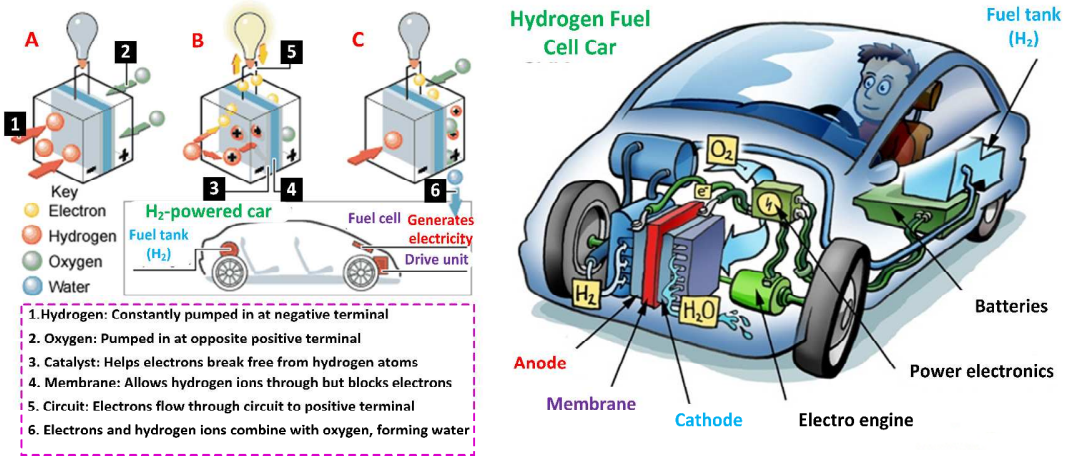

Figure 20. Schematic diagram of biohydrogen fuel cell vehicle

Bottlenecks and Prospective

In summary, biohydrogen production process generally faces two bottlenecks of low hydrogen yield in dark and high energy cost in case of photo-fermentation. The dark fermentation process yields only 4 mol of hydrogen per mole of glucose, whereas photo-fermentation produces 12 mol of hydrogen per mole of glucose. However, photo-fermentation requires external source of light energy. The researchers have proposed two-stage processes by integration dark fermentation with photo-fermentation (Fig.21). In dark-photo fermentation model 4 mol of hydrogen can be produced under dark and rest of the byproducts can be oxidized by photosynthetic bacteria to produce hydrogen. Another approach to degrade acetate (an intermediate product) is to use acetate containing biomass in microbial fuel cell (MFC) to produce 8 mol of hydrogen. Produced proton at cathode by fermentative bacteria will be reduced at cathode to produce hydrogen[379]. Logan et al. developed an electricity generation approach using fuel cell microbial, via acetate containing biomass[380]. This novel MFC referred as bio-chemically assisted microbial reactor has potential to generate pure hydrogen at the cathode. Domestic wastewater could be used as substrate. This way efficient and sustainable hydrogen production using microalgae is possible[380]. Another approach is to produce methane from these byproducts than hydrogen, but the output efficiency is not explored yet. Wastewater treatment by the use of microalgae has been studied long before; however, the application is not

commercialized yet. A wide variety of microalgal species are able to grow in wastewaters. The main difficulty to grow microalgae in wastewater is the presence of high concentration of ammonia inhibiting microalgae growth. Furthermore, it is required to determine whether or not this process is truly sustainable and carbon neutral in terms of the utilization[381-383].

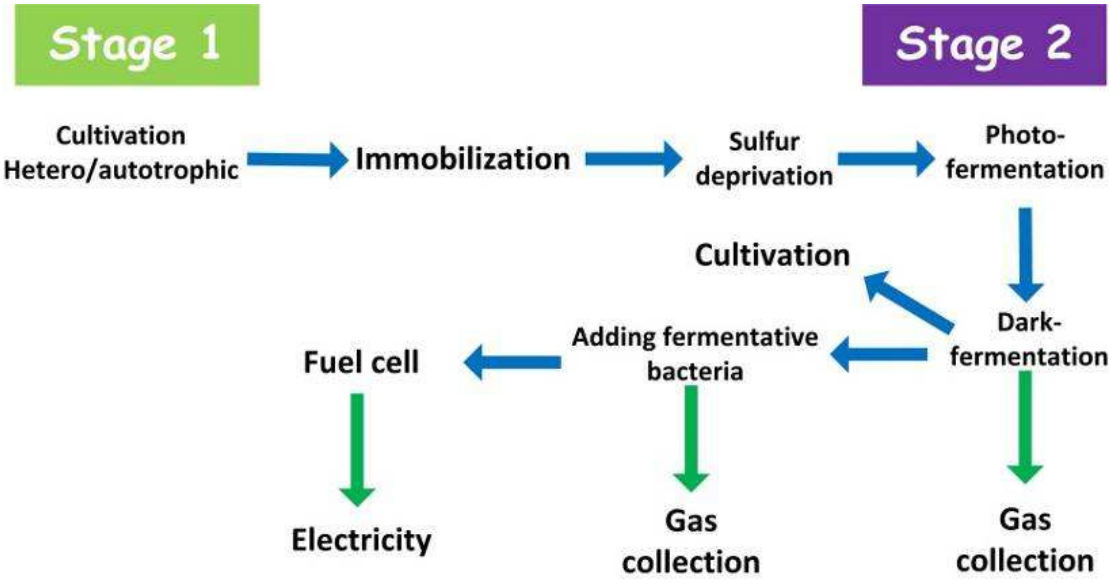

Figure 21. A new concept for enhanced hydrogen production and electricity generation by microalgae

Biohydrogen is usually produced via dark fermentation, which generates CO_2 emissions and produces soluble metabolites (e.g., volatile fatty acids) with high chemical oxygen demand (COD) as the by-products requiring further treatments. Liu et al.[384] successfully demonstrated the feasibility of a novel integration of dark fermentation and mixotrophic microalgae culture, allowing efficient biohydrogen production with minimal CO_2 emissions and no COD discharge by circulating the byproducts of dark fermentation and biomass from microalgae culture. The results showed that the production rate of H_2 was 205 mL/L/h with only 5 mL/L/h of CO_2 emission when this integrated system was performed. The microalgae-based COD removal of dark fermentation effluent was the most efficient when *C. vulgaris* was grown at a food to microorganism (F/M) ratio of 4.5 and a light intensity of 150 mmol/m^2/s. The addition of CO_2 for mixotrophic microalgae growth would improve overall microalgal biomass production performance but led to a decrease in butyrate consumption efficiency due to competition of the organic and inorganic carbon sources. Meanwhile, Kumar et al.[385] proved the pretreated algal biomass of 10 g/L with 2% (v/v) HCl-heat was found most suitable for hydrogen production yielding 9 ± 2 mol H_2 (kg COD reduced)$^{-1}$ and was found fitting with modified *Gompertz* equation. Furthermore, hydrogen energy recovery in dark fermentation was significantly enhanced compared to earlier report of hydrogen production by biophotolysis of algae.

To enhance the efficiency of H_2 production from pretreated feedstock, the optimization of

the pretreatment method and hydrolysis conditions may be required[386,387]. Yun et al.[386] optimized the individual pretreatments (acid and ultrasonic) and a combination of these pretreatments to enhance the efficiency of dark fermentative hydrogen production (DFHP) from microalgal biomass. It showed that the maximum H_2 production performance of 42.1 mL H_2/g dry cell weight (dcw) was predicted at 0.79% (v/w) HCl and at a specific energy input of 49,600 kJ/kg dcw in the combined pretreatment, while it was limited in both individual pretreatments. Besides, the combined pretreatment conditions for DFHP from microalgal biomass were successfully optimized by increasing the solubilization of the feedstock and by reducing the formation of the toxic 5-hydroxymethylfurfural (HMF).

Recently, Xia et al.[388] investigated for the first time the thermodynamic comparison in dark fermentation between amino acids and reducing sugars released from *Nannochloropsis oceanica*. A three-stage method comprising dark fermentation, photo fermentation and methanogenesis[388,389] was proposed to improve hydrogen and energy yields from *N. oceanica*. The total utilization efficiencies of amino acids and reducing sugars are both about 95% in dark fermentation. But the consumption time of most amino acids is about 2 times as long as that of most reducing sugars in dark fermentation. Overall, the maximum hydrogen yield of 183.9 mL/g-total volatile solids (TVS) and the methane yield of 161.3 mL/g-TVS are achieved from *N. oceanica* biomass through the three-stage method. The total energy yield of hydrogen and methane from microalgae biomass through the three-stage method is 1.7 and 1.3 times higher than those through the two-stage (dark fermentation and methanogenesis) and single-stage (methanogenesis) methods, respectively. During the stages of hydrogen production there are energy demands, mainly of electricity, and associated CO_2 emissions. Fig.22 shows the microalgal biomass production and whole fermentation process and corresponding inputs. The main stages considered were the microalgal biomass production, the fermentation medium preparation, which included BM1 preparation and hydrolysis of microalgal biomass, degasification and fermentation.

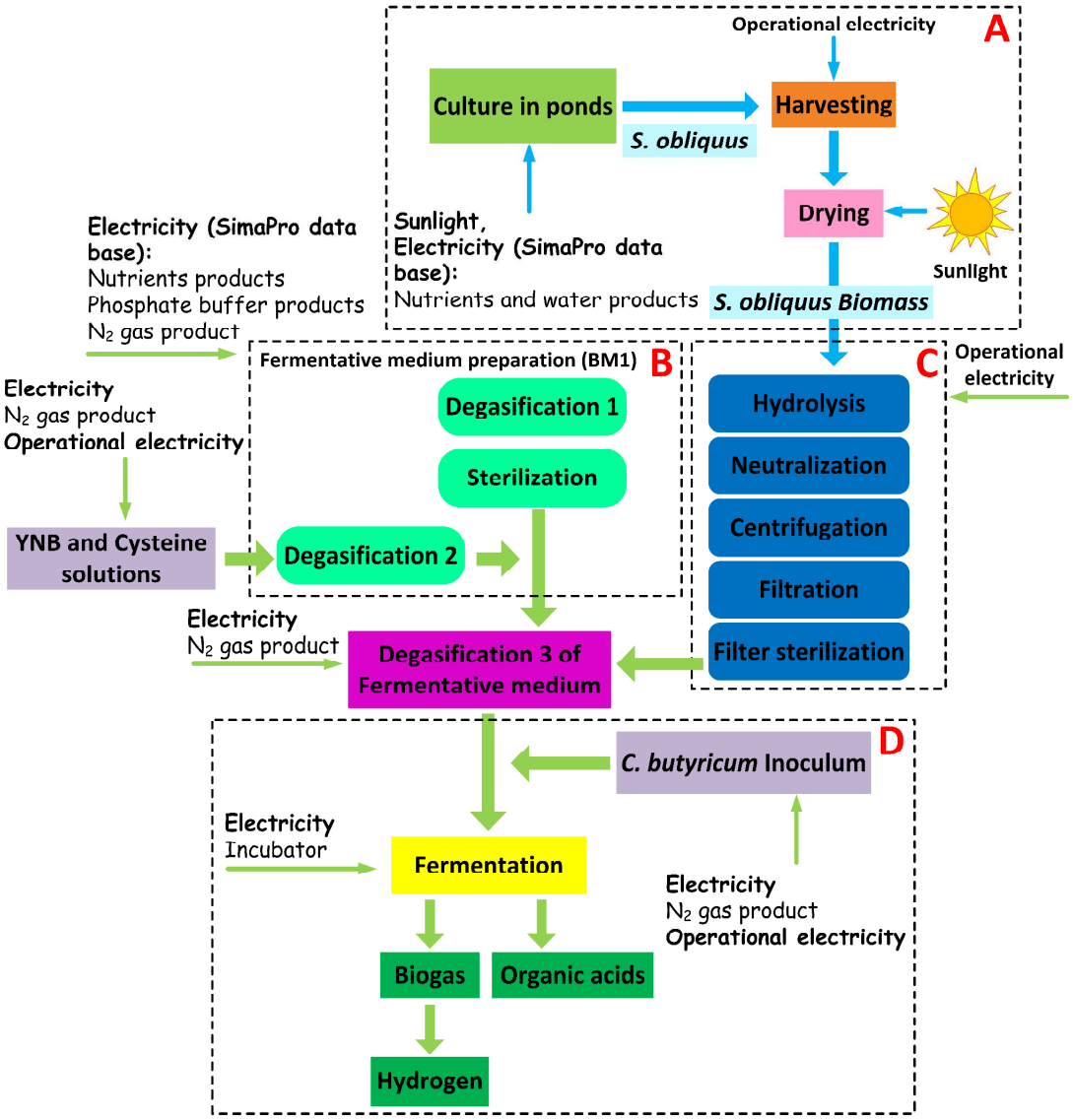

Figure 22. Scheme of the experimental stages of biomass production and the whole fermentation process and corresponding imputs/outputs: (A) *Scenedesmus obliquus* biomass production, (B) BM1 preparation, (C) Biomass hydrolysis and (D) Fermentation

Ferreira et al.[390] presented the life cycle inventory of hydrogen production by *Clostridium butyricum* fermentation of *Scenedesmus obliquus* hydrolysate to evaluate the potential of H_2 production from microalgae and the respective energy consumption and CO_2 emissions in the bioconversion process considering the microalga production, acid hydrolysis of *S. obliquus*, preparation of the inoculum and culture media, and fermentation. In this work, the H_2 yield was 2.9±0.3 mol H_2/mol sugars in *S. obliquus* hydrolysate. Results showed that this process of biological production of hydrogen can achieve 7270 MJ/MJ_{H2} of energy consumption and 670 kg CO_2/MJ_{H2}. The microalgal culture was the stage responsible for

98% of these total final values due to the use of artificial lighting. All stages and processes with the highest values of energy consumption and CO_2 emissions were identified for future energetic and environmental optimization.

In order to decrease the energy consumption and associated CO_2 emissions, the experimental procedure must be optimized aiming at processing a larger amount of biomass to be able to achieve production at an industrial scale. With the present results, it is possible to identify the most critical steps of the whole fermentation process that can be optimized in terms of energy saving and CO_2 emissions reduction. In this study the microalgae production was made indoor with artificial light. A possible solution to reduce energy consumption and CO_2 emissions in the experimental hydrogen production is to replace artificial light, used for the microalgal growth by sunlight with much less electricity consumption. The dryness process could also be done by wind or solar energy especially since this study is conducted in a country with good climatic conditions. Therefore, it would be possible to reduce the values obtained of 308-441 MJ/MJ_{H2} and 28.5-36.3 kg CO_2/MJ_{H2}, by reducing 98.5% of the total electricity used. In addition, other possible scenarios could include the substitution of the "degasification 1" (Fig.22) by a unique step of degasification of BM1 medium, rendering a 0.13% electricity saving. Moreover, the use of the whole acid-treated *S. obliquus* as carbon substrate would avoid the steps of centrifugation and filtration for the solid-liquid separation, resulting in a further decrease of 0.1% in the electricity consumption. With all these possibilities it would be possible to reduce the final energy consumption and CO_2 emissions by 98.7%. Fig.23 shows the scheme of the optimized microalgal biomass production and the whole fermentation process. Advanced techniques such as electrocoagulation for microalgae culture harvesting, dewatering of microalgal biomass in solar ovens and wind tunnels, the use of hybrid fermentation systems and recombinant microorganisms should also be considered for further process improvement.

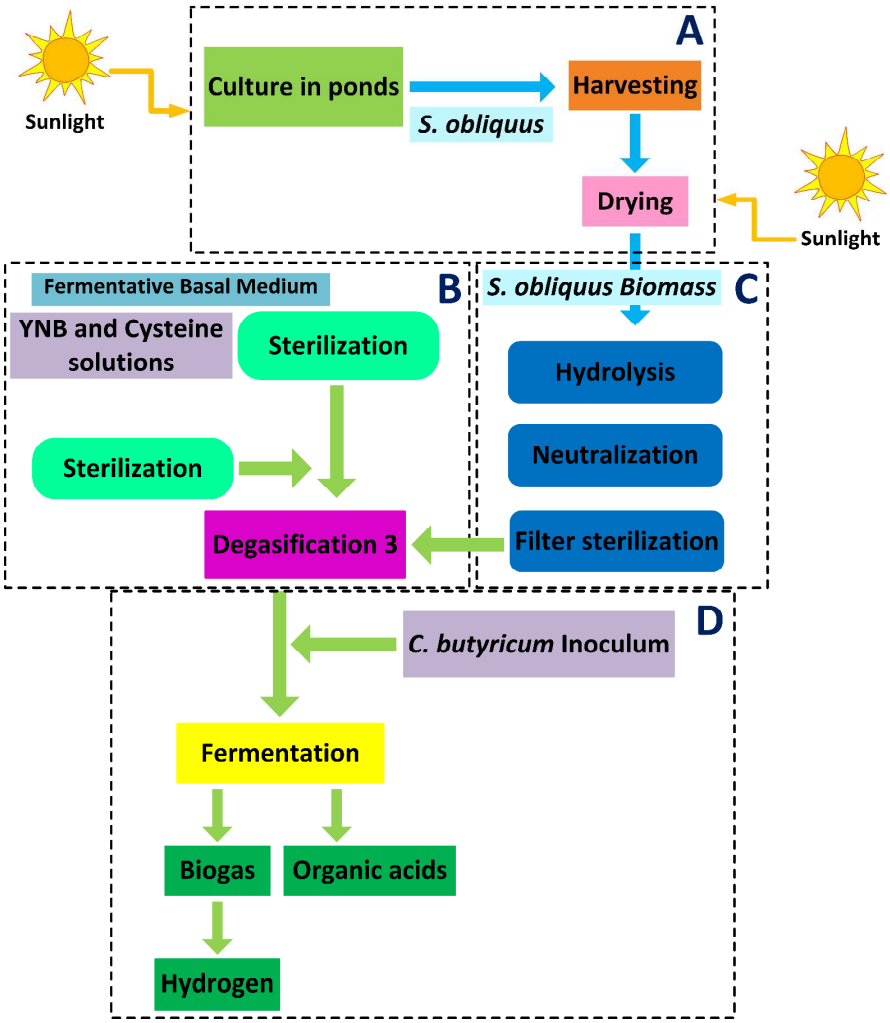

Figure 23. Scheme of the optimized of biomass production and the whole fermentation process and corresponding inputs/outputs: (A) *Scenedesmus obliquus* biomass production, (B) BM1 preparation, (C) Biomass hydrolysis and (D) Fermentation

Metabolic engineering is also a tool to bring a major breakthrough in biohydrogen process. By exploring the pathway of hydrogen production using molecular biology, this technique can eliminate bottlenecks, and increase carbon flow to hydrogen-producing pathway. It can also be favor to increase the substrate utilization by engineering more efficient and oxygen resistant hydrogen evolving enzymes[391]. The *C. reinhardtii* genome sequence showed several unexpected pathways, such as inorganic carbon fixation, fermentation, and vitamin biosynthesis[391-393]. Each of them can be exploited to improve the biohydrogen yield. Exploring nutrients limitation and substrate utilization can benefit to discover particular chromosomal genes in microalgae for hydrogen production enhancement[391]. Random and direct mutagenesis has succeeded in improving tolerance by 10-fold. One approach to address this problem is gene shuffling, which has been used to generate a diverse recombinant hydrogenase library to screen for enhanced O_2 tolerance and stability[391,394].

Algal hydrogenase (HydA) is in charge of catalyzing the reaction: $2H^+ + 2e^- \leftrightarrow H_2$ but usually inhibited by O_2, a byproduct of photosynthesis. Therefore, Lin et al.[395] studied to knockdown PsbO, a subunit concerned with O_2 evolution, so that it would lead to HydA induction. The green alga (*Chlorella* sp. DT) was then transformed with short interference RNA antisense-*psbO* (*siRNA-psbO*) fragments. The algal mutants were selected by checking for the existence of *siRNA-psbO* fragments in their genomes and the low amount of PsbO proteins. The HydA transcription and expression were observed in the PsbO-knockdown mutants. Under semi-aerobic condition, PsbO-knockdown mutants could photobiologically produce H_2 which increased by as much as 10-fold in comparison to the wild type.

A new strategy has been introduced to search natural diversity through the use of degenerate polymerase chain reaction (PCR) primers[391]. Developments are required for optimum design of PBRs. Another critical issue is to find a cheaper carbon source that could produce hydrogen efficiently. To address the economy of this process, the shortening of the total time of hydrogen production should be on top priority. The use of optical fiber is a striking approach to decrease the lag time for hydrogen production. Biohydrogen is still more expensive than other fuels. Thus, if technology improvements succeed in bringing down the costs, it can attain considerable attention as a sustainable biofuel. The optimization of key experimental factors, genetic modification, and metabolic engineering of microalgae are the ultimate approaches to make hydrogen production cost-effective and sustainable. Catabolism of glycogen stored by *cyanobacteria* occurs during anaerobic auto-fermentation and produces a range of C1-C3 fermentation products and hydrogen *via* hydrogenase. Kenchappa et al.[396] investigated both augmenting and rerouting this carbon catabolism by means of engineering the glycolysis pathway at the NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH-1), its major regulation site at the nexus of two pathways [e.g., oxidative pentose phosphate (OPP) pathway and glycolysis/gluconeogenesis] (Fig.24). Null (*gap1::aphII*) and overexpression (*gap1*$^+$ strains of *Synechococcus* sp. strain were constructed in order to produce more NADPH (*via* rerouting carbon through OPP) and more NADH (*via* opening the glycolytic bottleneck), respectively. For *gap1::aphII* quantitative analyses after four-days dark auto-fermentation showed undiminished glycogen catabolism rate, significant increases of intracellular metabolites in both OPP and upper-glycolysis, decrease in lower-glycolysis intermediates, 5.7-fold increase in NADPH pool, 2.3-fold increase in hydrogen and 1.25-fold increase in CO_2 *vs.* wild type (WT). These changes demonstrate the expected outcome of redirection of carbon catabolism through the OPP pathway with significant stimulation of OPP product yields. The *gap1*$^+$ strain exhibits a large 17% increase in accumulation of glycogen during the prior photoautotrophic growth stage (gluconeogenesis), in parallel with a 2-fold increase in the total [NAD^+ + NADH] pool, foreshadowing an increased catabolic capacity. Indeed, the rate of glycogen catabolism during subsequent dark auto-fermentation increased significantly (58%) *vs.* WT, resulting in increases in both NADH (4.0-fold) and NADPH (2.9-fold) pools, and terminal fermentation products, hydrogen (3.0-fold) D-lactate (2.3-fold) and acetate (1.4-fold). The overall energy conversion yield over four

days from catabolized glycogen to hydrogen increased from 0.6 mole of hydrogen mole^{-1} of glucose (WT) to 1.4 (*gap1::aphII*) and 1.1 (*gap1*$^{+}$) under headspace accumulation conditions (without hydrogen milking). It has demonstrated that metabolic engineering has a significant potential for redirecting carbon pathways on carbohydrate catabolism and hydrogen production in *cyanobacteria.*

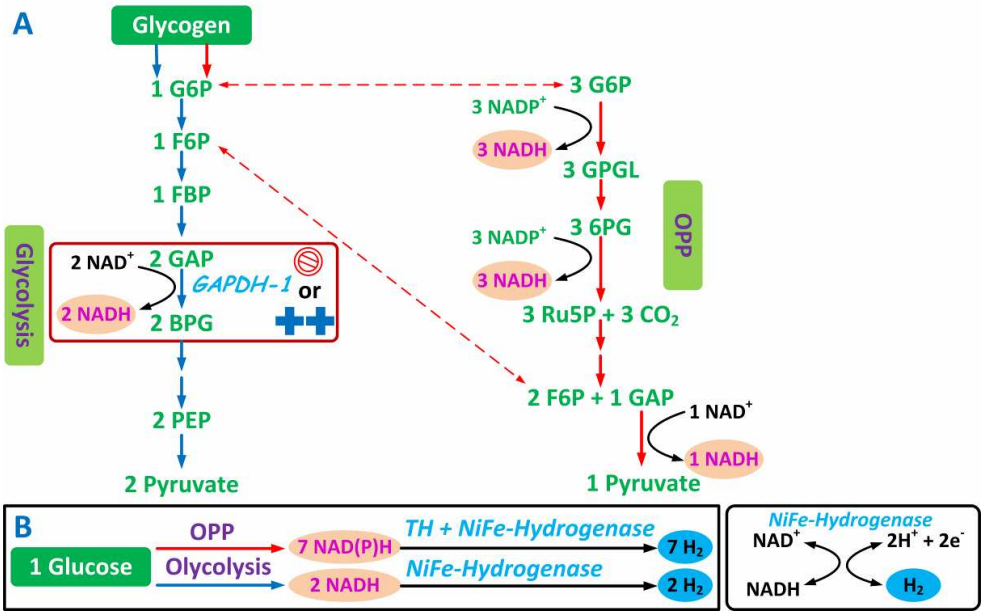

Figure 24. (A) Schematic representation of theoretical yields of NAD(P)H by glycolysis and oxidative pentose phosphate (OPP) pathways; (B) Possible yields of hydrogen per mole of glucose *via* glycolysis and OPP pathways. *Metabolites:* G6P=Glucose-6-phosphate; F6P=Fructose-6-phosphate; FBP=Fructose-1,6-bisphosphate; GAP=Glyceraldehyde-3-phosphate; BPG=1,3-bisphosphoglycerate; PEP=Phosphoenolpyruvate; 6PG=6-phosphogluconate; Ru5P= Ribulose-5-phosphate. *Enzymes:* GAPDH-1=NAD^{+}-dependent glyceraldehyde-3-phosphate dehydrogenase; TH=Transhydrogenase.

3.4. Biodiesel

First generation biofuels derived from agricultural edible crop oils accounting for more than 95% of biodiesel sources, have a great impact on food security and have the potential to increase the cost of food crops (i.e., soybean, corn) resulting in biodiesel production more expensive[397]. Second generation biofuels (i.e., jatropha oil, waste cooking oil and animal fats) do not affect food security and have significant advantages over first generation oil crops, but they are unsustainable. Moreover, production of crop-derived biofuels brings on new challenges. For example, poor cold flow properties and saturated fatty acids contained in animal fats may cause production difficulties and constitute a bio-safety hazard owing to their solid nature at room temperature[398]. In terms of social and economic acceptability and greater energy security, microalgal oil is regarded as third generation biofuels source. Algal can produce twenty times that of oilseed crops on a per hectare basis, so it is a more viable alternative[97,116,399,400]. Microalgae have faster growth rates than plants and are capable of growth in highly saline waters, which are unsuitable for

agriculture. They utilize a large fraction of solar energy making them effective solar to chemical energy converters[401,402]. Microalgae have greater photosynthetic efficiency than terrestrial plants and require very little simple nutrients supply for growth[400]. Normally, a dry cellular weight basis lipid content of microalgae generally varies between 20% and 40%, while lipid contents as high as 85% have been reported for certain microalgal strains[403-405]. The triglycerides productivity of microalgae could be 25-220 times higher than terrestrial plants[401], which can be readily converted to biodiesel by the transesterification process[404,406]. As compared to biomass from trees and crops, microalgal oil is more economical in that transportation costs are relatively low[398]. Algae-to-energy systems can be either net energy positive or negative depending on the specific combination of cultivation and conversion processes used. Conversion pathways involving direct combustion for bioelectricity production generally outperformed systems involving anaerobic digestion and biodiesel production[407]. Therefore, microalgae offer significant higher yield advantage as potential feedstock for biodiesel production[58,408-410]. Tang et al.[411] examined the influence of light, CO_2 concentration, and photoperiod on the growth of the *D. tertiolecata*. Moreover, the results indicated that white and red LEDs, and fluorescent lights all are good light sources and a higher light intensity can significantly improve the cell growth. CO_2 levels of 2-6% in air provided the highest growth rates. Continuous lighting also significantly increased the biomass productivity of *D. tertiolecta*. Differences in light source and intensity had no significant effect on the content and composition of fatty acid methyl esters (FAME, the components of biodiesel) from *D. tertiolecta* oil. Finally, a high content of C18:3 of *D. tertiolecta* biodiesel may lead to poor oxidative stability. However, the high growth rate and ability of these microalgae to grow in a brackish environment lead to it being a good candidate for biofuel production via other pathways.

Extraction of Algal Oil

Currently, algal oil extraction is a prevalent research topic because this process is one of the more costly features that can determine the sustainability of algae-based biodiesel. The process basis is that the algae are first grown, and then removed from the culture medium by some means. Ideally it is not necessary to dry the algae before extracting the oil, which is a real saving in terms of both energy and cost. Tried and trusted methods used to extract oil from oilseeds are adapted to doing the same job on algae, which are expellerypress, solvent oil extraction and supercritical fluid extraction. These and some other less familiar procedures are outlined below. The most direct process involves a simple mechanical crushing and pressing of the dried algae. However, different strains of algae exhibit appreciable differences in their physical properties and so the used particular press configurations (screw, expeller, piston, etc) are chosen to yield maximum effectiveness according to which strain exactly is being processed. Cost is paramount in this as in all alternative energy strategies, and it is reckoned that, in rough numbers, that for extracting oil from microalgae might be in the region of $1.80/kg (compared to $0.50/kg for palm oil)[412].

The first step for any biodiesel technology involves oil extraction from the biomass source. This step is relatively well-established for edible feedstocks and more troublesome for waste oils (the presence of water and free fatty acid impurities) and algae (lack of efficient methodologies for oil extraction). Vegetable oils, which are rich in triglycerides (TGs), are subsequently treated with methanol under mild temperatures (50-80 °C) and in the presence of a basic homogeneous catalyst (Fig.25A). The process is transesterification, which allows conversion of TGs in a mixture of FAME and glycerol (1,2,3-propanetriol). A large part of this co-produced glycerol is separated from FAME by simple decantation, although further washing/drying steps are required to remove traces of glycerol in order to comply with strict regulations for fuel grade biodiesel. This extra purification process increases production costs and generates great amounts of salts, soaps and waste water. Furthermore, the management of the large amounts of residual crude glycerol produced (100 kg per ton of biofuel) represents an important challenge for the biodiesel industry. Fig.25B shows a comparative scheme of transesterification and hydrotreating processes. Both technologies utilize TGs as feedstocks but they differ in the reactants utilized (methanol *vs.* hydrogen), the by-products generated (glycerol *vs.* propane), the final fuel product obtained (biodiesel *vs.* green hydrocarbons) as well as in the reaction conditions and catalysts used. Methanol and hydrogen are typically derived from fossil fuels and, consequently, efforts should be made to obtain these reactants from biomass sources in order to reduce the overall CO_2 footprint of biofuel. While solutions in the biodiesel industry involve replacement of methanol with biomass-derived ethanol as an esterification agent, hydrotreating technologies can drastically reduce external hydrogen consumption by employing sub-products and/or residues generated during the process as sources of this gas. For example, up to 75% of H_2 needs of hydrotreating can be covered by steam reforming and subsequent water gas shift (WGS) of the propane co-produced during the process[413], while the lignocellulosic soybean hull wastes discarded after oil extraction can provide hydrogen for hydroprocessing by means of microbial fermentation[414]. The higher cost of hydrogen compared to methanol should be a strong incentive to implement the mentioned solutions in commercial hydrotreating plants. The separation and subsequent management of the by-products generated during the process is also an important aspect determining the profitability of both technologies. In this sense, transesterification seems to be more sensitive to this parameter given the large amounts of glycerol generated and the difficulty to completely remove it from the biodiesel fuel (Fig.25A). However, once separated, this crude glycerol can serve as a cheap feedstock for the production of a large variety of high value-added chemicals and fuels[415], thereby, representing an opportunity to reduce overall biodiesel production costs[416]; On the other hand, hydrotreating generates a by-product gas stream enriched in propane, which is easily separable from the liquid hydrocarbon fuel but presents a lower chemical value compared to glycerol. Consequently, rather than to decrease the costs, this gas stream could be important to reduce the overall input of fossil fuels in the process by offering an internal source of hydrogen or heat/electricity[417].

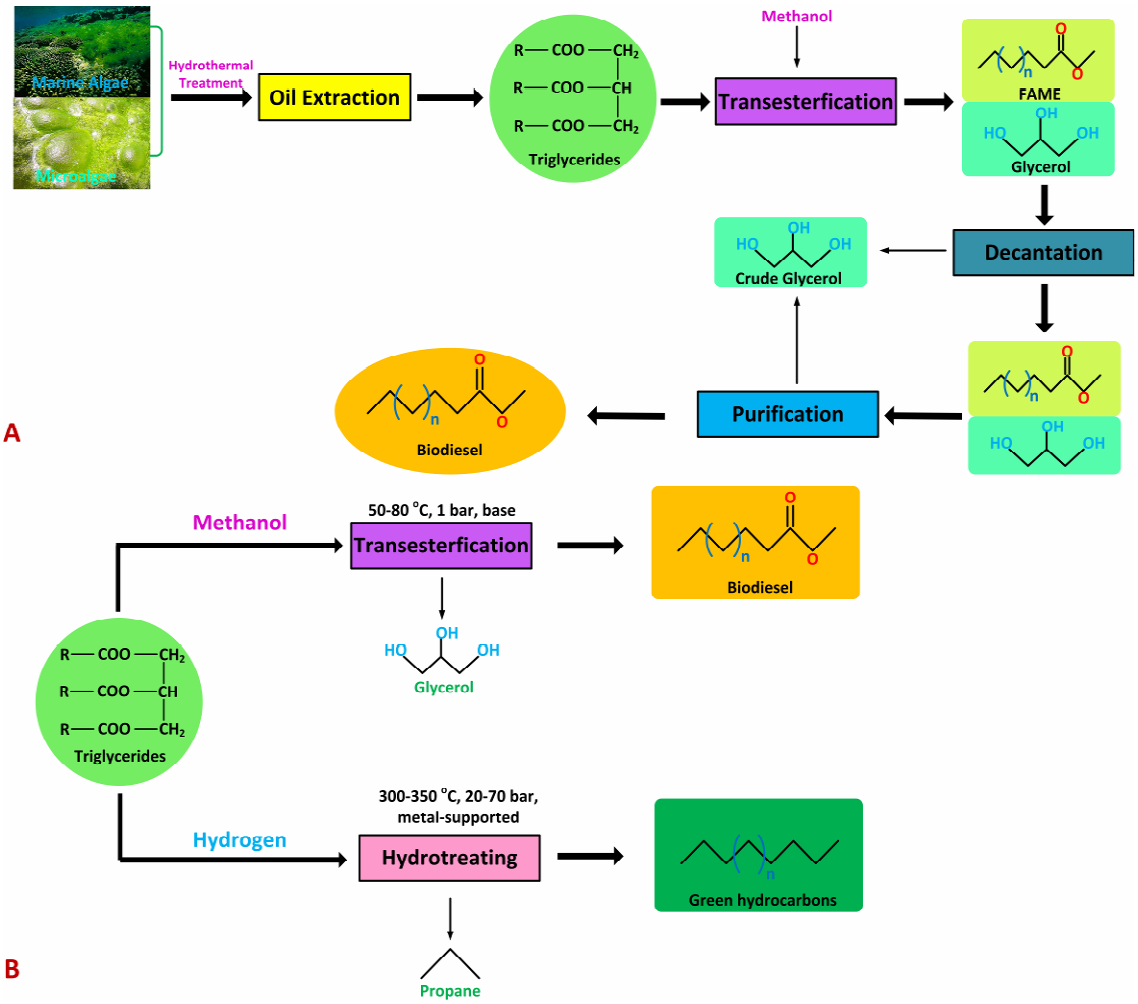

Figure 25. (A) Summarized scheme of transesterification process used for the production of biodiesel from algal biomass; (B) Comparative scheme of transesterification and hydrotreating processes for the conversion of triglycerides into biodiesel and green hydrocarbons, respectively.

Transesterification of lipid feedstocks requires milder temperature and pressure conditions compared to hydrotreating, so the operational costs are greater for the latter route. Nevertheless, hydrotreating conditions are similar to those used in hydrodenitrogeneration (HDN) and hydrodesulfurization (HDS) of petroleum, which provides the possibility to co-process lipids and fossil feeds in existing refinery facilities[418,419]. This synergy between hydrotreating and conventional oil refineries would greatly reduce capital costs[420], and represent one of the key advantages of hydrotreating *vs.* conventional transesterification. However, some key points on hydrotreating still require further research studies, such as the corrosion of the hydroprocessing reactor by free fatty acids, the detrimental cold flow properties of the diesel product as a consequence of the increased content of n-alkanes[421,422], and the effect of the presence of oxygenates over intrinsic HDN and HDS activities of commercial hydroprocessing catalysts. The simplified of the chemistry involved in transesterification and hydrotreating allows production of biodiesel

and green hydrocarbons with high yields. In this sense, both technologies benefit from the utilization of feeds with relatively low oxygen content (and thus low reactivity) like TGs to achieve the required transformations in a selective fashion, and this represents an important advantage compared with other biomass conversion routes managing more reactive feedstocks (i.e., sugars, lignocellulosic biomass). However, the latter feedstocks are more abundant and cheaper than vegetable oils. So far, the limited availability of lipids to satisfy the growing demand for both biodiesel and green hydrocarbon fuels is the most important issue facing both transesterification and hydrotreating technologies. Therefore, it is imperative to search for additional and preferable non-edible lipids sources that can ensure sustainable supply without affecting food markets or requiring large land extensions. Hydrotreating presents higher flexibility to cope with different kinds of feeds compared to transesterification, which is more sensitive to the presence of impurities or free fatty acids. In this sense, hydrotreating is better positioned for the implementation of new, more abundant and non food-competitive feedstocks (e.g., algae) in near future.

Transesterification of algal biomass or lipid to yield biodiesel can be performed by the following common methods, such as conventional heating[71], supercritical methanol conditions[274], enzyme-catalyzed method[423], and microwave irradiation[424]. Among these methods, conventional heating requires longer reaction times with higher-energy inputs and losses to the environment. Supercritical methanol processing operates in expensive reactors at high temperatures and pressures resulting in higher-energy inputs and higher production costs. The enzymatic transesterification reaction proceeds with a slower reaction rate and there is a strong possibility of enzyme inactivation by methanol during the process. Of the four methods, microwave-assisted transesterification, is the most energy-efficient, quick and reliable process to produce biodiesel from algal biomass. The two basic mechanisms of oil extraction from algal biomass observed during a microwave irradiation process are reported as diffusion of lipids across the cell wall into the solvent due to selectivity and solubility, and disruption of the cell wall with a release of contents into the solvent[425]. The direct conversion (*in situ* transesterification) of algal biomass under microwave irradiation conditions has proven to be an effective method for biodiesel production as this method achieves a high degree of oil-lipid removal from the dry algal biomass and efficiently converts oils-lipids to biodiesel[424,426]. It also reduces the reaction time and the solvent volume as compared with the separate lipid extraction and transesterification processes. However, the application of suitable power dissipation control in microwave-assisted transesterification reactions may result in greater benefit in terms of energy efficiency and reaction product yield. Furthermore, Patil et al.[427] studied the effects of power dissipation on microwave-enhanced *in situ* transesterification of dry algal biomass (*Nannochloropsis salina*) to biodiesel fuel as well. The microwave for the transesterification reaction has twofold effects of enhancing reaction by a thermal effect and evaporating methanol due to the strong microwave interaction of the material[428,429]. The microwave interaction with the reaction compounds (triglycerides and methanol) results in a large reduction of activation energy due to an increased dipolar polarization phenomenon. This is achieved due to molecular-level interactions of the microwaves in the

reaction mixture resulting in dipolar rotation and ionic conduction[430]. The reduction in activation energy is essentially dependent on the medium and reaction mechanism[431]. Methanol is a strong microwave absorption material and, in general, the presence of an OH group attached to a large molecule behaves as though it were anchored to an immobile raft, and the more localized rotations dominate the microwave spectrum and result in localized superheating, which assists the reaction to complete faster[432]. The microwave enhanced transesterification reaction of algal biomass to yield methyl ester is illustrated in Fig.26. The base-catalyzed microwave transesterification mechanism is described elsewhere[426].

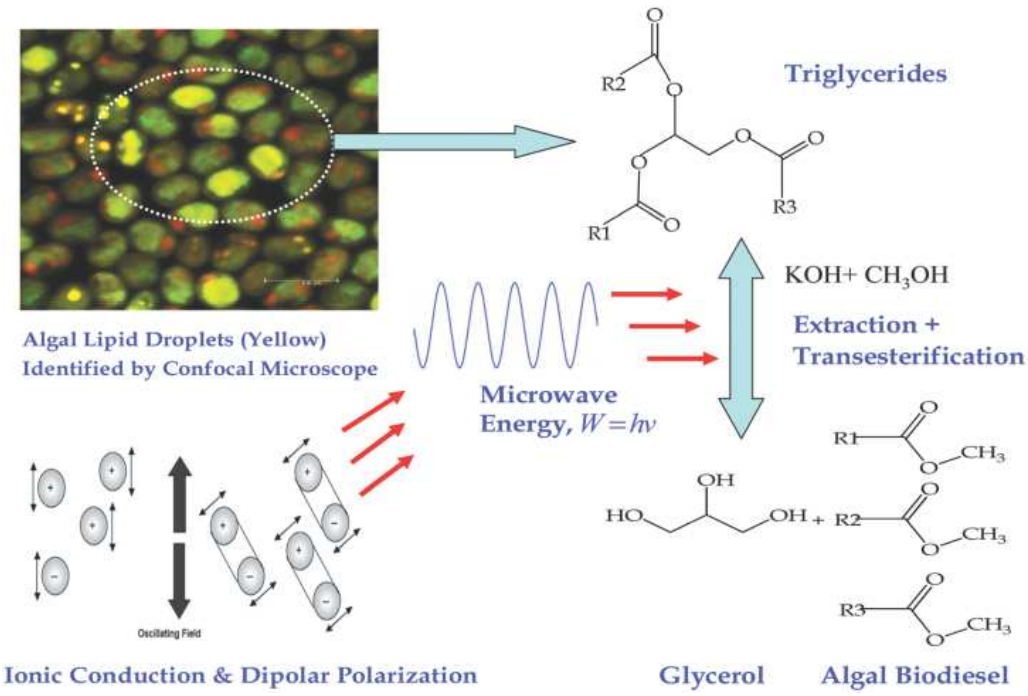

Figure 26. Microwave-enhanced *in situ* transesterification of algal biomass [427]

Emulsified Algal Biodiesel

It is well known that emulsified biodiesel could reduce both nitrogen oxide (NO_x) and smoke emissions. The addition of water with the fuel affects the density and viscosity, while an improvement in mixing process is induced inside the cylinder due to microexplosions of water and improves the combustion efficiency and brake thermal efficiency[433]. Surfactant is required to emulsify the fuel and ensure stability for long duration by reducing the interfacial tension[434,435]. Yoshikawa and his colleagues [436] have successfully excluded the necessity of surface active agents by mixing oils and water just before combustion. The production and principle of emulsified biodiesel production are shown in Fig.13. The emulsification unit consists of an injector and a line mixer. The production process of the water/oil emulsified fuel is: first oil and water are supplied from each supply unit at a constant flow rate before being mixed. Thereafter the supplied oil and water are mixed and emulsified by the emulsification unit to produce the water/oil emulsified fuel. The emulsification unit is installed just upstream of the burner, which

enables the emulsified fuel to be combusted before separation of oil and water, and therefore excludes the necessity of adding any surface active agents. An application test of this emulsified fuel to a boiler effectively indicated that suppression of NO_x and dust emissions is possible and improvement of thermal efficiency is also possible by adequately controlling the excess air ratio and water content in the emulsified biofuel. In addition, periodical maintenance inspection revealed that the inner surface of boilers became remarkably clean after using the emulsified fuel, possibly caused by the improvement of thermal efficiency[436]. Compared to a mechanical mixer, more fine and uniform droplets of methanol can be generated by using the static mixer. This resulted in the increase of the interface surface area between raw oil and methanol, and greater yield of FAME product in the initial stage of the reaction. Furthermore, the static mixer can accelerate the transesterification significantly, and thereby enhancing the reaction efficiency associated with biodiesel production[437].

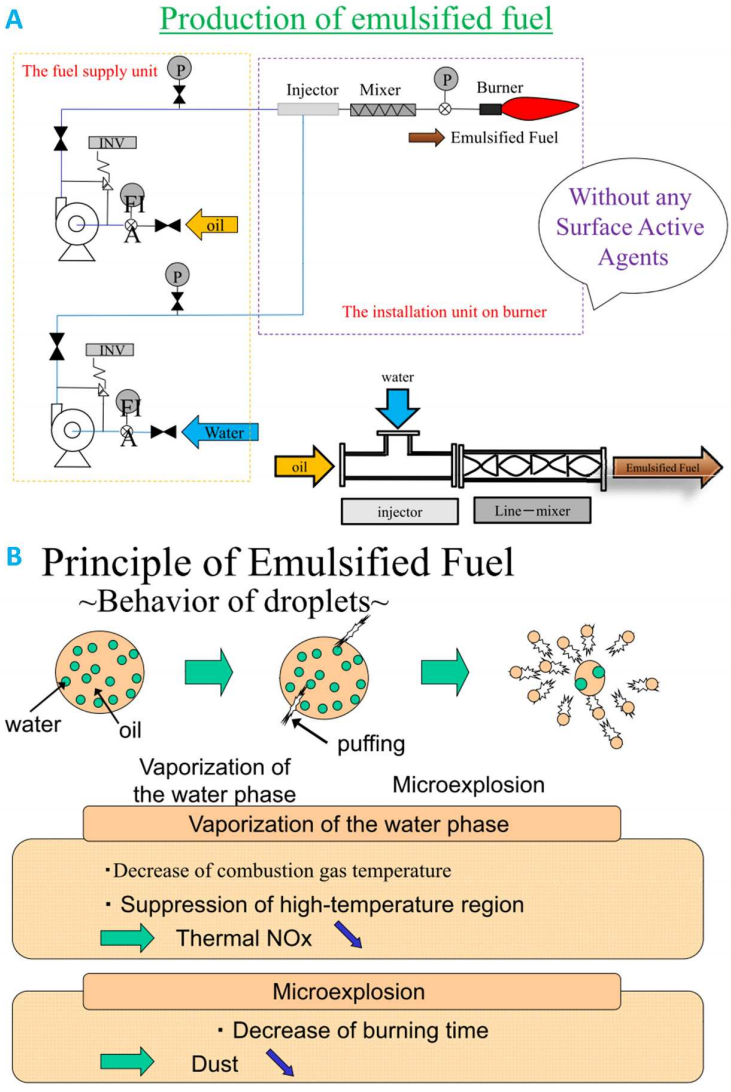

Figure 27. Schematic diagram of production (A) and principle (B) of emulsified biodiesel production

Life-cycle Assessment of Algal Biodiesel

Algal biofuel pipeline shows the major stages in the process, together with the inputs and outputs that must be taken into consideration by life-cycle assessment (LCA). At each stage, there are many factors to be considered and optimized, including energy and material inputs (e.g., nutrients, and energy for mixing during growth), and appropriate treatment of waste products, such as spent media and residual biomass (Fig.28)[438]. LCA is a modeling tool to quantify the impacts of products and processes along multiple environmental categories[439]. Multiple LCA studies of algal production have been conducted recently that highlight environmental challenges for algal biofuels, including large fertilizer and nutrient requirements[440,441], significant energy required to dewater the algae prior to lipid extraction[271,439,442] and for production and delivery of CO_2[440], and high water intensity relative to land-based bioenergy sources[440,441]. Techniques to mitigate this concerns have also been assessed using LCA, including using alternate sources of CO_2 from ammonia production or power plants[443,444], using wastewater for nutrients[440,444-446] or coupling algae cultivation and biogas production to reduce overall energy demands[447]. Multiple reactor designs for algae cultivation have been evaluated in the literature, in general finding that open raceway ponds (ORPs) have a lower energy use and GHG emissions profile compared to PBRs[361,448], although the choice of materials for the PBRs has a significant influence on the results[444]. Compared to petroleum and soy-based biodiesel, algal biodiesel produced using some PBR systems has been found to have a favorable energy and GHG balance[155].

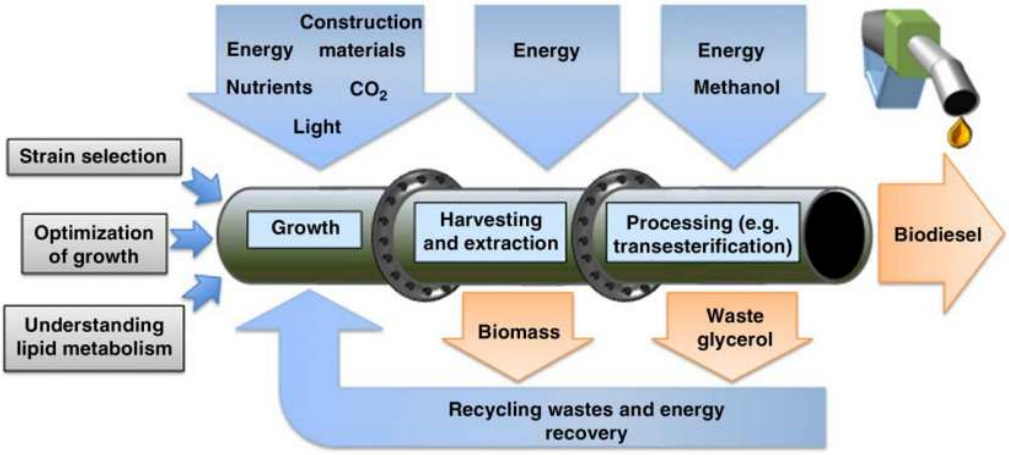

Figure 28. Algal biofuel pipeline, showing the major stages in the process, together with the inputs and outputs that must be taken into consideration by LC [438].

Although algae-based biodiesel production is still at the research and development stage, it is reasonable to expect that algal biofuel production, when commercially implemented, will resemble existing industrial processes. Hence, some process steps within the system boundary (e.g., dewatering and drying of algae) are modeled using data for other similar processes being currently practiced. Fig.29 shows the system boundary of the biodiesel production process. The life cycle impacts were assessed for an integrated microalgal

biodiesel production system that facilitates energy- and nutrient- recovery through anaerobic digestion, and utilizes glycerol generated within the facility for additional heterotrophic biodiesel production[449]. Efforts to increase productivity but reduce input and cost through process engineering and the use of transgenic methods, and classical breeding aimed at developing domesticated algal crop strains, will benefit both strategies[450]. The successful, large-scale generation of biodiesel from microalgal feedstocks will require viewing the algal production facilities as biologically diverse bioreactors that will obey the known rules of ecology. In the three subsections below we illustrate how the application of core concepts and principles from ecology and ecological physiology can provide important new insights into the design and operation of these systems[451,452].

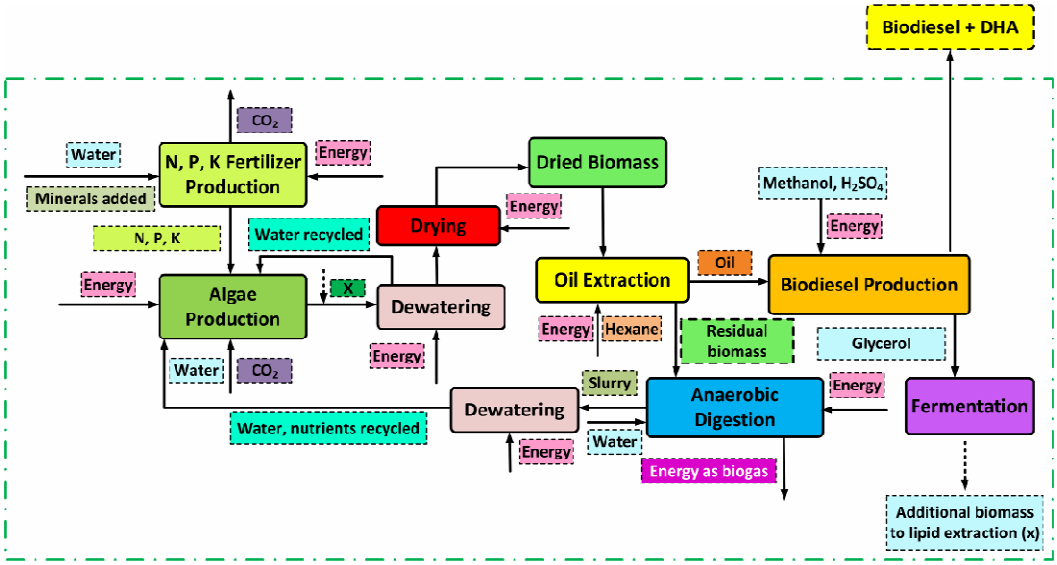

Figure 29. System boundary of the biodiesel production process

Utilization as a potential feedstock for biodiesel production, microalgae need to be overcome a few limitations of those are low growth rates of photoautotrophic algae and biomass concentrations. The algal species can grow only in a specified temperature range (15-30 °C) and fluctuation of temperature beyond the optimum range results in inhibition of growth of the microalga or its death. To achieve the desired temperature range in open ponds may be difficult as temperature at surface go high to about 40 °C. Therefore, closed bioreactors are fabricated for the microalgae culture to minimize temperature fluctuations. However, closed bioreactors too, if operated in hot areas, may observe an increase in temperature, which has to be controlled by using water for evaporative cooling, heat exchangers, reflection of infra-red radiation, or light dilutions. These processes make the microalgal biodiesel cost intensive. Synthesis of biodiesel from microalgae at present is low and need further improvement in the cultivation process. Krohn et al.[453] found that though the total lipids comprised 19% of algal dry weight, the synthesized biodiesel from the lipids were only 1% of dry weight. Algal lipids possess high free fatty acid content

which is not saponifiable and so transesterification cannot be done with the conventional homogeneous base catalyst[453]. The option available is to reduce the acid value by esterification or employing a solid acid catalyst. The deprivation of nitrogen on the accumulation of lipids in microalgae varies among the various species. A limitation of synthesis of biodiesel from microalgae is a high alcohol to oil molar ratio (up to the extent of 315:1) required during the synthesis process that enhances the production cost of biodiesel[454]. Another major limitation of the oil obtained from microalgae, yeast, of fungi is the lipid contents (broadly classified as neutral lipids, total lipid). Only a part of the neutral lipid that comprises of triglycerides and free fatty acids can be converted to fatty acid methyl esters (i.e., biodiesel) and many of microalgae tried as feedstock for oil comprises of constituents that cannot be converted to biodiesel[455].

3.5. Other Biofuels Production from Algae

Bioethanol from algae holds significant potential due to their low percentage of lignin and hemicellulose compared to other lignocellulosic plants[456]. With a low lignin content, macroalgae contain amounts of sugars (at least 50%) that could be used in fermentation for bioethanol production[457,458]. However, in certain marine algae such as red algae the carbohydrate content is influenced by the presence of agar, a polymer of galactose and galactopyranose. Current research seeks to develop approaches of saccharification to unlock galactose from the agar and further release glucose from cellulose leading to higher ethanol yields during fermentation[458,459]. Up to now, only a handful of research work has been reported on bioethanol production from microalgae due to several reasons. Firstly, more attention has been diverted to biodiesel production from microalgae since certain strains are capable to accumulate large quantity of lipid naturally inside their cells; Secondly, through nitrogen-deficient cultivation method (to save energy and cost), lipid content inside the microalgae cells is boosted up significantly by blocking carbohydrate synthesis pathway, while carbohydrate is the main substrate to produce bioethanol; Besides, biodiesel has a higher calorific value than bioethanol, 37.3 MJ/kg and 26.7 MJ/kg, respectively. Nonetheless, microalgae are found to be a superior feedstock to produce bioethanol in comparison with other first and second generation bioethanol feedstock. First generation bioethanol is derived from food feedstock such as sugar cane and sugar beet, in which over exploitation of this feedstock creates the "food versus fuel" issues and raised several environmental problems including deforestation and ineffective land utilization. Second generation bioethanol is produced from lignocellulosic biomass such as wood, rice straw and corn stover, which should be subjected to pretreat initially to break down the complex structure of lignin and to decrease the fraction of crystalline cellulose by converting to amorphous cellulose[460]. However, most of the pre-treatment methods, i.e., steam explosion and alkali or acid pre-treatment, are energy intensive and bring negative impact towards the environment.

In contrast, microalgae cells are buoyant not requiring lignin and hemicelluloses for structural support[461]. Therefore, it is expected that the overall bioethanol production

process can be simplified due to the non-requirement of chemical and enzymatic pre-treatment step. Nevertheless, it should be noted that high concentration of carbohydrates are actually entrapped within the microalgae cell wall, in which an economical physical pre-treatment process such as extrusion and mechanical shear is still required to break down the cell wall so that the carbohydrates can be released and converted to fermentable sugars for bioethanol production[461].

Green algae including *Spirogyra* sp. and *Chlorococum* sp. have been shown to accumulate high levels of polysaccharides both in their complex cell walls and as starch. This starch accumulation can be used in the production of bioethanol[456,462]. Harun *et al*.[456] proved that the green algae *Chlorococum* sp. produces 60% higher ethanol concentrations for samples that are pre-extracted for lipids versus those that remain as dried intact cells. This indicates that microalgae can be used for both lipid-based biofuels and ethanol biofuels production from the same biomass as a means to increase their overall economic value. On the other hand, simultaneous biodiesel and bioethanol production from microalgae is also possible, in which microalgae lipid is extracted prior to fermentation process[463,464]. This concept has been proven viable in a recent study in which lipid from *Chlorococum* sp. was extracted with supercritical CO_2 at 60 °C and subsequently subjected to fermentation by the yeast *Saccharomyces bayanus*[456]. From the report, microalgae with pre-extracted lipid gave 60% higher ethanol concentration for all samples than the dried microalgae without lipid extraction. This is because supercritical CO_2 can act as a superior pre-treatment method to breakdown microalgae cell wall causing the simultaneous release of lipid and carbohydrates embedded within the cell wall. Maximum bioethanol yield of 3.83 g/L was achieved from 10 g/L of lipid-extracted microalgae residue. In other words, lipid extraction from microalgae for biodiesel production and pre-treatment step to release carbohydrates for bioethanol production can occurs in just one single step which greatly enhanced the viability of microalgae biofuels production in commercial scale. Apart from supercritical CO_2, other lipid extraction methods such as ultrasonication, chemical solvent, microwave and bead-beater have not been studied to get a comprehensive comparison between the methods[463].

Fig.30 shows the block diagram of the superstructure for the integrated production of bioethanol and biodiesel from algae. The actual flowsheet including all the different units can be reconstructed using the detailed figures presented along the text. Firstly, algae are grown in ponds. After that, the oil is extracted by using an organic solvent. Finally, the starch is separated, which is saccharified and liquefied for the production of ethanol. In parallel, the oil is transesterified using the dehydrated ethanol. Two most promising alternatives were considered for the transesterification of oil using bioethanol[465], the use of a homogeneous alkali catalyst or the enzymatic catalyzed reaction. The ethanol is recovered, recycled, and mixed with part of the ethanol produced from the starch and the glycerol is separated from the product biodiesel, in this case fatty acid ethyl ester (FAEE). Then, Martín et al.[464] also presented two alternative technologies for the biodiesel

synthesis from algae oil, enzymatic or homogeneous alkali catalyzed that are coupled with bioethanol production from algae starch. To determine the optimal operating conditions, they not only couple the technologies, but also simultaneously optimize the production of both biofuels and heat integration while optimizing the water consumption. Multi-effect distillation is included to reduce the energy and cooling water consumption for ethanol dehydration. In both cases, the optimal algae composition results in 60% oil, 30% starch, and 10% protein. The best alternative for the production of biofuels corresponds to a production price of 0.35 $/gal, using enzymes, with energy and water consumption values (4.00 MJ/gal and 0.59 gal/gal). Even though the integrated process requires higher energy and water consumption, the simultaneous production of ethanol and biodiesel is more advantageous than the production of biodiesel using ethanol alone as it reduces the biofuel production cost around 20%, mostly because of the raw material cost reduction.

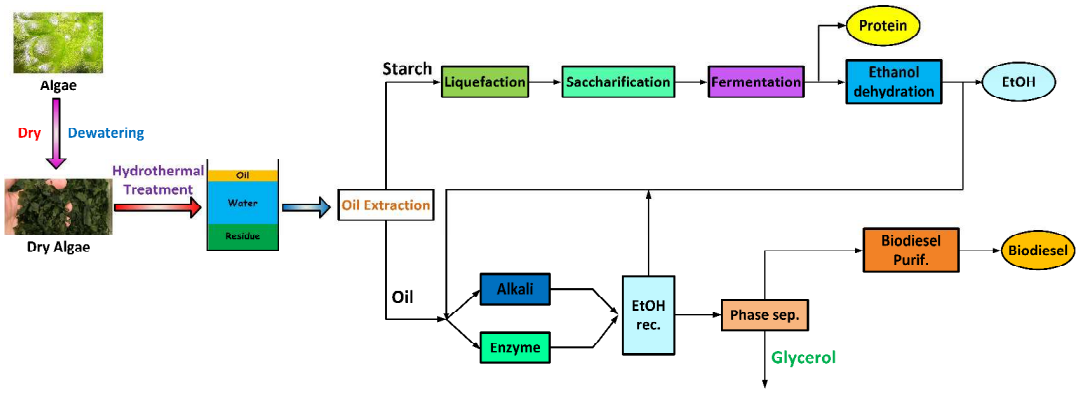

Figure 30. A integrated concept of production of bioethanol and biodiesel from algae

Microalgal biomass is still not a viable choice for commercial biofuels production due to the extensive energy input compared to current terrestrial energy crops. Nevertheless, several energy hotspots have been indicated in the overall microalgae process chain, including inorganic nitrogen source production, operation of photobioreactor and harvesting/dewatering of microalgal biomass. It is recommended that culturing microalgae for biofuels production should be coupled with wastewater treatment and waste CO_2 to minimize heavy dependency on the inorganic nutrients and carbon sources. For the downstream processes, extraction of lipid from microalgae presents a complicated task, as there is no single method that can give optimum lipid extraction for all types of microalgae strains. Thus, breakthrough technologies such as supercritical extraction/transesterification, in-situ transesterification, hydrothermal treatment and transesterfication assisted with ultrasonication or microwave have a great potential to significantly enhance the production of microalgae biodiesel. Additionally, the simultaneous production of bioethanol and biodiesel is more advantageous than the biodiesel production using ethanol alone, thereby reducing biofuel production cost around 20%. For long-term sustainability and environmental benefits, all the processing stages of microalgae biofuels should be simplified without involvement of extensive energy input. In addition, the processes should be easily adopted in the existing biofuels industry and can be implemented especially in third world countries, for culturing microalgae for biofuels production is not only meant

for profit making and benefiting the environment, but also to help people from the bottom billions in terms of food and energy security. The integrated process of microalgae biofuels production via combining wastewater treatment with CO_2 bio-mitigation has been attracted more and more attentions by researchers, which will be discussed in the following part.

4. Wastewater Treatment and Green Algae-to-biofuel Technology

Algae require considerable amounts of water in order to grow and thrive. The organisms themselves are typically 80-85% water[466] and the photosynthetic process results in the dissociation of roughly one mole of water per mole of CO_2[467]. This means that approximately 5-10 kg of water are consumed per kg of dry algae biomass produced. In addition to water incorporated within the cell, most algae grow and reproduce in aqueous suspension. When algae blooms are observed, it appears that there are copious amounts of biomass; indeed a thin suspension of *Chlorella* contains 2×10^{10} individual cells per liter of water[466]. However, the percentage of suspended solids is actually quite low, typically less than 0.5% wet biomass (0.1% dry). Thus, for every gram of dry algae biomass generated, more than a kilogram of noncellular water is required to produce and support it. Water not only provides a physical environment in which the algae live and reproduce, it also delivers nutrients, removes waste products, and acts as a thermal regulator. Unlike natural environments, mass cultivation systems require that the water be acquired, contained, circulated, and pumped to and between desired locations. All of these activities entail inputs of energy, both direct and indirect, and the amount of energy expended is tightly coupled to the volume of water involved. The volume of water involved depends upon system geometries, losses from the system, and most importantly, the ability to reclaim and reuse water. The latter is affected by the efficiency of the separation process, the quality of the return water, and the sensitivity of the specific culture to changes and/or impurities in the return water, including waste products introduced by the algae themselves[468]. Microalgae also have a significant role in wastewater treatment plants[469]. As indicated in Fig.31, the algal biomass grown to recover nitrogen phosphorus from wastewater can be utilized in several ways such as for a fertilizer or as a food source in its own right. In order for this biomass to replace traditional phosphorus fertilizers the harvesting, transportation, stability, application techniques and the proportion of phosphorus availability to crops must be considered[470]. Unlike bacterial biomass from enhanced biological phosphorus removal systems which quickly re-releases stored phosphorus under anaerobic conditions, Powell[471] showed that algal biomass can retain stored phosphorus for some days. Furthermore, with regard to its fertilizer potential, Mulbry et al.[472] compared seedling growth using dried algal biomass to commercial fertilizer and showed growth at comparable levels. However, overall these issues from harvest to application are currently quite poorly covered by the literature for both algae and macrophytes.

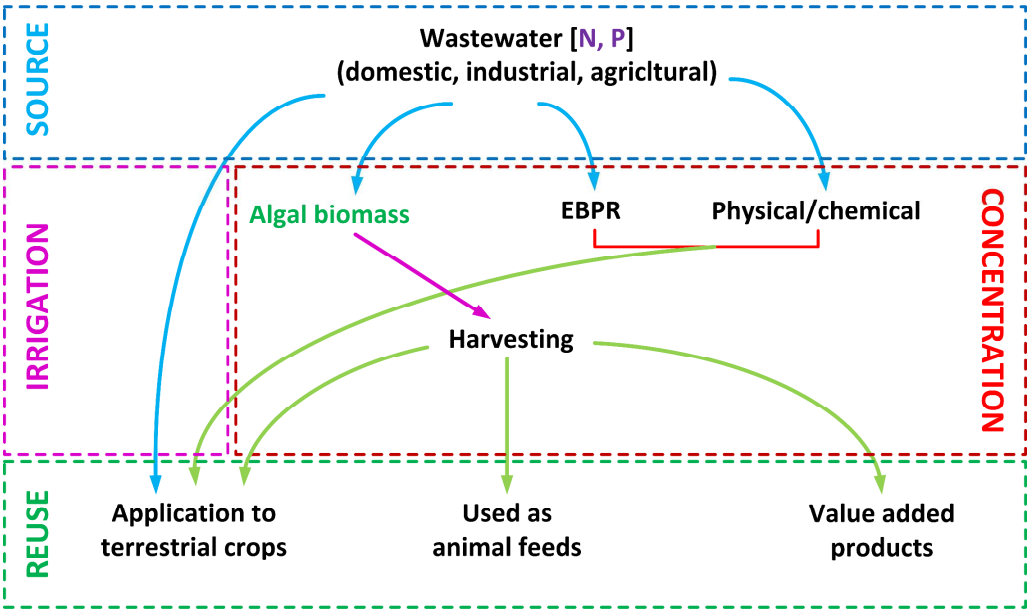

Figure 31. Overview of options to utilize algae to recover nitrogen phosphorus from wastewater[470]

4.1. Nutrients Recovery from Wastewater

One promising way to make algal biofuel production more cost-effective is to couple wastewater treatment[440]. Furthermore, many of the algal species in wastewater treatment processes form large colonies (50-200 μm), and cell aggregation might be achieved through nutrient limitation or CO_2 addition[473], which will further lower the cost of algae harvesting. However, knowledge on growing algae on wastewaters such as municipal wastewater for algae harvesting by self-sedimentation is still limited. Wastewaters derived from industrial, municipal, agricultural resources (e.g., animal manure) have been studied in terms of algae growth and nutrient removal efficiency[440,472-476]. However, the nutrient removal efficiencies achieved did not meet increasingly stringent regulations and limits on wastewater discharge. Therefore, further exploration is needed for improved wastewater treatment and cost-effective microalgae-based biofuel feedstock production.

As above, microalgae harvesting employs some typical methods such as filtration, sedimentation, centrifugation, or flocculation, which can be technically and economically challenging for larger production scales. Macroalgae are multicellular and can be more easily harvested, either manually or mechanically, which may suggest that macroalgae is a better candidate for nutrient removal from aquatic environments. However, microalgae usually have higher lipid productivity per cultivation area contributing to a greater potential for liquid fuel production (Table 1a). As macroalgae generally do not contain lipids and have high carbohydrate contents, they are more favored for biogas or alcohol-based fuels production. Table 1b shows the levels of the nitrogen and phosphorus in different wastewaters. Compared with animal wastewater, municipal wastewater has less

nitrogen and phosphorus. However, there are often considerable amounts of heavy metals such as lead, zinc, and copper in raw municipal sewage. Selection of microalgae strains with high metal sorption capacity is crucial to achieve high metal removal efficiency. So far, only a few algal species have been studied for metal sorption ability. Compared with typical agricultural, municipal, and industrial wastewater, anaerobic digestion (AD) effluent has relatively lower carbon levels because microbial activity during the digestion converts the carbon to methane[477]. The nitrogen in AD effluent is mainly in the form of ammonium[478]. Dilution of AD effluent is usually needed before feeding to algae in order to avoid the potential inhibition of algal growth due to high ammonium concentration and turbidity[479]. In addition, as there is a significant amount of bacteria in AD effluent, proper pretreatment, such as filtration and autoclave, may be necessary to prevent the contamination of algae production systems[477].

Chlorophytes is one of the largest phyla of microalgae, with a vast array of species and a wide geographical distribution. As shown in Table 1c, *Chlorella* sp. has been used in numerous studies and shown to be effective in removing nitrogen and phosphorus from different wastewater streams with a wide range of initial concentrations. Nitrogen and phosphorous removal efficiencies from the growth of *Chlorella* sp. range from 8% to 100%. Studies in Table 1c also confirm that *C. vulgaris* has higher nutrient removal efficiencies than that of *Chlorella kessleri* when comparing their performances in artificial media. An exceptionally low nutrient removal was found in the growth of *C. kessleri* in which the microalgae were subjected to artificial wastewater for a relatively small amount of time[500]. In other studies, *Chlorella* sp. nitrogen removal efficiency was 23-100%, while phosphorus removal efficiency was 20-100%[477,501-506]. In addition, it has been reported that *Chlorella* sp. is tolerant to NH_4^+-N [477].

To utilize simultaneously both nitrogen and phosphorus, the N/P ratio should be with in a proper range. The optimal ratio differs among cultures due to strain-varying metabolic pathways. The N/P ratio can be up to 250 for healthy freshwater environments, but in most wastewater streams ratios may be as low as 4-5[514]. An optimal N/P ratio for *C. vulgaris* was reported to be 7[515], in agreement with the N/P ratio of 7.2 calculated from the *Stumm* empirical formula for microalgae ($C_{106}H_{263}O_{110}N_{16}P$). These ratios indicate that the removal rate of nitrogen would be faster than that of phosphate, since a larger proportion is required. The faster removal of nitrogen over phosphorus was observed in the growth of *Chlorella pyrenoidosa* in soybean processing wastewater[504]. It was observed that the removed nitrogen was mainly used for algal cell synthesis, whereas 17% of the phosphorus was removed via precipitation rather than by assimilation.

Some *Chlorella* species are heterotrophic or mixotrophic and can consume organic forms of carbon in addition to inorganic nutrients as part of their metabolic process. This can be an advantage when using wastewater streams containing carbon residues, such as digested

dairy manure[477]. Acetate, found in some wastewaters, was shown to be effectively consumed during heterotrophic or mixotrophic microalgae cultivation[516]. Anaerobically pretreated soybean processing wastewater was shown to improve the growth of *C. pyrenoidosa* by means of providing additional acetate and small organic molecules[504]. Heterotrophic growth is not an advantageous strategy in wastewaters deficient in organic carbon. Under heterotrophic conditions, the addition of carbon in the form of sodium acetate or glucose was necessary to achieve ammonium removal at a level equivalent to that under autotrophic conditions for the growth of *C. Vulgaris*[517]. Another chlorophyte widely used for nutrient removal studies is *Scenedesmus* sp. (small non-motile green algae) often clustered in colonies consisting of 2, 4, 8, 16 or 32 cells. The cells are equipped with spines and bristles, which make the colonies more buoyant and allow increased light and nutrient uptake while deterring predation in the water. Table 1c shows that the nitrogen and phosphorus removal efficiency of *Scenedesmus* sp. was 30-100%. Its nutrient uptake behavior was not remarkably different from that of some *Chlorella*, i.e., *Scenedesmus dimorphus* versus *C. Vulgaris*[503]. However, the removal of ammonium by *S. dimporphus* was significantly greater than that of *C. vulgaris* at an incubation time of less than 9 days (220 h); while immobilized in alginate, the ammonium removal efficiency of *Scenedesmus obliquus* was higher than that of *C. Vulgaris*[501]. It was reported that *Scenedesmus* sp. requires an N/P ratio of approximately 30 to grow without limitation by either nutrient[518]. When grown in an environment with N/P ratios between 12 and 18, the microalgae had a continuous nitrogen limitation, resulting in a high internal phosphate pool[519]. Thus, the subsequent nitrogen removal rates were always shown to be greater than that of phosphorus. The high N/P ratio requirement could possibly explain the low phosphorus removal of 20-55% from agricultural wastewater by *S. Dimorphus*[503]. Other genera of green algae are also capable of effectively removing nutrients from wastewater. Sawayama et al.[520]554 found that *Botryococcus braunii* grown in treated sewage from municipal wastewater was able to consume nitrate and nitrite, but did not remove ammonium. Ammonium was reported to be inhibitory to cell growth in this particular culture. *Chlamydomonas reinhardtii* was capable of removing 42-55% of ammonium and 13-15% of phosphorus from an artificial medium with an N/P ratio of approximately 1[506]. The removal efficiency was slightly increased when scaling up the process 45- or 90-fold in a biocoil reactor[506]. Non-axenic cultures, which are a mixture of different algae species, can also be used to remove nutrients from wastewater. A combination of *C. vulgaris*, *Scenedesmus falcatus*, *Chlamydomonas mirabilis*, and *Microcystis aeruginosa* showed a 58% reduction in ammonium and 34% reduction in phosphates during the algal treatment phase of a city sewage treatment process[521]. Table 1d shows the algal biomass productivity, N and P removal rates of the four different unicellular microalgae species. It was clearly observed that the three green microalgae *C. reinhardtii*, *C. vulgaris* and *S. rubescens* were suitable for integration of wastewater treatment and algae cultivation in terms of biomass settleability, nutrient removal rate and biomass productivity.

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Table 1

(a). Comparison Between Typical Microalgae and Macroalgae Species

Algae category	Representative species	Composition (%w/w)			Lipid productivity [g/(m^2·d)]	Cultivation methods	Harvesting methods	Ref. (s)
		Carbohydrates	Protein	Lipid				
Microalgae	*Scenedesmus obliquus*	10-17	50-56	12-14	2.4-13.5	Open ponds; PBRs	Filtration; Sedimentation; Centrifugation; Flocculation	480,481
	Chlorella sp.	12-17	51-58	14-22	1.6-16.5			
	Euglena gracilis	14-18	39-61	22-38	7.7			
Macroalgae	*Laminaria* sp. (brown seaweed)	60	12	2	0.7-0.9	Natural stocks; Aquaculture	Manual; Mechanization	480,482
	Ulva sp. (green seaweed)	23-78	10-33	0-6	0.6			

(b). Total Nitrogen (TN) And Total Phosphorus (TP) Content of Different Waste Streams

Wastewater category	Description	TN (mg/L)	TP (mg/L)	N/P	Ref. (s)
Municipal wastewater	Sewage	15-90	5-20	3.3	483
Animal wastewater	Dairy	185-2636	30-727	3.6-7.2	484,485
	Poultry	802-1825	50-446	4-16	485,486
	Swine	1110-3213	310-987	3.0-7.8	485,487
	Beef feedlot	63-4165	14-1195	2.0-4.5	485,486
	Piggery	56	13.5	4.1	488
Industrial wastewater	Textile	21-57	1.0-9.7	2.0-4.1	489,490
	Winery	110	52	2.1	491
	Tannery	273	21	13	492
	Paper mill	1.1-10.9	0.6-5.8	3.0-4.3	493
	Olive mill	532	182	2.9	494
Anaerobic digestion effluent	Dairy manure	125-3456	18-250	7.0-13.8	477,495
	Poultry manure	1380-1580	370-382	3.6-4.3	496,497
	Sewage sludge	427-467	134-321	-	498
	Food waste and dairy manure	1640-1885	296-302	-	499

(c). Nitrogen And Phosphorus Removal By Barious Genera of Microalgae and Cyanobacteria In The Axenic Batch Processes of Different Waste Streams.

Algae category	Genus & species	Waste stream	Process type	Removal time (d)	Total nitrogen (TN)		Total phosphorus (TP)		Ref. (s)
					Initial conc. (mg/L)	Removal efficiency (%)	Initial conc. (mg/L)	Removal efficiency (%)	
Chlorophyte	*Chlorella* sp.	Digested manure	Batch	21	100-240	76-83	15-30	63-75	477
	C. kessleri	Artificial medium	Batch	3	168	8-19	10-12	8-20	500
	C. pyrenoidosa	Industrial wastewater	Fed-batch	5	267	87-89	56	70	504
	C. sorokiniana	Municipal wastewater	Batch	10	-	-	22	45-72	507
	C. vulgaris	Artificial medium	Batch	1-10	13-410	23-100	5-8	46-94	505

	C. vulgaris	Industrial wastewater	Batch	5-9	3-36	30-95	112	20-55	503
	C. vulgaris	Municipal wastewater	Batch	2-10	49-1550	55-88	4-42	12-100	501,502
	C. reinhardtii	Artificial medium	Batch	10-30	129	42-83	120	13-14	516
	Scenedesmus sp.	Artificial medium	Batch	0.2-4.5	14-44	30-100	1.4-6.0	30-100	517
	S. dimorphus	Industrial wastewater	Batch	9	-	-	112	20-55	503
	S. obliquus	Municipal wastewater	Batch	0.2-8	27	79-100	12	47-98	501,519
Caynobacteria	*Arthrospira* sp.	Animal wastewater	Semi-cont.	-	-	84-96	-	72-87	522
	A. platensis	Industrial wastewater	Batch	15	2-3	96-100	18-21	87-99	509
	Oscillatoria sp.	Municipal wastewater	Continuous	14	498	100	76	100	510
Diatom	*P. tricornutum*	Municipal wastewater	Continuous	14	498-835	80-100	76-116	50-100	507,508
Haptophyte	*I. galbana*	Artificial medium	Batch	8	377	99	-	-	512

(d) Nutrient And Phosphorus Removal Rates With Microalgae Productivities

Algae category	Algal biomass productivity ($g/m^2/d$)	Daily removed per reactor volume (mg/L/d)		Ref.(s)
		N	P	
Phormidium sp.	2.71 ± 0.7	3.66 ± 0.17	0.56 ± 0.07	513
Chlamydomonas reinhardtii	6.06 ± 1.2	6.39 ± 0.20	0.89 ± 0.06	513
Chlorella vulgaris	6.28 ± 0.8	4.39 ± 0.06	0.76 ± 0.09	513
Scenedensmus rubescens	6.56 ± 0.8	4.31 ± 0.18	0.60 ± 0.05	513

4.2. Integrated Algae Systems for Biofuel Production

Post-hydrothermal liquefaction wastewater (PHWW) is a high-strength wastewater that can accumulate most of the feedstock nutrients (80% or so) and some of the organics up to 40%[523], which provides a significant opportunity for nutrient and carbon recycling. PHWW recycled back to the algae culturing system can allow for multiple cycles of algae growth on each aliquot of incoming nutrients, which maximizes bioenergy production per unit of nutrient inputs. This approach has been investigated in recent studies using HTL wastewater[524-526] and an earlier study suggested a similar approach but used a recondensed wastewater from gasification[527]. These studies show that nutrients in wastewaters from thermochemical conversion processes can be used for algae cultivation, but that significant dilution was required (50-500 times). However, these studies did not identify a viable and sustainable source of dilution water and raised other important questions about how this nutrient recycling can be incorporated into an algae biofuel production system. In Zhou et al.'s study[528], it addressed these issues in pursuit of an optimized system integrating algal wastewater treatment and bioenergy production including original process modeling to quantify the specific benefits of nutrient recycling and analyze the national implications for sustainable biofuel production. Specifically, this

study investigated a novel integrated waste-to-energy system referred to Environment-Enhancing Energy (E^2-Energy) that synergistically combines algal wastewater treatment with large-scale bioenergy production via HTL as shown in Fig.32.

In the proposed E^2-Energy system, wastewaters from a variety of sources (e.g., municipal, livestock, food processing) can be initially separated into a concentrated biosolids fraction and a dilute liquid fraction by common physicochemical processes (e.g., sedimentation, filtration). Then, mixed cultures of low-lipid, fast-growing algae and bacteria are cultivated in a combination of the dilute liquid wastewater fraction and recycled PHWW (Key Step 1). As the algae and bacteria grow symbiotically, the wastewater is treated by providing removal of organics and nutrients (Key Step 2). Note that the energy input for aerobic breakdown of wastewater contaminants is reduced by the oxygen provided during algal photosynthesis. Subsequently, the mixed culture biomass is harvested, and combined with the concentrated biosolids separated from the initial wastewater. This mixture is then fed into a HTL reactor for biofuel production (Key Step 3). The HTL process also generates a CO_2-rich gaseous product and strong wastewater with re-released organics/nutrients (Key Step 4), which is recycled back to the algal-bacterial cultivation system for reuse. This recycling can repeat again and again over multiple cycles of algal growth, harvesting and biofuel conversion that leverages the nutrient content of wastewater into maximum bioenergy quantities, which can be many times the original wastewater energy content. The E^2-Energy system elegantly has resolved several key bottlenecks in other current approaches to algal biofuel production, and provided significant environmental benefits, including carbon capture and wastewater nutrient removal. This novel approach embodies a significant paradigm change and brings together two important goals of modern society-"energy production" and "environmental protection"-into a complementary relationship, whereas historically these goals have most often been antagonistic. This harmonious combination is critically important for addressing the major challenges of a growing world population including energy security, climate change, quality of water resources and sustainable development[529].

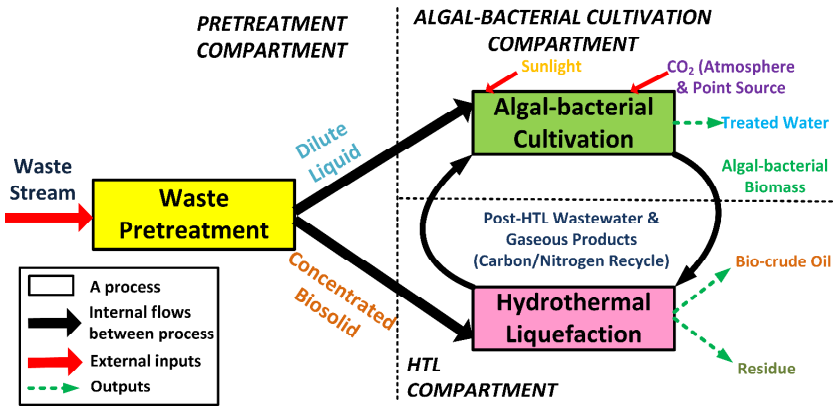

Figure 32. Simplified schematic of the Environment-Enhancing Energy (E^2-Energy) process for integrated wastewater treatment and biofuel production

Some microalgae species can grow in a photoautotrophic mode (PM) under light, or in a heterotrophic mode (HM) in the presence of organic carbon or in a mixotrophic culture mode (MM) when supplied with both organic and inorganic carbon under light/dark conditions[530]. Many different cultivation strategies have been developed to explore the potential of algae as feedstock for biofuel, metabolites and other high-value bio-products based on these growth modes. Oyler[531] developed a process of sequential photoautotrophic and heterotrophic growth (PHM) for algal biofuel production. Das et al.[530] studied a phototrophic-mixotrophic two phase culture model (PMM) for algae-based biodiesel production using glycerol, glucose and sucrose as organic carbon. Xiong et al.[532] developed a similar photoautotrophic-heterotrophic culture mode (PHM) for high algal cell density production. Furthermore, developing a hetero-photoautotrophic culture mode (HPM) to effectively couple treatment of organic-rich wastewater such as concentrated municipal wastewater (CMW) with low-cost biofuel production has been also reported by Zhou et al.[533]. The success of such an algae-based treatment system for organic-rich wastewater relies on the ability of the algal cells to effectively assimilate both organic carbons (heterotrophic growth) and nutrients, such as N, P from wastewater and inorganic carbon (e.g., CO_2) from flue gas for maximal algal biomass and lipid production and efficient nutrient removal as well as CO_2 sequestration[440,534-536]. The locally isolated strain *Auxenochlorella protothecoides* UMN280[476] is facultative heterotrophic and adapts well to CMW. In Zhou's study, a biological system was utilized in order to treat municipal wastewater and the sludge generated at the Metro plant are dewatered using centrifuges and then combusted in fluid bed incinerators equipped with heat recovery boilers[533]. The process requires no additional inputs of fuel and creates heat and electricity for the buildings. The CO_2-rich flue gas during combustion could be sequestered by sparging into an algae bioreactor and the electricity produced could be used to run the bioreactor and harvest the algae as well as to convert algae to refined bio-oil directly by thermochemical processes[537] or biodiesel by transesterification. Therefore, the integrated process could be incorporated into the typical Metro plant municipal wastewater treatment to achieve significant cost reductions of algae-based biofuel (Fig.33).

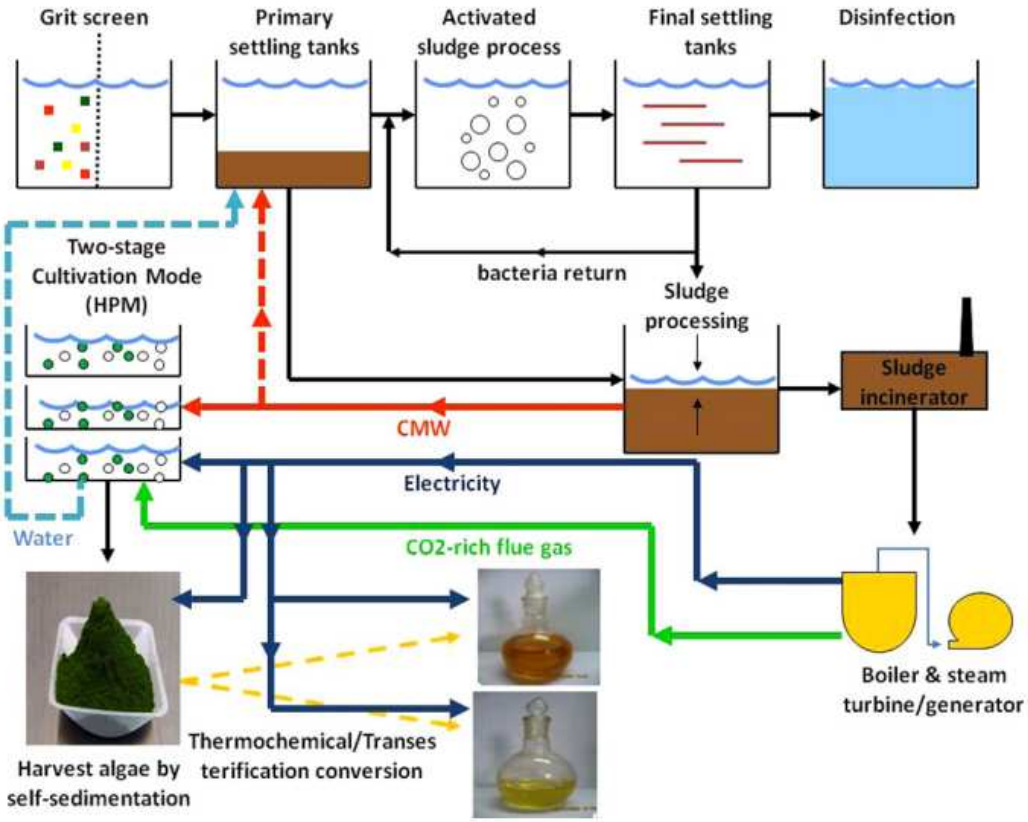

Figure 33. Integration of Pilot-scale HMP algae cultivation system into Metro Plant Municipal Wastewater Process Flow [532]

Integrated algal systems can be employed for wastewater treatment and bioremediation to capture carbon, nitrogen and phosphorus from the specialty industrial, municipal and agriculture wastes (Fig.34). A *Green Wisdom Inc. Plans (G-WISP)* in Arkansas has been developed to implement an integrated algal production system to recycle agricultural wastes (i.e., cotton plants) for biofuel. The future communities can create processing facilities that support algae production as their common core goal by developing local sustainable systems, like G-WISP. It means that using existing crops or rotational crops which are desirable for their waste-to-energy value. Using local, multiple mix agricultural waste and alternative technologies in near closed-loop systems, these communities can create cost effectiveness and produce jobs. Algal production, combining and integrating alternative energy technologies, will foster synergistic development supporting a self-sustaining community system. This system uses anaerobically digested agricultural waste materials including catfish processing waste to feed algal cultures. It shows multiple environmental abatements of CO_2 (e.g., from the anaerobic digester), and reclamation and use of other waste streams, such as water and heat (e.g., from the digester and co-generation), to support optimum sustainable algal growth. Furthermore, this system demonstrates how near-closed-loop sustainable systems can create products, algal oil and

methane energy, and by-products (e.g., fertilizer), and even multiple resulting business spin-offs, companies to help market or distribute the products and byproducts, which, in turn, create jobs for communities of the future. This sustainable community plan would insure rural revitalization and, ultimately, global economic development, while curbing the US dependence on fossil fuels[538]. Although nitrogen and phosphorous are elements key to algal growth, they are serious pollutants in many waterways. Algae can thrive in nitrogen- and phosphorus-rich conditions common to many wastewaters[381,539,540], and this feature may be harnessed to not only remove[540], but also capture these important nutrients with the aim of returning them to the terrestrial environment as agricultural fertilizer, providing a high value by-product for algae that are primarily being grown for biofuel.

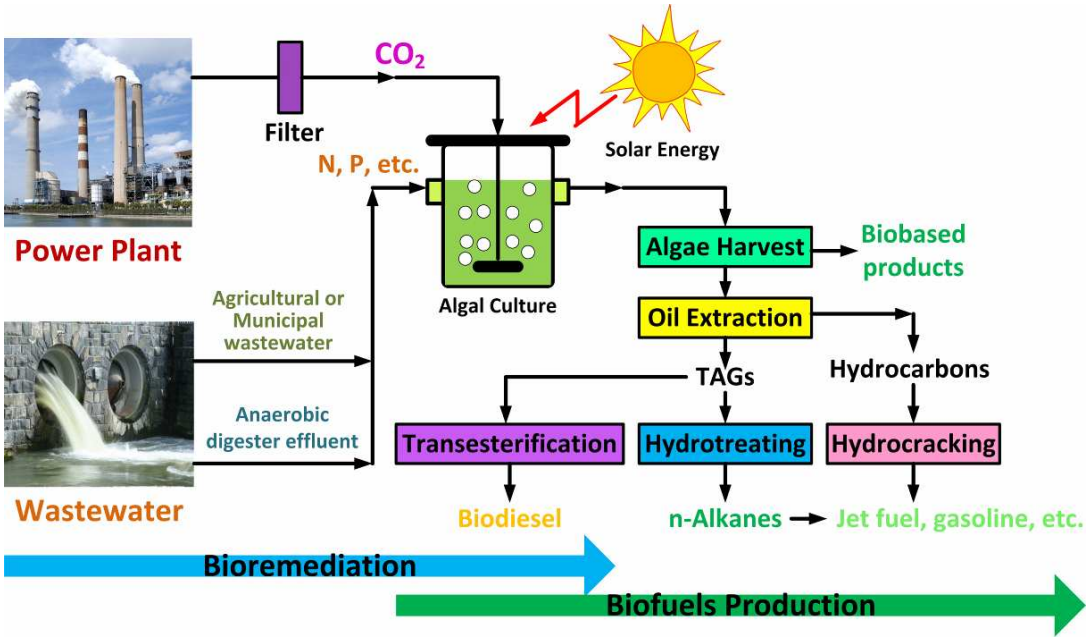

Figure 34. Schematics of an integrated algal culture system for bioremediation and biofuel production

In an environmental point of view, microalgae culture systems should be studied to capture CO_2 and consume the nutrients in wastewaters, simultaneously. In an engineering point of view, the costs associated with all different processes should be reduced. For instance, harvesting and dewatering are processes with high energy requirements mainly because of small cell size and low cell biomass levels in microalgal cultures; thus, research efforts should be performed to achieve high cell densities. This limitation is related with the access of the cells to gas and light. Air-lift bioreactors with light distribution through optical fibbers (increasing the ratio between the illumination surface and reactor volume) and membrane integrated microalgal cultivation processes may resolve these kinds of problems. Apart from the advances in PBR engineering, the application of biorefinery concepts (to exploit the full potential of commercial products derived from microalgal biomass) can make this CO_2 capture process economically feasible[541,542].

5. Concluding Remarks and Outlook

The high growth rates, reasonable growth densities and high oil contents have all been the advantages to invest significant capital to turn algae into biofuels. The algal biofuels production chain shows that the major challenges including strain isolation, nutrient sourcing and utilization, production management, harvesting, co-product development, fuel extraction, refining and residual biomass utilization. Improved engineering will make a significant impact on algae biofuel production. There are important challenges for engineers and biologists to either design cheap PBRs for large-scale deployment, or to combine forces to develop species that grow efficiently in low-cost open systems. PBRs have advantages over open systems as they can more easily maintain axenic cultures, controlled growth environments, which may lead to increase in productivity and decrease in contamination; however, contained systems are challenged by efficiencies in gas exchange and a requirement for supplemental cooling. Regardless of the growth strategy employed, substantial improvements over current technologies for the growth, harvesting and extracting oil from algae need to be made, and coordinated efforts will be needed to couple engineering advances with improved production strains. Oil extraction is another challenge. There are three major strategies (i.e., oil press/expeller, hexane extraction, and supercritical CO_2 fluid extraction) for extracting oil from algae. These technologies have been successfully demonstrated but are relatively expensive, either in terms of equipment needed or energy required to extract the oil[543]. Therefore, large-scale cultivation of algae for biodiesel production is still in the research and development phase. The long term potential of this technology can be improved by the following approaches[544]: (1) Cost saving growth technologies of oil-rich algae should be identified and developed; (2) Integrated bio-refineries can be used to produce biodiesel, animal feed, biogas and electrical power thereby reducing the cost of production; (3) Enhancing algal biology by genetic modification and metabolic engineering has the greatest impact on improving the economics of microalgae biodiesel; (4) Area efficient techniques to capture CO_2 from industrial power plants need to be identified; (5) Recycling of nutrients from municipal sewage and industrial wastewaters are required to reduce the demand of fertilizers to grow algae; (6) Economics of microalgae production can be improved by additional revenues from wastewater treatment and greenhouse gas emissions abatement.

Algae can be grown in many ways in freshwater, saltwater or wastewater; in closed PBRs or open ponds. One key advantage of algae is that its cultivation does not require cropland. But other resources are needed, and the amounts of these resources vary widely from one algae production pathway to another. For instance, it was reported that between 3.15 and 3,650 liters of freshwater are needed to produce the algal biofuel equivalent to 1 liter of gasoline using current technologies. For comparison, 5-2,140 liters of water are needed to produce a liter of corn ethanol and 1.9-6.6 liters are needed to produce a liter of petroleum-based gasoline. The national research council report notes that none of the sustainability concerns will be a definitive barrier to future production of algal biofuels, significant biological and engineering innovations are needed to mitigate demands on resources[545,546]. Thus, the integration of upstream production and downstream processing

of microalgae, and the framing of these in the context of water savings and net energy gain, is needed to build up credibility and withstand scrutiny. Otherwise, microalgae biofuels could go from 'hero to zero' in a very short space of time in this age of advanced communications[546]. The latest research indicated that biomass impregnated into seawater (saltwater, i.e. $MgCl_2$). Then, the $MgCl_2$ preloaded biomass can be fabricated into the mesoporous carbon stabilized MgO nanoparticles for highly efficient CO_2 capture[372]. Thus, in our points, if microalge are grown in seawater, it has one possibility that the solid products containing amounts of alkaline or alkaline earth metallic salts can be synthesized into the value-added mesoporous carbon materials for CO_2 capture. There is an increasing emphasis on ensuring that bio-based products do not have negative effects on the natural environment and, as such, it is crucial that any issues surrounding the environmental impacts of biofuels, bioenergy and commodity chemicals production are addressed prior to the commercialization of products. Among biofuel feedstocks, algae can hold the promise to offset much or all of our fossil fuels utilization. While many of the outstanding challenges are daunting, there are many reasons to be optimistic. Investment in research and development has been steadily increasing, and new multi-stakeholder collaborations bode well for innovation. The further development of co-products for algal fuels will help increase the likelihood of success. The criteria for which chemicals are most promising as value-added algal biorefinery co-products would be scalability, demand and, most importantly, raw materials. Algal biomass serving as the feedstocks for chemical co-products is likely to have a unique and somewhat tunable chemical composition compared with traditional plants. The absence of lignin, the presence of phospholipids and the unique carbohydrate fractions of algae, as well as the variability between and within algal species, will require new product platforms and technological adaptations beyond those currently realized in conventional biorefineries. However, these challenges can easily be viewed as opportunities. The biorefinery is an ideal setting for innovation, and the creativity of the green chemistry and green engineering community with respect to biomass transformations would be critical in improving the future prospects for our energy and material economy.

Up to now, many microalgae projects can achieve maximal lipid yields only under stress conditions hindering growth and providing compositions not ideal for biofuel applications. Metabolic engineering of algal fatty acid biosynthesis promises to create strains capable of economically producing fungible and sustainable biofuels. The algal fatty acid biosynthetic pathway has been deduced by homology to bacterial and plant systems, and much of our understanding is gleaned from basic studies in these systems. However, successful engineering of lipid metabolism in algae will necessitate a thorough characterization of the algal fatty acid synthase including protein-protein interactions and regulation. Thus, many efforts have been made for improving engineer fatty acid biosynthesis toward optimizing microalgae as a biodiesel feedstock. Algal bioresource generation can be integrated with human communities to form a sustainable permaculture ecosystem, or an algae-based bioresource cycle. Local algae species are sourced and studied from 'nature's culture collection' for bioresource production. Algal farmers can utilize locally available waste resources (e.g., wastewaters, CO_2 and heat) to cultivate desired native algal biomass, which

is harvested and processed at an algae-based biorefinery into consumable products. Algal cultivation integrated with algae-based biorefineries can yield a diversity of bioresources (biodiesel, green gasoline, bio-jet fuel, isolated proteins, food starches, textiles, organic fertilizers, etc.), which mitigate the cost of biofuel production. For example, the alga could be an indigenous variety of *Chlorella* that is grown on local nutrients from municipal wastewater treatment plant effluent and captures CO_2 derived from nearby sources such as the combustion of fossil fuels, fermentation and industrial facilities, cement plants, landfill gas, or biogas from anaerobic digestion. Algal biomass produce lipids, proteins or starches that could be processed into biodiesel, nutritional supplements, and food products. The organic residuals produced during processing and after consumption can be anaerobically digested to produce biogas (methane and CO_2) and solubilized mineral nutrients. The CO_2 and the nutrients can be reused directly by the algal culture, avoiding the costs associated with supplying these external inputs. In addition to community use as a renewable fuel, the methane can provide energy for *on-site* processing, including harvesting, drying, heating, or mixing the algal culture. Utilizing the energy, nutrients and CO_2 held within residual waste materials to provide all necessary inputs except for sunlight, the cultivation of algae becomes a closed-loop engineered ecosystem. Developing this biotechnology is a tangible step towards a waste-free sustainable society. Significantly, utilizing industrial wastewaters for algae cultivation, the biological effects of the emergent pollutants (i.e., engineered nanoparticles, high-concentration heavy metal) to aquatic ecosystems should be evaluated.

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Graphical Abstract

Utilizing the energy, nutrients and CO_2 held within residual waste materials to provide all necessary inputs except for sunlight, the cultivation of algae becomes a closed-loop engineered ecosystem. Developing this green biotechnology is a tangible step towards a waste-free sustainable society.