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"Applied Biology and Environmental Safeguard"

"*Green microalgae biohydrogen production*"

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“Non come chi vince sempre, ma come chi non si arrende mai.”

(Frida Khalo)

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Antonina

Sintesi

La ricerca nell'ambito delle energie rinnovabili è attualmente uno dei temi più dibattuti dalla comunità scientifica internazionale. L'uso sfrenato dei combustibili fossili ha sollevato questioni rilevanti sulla sua sostenibilità a lungo termine e sugli effetti ambientali. L'idrogeno (H_2) è considerato un combustibile promettente grazie alle sue proprietà termodinamiche e alla sua combustione priva di prodotti carboniosi come l'anidride carbonica (CO_2). L'idrogeno viene principalmente ottenuto da processi dipendenti dalle fonti fossili, ma solo l'idrogeno "verde" identifica una produzione esclusivamente basata su processi non inquinanti. Attualmente, diversi microrganismi sono noti per la loro capacità di produrre H_2 come caratteristica metabolica. Per quanto riguarda le microalghe, la maggior parte delle informazioni relative a questo tipo di processi proviene dalla specie *Chlamydomonas reinhardtii*. In questa microalga verde sono stati descritti diversi pathway biochimici per la produzione, seppur transitoria, di H_2: due collegati al processo fotosintetico e uno di natura fermentativa. Per innescare la produzione di H_2 un requisito indispensabile è la creazione di un ambiente ipossico. Ciò si verifica o quando l'attività fotosintetica rallenta o se vi è un aumento della frequenza respiratoria mitocondriale. Al contempo, il flusso degli elettroni fotosintetici deve essere diretto preferenzialmente verso l'enzima responsabile della produzione dell'H_2, l'idrogenasi.

Per quanto riguarda il condizionamento fisiologico, una delle strategie più promettenti per l'incremento della produzione di H_2 è la deprivazione dallo zolfo dal terreno di coltura. In questo modo, in condizioni di luminosità elevata, entro tre giorni si sviluppa una condizione di anaerobiosi. *Chlamydomonas* è stato anche un ottimo organismo modello per lo sviluppo di strategie molecolari che hanno permesso di superare alcune limitazioni ed estendere la produzione di H_2. Una di queste è legata alla saturazione e alla dissipazione della luce solare, condizioni che influiscono negativamente anche sulla produzione di H_2. Un'antenna di dimensioni ridotte nel cloroplasto dei mutanti *truncated light-harvesting antenna* (*tla*) è invece soggetta a

minori fenomeni di saturazione e foto-inibizione. Un'altra limitazione è legata ai pathway competitivi che sottraggono elettroni all'idrogenasi. Il mutante *Pgrl1* (*protein gradient regulation like 1*) ha mostrato una migliore produzione di H_2 riducendo questo fenomeno. Un altro problema rilevante è la sensibilità all'ossigeno dell'enzima idrogenasi. Una [FeFe]-idrogenasi di un batterio afferente al genere *Clostridium*, meno sensibile all'O_2, ha infatti mostrato una migliore attività enzimatica, anche quando espressa eterologamente in *C. reinhardtii*.

Il lavoro svolto nel triennio di dottorato è stato principalmente finalizzato all'isolamento e alla caratterizzazione di specie microalgali nella regione Basilicata per l'identificazione di ceppi idonei alla produzione di bioidrogeno. Particolare attenzione è stata dedicata alla ricerca di specie con buoni tassi di crescita e in grado di esibire versatilità nell'uso delle diverse fonti di carbonio. In secondo luogo, è stato analizzato il comportamento fisiologico di ceppi mutanti di *Chlamydomonas*, singoli e doppi, per quanto riguarda la produzione di H_2, modulando le condizioni di luminosità senza ricorrere all'applicazione di situazione di stress, come la deprivazione di zolfo.

Campioni di acqua dolce raccolti in diversi centri della regione Basilicata sono stati utilizzati per isolare diverse morfologie microalgali. Le osservazioni microscopiche e l'identificazione molecolare hanno permesso di identificare il genere di appartenenza delle colonie isolate. La crescita dei vari ceppi è stata monitorata con metodi diversi: la densità ottica e il contenuto di clorofilla si sono rivelati metodi efficaci e veloci per seguire la crescita cellulare nel corso dei giorni. Questo ha permesso di valutare i tassi di crescita delle specie in esame.

Sono stati effettuati diversi test per rilevare l'eventuale produzione di H_2. I bioreattori sono stati mantenuti al buio, con luce limitata (12 PAR) o in condizione di deprivazione di zolfo (con luce intensa a 100 PAR). Tutti gli esperimenti sono stati ripetuti testando anche diverse fonti di carbonio. I livelli di H_2 gassosi sono stati poi valutati mediante gas-cromatografia prelevando quotidianamente un campione dallo spazio di testa a contatto con la coltura liquida posta all'interno dei bioreattori ermetici. I ceppi di *Desmodesmus* sp. ed *Haematococcus* sp. hanno dimostrato una produzione di H_2 simile ai ceppi di *Chlamydomonas* usati come controllo, nelle stesse condizioni (5-10 ml/litro di coltura). Inoltre, la produzione è stata registrata sia utilizzando acetato che glucosio, come fonte di carbonio.

Per i mutanti di *Chlamydomonas*, gli esperimenti sono stati condotti in collaborazione con l'Università di Córdoba (Spagna). I mutanti oggetto di studio sono stati *tla3*, *pgrl1*, uno ingegnerizzato con l'idrogenasi batterica di *Clostridium* (*clostr*) e le relative combinazioni *tla3+pgrl1* e *clostr+pgrl1* ottenute tramite *genetic cross*.

In questo caso, il *wild-type* e i ceppi mutanti singoli e doppi sono stati sottoposti a diverse condizioni di illuminazione (12, 50, 100, 450 PAR). In particolare la combinazione *tla3+pgrl* si è rivelata più efficace dei rispettivi singoli mutanti essendo in grado di produrre H_2 anche ad intensità luminose generalmente meno tollerate, aprendo nuovi scenari applicativi. Il mutante *Clostr* ha invece mostrato una rapida attivazione dell'enzima idrogenasi anche proporzionalmente all'aumento dell'intensità luminosa.

In conclusione, le alghe isolate durante il triennio di dottorato hanno mostrato interessanti risvolti per la produzione di H_2, come la versatilità metabolica rispetto all'uso delle diverse fonti di carbonio. Ciò apre nuovi scenari e spinge a voler ulteriormente approfondire i meccanismi alla base del metabolismo di queste microalghe sia dal punto di vista fisiologico che molecolare. Per quanto riguarda i singoli e doppi mutanti di *Chlamydomonas*, sono state invece ampliate le conoscenze sulla fattibilità della produzione di H_2 anche in condizioni non soltanto legate all'applicazione di stress.

Summary

Nowadays, renewable energy is one of the most discussed issues by the international scientific community. The unrestrained use of fossil fuels has raised relevant questions about sustainability and effects on the environment. Hydrogen (H_2) is considered a promising fuel due to its thermodynamical properties and CO_2-free combustion. Nevertheless, environmental problems arise when H_2 is produced using energy deriving from fossil sources: only "green hydrogen" identifies a production 100% based on renewable energy. Currently, several microorganisms are known for their ability to produce H_2 as a metabolic feature. Regarding microalgae, most of the information comes from *Chlamydomonas reinhardtii*. In this green microalgae, two different photosynthetic production pathways and one fermentative-like metabolism have been described concerning transitory H_2 production. To extend H_2 production one requirement is the creation of a hypoxic environment. This occurs when photosynthetic activity slows down or if there is an increase in mitochondrial respiration rates. Moreover, the electron flow should be directed preferentially towards the H_2 evolution enzyme, hydrogenase.

Concerning physiological conditioning, one of the most promising strategies for H_2 production is sulphur deprivation from cultivation medium: within three days, anaerobiosis develops under saturated light. *Chlamydomonas* has also represented an excellent example for the development of different molecular strategies, that allow to overcome some limitations and extend the H_2 production. One of the limitations is related to sunlight saturation and dissipation that also affect H_2 production. Mutants with the truncated light-harvesting antenna (*tla*) in the chloroplast are subject to fewer phenomena of photoinhibition and light saturation. Another limitation is linked to the competitive pathways that remove electrons from hydrogenase. *Pgrl1* (protein gradient regulation like 1) mutant showed improved H_2 production reducing this phenomenon. Another relevant issue is the oxygen sensitivity of the hydrogenase enzyme. The use of an O_2-tolerant clostridial [FeFe]-hydrogenase, expressed in *C. reinhardtii*, showed better enzymatic rate, as the bacterial hydrogenase had a lower inactivation rate in aerobiosis.

The work carried out over the past three years was aimed primarily at the isolation and characterization of microalgal species in the Basilicata region for the identification of new biohydrogen producers. Particular attention has been given to the search for strains with good growth rates and able to use different carbon sources. Secondly, the physiological behaviour of single and double mutants of *Chlamydomonas* was analyzed concerning H_2 production by modulating light condition without resorting to stress application, such as sulphur deprivation.

Freshwater samples collected in different villages of the Basilicata region were used to isolate microalgae with different morphologies. Microscopical observations and molecular identification made it possible to identify the genus of the isolated pure colonies. The growth of the various strains was followed by different methods: absorbance and chlorophyll content proved to be effective and fast for monitoring cell growth over the days. This made it possible to evaluate the growth rates of the species under examination.

Various tests were carried out to detect the production of H_2. Bioreactors were kept in dark, limited light (12 PAR) or sulfur deprivation (with intense light, 100 PAR). All the experiments considered different carbon source too. The levels of H_2 gas produced were daily assessed by gas chromatography by taking a sample of the airspace in contact with the liquid culture in the airtight bioreactors. *Desmodesmus* sp. and *Haematococcu*s sp. strains demonstrated production of H_2 similar to wild type *Chlamydomonas* (5-10 ml/litre of culture). Furthermore, the same production occurred similarly using acetate or glucose.

For *Chlamydomonas* mutants, the experiments were conducted in collaboration with the University of Córdoba (Spain). Investigated mutants were *tla3*, *pgrl1*, and one engineered with *Clostridium* bacterial hydrogenase (*clostr*) and the relative combinations *tla3 + pgrl1* and *clostr + pgrl1* from genetic cross. In this case, the wild type, single and double mutant strains were subjected to different lighting conditions (12, 50, 100, 450 PAR). In particular, the combination *tla3 + pgrl* proved to be the best as it is capable of producing H_2 even at light intensities that are generally less tolerated, opening up new application scenarios. The single mutant *Clostr* showed instead a fast hydrogenase activity in a replete media also proportionally with the increase of light.

In conclusion, the algae isolated during the PhD project have shown interesting implications for the production of H_2 such as the metabolic versatility regarding the use of the different carbon sources. This leads to the need to carry out a more in-depth investigation of the mechanisms underlying the metabolism of these microalgae both from a physiological and a molecular point of view. Regarding single and double *Chlamydomonas* mutants, knowledge about their behaviour in different light conditions and the feasibility of H_2 production has been expanded.

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CHAPTER 1 - HYDROGEN: THE FUEL OF THE FUTURE

1.1 Global energy overview

Nowadays, the search for renewable energy is one of the most discussed issues by the international scientific community. The increase in the world population, on one hand, and the growing industrial activities, on the other, has revolutionized how man relates to Earth, especially from the 18th century onwards.

Predicting exactly how the energy situation will evolve on a global scale in the coming decades, is not an easy task, as sudden and unexpected social, political, and economic changes may have a significant impact. According to the most recent estimates, the world population may increase up to 8-10 billion by 2040, and, by the same year, global energy demand, required for maintaining all population activities and industrial activities to support technological development, could increase by an additional 170 qBtu[1] [1,2].

The unrestrained use of fossil fuels has raised relevant questions about sustainability and the short and long-term environmental effects. Fossil fuel has been the most exploited energy sources since the industrial revolution and still plays a leading role: today, it is estimated that fossil sources satisfy more than 80% of the world's energy demand (Figure 1.1). The combustion of oil, coal and natural gas is notoriously associated with the emission of various pollutants into the Earth's atmosphere. Carbon dioxide (CO_2) is the dominant greenhouse gas (GHGs) and is the main responsible for global warming. CO_2 emissions increased by almost 50% in the years between 1998 and 2018.

In an attempt to remedy the environmental problems in progress, various measures, such as the 2015 “Paris Climate Agreements”, were launched to contain polluting emissions and encourage renewable energy use [3,4].

[1] British Thermal Unit (Btu) is an energy unit of measure (1 BTU = 252 cal, 1 BTU = 1.055056 kJ)
1 quad equals 10^{15} Btu, approximately 1.055×10^{18} J.

In 2020, the year in which the Covid-19 emergency exploded, global energy demand suffered a significant drop of 4.5% due to various lock-downs and slowdown of general activities: a similar collapse had not occurred since the Second World War. In particular, among the fossil sources, it was oil that had the worst. Unexpectedly, renewable energy demand still increased, led by solar and wind ones, until the overall generation value of 358 TWh, the highest ever achieved [5].

CO_2 emissions also decreased by 6.3% in the same year. The observed decrease is similar in magnitude to what has been set annually by the Paris Climate Agreements to avoid irreversible damages in the next decades, further underlining the importance and urgency of finding alternative energy measures to mitigate global warming effects [5].

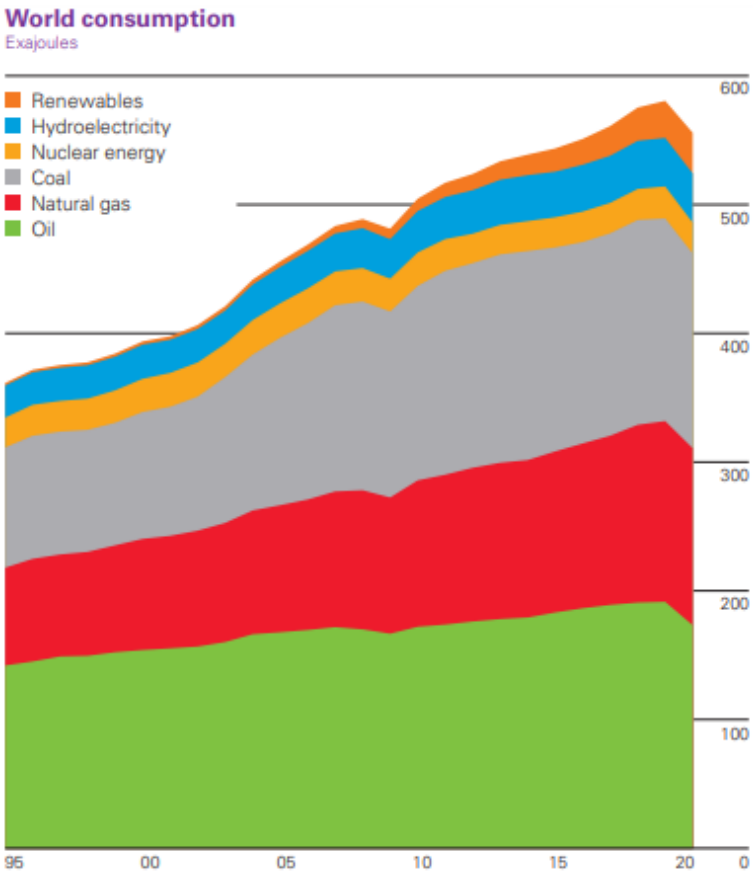


Figure 1.1 - Primary energy consumption in 2020
(adapted from Statistical Review of World Energy, 2021 [5])

1.1.1 Fossil fuels and non-renewable energy

Fossil sources derive from the transformation of organic matter deposited in the layers of the earth's crust over the course of geological eras. Generally, the most common applications of fossil fuels are heat production, raw materials in the petrochemical industry, and fuel in combustion engines. Their use, however, poses various problems. The respective extraction sites are in limited geographic areas, and, as a consequence, some countries are energetically dependent on others. The issues that, in recent years, have shifted the attention to renewable sources include their future availability and the environmental problems associated to their use.

Among fossil fuels, oil is the most used. Oil is a viscous and flammable liquid, a mixture of hydrocarbons, mainly alkanes. After the extraction processes, crude oil undergoes various refining processes to be effectively ready to use.

Coal is a mixture of woody material residues that have not undergone decomposition processes. Peat, lignite, anthracite, coke are additional types distinguished according to time and type of formation. Its use was crucial following the first industrial revolution, representing the fuel of the first steam vehicles. Like oil, coal also undergoes refining processes that make it liquid or gaseous for more practical use.

Natural gas is a by-product of the anaerobic decomposition of organic material. It is mainly composed of methane, but heavier hydrocarbons are also present to a lesser extent. Unlike the previous ones, natural gas generally encounters several transport issues, considering the path from the extraction sites to those of distribution or use.

On the other hand, nuclear energy is not a fossil fuel but is equally non-renewable. Processes such as nuclear fission, nuclear fusion, and radioactive decay, allow for the release of electrical, kinetic, or thermal energy. Although there are no carbon emissions, the product of their processing is harmless radioactive waste only after appropriate treatments and long periods [5,6].

1.1.2 Renewable sources

Renewable energy sources have the characteristic of not running out and are generally associated with a lower environmental impact. Among them, the most abundant and cleanest is the solar one. Although the great potential and the quantities that could

adequately and abundantly satisfy the current energy needs, the initial investment costs are still too high and the areas dedicated to its exploitation are of considerable size.

Closely related to solar energy is wind power. Sun heats the atmosphere by creating temperature gradients responsible for the formation of winds. Other generation factors are the rotational motion of the Earth and the irregularities present on its surface. Wind power is based on the conversion of the movement of special turbines into mechanical or electrical energy. This makes wind energy one of the cleanest one.

Geothermal energy, on the other hand, uses thermal energy from inside the Earth. In the dedicated areas, heat is used to generate hot water or steam. Although the technologies in this sector are efficient, there are few sites and far from meeting the current energy demand.

Another renewable energy sector with well-established technologies is hydroelectricity. Although the initial costs and environmental impact is high, once these plants are set up, they are easy to manage and maintain. The movement of water is commonly used to generate electricity. Similarly, technologies to exploit water masses in the oceans are also under study. However, in this case, the energy released is hardly produced and captured constantly.

One of the most recent proposals to produce green energy is the use of biomasses. This term generally indicates the organic matter produced by plants, microorganisms, or animals. Organic matter is created from atmospheric CO_2, which is concentrated and fixed in carbohydrates by photosynthetic organisms. Compared to fossil fuels and their exceptionally long formation times, this is also called the "short carbon cycle".

Various types of biomasses can be exploited to produce energy or fuels. It is possible to have a thermal conversion of biomass through processes such as pyrolysis or gasification, or an enzymatic conversion, through the metabolism of living organisms that transforms biomass. However, the costs are still high and research has not yet produced technologies that are easy to use on large scale. Products in this area include bioethanol, biodiesel, biogas, and biohydrogen [5,7].

The following sections will deal in more details on why bio-hydrogen is considered a fuel of the future and why green hydrogen production is necessary. In this context, microalgae represent one of the most remarkable solutions.

1.2 Hydrogen

Hydrogen is the most common element in the entire universe, being part of the water and organic compounds molecules. It is the simplest and lightest component of the periodic table, characterized by an atomic structure with one proton in the nucleus and one electron in the orbitals. The identification of hydrogen is due to Henry Cavendish, who presented his investigation to the Royal Society of London in 1776. Antoine Lavoisier in 1788 called it for the first time hydrogen (*Hydrogenium*) with Greek roots "hydro" (water) [8].

Hydrogen is colorless, odorless, and not toxic in the atmospheric gaseous form but, it 'is easily flammable (Table 1.1).

Hydrogen is an energy carrier with a calorific value of 122 kJ/g, almost 3 times that of fossil fuels. Furthermore, contrary to them, it is an eco-friendly fuel as the by-product of its combustion is water. It is not surprising that the environmental impact factor (EIF), namely the ratio between artificial and natural emissions of a compound, for hydrogen has been estimated at 1, while that of hydrocarbons is about 20 times higher [9,10].

The gaseous hydrogen (H_2) in the Earth's atmosphere is not retained and is present only in trace amounts. For this reason, specific techniques are necessary for its production [8,11].

Table 1.1 - Hydrogen properties and thermodynamical parameters
(adapted from Dawood *et al*., 2020 [8])

Properties	**SI units**
Discovery date/by/Chemical formula	1766/Henry Cavendish/H_2
Isotopes	^{1}H (99.98%), ^{2}H, ^{3}H, (^{4}H-^{7}H Unstable)
Equivalences; Hydrogen solid, liquid and Gas at 981 mbar and 20 °C	1kg = 14,104 l = 12,126 m^3
Molecular weight	1.00794
Vapor pressure at (- 252.8 °C)	101.283 kPa
Density of the gas at boiling point and 1 atm	1.331 kg/m^3
Specific gravity of the gas at 0 °C and 1 atm (air=1)	0.0696
Specific volume of the gas at 21.1 °C and 1 atm	11.99 m^3/kg
Specific gravity of the liquid at boiling point and 1 atm	0.0710
Density of the liquid at boiling point and 1 atm	67.76 kg/m^3
Boiling point at (101.283 kPa)	-252.8 °C
Freezing/Melting point at (101.283 kPa)	-259.2 °C
Critical temperature	-239.9 °C
Critical pressure	1296.212 kPa, abs
Critical density	30.12 kg/m^3
Triple point	-259.3 °C at 7.042 kPa, abs
Latent heat of fusion at the triple point	58.09 kJ/kg
Latent heat of vaporization at boiling point	445.6 kJ/kg
Solubility in water vol/vol at 15.6 °C	0.019
Dilute gas viscosity at 26 °C (299 K)	9 x 10^{-6} Pa s
Molecular diffusivity in air	6.1 x 10^{-5} m^2/s
C_p	14.34 kJ/(kg) (°C)
C_v	10.12 kJ/(kg) (°C)
Ratio of specific heats (C_p/C_v)	1.42
Lower heating value, weight basis	120 MJ/kg
Higher heating value, weight basis	141.8 MJ/kg
Lower heating value, volume basis at 1 atm	11 MJ/m^3
Higher heating value, volume basis at 1 atm	13 MJ/m^3
Stoichiometric air-to-fuel ratio at 27 °C and 1 atm	34.2 kg/kg
Flammable limits in air	4%-75%
Explosive (detonability) limits	18.2 to 58.9 vol% in air
Maximum combustion rate in air	2.7/3.46 (m s-1)
Maximum flame temperature	1526.85 °C
Autoignition temperature/in air	400 °C/571 °C

1.2.1 Production methods and uses

Although hydrogen is a fuel with interesting parameters and its combustion produces water and heat as a product without producing CO_2, the environmental problem can instead arise when hydrogen is produced using energy deriving from fossil sources (Figure 1.2).

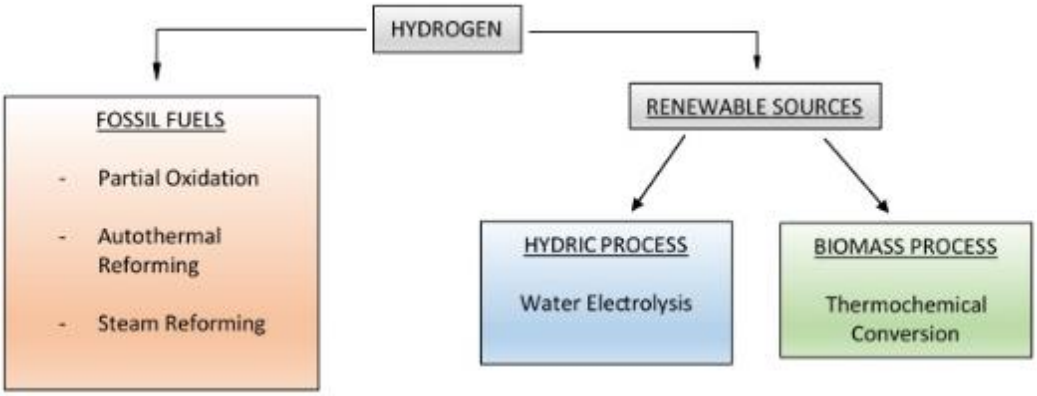


Figure 1.2 - Hydrogen production methods
(adapted from Limongi *et al*., 2021 [12])

The production methods are generally based on high energy and temperature processes. Water electrolysis is the inverse of combustion. The reaction is not spontaneous and it takes a large amount of energy to support it. If the energy comes from renewables such as wind or photovoltaic, the environmental impact is considerably reduced.

A variant of this technique is steam electrolysis. The process is carried out in the same way but at higher temperatures. This allows to significantly reduce energy consumption.

The hydrocarbon reforming process involves the treatment of hydrocarbons, such as methane, at high temperatures, around 900 °C. In this case, the yields are higher but there is always the problem dictated by the use of fossil fuels.

Coal gasification also proceeds using similar temperatures. However, the high temperatures used together with vegetable carbon give a gaseous mixture which must then be purified to obtain only pure hydrogen.

In general, the cleanliness of hydrogen is indicated by a code based on three different colours. Gray indicates hydrogen produced with polluting methods. Blue hydrogen indicates the production of hydrogen associated with Carbon Capture and Storage (CCS)

technologies that minimize or cancel the polluting impact. However, the CSS approach has significant design and operational costs. In the long term, its application is not feasible.

Finally, green hydrogen identifies a production based on 100% renewable energy [8,11]. The production of hydrogen has been in continuous and constant growth from 1975 to today (Figure 1.3). In addition to its use for energy, hydrogen is mainly involved in industrial uses, such as ammonia or petrochemicals production or in metal and refining processing [13].

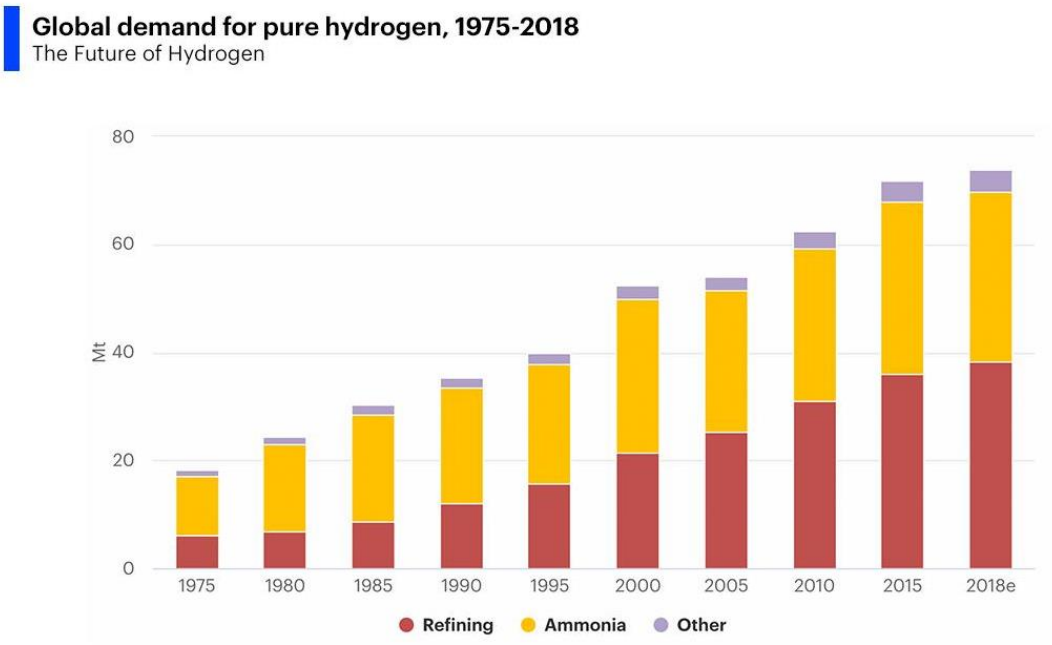


Figure 1.3 - Global hydrogen demand in the last decades
(adapted from International Energy Agency, 2019 [13])

1.2.2 Fuel cells and technical issues

The fuel cell, a sort of modern battery, is often used to exploit the energetic properties of hydrogen (Figure 1.4). This electro-chemical technology provides two separate chambers containing fuel, such as hydrogen, and the oxygen comburent. There are also two electrodes and an electrolyte that carries the ions released in the solution. These elements are always present inside the fuel cell even if various fuels, catalysts, or materials can be used.

In the hydrogen fuel cell, the combustion is not of a thermal nature and carbonaceous by-products do not develop. Redox reactions cause chemical energy to transform into electrical energy. At the anode, the hydrogen undergoes an oxidation reaction. Protons are produced which will join with oxygen to give water and electrons. The electrons are transferred to the external electric circuit and as they go through it, they release part of their energy.

The efficiency rates of a fuel cell are around 40-60%. However, several limitations hinder large-scale use such as the high cost and durability of the component materials [14–16].

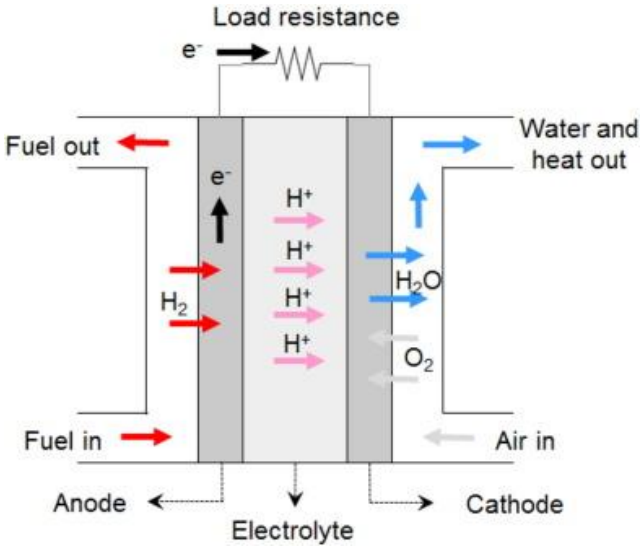


Figure 1.4 - Schematic functioning of a fuel cell
(adapted from Le *et al.*, 2016 [17])

Despite this type of technology and the research that continues to adapt the use of hydrogen to different types of vehicles, one of the biggest problems concerns the widespread presence on the territory of distribution systems for this new fuel.

Moreover, hydrogen storage is a complex topic considering the flammability and low volumetric density. To date, the techniques used are mainly liquefaction and, above all, compression. Some solid-state storage methods with metal hydrides are currently under study [18].

CHAPTER 2 – THE WORLD OF MICROALGAE

2.1 Biodiversity and ecological relevance

Algae are a complex group of photosynthetic and, mainly, aquatic organisms. The exceptional algal biodiversity has allowed them to colonize different habitats, even those considered extreme for life. Because of this, it is not surprising that microalgal species are largely spread across the tree of life branches (Figure 2.1). The term “alga” does not represent a taxonomic category but a heterogeneous group of multicellular and unicellular organisms. Multicellular organisms are macroalgae or seaweeds, while microalgae comprise unicellular organisms which include both prokaryotes and eukaryotes. To date, about 40,000 microalgal species have been described and identified, but probably this number largely underestimates the real existing ones [19,20].

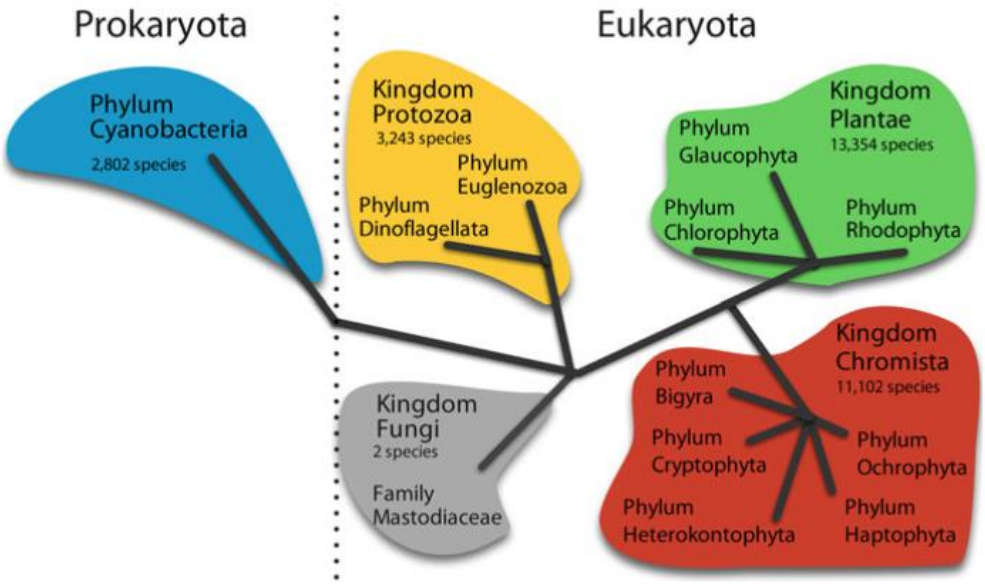


Figure 2.1 - Algae groups distribution in the tree of life
(adapted from Leite and Hallenbeck, 2012 [20])

Their role as primary producers in the food chain of marine and freshwater ecosystems is fundamental. These organisms are in fact the main constituents of phytoplankton, able to exploit solar energy, water, and a few nutrients to build organic carbon compounds starting from atmospheric carbon dioxide. Algal organisms are the main source of atmospheric oxygen: they were the main responsible for its significant increase 1-2 billion years ago, giving a significant input to the development of all other life forms that appeared subsequently. The photosynthetic activity is made possible by a wide range of photosynthetic pigments present in different combinations across the species. And it is precisely the characteristic pigmentation that gave rise to one of the first classification attempts [21].

2.2 Morphology and classification

Microalgal species show great heterogeneity although some morphological and metabolic characteristics are common. Photosynthesis is the predominant metabolic trait, realized employing a various range of pigments. A first differentiation is between chlorophylls and accessory pigments, such as carotenoids.

Cell size and morphologies change according to species (Figure 2.2). The cellular diameter can reach up to 50 µm. On the other hand, the cells of the *Ostreococcus tauri* species are the smallest ever described among eukaryotes with a diameter of 0.8 µm. Microalgae can also have different organizations, including single cells or colonies.

Among the morphologies described there are coccoid, filamentous, flagellate, amoeboid, and palmelloid. The morphology can also vary in the same species depending on the reproductive cycle or environmental conditions [22–25]. *Haematococcus pluvialis* shows a distinct motile phase from a non-motile one. Under unfavourable environmental conditions, green cells lose their flagella and become non-motile. There is also an increase in cell size and an accumulation of various stress-induced compounds. The carotenoid astaxanthin is the most relevant, responsible for its characteristic red colour [26]. *Chlamydomonas reinhardtii* is characterized by a life cycle in which both meiotic and mitotic division can occur. The zoospores of this species are motile and flagellated and behave like two different gametes of mating type + and -. From their union, the cells form a haploid non-flagellated zygote which, in unfavourable conditions, can resist entering a state of quiescence [27].

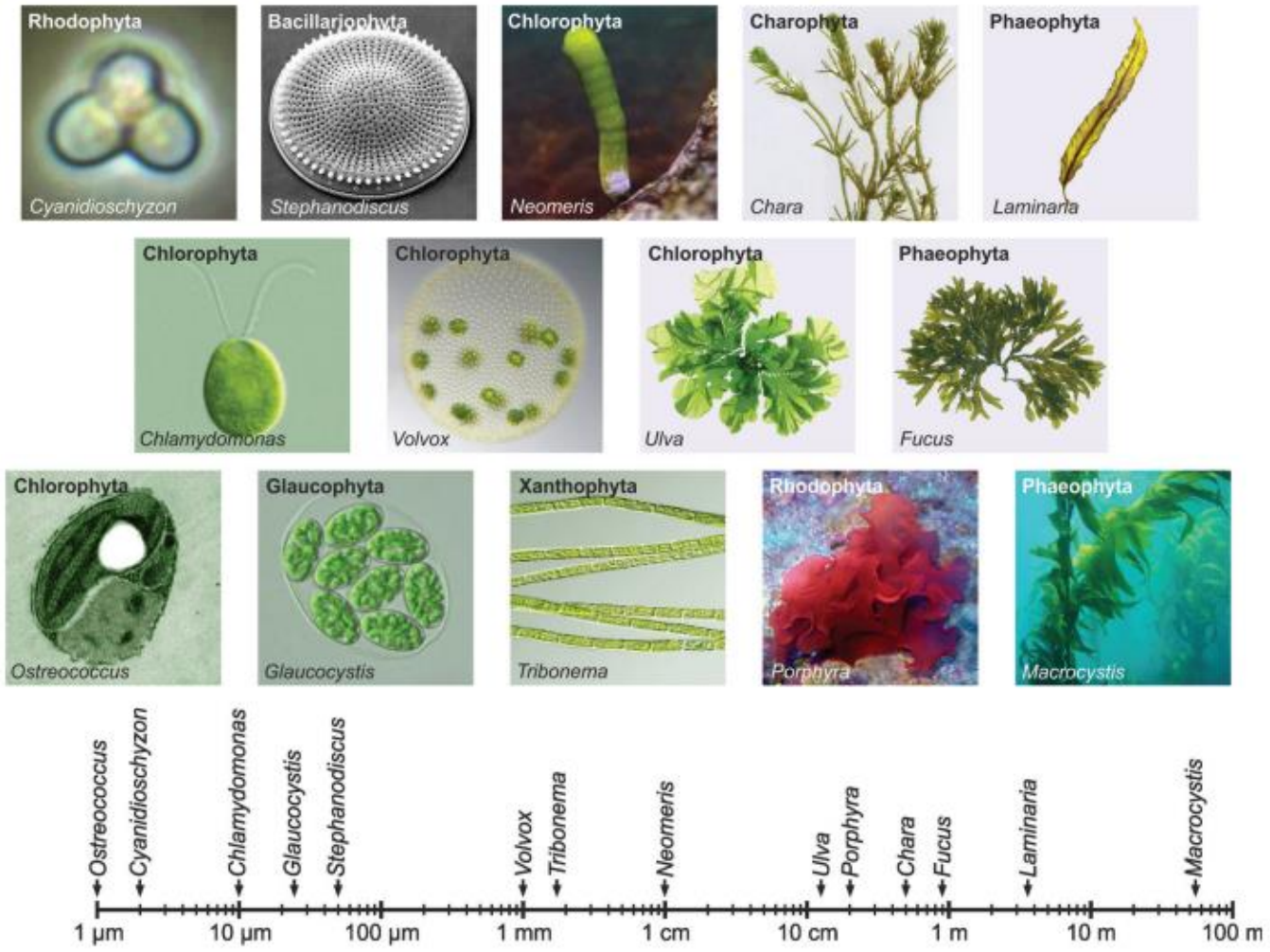


Figure 2.2 - Examples of algae with different sizes and morphologies
(adapted from Hallmann *et al.*, 2015 [25])

The first taxonomic classification of algae mainly considered the presence of photosynthetic pigments. However, it was progressively necessary to evaluate other aspects connected to morphology, such as the presence or absence of the flagellum, or metabolism, such as the presence of the stored compound. It is now possible to exploit some molecular criteria: highly conserved gene or protein sequences are used to obtain phylogenetic relationships between species [28]. To date, microalgae can be divided into prokaryotic and eukaryotic domains. In the prokaryotic domain, there are only *Cyanophyta* and *Prochlorophyta*. The eukaryotic one, the most abundant, has nine phyla: *Glaucophyta*, *Rhodophyta* (red algae), *Heterokontophyta*, *Haptophyta*, *Cryptophyta*, *Dinophyta*, *Euglenophyta*, *Chlorachniophyta*, and *Chlorophyta* (green algae) [23].

Cyanobacteria, gram-negative bacteria previously identified as blue-green algae, are included in the *Cyanophyta*. These photosynthetic bacteria owe their characteristic colouring to chlorophyll a and various phycobiliproteins. These organisms are also capable of fixing atmospheric nitrogen, playing an important role in the cycles of the various elements at the level of aquatic ecosystems. Their appearance was decisive for the increase in oxygen levels in the earth's atmosphere and also for the appearance of subsequent eukaryotic microalgae [29].

A primary endosymbiosis event saw a eukaryotic cell engulf a cyanobacterium leading to the appearance of *Glaucocistophyta*, *Rhodophyta*, *Chlorophyta* phyla (Figure 2.3). From the latter two, there were further secondary endosymbiotic events leading to the appearance of numerous and different microalgal species [30].

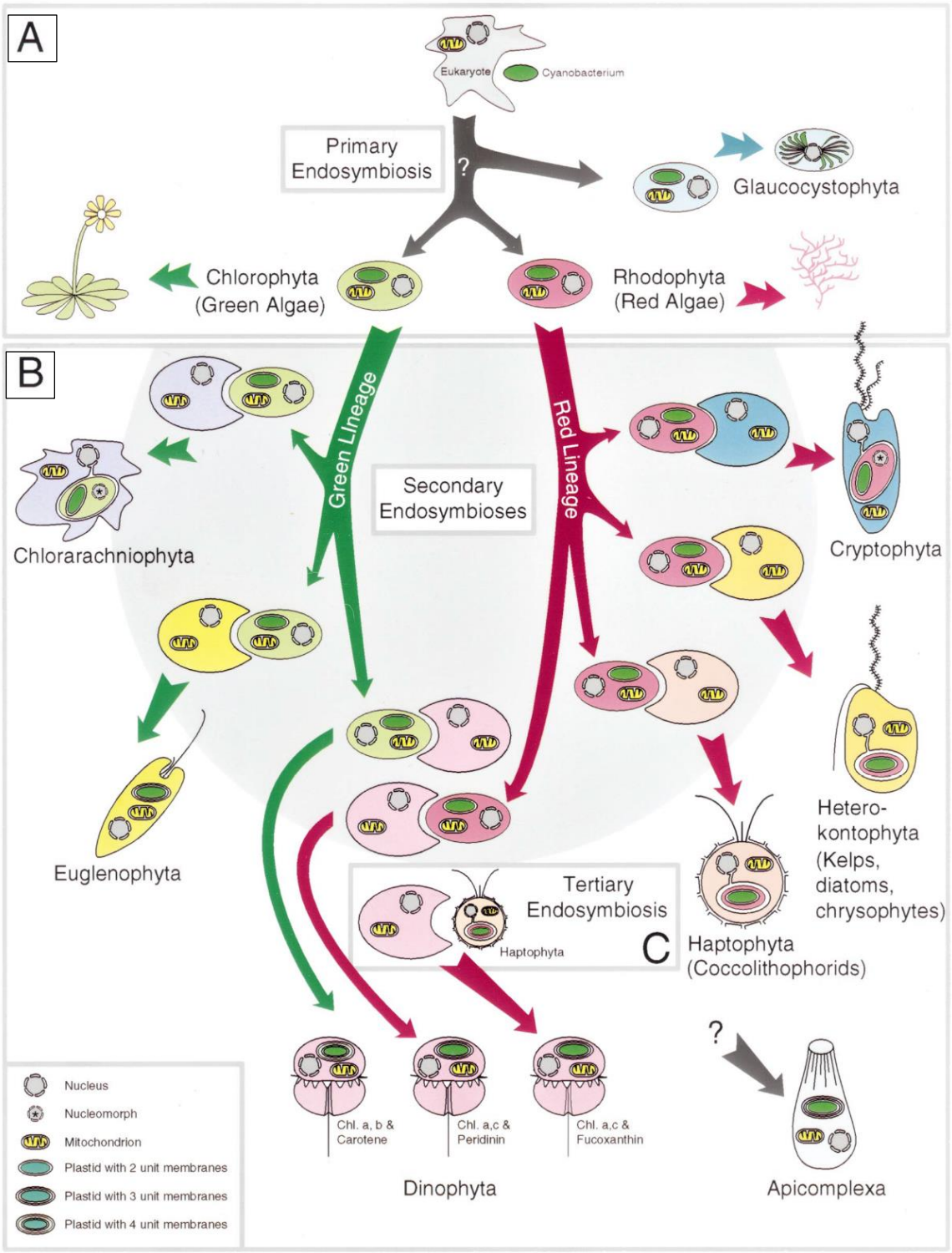


Figure 2.3 - A) Primary and B) secondary endosymbiosis in algae
(adapted from Delwiche, 1999 [30])

2.3 Main metabolic features

The growing interest in microalgal products and applications has led to an increase of their overall knowledge in order to use them optimally and to their maximum potential.

Microalgae have great metabolic versatility. As photosynthetic organisms, they can carry out autotrophic metabolism. They convert CO_2 from the atmosphere into structural and storage carbon-based molecules. To carry out this process they need a few other factors: water, sunlight, and a few elements. These include macro elements such as nitrogen, phosphorus, potassium, and microelements such as molybdenum, zinc, iron, copper. Nutrient-rich wastewater can also represent a valid alternative as a substrate for algal growth, enhancing products otherwise not reusable for other purposes.

Some microalgae can shift in the presence of an organic source of carbon towards a heterotrophic metabolism. Acetate, glucose, and sucrose are among the organic carbon source more frequently used. Moreover, algae can combine autotrophic metabolism with heterotrophic one: this condition defines the mixotrophic metabolism [31].

2.4 Cultivation: laboratory research and scaling-up

In experimental *in vitro* conditions, microalgae follow a growth curve similar to bacterial cultures. After a preliminary phase of adaptation, the lag phase, in which cells prepare themselves for division, an exponential growth phase follows. The latter continues until the nutrient level is optimal. Normally, after a few days in culture, the cells experience a stationary phase, in which growth progressively slows down, followed by a phase of decline or cell death, in which the production of waste substances increases [32]. In general, microalgae growth achieves best performance in a range of pH comprised between 6 and 8, although some species can adapt in the larger interval. Furthermore, extreme salinity levels should be avoided to keep physiological conditions [19].

In general, at the laboratory level, more efficient management of the variables involved in the cultivation of algae is possible. In addition to the composition of the cell medium, salinity, and pH, the control of the light intensity, temperature, stirring, and mixing is also important.

Photosynthetic organisms convert light energy into chemical energy as described in the next chapter. Their pigments and metabolic structures have evolved to capture

electromagnetic radiation in the spectral region of the visible (380-750 nm). In the natural environment, the different pigments make an algal species more or less likely to colonize a given environment depending on the amount and quality of light available [33].

Managing culture productivity is an essential step. If a culture reaches too high densities, the cells risk not being exposed to light properly. On the other hand, under high light intensity, microalgae trigger a series of mechanisms that aim to disperse excess light absorbed in the form of heat. This can lead to a stressful temperature increase for the cell population [34].

Laboratory artificial lighting uses LED lights and intensities of around 200-400 µM photons/m^2/s. In trying to adapt the growth cycles to natural conditions, light-dark cycles are often reproduced. Most microalgae achieve optimal growth levels following cycles of 16 hours of light/8 hours of dark [35,36].

The stirring is, therefore, necessary to ensure adequate light distribution, and to avoid algae to settle and/or create aggregates, reducing the overall productivity. In this way, gas exchanges are also facilitated [37].

Temperature control has a deep impact on biochemical processes. In microalgae, a decrease in optimal temperature reduces carbon assimilation, while, an increase reduces photosynthetic activity. Most algae in nature grow at temperatures between 15 and 30 °C. Extremophiles microalgae, whose physiology has adapted to polar or desert climates, are an exception. Most of the species grown *in vitro* grow at temperatures between 20 and 25 °C. Maintaining temperature issue is particularly evident in open cultivation systems, often unsuitable for mutant algal strains, sometimes weaker than wild type ones [38,39].

Although large-scale algal cultivation presents several technical challenges, such as the control of growth parameters and the presence of contaminants, it is undoubtedly a goal to be pursued for various reasons. From an economic point of view, large-scale cultivation does not require large investments. At the same time, it is possible to use cultivation systems even in areas that cannot be used for other purposes such as agriculture. Microalgae require less water than the traditional crops. They can also use seawater or wastewater, having an overall high productivity per area. Moreover, they do not show seasonality [40].

Before moving to a large-scale cultivation system, the behaviour of the microalgae must be assessed on a small scale at the laboratory level and the growth parameters finely

optimized. It is not always possible to observe the same behaviour in the strains during scaling up.

Photobioreactors that provide closed systems, although more expensive, are required for the production of high-quality metabolites whose production requires greater reproducibility and purity. Among the most considered photobioreactor options, tubular, vertical column, and flat-panel devices are available (Figure 2.4) [41–43].

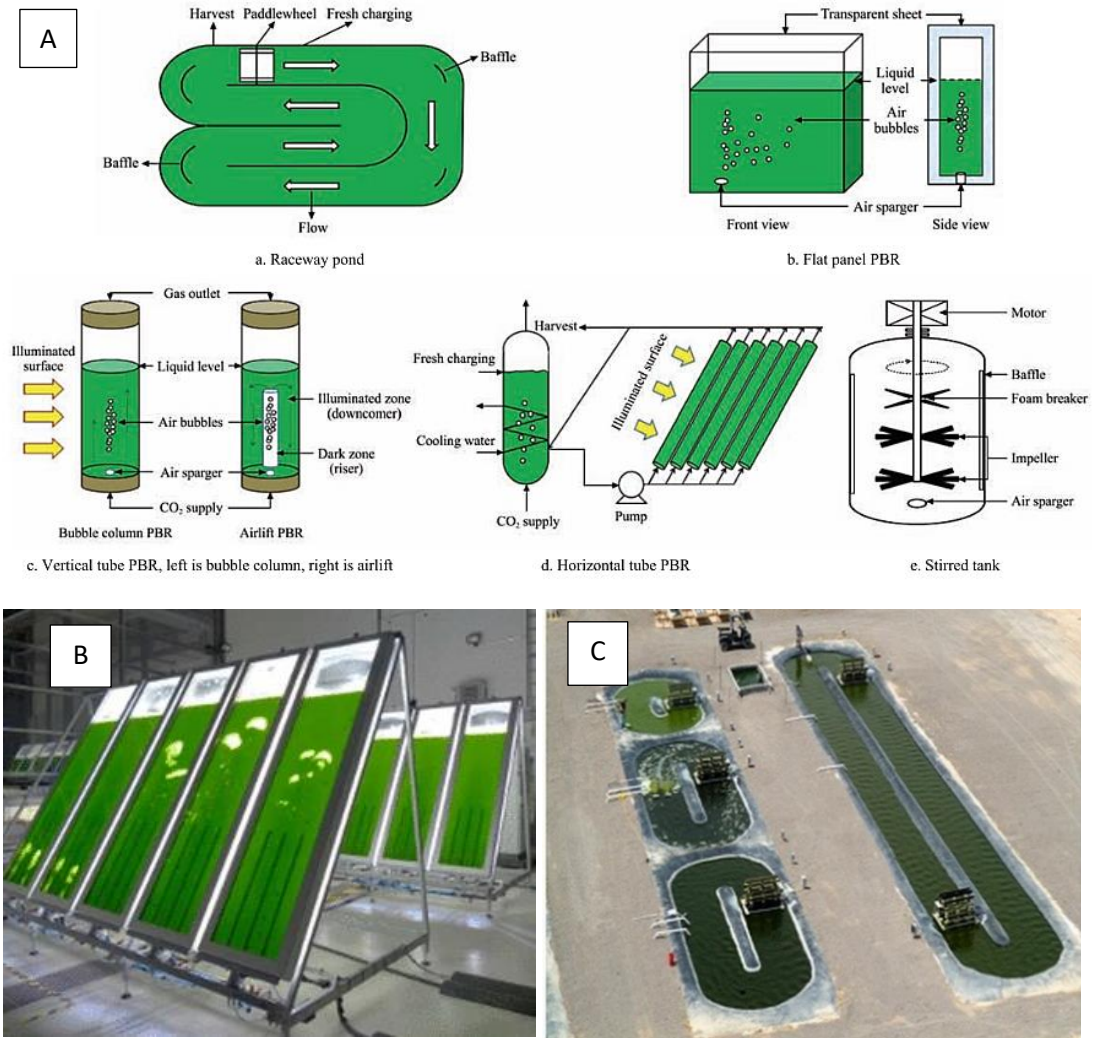


Figure 2.4 - A) Schematic illustration of closed bioreactors and open pond cultivation system functioning; B) flat panel bioreactor; C) open pond cultivation
(adapted from Ting *et al.*, 2017 [41]; Koller *et al.*, 2018 [42]); Lammers *et al.*, 2017 [43])

2.5 High value products and applications

Microalgae biodiversity is responsible for the wide range of products obtained and used in different fields and applications. Depending on the microalgal species, a different cellular composition makes an organism more suitable for specific metabolites extraction (Table 2.1). Furthermore, by changing cultivation conditions, the same organism can direct its metabolism towards slightly different sets of products [44].

Table 2.1 - Biochemical composition of some characterized strains
(adapted from Russel *et al.*, 2021 [44])

Species	Protein (w/w)	Carbohydrates (w/w)	Lipids (w/w)
Botryococcus braunii	40%	6%	33%
Chlorella luteoviridis	47%	12%	22%
Chlorella sorokiniana	56%	17%	22%
Haematococcus pluvialis	68%	9%	26%
Chlorella pyrenoidosa	57%	26%	2%
Chlorella vulgaris	58%	17%	22%
Scenedesmus obliquus	56%	17%	14%
Dunaliella salina	57%	32%	6%
Chlamydomonas reinhardtii	48%	17%	21%

One of the eldest microalgal applications is the direct use in the food field, practiced by ancient populations such as the Aztecs. Given the actual and rapid population growth, microalgae are taking on an increasingly prominent role as an unconventional food source due to the quality of nutrients and the relative ease of production with the approval of the US Food and Drug Administration (FDA). The species that dominate food sector markets are *Spirulina* and *Chlorella* spp. Microalgae are a valuable source of protein (over half of the entire biomass in some species), as well as essential amino acids. Microalgal lipids usually accumulate in the stationary phase of growth. Omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) are among the best known and investigated molecules for their beneficial effects on human health, in particular for the cardiovascular and immune systems. Among PUFAs, microalgae are rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [23,44,45].

Another advantageous use concerns the animal feed sector. Farmed fishes and animals can benefit from a diet with microalgae extracts, gaining an overall superior food quality. In particular, microalgae can replace the corresponding animal proteins in feed [21,44].

Other valuable molecules that can be found are sterols, vitamins, and pigments such as carotenoids. These molecules are interesting in the pharmaceutical field for their anti-inflammatory action, the modulation of cholesterol levels, the antioxidant activity. On the other hand, microalgal polysaccharides are known for interesting antiviral and antimicrobial activities. All these properties make the extracts and algal molecules interesting also in the cosmetic field [21,23,46,47].

Microalgae-derived compounds also find applications in the chemical industry. The algal pigments, such as the widely used phycocyanin (Figure 2.5), have proved to be good dyes, as well as an excellent alternative to the respective synthetic compounds used for the same purpose, often polluting and toxic to humans.

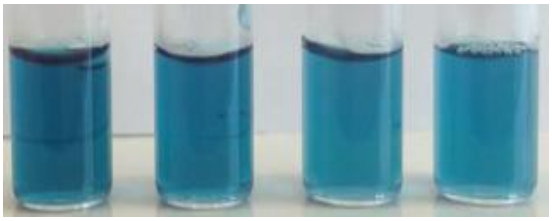


Figure 2.5 - Liquid phycocyanin extract from *Arthrospira platensis*
(adapted from Li *et al.*, 2020 [48])

Biomaterials obtained from algae are also of great interest. Bioplastics such as polyhydroxyalkanoates (PHA) have been obtained from microalgae although the process costs are still excessive. Similarly, algae have also been used in agriculture as a raw material for so-called biofertilizers. These compounds have proved effective and their use also has the dual advantage of reducing soil pollution [45].

Industrial applications include bioremediation. The ability of algae to grow also by exploiting the nutrients contained in wastewater has already been mentioned. Microalgae in different contexts have shown the useful ability to absorb toxic metal ions from processing water. Treatments of this type can prevent the release of these wastes into the environment and therefore having excessively harmful impact on ecosystems [49].

Although there are numerous technical challenges still to be addressed, microalgae are also the protagonists of the so-called "feedstock of the third generation" among the green energy options. Microalgae can represent sources of bioethanol, biodiesel, biogas and biohydrogen [50].

CHAPTER 3 – BIO-HYDROGEN FROM MICROALGAE

3.1.1 Biological hydrogen production in microorganisms

Currently, several microorganisms are known for their ability to produce H_2 as a metabolic feature. Yields and production rates are still far from being attractive for industrial applications, but this field of research has shown a notable development in recent years that is worth investigating further. The main bottlenecks that hinder the achievement of significant production levels are mainly related to the fact that the production of H_2 is a transitory phenomenon that occurs in particular situations. For example, microalgal hydrogenases perform their activity only in almost anoxic conditions. Furthermore, the presence of metabolic pathways that compete for the electrons that supply the H_2 - producing enzymes, further decreases the theoretical yield [51].

Microalgae and cyanobacteria are among the best-known producers. Regarding microalgae, most of the information available to date comes from the *C. reinhardtii* model. As detailed in the next sections, pathways associated with the photosynthetic electron transport chain have been highlighted, and, to a lesser extent, even an activity linked to fermentation-type metabolism [52,53].

Cyanobacteria have long been included among algae because for the similar morphology and metabolic activities, mostly based on the photosynthetic process. In addition, these microorganisms perform nitrogen fixation *via* nitrogenases. This enzyme is mainly responsible for the production of H_2 in these organisms although the efficiency does not reach that of microalgal hydrogenases.

Other relevant bacterial species are non-oxygenic photosynthesis bacteria. The Purple Non-Sulfur Photosynthetic (PNSP) bacteria are among the most efficient in this group and the production is linked mainly to nitrogenase activity. These bacteria exploit light to produce energy in the photo-fermentation process, but, at the same time, there is no

production of oxygen. Interacting with light, organic acids, such as butyrate, become an electrons source, which is also available for H_2 production.

Heterotrophic bacteria operate different fermentative metabolisms starting from sugars. In some cases, they lead to a wide range of products which also include H_2. The process is also called dark fermentation H_2 production. Anaerobic bacteria such as *Clostridium* spp. or facultative anaerobes such as *Escherichia Coli* are among the most investigated groups [54].

3.1.2 Biological hydrogen production in microalgae

The discovery of H_2 production in microalgae dates back to 1939. The German physiologist Hans Gaffron (1902-1979) conducted studies on microalgal photosynthesis and observed a transient H_2 production that alternated with the oxygen evolution [55]. Indeed, production occurs as a biological response to several conditions characterized by low oxygen levels as illustrated in the next paragraphs.

3.1.2.1 Photosynthesis and electron transport chain

In microalgae, photosynthesis occurs in a similar way to higher plants. The process takes place at the level of the thylakoid membranes in chloroplasts. The first phase is the so-called light-dependent phase. The protagonists of this first set of reactions are various protein complexes along which a flow of photosynthetic electrons is generated: photosystem II (PSII), plastoquinone (PQ) pool, cytochrome b6f complex (Cyt b6f), and photosystem I (PSI) (Figure 3.1). The flow of electrons is triggered thanks to the numerous photosynthetic pigments that form the light-harvesting complexes I and II (LHCI and LHCII) at the level of the photosystems (PSs). The electrons leave the chain via PSI and convey to the ferredoxin (Fd). In physiological conditions, they are involved in carbon fixation through the transfer to ferredoxin-NADPH oxidoreductase (FNR) to produce NADPH.

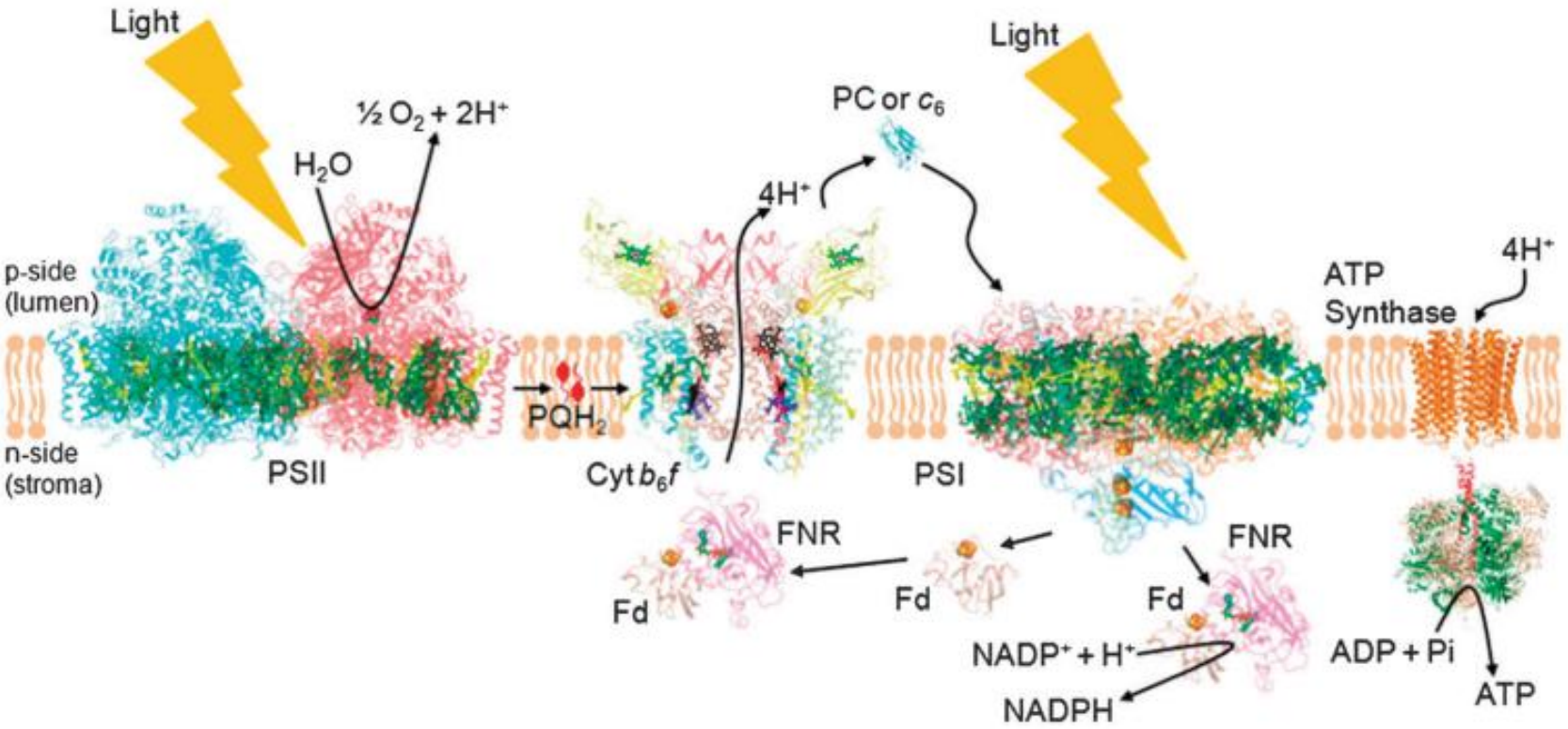


Figure 3.1 - Photosynthetic electron chain proteins
(Cytb6f, cytochromeb6f; Fd, ferredoxin; FNR, ferredoxin–NADP+–reductase; PC, plastocyanin; PSII and PSI, photosystems I and II adapted from Hasan and Cramer, 2012 [56])

In the second dark phase, ATP and NADPH molecules are used to fix CO_2 and obtain carbohydrates. The pivotal enzyme of this phase is RuBiSco, namely ribulose-1,5-bisphosphate carboxylase/oxygenase, the most abundant protein on Earth. The process cyclically requires a series of reactions (Figure 3.2). First, the reduction of CO_2 occurs thanks to the Rubisco enzyme and the combination with the substrate ribulose-1,5-bisphosphate (RuBP), with the consumption of one water molecule. It leads to the subsequent formation of an unstable 6-carbon compound, which splits into two molecules of 3-phosphoglycerate (3-PG). Then, ATP and NADPH favour their reduction in glyceraldehyde-3-phosphate (G-3-P). Finally, part of the latter molecules is reconverted into RuBP to allow the cycle to continue while the residual amount is used to form glucose molecules [56–58].

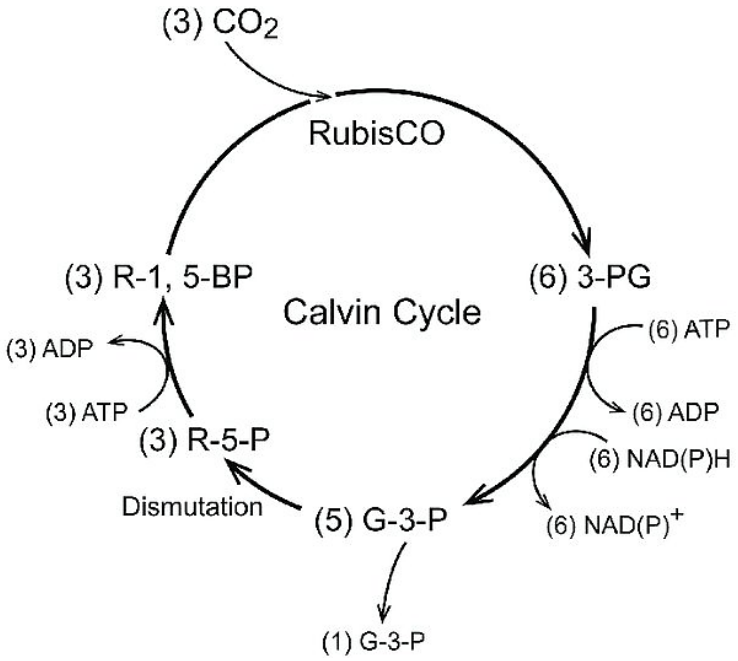


Figure 3.2 - Schematic representation of CO_2 fixation pathway, also known as Calvin cycle (adapted from Wilson *et al.*, 2021 [58])

3.1.2.2 Direct bio-photolysis

The direct bio-photolysis process is also referred to as PSII dependent pathway. It provides a linear electron transfer (LET) from PSII and PSI, after PSII light excitation. Nearby PSII, the release of electrons occurs thanks to the water-splitting process. For these electrons to be directed towards H_2 production, a decrease in oxygen levels is necessary.

In hypoxic state, the electrons arriving at PSI are transferred to Fd and finally to the enzyme hydrogenase. Overall, the use of 8 photons that interact with the PSs are necessary to form two H_2 molecules.

Direct bio-photolysis occurs simultaneously with other pathways that lead to H_2. To determine the contribution deriving from the activity of the single PSII dependent pathway, the DMCU, 3- (3,4 - dichlorophenyl) -1,1- dimethylurea, an herbicide that specifically inactivates the PSII, can be used. Although the process is conditioned by the experimental variables and the algal species, the flow of electrons fed by the activity of PSII is responsible for the production of about 80% of the total H_2 photo-production.

The main obstacle of this process is linked to the water-splitting that produces together with protons and electrons also the O_2 which is normally coupled to the photosynthetic process [59,60].

3.1.2.3 Indirect bio-photolysis

The indirect bio-photolysis process is also referred to as PSII independent pathway. In this case, the electron flow reaches the hydrogenase starting from the non-photochemical reduction of the PQ pool. This step occurs in *C. reinhardtii* thanks to a type-II calcium-dependent NADH dehydrogenase type II (NDA2). The reductants in circulation, on the other hand, come from the glycolytic degradation of glucose or starch. Specifically, the process is not related to PSII activity or O_2 production. However, it has a lower overall yield than direct bio-photolysis.

The main underlying reason is related to the presence of numerous metabolic competitive routes (e.g., CEF, Cyclic Electron Flux, as detailed below) that subtract reductants from the hydrogenase pathway [59,61–63].

3.1.2.4 Hydrogenase enzyme

Hydrogenases are enzymes of the oxidoreductase family, involved in a reversible reaction between protons, electrons, and molecular hydrogen, as stated in the equation below:

$$2H^+ + 2e^- \leftrightarrow H_2$$

The catalytic site can have several metal ions: this is a criterion commonly adopted for their classification. Nickel-Iron [NiFe] hydrogenases are more widespread, being present in cyanobacteria, archaea, and anaerobic bacteria. In [NiFe] hydrogenases there is a greater tendency to H_2 uptake. The enzyme typically present in green microalgae is a di-iron [FeFe] hydrogenase (Figure 3.3). In [FeFe] hydrogenase there is a more marked tendency towards H_2 production rather than its consumption [52,64].

Most of the information from microalgal hydrogenases has been obtained from the model alga *C. reinhardtii*. Two genes encode the two different isoforms of the enzyme: *hydA1*, the major contributor, and *hydA2* [65,66]. Thanks to a specific transit peptide they reach the chloroplast, where they perform their function [67]. The maturation process is driven by proteins encoded by the *HydEF* and *HydG* genes to obtain a final 47-48 kDa protein [68–70]. The transcription process and the enzymatic activity are strongly inhibited by oxygen presence. Loss in activity is due to the inactivation of H cluster, the catalytic site that consist of highly sensitive [4Fe4S] unit cysteine-linked to a di-iron unit that receives electrons via Fd [71–74].

Other studies have investigated, albeit to a lesser extent, the same activity in other green microalgae. In the species *Scenedesmus obliquus* and *Chlorella fusca*, various analyses confirmed the presence and enzymatic activity of a hydrogenase similar to that of *C. reinhardtii* by molecular weight and catalytic site [75–77].

Studies on the *Chlorella variabilis* species have instead highlighted a peculiarity in the same enzyme. Sequencing data suggested the presence of a [FeS] cluster-binding domain, or “F cluster”, not currently described in any other alga. The interactions between the hydrogenases of this species (CvHydA1) and Fd have highlighted another newsworthy feature. *In vitro* studies have shown a poor interaction between the enzyme and a plant-type ferredoxin (PetF) but a greater affinity with a 2 [4Fe-4S] bacterial

ferredoxin type. This evidence may indicate that this enzyme is the oldest from an evolutionary point of view [78].

A different evolutionary history could be at the basis of adaptation to anaerobiosis, that induces different patterns of enzymatic activity in hydrogenase. In *Chlamydomonas moewusii, in vitro* tests showed hydrogenases capable of rapid activation, while in *Lobochlamys culleus* pulsed light activates the enzyme, whose activity in vivo does not reach considerable levels [79].

There is a smaller amount of information on marine-species hydrogenases. In species such as *Chlorococcum littorale* or *Tetraselmis subcordiformis*, what has already been shown for *C. reinhardtii* is confirmed, while in the species *Tetraselmis kochinensi*, a [NiFe] hydrogenase seems to be responsible for H_2 production in anaerobic conditions [80–83].

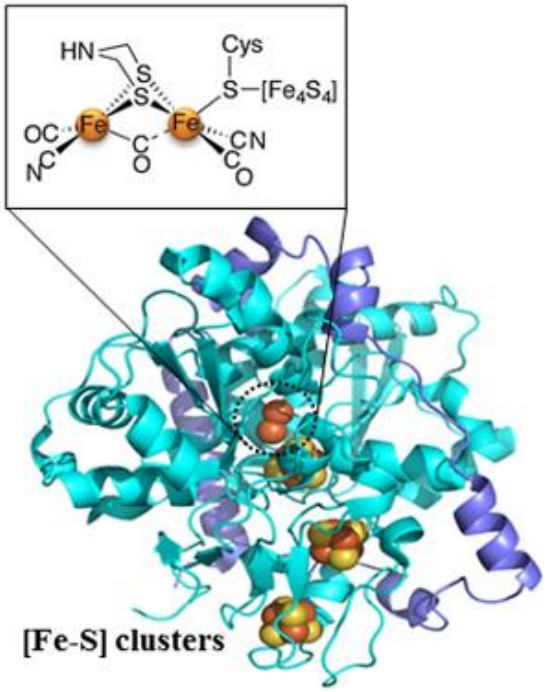


Figure 3.3 - [FeFe] Hydrogenase
(adapted from Khetkorn *et al.*, 2017 [52])

3.1.2.5 Fermentative metabolism

In dark anoxic conditions, it is possible to have a H_2 production linked to a fermentative-type metabolism from the degradation of starch reserves. In *C. reinhardtii* this production is very scarce but it is not entirely negligible. Nevertheless, this process could be crucial in responses needed by the alga to maintain a redox balance useful for survival during prolonged anaerobiosis. In general, the use of stored carbohydrates through fermentative pathways is a common way to produce ATP molecules and sustain life cell processes.

The reduction of Fd is in this case carried out by the electrons produced by the decarboxylation of pyruvate to acetyl CoA by means of the algal enzyme pyruvate ferredoxin oxidoreductase (PFR) (Figure 3.4).

In the fermentation processes, H_2 and CO_2 are in any case produced in lower quantities than other fermentation products, such as formate, acetate, and ethanol [53,59,84,85].

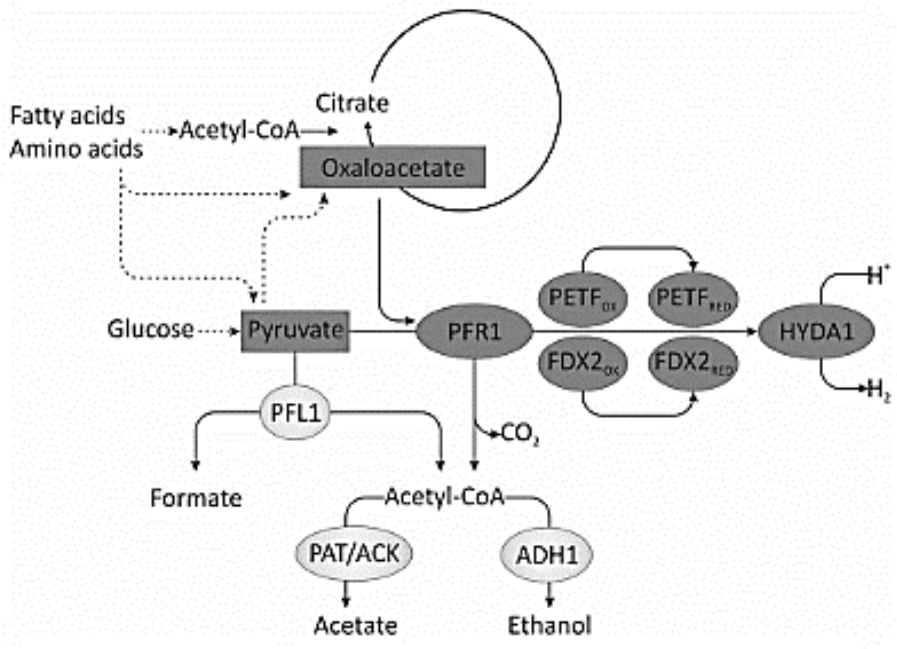


Figure 3.4 - *C. reinhardtii* fermentative pathway occurring in dark anoxic condition (With PETF, plant type ferredoxin PETF/FDX1, PFL1, pyruvate formate lyase 1, PAT/ACK, phosphotransacetylase and acetate kinase pathway, ADH, acetaldehyde-alcohol dehydrogenase; adapted from Noth *et al.*, 2013 [53])

3.2 Strategy to improve the production

As previously stated, to favor the H_2 production one of the main requirements is the creation of an anaerobic or hypoxic environment. In general, this occurs when photosynthetic activity slows down or if there is an increase in mitochondrial respiration rates. Another important point concerns the metabolic activity of the chloroplast. In this organelle, in conjunction with the H_2 production, the flows of protons and electrons should be directed preferentially towards the hydrogenase and H_2 evolution pathways.

3.2.1 Physiological approaches

3.2.1.1 Depletion of macronutrients and carbon sources

Concerning physiological conditioning, the approaches tested are mainly based on the depletion of some macronutrients in the algal cultivation media [86].

In such a condition, the CO_2 fixation process slows down as well as the anabolic metabolic reactions. An increase in lipid and carbohydrate (starch) reserves is generally observed. If necessary, the latter can be mobilized and directed towards the chloroplast. The increased traffic of reductants represents a good possibility for H_2 production too. A non-photochemical reduction of the PQ pool is established, which increases the activity of the PSI. At the same time, the load on PSII decreases, and the photoproduction of oxygen decreases [87,88].

Melis and collaborators identified S (sulphur) deprivation as a promising strategy for producing H_2 [89]. By applying this starvation, a multitude of effects (Figure 3.5) are recorded on different cellular activities [90]. Among the most important, a decrease in PSII activity and O_2 levels is obtained. Mitochondrial activity, as well as activity at the PSI level, does not undergo variations. In *C. reinhardtii,* this biochemical behaviour is supported by the starch reserves. Within three days, anaerobiosis develops in the presence of saturated light [90]. This phenomenon is transitory and, by supplying sulfur again, the culture PSII restores, but 30-40% in its activity is irremediably lost [91].

To assess the contribution of PSII to H_2 production DMCU was used. A reduction of 70% in H_2 production occurs when PSII is not functional. The remaining 30% is attributable to the PSI [63,92].

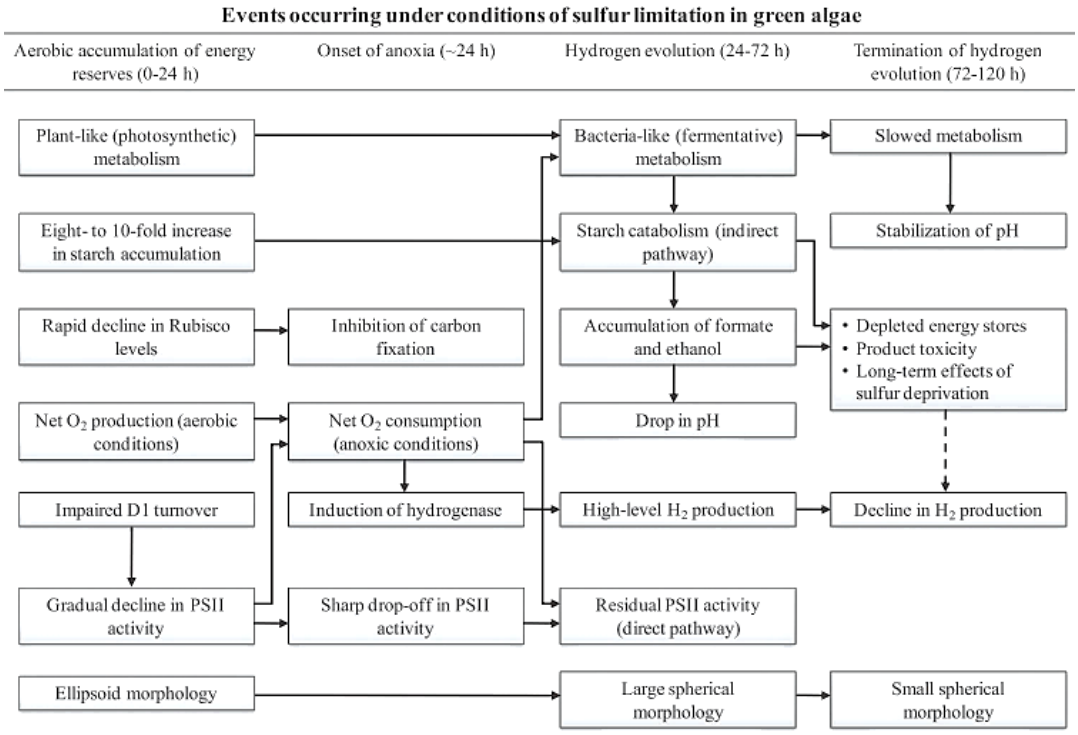


Figure 3.5 - S deprivation effects in a time scale
(adapted from Srirangan *et al.*, 2011) [90]

The N (nitrogen) deprivation strategy also affects H_2 production. There is a decrease in photosynthetic activity and there is an even more marked increase in the reserves of lipids and starch than in S deprivation. Anyway, the production is delayed in time and the overall yields are scarcer. In microalgae *Scenedesmus obliquus*, this strategy has often been explored to direct metabolic activities towards the lipid production and accumulation rather than H_2 production [93]. Specifically, DMCU does not create any effect in N deprivation strategies: production may be PSI-dependent. Changes in starch reserves do not produce significant effects. Protein degradation would appear to contribute more in providing equivalents [94].

Also, a decrease in oxygen levels is observed in phosphorus (P) deprivation too. Although more slowly than in S deprivation, it leads to the production of H_2. This type of strategy has been applied in some marine species of *Chlorella* spp. The use of culture media seawater-like makes difficult to apply other strategies such as sulfur deprivation. However, the presence of cellular reserves, such as polyphosphates, makes difficult to apply an effective phosphorous starvation. A potential solution could be the use of very dilute cultures with low starting cell and phosphorus concentrations. In this process, the

mobilisation and consumption of starch reserves also increase during the H_2 production phase. The respective contributions of PSII and PSI in the process are not known to date [95].

Deprivation of other elements has been poorly investigated. Among the reported, the absence of elements such as potassium, copper, manganese and magnesium was evaluated. For the latter, significant H_2 production has been reported in *Chlamydomonas*, but the molecular reasons behind it remain to be elucidated [86,96].

Overall, the effects related to N-deprivation are much more lethal than S-deprivation by involving multiple cell processes simultaneously. In the case of deprivation from S, the effects mainly concern PSII. Photosystem protein D1degradation is responsible for the collapse of its activity. However, this response may not simply be due to damage or simply stress. Protein D1 is degraded by the FstH protease and could be the result of an adaptive response to anaerobiosis rather than a phenomenon resulting from effective damages. An interesting fact emerged from transcriptome analysis which highlighted the physiological changes induced by sulphur deprivation. One transcript, LHCBM9, was 1000-fold more expressed in the above conditions but not in replete medium condition. It has been observed that, in mutants that do not express this transcript, cells show greater suffering from oxidative stress [97–102].

Most of the information relating to the impact of the composition of the medium on H_2 production derives from *C. reinhardtii* and consequently by using acetate as a carbon source. Some studies used autotrophic conditions (Figure 3.6), but yields were lower than the corresponding heterotrophic ones.

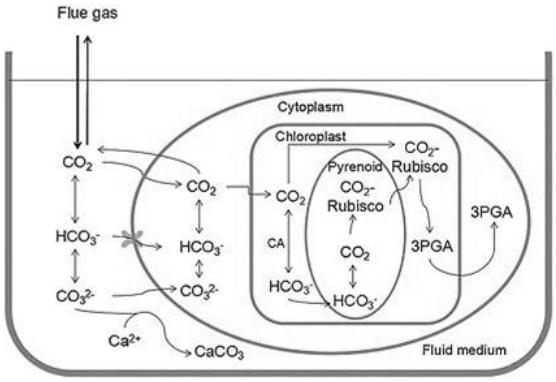


Figure 3.6 - Inorganic carbon uptake in microalgae
(adapted from Chen et al., 2017 [103])

Acetate in a sealed culture can promote anoxia in low light levels. In general, the presence of acetate causes PSII activity to decrease as well as oxygen levels and CO_2 fixation. Instead, mitochondrial activity is stimulated. In dark conditions, on the other hand, the presence of acetate has no influence.

The acetate is assimilated by the tricarboxylic acid cycle (TCA) and the glyoxylate cycle (Figure 3.7). So, it is incorporated as CO_2 into lipids and carbohydrates, while H_2 is released. Following the onset of anaerobiosis and the cessation of acetate assimilation, starch reserve begins to be mobilized and H_2 production is more sustained. Acetate likely supports H_2 production via PSI by reducing equivalents from starch. Furthermore, it is not excluded that it also plays a role at the level of the fermentation route [53,59,89,104,105].

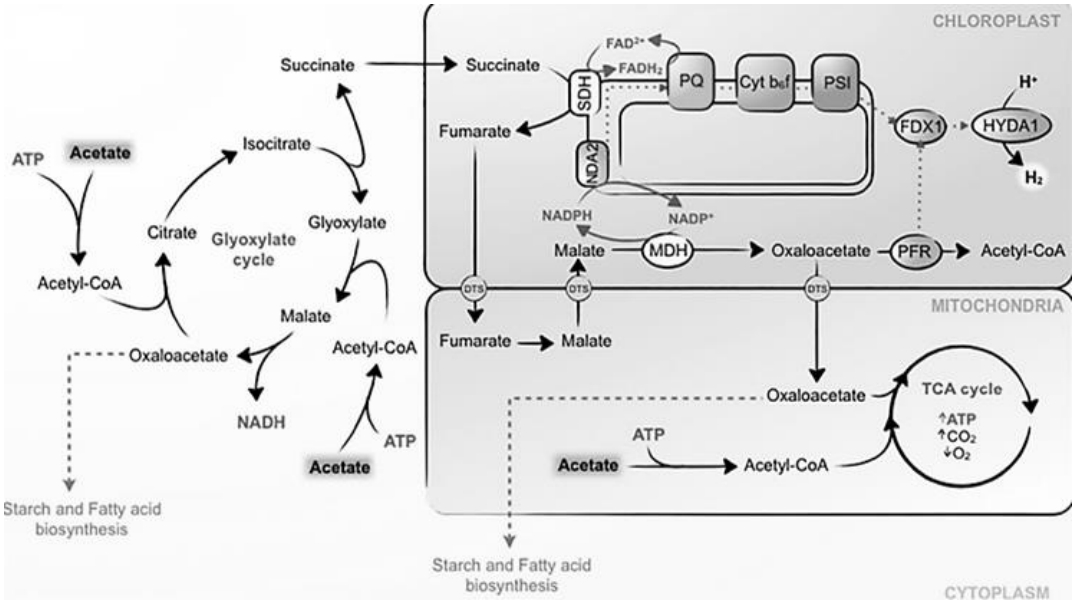


Figure 3.7 - Acetate uptake in *C. reinhardtii*
(adapted from Jurado-Oller et *al.*, 2015 [106])

Considering a major metabolic versatility, different carbon sources were tested in other microalgae. Several microalgae, such as *Chlorella pyrenoidosa* or *Scenedesmus obliquus*, have demonstrated efficient use of glucose (Figure 3.8) which, added to the culture medium, has been shown to support the production of H_2 [93,107,108].

Albeit to a lesser extent, further carbon sources have also been experimented with *Chlorella* spp. strains recently isolated. Although acetate and glucose tend to show the

best yields, good results have also been obtained from sucrose and fructose. They had a positive impact on the volume and rates of H_2 production, respectively. Inorganic carbon sources, such as sodium bicarbonate or potassium carbonate, have not yielded interesting production levels [109–111].

However, the metabolic pathways involved in the use of the different carbon sources and the impact of the other parameters used in the production process on the overall yields remain to be elucidated.

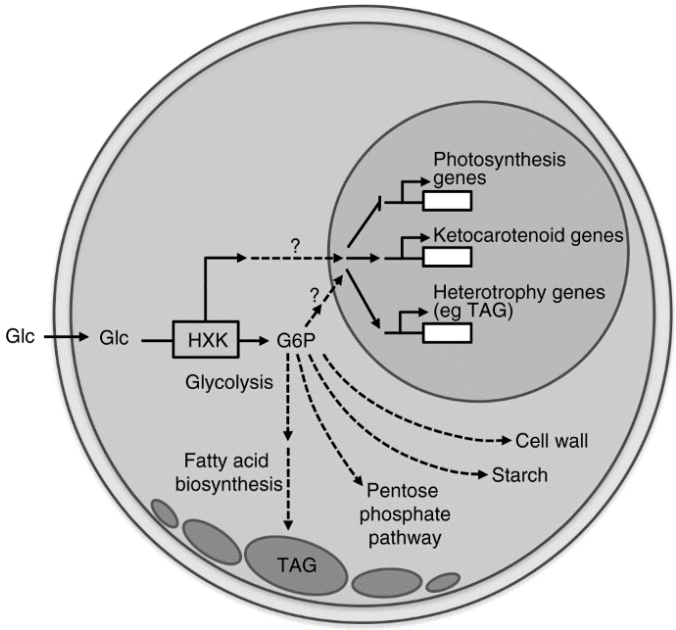


Figure 3.8 - Model of glucose uptake through algal hexokinase (HXK) enzyme (adapted from Roth *et al.*, 2019) [112]

3.2.1.2 Immobilization systems and photobioreactors

Acting on the cultivation parameters allows to obtaining relatively fast responses from the cells. Nonetheless, several operational problems still need to be addressed.

For example, using media without elements, such as S, N, P, implies that the culture undergoes several centrifugation/washing steps to remove the old complete culture medium and replace it with the new one. In overcoming this issue, various strategies have been tried. Regarding S deprivation, incomplete starvation of the culture medium has been tried using lower concentrations of the S compounds. In this way, the deprivation is simulated without transferring the biomass into different media [113,114].

To better manage any transfers of cells in culture, the use of different cell immobilization systems has been tested. Among the various attempts made, mainly in *C. reinhardtii*, thin alginate films, fiberglass matrix, or, recently, sodium alginate beads have tested [115–117]. In the latter case, the sodium alginate spheres reach diameters of just a few millimetres (Figure 3.9).

However, these new strategies raise the question of having to use more appropriate reactors than the classic ones used for liquid cultures.

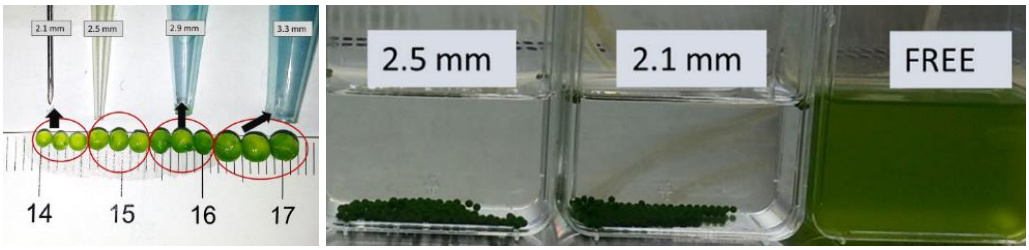


Figure 3.9 - Microalgal beads used for hydrogen production
(adapted from Canbay *et al*, 2018 [117])

Finally, the characteristics of the photobioreactors are also of great importance. Almost all of the scientific constructions related to the production of H_2 from microalgae are based on closed systems. In these systems, all growth parameters are carefully monitored over time. In addition, gas leaks are avoided in this way.

Tubular, vertical column and flat-panel bioreactors have been investigated for H_2 production. The flat system seems to be the best solution providing the highest surface/volume ratio [118–120]. Fifty litres horizontal tubular photobioreactors (Figure 3.10) were utilised in the only attempt made to produce H_2 outdoors using sunlight, *C.*

reinhardtii and sulfur deprivation: lower yields were obtained compared to laboratory ones [121].

However, this production methods do not solve all technical issues, and this area of research still faces numerous challenges in order to help this research field in achieving the scale-up level.

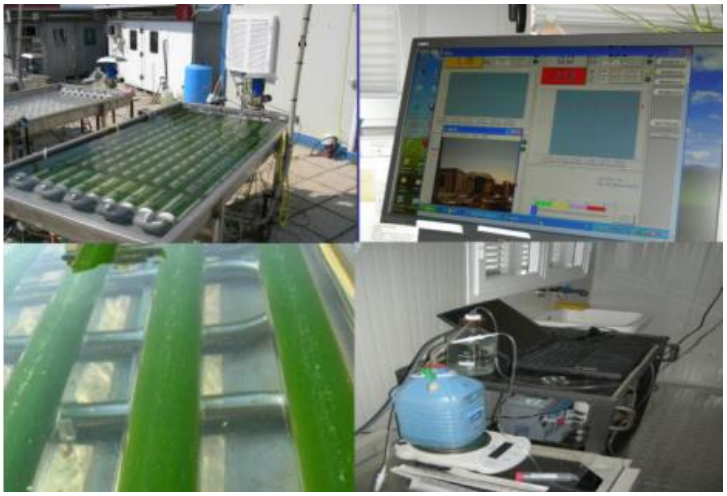


Figure 3.10 - Outdoor microalgal hydrogen production
(adapted from Touloupakis *et al*., 2021 [122])

3.2.2 Molecular approaches

The strategies described so far do not represent a permanent solution to the improvement of H_2 production, as long-term stresses culminate in suffering or cell death leading to overall yields that are not advantageous.

Different molecular tools can be adapted to direct the algal metabolism in a specific direction. However, similar approaches can only be used where it is possible to have a thorough knowledge of the organism under study. Furthermore, other problems to be addressed are related to the fact that the changes are not always well tolerated within the recipient organism.

The model algae *Chlamydomonas* has represented an excellent example for the development of different molecular strategies, which have made possible to extend the production of H_2 by reducing the effects of the barriers that otherwise are an obstacle to a more conspicuous production [123].

3.2.2.1 Random mutagenesis and clone screening

The first approach concerns the use of mutagenic agents, physical or chemical. Mutations can lead to the onset of new characteristics compared to the wild-type organism. Since this approach does not have a defined target, a subsequent screening and study phase is necessary. The mutants of interest must be selected based on the physiological parameters of interest. Subsequently, molecular and biochemical investigations help in understanding the newly acquired biological behaviour.

In a recent approach, *Chlamydomonas* mutants were obtained through atmospheric and room temperature plasma (ARTP) mutagenesis. First of all, mutants with a light green colour, indicating a lower chlorophyll content compared to wild types, were selected. Transcriptomic analyses, together with physiological assessments, confirmed a significant H_2 production. It is observed that a reduced amount of chlorophyll translates into efficient photosynthetic performances, and consequently H_2production because both the light transmittance and the conversion of solar energy into chemical energy are probably improved [124].

However, the success of a mutagenesis process is closely linked to the screening phase of the generated clones. Otherwise, the selection of phenotypes that produce the

highest H_2amount from thousands of clones, risks to being a time-consuming and costly process. Among the first attempts, chemo-chromic screening systems were based on substances that, in the presence of H_2, change colour. For example, a tungsten oxide film undergoes a reduction process and even changes colour when in contact with H_2 [68,125]. A more recent and sophisticated system is based on the photosynthetic bacterium *Rhodobacter capsulatus*. A green fluoresce reporter protein, upstream of the bacterial hydrogenase uptake promoter, is capable of emitting a fluorescence signal if nearby algal cells produce H_2 [126].

3.2.2.2 Targeted mutagenesis

Specific approaches were used to overcome the main bottlenecks of H_2 production process. One of the limitations is related to light saturation. Green algae can use solar energy thanks to the chlorophylls of the PSs. In particular, the spectral region exploited is between 400 and 700 nm, also called photosynthetically active radiation (PAR). This means that only one part of the solar energy can be exploited by the light-receptive algal components. The light energy involves excitation of the photosystems which results in a separation of charge at the level of the photosystems, but this mechanism is unable to fully exploit all the energy: most of it is dissipated by the emission of fluorescence and heat (Figure 3.11). All these phenomena contribute to an effective use of solar radiation of only 2% [59].

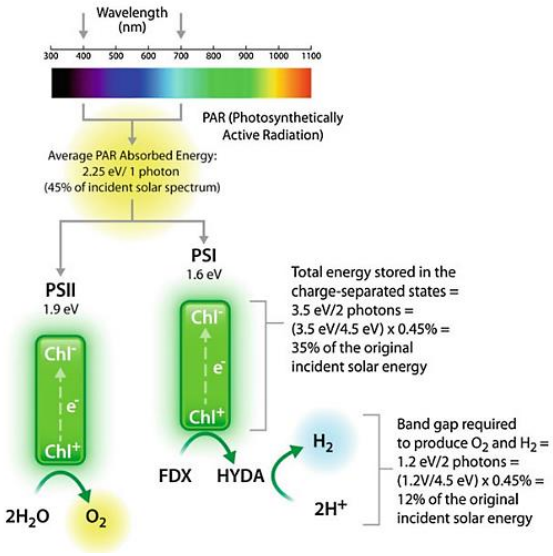


Figure 3.11 - Transformation of solar energy into chemical energy trough PSs
(Adapted from Dubini and Gonzalez Ballester, 2016 [59])

Furthermore, in high-density cultures, most exposed cells absorb more light, but dissipate more too. Conversely, darker cells are less able to absorb light and, therefore, convert solar energy. A solution to this problem came from mutants with the truncated light-harvesting antenna (tla) complex. An antenna of reduced dimensions is subject to fewer phenomena of photoinhibition and light saturation. Regarding the production of H_2, *C. reinhardtii* mutant tla1 CC-4169, with a light intensity of 350 μE m^{-2} s^{-1}, has been shown to produce up to six times more H_2 than the wild type strain [127,128]. Furthermore, the simultaneous knock-down of three light-harvesting complex proteins (LHCBM 1, 2, 3) similarly contributed to increase photosynthetic production of H_2 [129].

Another limitation is linked to the formation of a proton gradient in anaerobic conditions. Indeed, over-reduction of the PQ pool and the consequent displacement of LHC proteins from PSII to PSI favours the entry of electrons in the CEF circuit (Figure 3.12). This pathway stimulates the synthesis of ATP and, at the same time, the formation of a proton gradient. It affects photosynthetic efficiency removing electrons from the Fd - hydrogenase axis. Pgrl1 (protein gradient regulation like 1) mutant showed improved H_2 production as it was engineered to lack the CEF-mediating protein. In this, fewer electrons are removed from the hydrogenase [130].

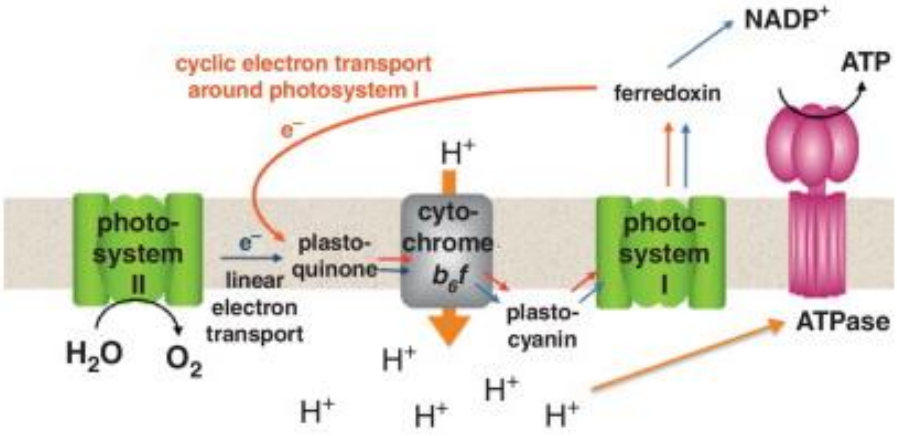


Figure 3.12 - Linear electron transport and cyclic electron transport
(adapted from Shikanai, 2014 [131])

A further obstacle is represented by the competitive path that directs the photosynthetic electrons towards the fixation of CO_2.

A mutant was obtained through a fusion protein between ferredoxin and hydrogenase, to direct electrons preferentially towards this latter. It showed improvements in H_2 *in vivo* production and oxygen tolerability [132]. Again, with the intent of hindering CO_2 fixation, several approaches have targeted its key enzyme, rubisco. In mutants with reduced enzyme levels or altered subunits, there were increases in H_2 production [133,134].

Mutants of *C. reinhardtii*, knock-out for the flavodiiron proteins (FDPs), also showed increased photoproduction of H_2. Once again, it is the elimination of a competing pathway (the FDP-mediated O_2 photoreduction pathway) that directs the traffic of electrons towards the hydrogenase. Furthermore, these mutants exposed to prolonged light pulses do not direct the metabolism towards CO_2 fixation. This proves how genetic engineering and physiological conditioning approaches synergistically produce beneficial results [135].

To add a new electron source usable by hydrogenases, mutants of *C. reinhardtii* were engineered to have an additional protein for hexose uptake as naturally happen in the microalgae *Chlorella kesslerii*. This has allowed these mutants to utilize the otherwise un-metabolized glucose [136].

Another relevant issue is the oxygen sensitivity of the hydrogenase enzyme. A double amino acid substitution (L159I - N230Y) at the level of the D1 protein of *C. reinharditii* gave the mutant several new characteristics, including greater oxygen tolerance than the wild strain [137]. The use of an O_2-tolerant clostridial [FeFe]-hydrogenase, expressed in *C. reinharditii*, showed better H_2 production performances, as the bacterial hydrogenase had lower inactivation rate in aerobiosis [138]. Another solution instead sees the insertion of metabolic pathways and related enzymes such as pyruvate oxidase from *Escherichia coli* which metabolizes oxygen favouring the establishment of an anoxic environment [139].

3.3 Future perspectives

Among the most recent molecular strategies, there is the use of non-coding but regulatory RNA molecules such as microRNA (miRNA) or long non-coding RNA (lncRNA). In particular, miRNAs can modulate the translation process by binding or degrading messenger RNA. Several studies have pointed out that these molecules accumulate in microalgae in stressful situations to ensure immediate responses in the cells [140]. These observations led to the design of several artificial miRNAs (amiRNAs) to stimulate H_2 production, making oxygen consumption faster or repressing the expression of the *psbA* gene that encodes the PSII D1 protein [141,142]. Other promising tools are the optogenetic systems that use blue light-inducible expression amiRNAs, enhancing H_2 production [143].

Molecular strategies require careful planning as well as considerable resources. Support tools also include computer resources and modelling that allow prediction of responses of an organism to varying cultivation parameters or by engineering certain metabolic pathways. This is undoubtedly a further incentive to persevere in this area of research [144,145].

Recently, *Chlamydomonas* and bacteria co-cultures have provided new insights to improve biohydrogen production that could also benefit from waste. Bacterial metabolism produces acetic acid from sugars, while, algae consume the excreted bacterial acetic acid. This approach highlights the integration of photobiological and fermentative H_2 production [54,146].

In conclusion, Table 3.1 summarizes the main and most effective strategies adopted to produce biohydrogen from microalgae.

Table 3.1 - Highest H_2 production achieved by *C. reinharditii* with different approaches (adapted from Fakhimi *et al*., 2020 [54])

Strategy	**Parental alga strain**	**Mutant strain**	**Condition**	**Reported H_2 production**	**Estimated H_2 production (mL/L)**	**Estimated average H_2 production rate (mL/L·d)**
Monoculture/Genetic modification/S deprivation	cc124	pgrl5	TAP-S, 60 PPFD	850 mL/L (9 days)	850	≈94.4
Monoculture/Genetic modification/S deprivation	cc1618	stm6	TAP-S, 100 PPFD	540 mL/L (14 days)	540	≈38.6
Monoculture/Genetic modification/S deprivation	11/32b	L159I-N230Y	TAP-S, 70 PPFD	504 mL/L (12 days)	504	≈42
Monoculture/Genetic modification/S deprivation	137c(cc124)	pgrl1	TAP-S, 200 PPFD	≈1.5 mmol/mg chl (5 days)	≈437	≈87.4
Monoculture/Genetic modification/S deprivation	cc1618	Stm6Glc401	TAP-S + 1 mM glucose, 450 PPDF	361 mL/L (≈8 days)	361	≈46
Consortia/*Pseudomonas* sp. /S deprivation	FACHB-265	–	TAP-S, 200 PPFD	170.8 mL/L (13 days)	170.8	13.1
Consortia/*Bradirizhobium japonicum*/S deprivation strain	cc849	Transgenic lba strain	TAP-S, 60 PPFD	298.54 µmol/40 mL (14 days)	≈170.5	≈11.95
Consortia/*Bradirizhobium japonicum*/S deprivation strain	cc503	–	TAP-S, 200 PPFD	310 µmol/mg chl (16-days)	≈164.9	≈10.3
Monoculture/S deprivation	137c (cc125)	–	TAP-S	≈155 mL/L (≈4 days)	≈155	≈38.75
Monoculture/Mg deprivation	137c (cc125)	-	TAP-Mg, 80 PPFD	6.3 mmol/L (≈8 days)	≈141.1	≈16.9
Monoculture/S deprivation/acetate free	UTEX 90 (cc1010)	-	T(A)P-S3, 50 PPFD, N_2 purging	118 mL/L (4.5 days)	118	26.2
Monoculture/O_2 scavenging	cc503	-	TAP + $NaHSO_3$, 200 PPFD	≈150 µmol/30mL (3 days)	≈112.05	≈37.3
Monoculture/Genetic modification	cc849	*hemHc-lbac*	TAP-S, N2 purging, dark incubation, 50 PPFD	3.3 mL/40 mL (5 days)	82.5	16.5
Monoculture/Light modulation	cc124/cc4533	–	TAP, 1 s light pulses (180 PPFD) + 9 s dark periods under Argon atmosphere	3.26 mmol/L (2.25 days)	≈73.06	≈32.5
Monoculture/acetic acid supplementation/ Light Modulation	704	–	TAP + acetic acid supplementation, daily aeration, 12 PPFD	65 mL/L (9 days)	65	≈10
Consortia/*E. coli* (*hypF*)/S deprivation	cc124	–	TAP-S, 50 PPFD	47.2 mL/L (7 days)	47.2	6.75

CHAPTER 4 - OBJECTIVES OF THE RESEARCH

Nowadays, hydrogen is considered a promising fuel due to CO_2-free combustion. Currently, several microorganisms are known for H_2 production as a metabolic feature, even if transiently or after harmful stresses application.

Regarding microalgae, most of the information related to H_2 production comes from the model organism *Chlamydomonas reinhardtii*. The need to extend the production and realise a feasible industrial scale-up has led to further investigating the microalgal H_2 bioproduction.

The present PhD project was aimed primarily at the isolation and characterization of microalgal species in the Basilicata region for the identification of new biohydrogen producers. Particular attention has been given to the search for strains with good growth rates and able to use different carbon sources.

Secondly, the physiological behaviour of single and double mutants of *Chlamydomonas* was analyzed concerning H_2 production by modulating light conditions without applying stress conditions.

CHAPTER 5 – MATERIALS AND METHODS

5.1 Microalgae

5.1.1 Freshwater sample for microalgae isolation

Freshwater samples for microalgae isolation were collected in the Basilicata region, Potenza (PZ) district. The sampling took place between November and December 2018 in the villages indicated in the Table 5.1 with the relative geographical coordinates. Samples were stored at room temperature before isolation in the laboratory.

Table 5.1 - Freshwater sample collected

Villages	Coordinates	Sampling Date
Atella (PZ)	40°53'21.9"N 15°38'24.2"E	November 2018
Maratea (PZ)	40°00'02.8"N 15°42'43.7"E	November 2018
Potenza (PZ)	40°40'06.5"N 15°47'28.4"E	December 2018
Rapone (PZ)	40°51'53.9"N 15°30'16.8"E	November 2018
San Chirico Nuovo (PZ)	40°40'47.1"N 16°04'47.3"E	December 2018
Trivigno (PZ)	40°34'43.5"N 15°58'56.8"E	December 2018

5.1.2 *Chlamydomonas* strains

The strains of *Chlamydomonas* used in this project were kindly provided by Professor David Gonzalez Ballester from the University of Córdoba. The following Table 5.2 summarized the different strains and the purposes they were used for.

Table 5.2 - Chlamydomonas strains used in the project

Control strains	**Characteristics**
137c	From wild type strain CC-125 mt^+. It lacks *nia1* and *nit2* functional genes related to nitrogen assimilation: it able to grow only in NH_4^+ containing media [147]
704	Wild type strain for nitrate assimilation containing reporter gene ARS encoding Arylsulfatase (Ars) under the control of the *nia1* gene promoter (mt+ cw15 ARG7- pNIA1: ARS+ WT) [148]
Single mutant strains	
pgrl1	Strain defected (through insertional mutagenesis) in the Proton gradient regulation like1 protein expression [130]
tla3	Truncated light-harvesting antenna3 through insertional mutagenesis [149]
Clostridium	*C. reinhardtii* strain defective in the expression of native [FeFe]-hydrogenases, (D66 ΔHYD) used to host the [FeFe]-hydrogenase I (CaI) gene from *Clostridium acetobutylicum* ATCC strain 824 [138]
Double mutant strains	
pgrl1 + tla3	From genetic cross
Clostridium + pgrl1	From genetic cross

5.2 Liquid and Solid Media

5.2.1 Stock solution for liquid media

According to Harris, 1989, [150] stock solutions for preparing liquid culture media were prepared by dissolving each combination of the following salts in one litre of distilled water (dH_2O):

- **Tris Solution**: 242 g Tris;
- **A Solution**: 5 g $CaCl_2 \cdot 2H_2O$, 10 g $MgSO_4 \cdot 7H_2O$ and 40 g NH_4Cl;
- **B Solution**: 115 g K_2HPO_4 and 46 g KH_2PO_4;
- **Trace Elements Solution**: 50 g EDTA (free acid) and 21 g of KOH were first dissolved in 250 mL dH_2O. Separately, 11.4 g H_3BO_3, 22 g $ZnSO_4 \cdot 7H_2O$, 5.1 g $MnCl_2 \cdot 4H_2O$, 5 g $FeSO_4 \cdot 7H_2O$; 1.6 g $CoCl_2 \cdot 6H_2O$, 1.6 g $CuSO_4 \cdot 5H_2O$, 0.214 g $MoO_4Na_2 \cdot 2H_2O$ were added in this order in 200 mL dH_2O. After dissolving all the components, the solutions were mixed and heated up to 90ºC. The pH was adjusted to 6.8 with KOH and dH_2O was added up to 1 L. Finally, the solution was transferred to a dark bottle waiting at least two days before use.

5.2.2 Liquid and solid standard TAP and Minimal Media

TAP (Tris-acetate-phosphate) medium was used for growth and hydrogen production in *Chlamydomonas* and in the isolated strains. It was prepared using the following amount of the previously described stock solutions:

- 10 mL Tris solution;
- 10 mL A solution;
- 1 mL B solution;
- 1 mL trace elements solution;
- 0.95 mL glacial acetic acid (17.46 mM)
- dH_2O up to one litre

The corresponding sulfur-deprived media were obtained in the same way but using a stock solution A-minus S (A-S), identical to the stock solution A already described above, but without addition of $MgSO_4 \cdot 7H_2O$.

Minimal media (MM) was prepared in the same way excluding glacial acetic acid in the preparation to reduce the heterotrophic microalgal growth impact shifting the metabolism towards the usage of the inorganic carbon sources.

To obtain solid media, 10 g of agarose (1% w/v) was added for each litre of media. The solution was autoclaved and then transferred to appropriate containers to solidify.

Tubes were used for long-term storage of the algal strains (six months), while Petri dishes were used to store the cultures for short times between experiments.

Furthermore, solid media supplemented with antibiotics (ampicillin 100 µg/mL), were used in the early stages of algal plating and isolation from freshwater samples to remove non-algal microorganisms.

5.2.3 Liquid media with other carbon source

To determine the working concentration of every carbon source, a preliminary qualitative screening was performed for every isolated microalga. Several concentrations were taken from literature and were tested for each carbon compound [108,109,111,151,152]. This first qualitative survey, provided fast responses in terms of algal survival and adaptability, to guide the choice of the subsequent concentrations to use for hydrogen production.

Glucose, sucrose and sodium bicarbonate were considered to evaluate hydrogen production in the isolated strains.

- **Glucose Media (Tris-glucose-phosphate, TGP)** was prepared using (per litre): Tris Solution (10 mL), A solution (10 mL), B solution (1 mL), Trace Elements Solution (1 mL) and 3 g of glucose (16 mM).
- **Sucrose Media (Tris-sucrose-phosphate, TSP)** was prepared using (per litre): Tris Solution (10 mL), A solution (10 mL), B solution (1 mL), Trace Elements Solution (1 mL) and 5 g of sucrose (14.60 mM).
- **Sodium Bicarbonate Media (Tris-bicarbonate-phosphate, TBP)** was prepared using (per litre): Tris Solution (10 mL), A solution (10 mL), B solution (1 mL), Trace Elements Solution (1 mL) and 1.5 g of sodium bicarbonate (17.85 mM).

Every corresponding sulfur-deprived media were obtained in the same way but using a stock solution A-minus S (A-S), identical to the stock solution A already described above, but without addition of $MgSO_4 \cdot 7H_2O$.

5.3 Isolation

Small aliquots, 10 µL, of the collected freshwater samples were placed on Petri dishes with MM medium supplemented with ampicillin (Figure 5.1). The plates were placed under LED cold light 100 µmol·m^{-2}·s^{-1} (Philips TLD 30W/55 lamp) at 25 °C.

Following the removal of fungi and bacteria, the algal colonies were transferred to Petri dishes with TAP. This allowed isolation of pure colonies, suitable for subsequent transfer in liquid media.

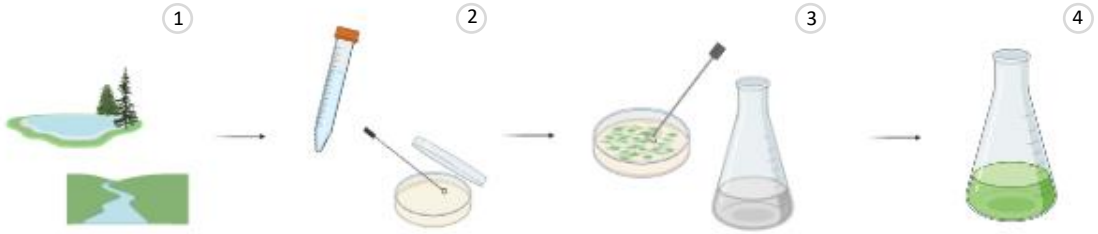


Figure 5.1 - Schematic workflow regarding the isolation of microalgae from the environment.
1) Samples were collected from natural fresh water sources.
2) Aliquots of the samples were spread on Petri dishes.
3) The pure colonies obtained by subsequent plating were transferred to the liquid culture medium.
4) Liquid culture ready for subsequent experiments and evaluations.

5.4 Identification

5.4.1 Morphological observation

To identify the algal species, microscopic observations were first conducted at 10X, 20X, 40X magnifications (Leica DM750) in order to highlight structural peculiarities, the presence/absence of different morphologies indicative of different phases in the growth cycle.

5.4.2 Molecular identification

Sequence analysis of the 18 S rRNA gene was performed to perform a molecular identification according to Khaw *et al.*, 2020 [28].

Phire Plant Direct PCR Master Mix (Thermo Fisher Scientific) was used to perform the PCR reaction (Peqlab Primus 25 Advanced Thermocycler). A colony of microalgae was removed from the solid growth medium and treated with 50 μL of the kit lysis buffer as indicated by the manufacturer's instructions. After three minutes incubation at room temperature, the pellet was removed by centrifugation at 1000 g for 1 minute. One μL of the obtained supernatant was added to the reaction mix. Details of primers, reaction mix components and timing are summarized in Table 5.3, Table 5.4 and Table 5.5.

The samples obtained from the amplification were visualized by electrophoretic run on a 1.5 % agarose gel (GellyPhor®LE agarose EMR010001, EuroClone S.p.a., lot no. 468654). The required amount of agarose was dissolved in TBE buffer (Tris-Borate 0,045 M – 0,01 M EDTA). Once the solution had cooled, 1 μg/mL of ethidium bromide intercalator (Ethidium bromure 3H000294 - Euroclone S.p.a.) was added to highlight the presence of DNA after electrophoresis run. The samples were run with a voltage of 80 V for about an hour using a molecular weight marker ranging from 100 bp to 5000 bp (O 'GeneRuler Ladder, Thermo Fisher Scientific).

Following the visualization of the amplification, the samples suitable for sequencing were conditioned in accordance with the guidelines of the external sequencing service Microsynth Seqlab, Germany. Each sample sent for sequencing consisted of 12 μL of amplified and 3 μL of 20 μM forward primer.

The sequences obtained were then compared using the Basic Local Alignment Search Tool (BLAST) algorithm (NCBI, https://www.ncbi.nlm.nih.gov/) with the sequences of the microalgal 18S rRNA gene present in the database.

Table 5.3 - Details of the primers used for molecular identification
(from Khaw *et al*., 2020 [28])

Gene	**Primers (5'-3')**	**Product Length (bp)**	**Annealing t (°C)**
RNA 18S	Forward primer: GGTGATCCTGCCAGTAGTCATATGCTTG	1823	50 °C
	Reverse primer: GATCCTTCCGCAGGTTCACCTACGGAAACC		

Table 5.4 - PCR components mix

Components of mix reaction	**Volume**
2X Phire Plant Direct PCR MasterMix	10.00 µL
Primer Forward 10 µM (BMR Genomics, Italy)	1.00 µL
Primer Reverse 10 µM (BMR Genomics, Italy)	1.00 µL
Sterile dH_2O	7.50 µL
Sample	1.00 µL

Table 5.5 - PCR Cycles

	Temperature	**Time**	**Cycles**
Initial denaturation	98 °C	5 minutes	1
Denaturation	98 °C	5 sec	40
Annealing	50 °C	5 sec	
Extension	72 °C	75 sec	
Final extension	72 °C	1 minutes	1

5.5 Microalgal growth

Microalgal colonies from the plates were transferred to a liquid medium to evaluate the adaptability to growth *in vitro*.

Flasks of different capacities were used depending on the amount of biomass required for the downstream analyses or experiments. For larger volumes, a gradual transition into progressively larger volumes up to the required culture volume has been planned.

The flasks were placed under constant stirring at 70-90 rpm (ASAL s.r.l. shaker) to favor a homogeneous exposure to light and avoid biomass sedimentation. The conditions of light and temperature are those previously described in section 5.3.

5.5.1 Optical density

Optical density (OD) allows for the quantification of cell density in the growth medium. For single-celled microorganisms, the absorbance tends to be proportional to the number of cells and can be used to replace the count using an optical microscope. The optical density or absorbance (A) is calculated as the logarithm of the ratio between the intensity detected for the blank signal (Io), the growth medium only, and that relative to the cell sample (I) in the growth medium ($A = \log I / I$).

The measurements were carried out using the SPECTROstar® Nano spectrophotometer (BMG Labtech). The samples were diluted if necessary to avoid absorbance values higher than one unity, where the relationship between optical density and the number of cells ceases to be linear. The wavelength used for the spectrophotometric measurement was 750 nm, following the example of the studies conducted by Griffiths *et al.*, 2011 [153]. This spectral region falls outside the absorption peaks of the microalgal pigments and allows for the estimation of cell density.

5.5.2 Cell counting

The cell count was carried out using a Burker chamber. A small aliquot of the sample (10 μL) was made to diffuse by capillarity covering the entire surface of the counting grid. It is made up of nine delimited squares, each of which contains sixteen smaller delimited squares. By convention, the cells located in the four corner squares of the chamber are counted.

The counting operation was carried out with an optical microscope (ZEISS - Axioplan) at a 10X magnification. The number of cells in the sample per millilitre of suspension was obtained by multiplying the average of the cells counted in the four squares considered, by the chamber volumetric conversion factor, 10^4, and the dilution factor used.

5.5.3 Wet Biomass

Wet biomass at the end of the growth cycle (plateau phase) was also quantitatively assessed. In particular, 5 mL of sample was taken after one week for the analysis. The samples, collected in falcon tubes, were centrifuged (Thermo Fisher Scientific MR23i Centrifuge) at room temperature and 4,500 g for 3 minutes to separate the pellet, from the liquid phase. The pellet was then weighted.

5.5.4 Chlorophyll content

Growth was also quantified in terms of chlorophyll content. The extraction of total chlorophyll took place using an organic solvent.

Specifically, ethanol was used for *Chlamydomonas* strains [154]. For each sample, 200 µL of culture was collected and added to 800 µL of pure ethanol. The samples were then incubated for 5 minutes in the dark at room temperature, before centrifuging them at 13000 g for 75 seconds. At this stage of the procedure, the pellet appeared completely bleached and the supernatant containing the extracted chlorophylls was recovered and dosed. The absorbance values were measured at 649 nm and 665 nm. Finally, the concentration (µg/mL) of total chlorophyll (C_{a+b}, chlorophyll a plus chlorophyll b content) was calculated with the following formula, obtained by Wintermans *et al.*, 1965 [154]:

$$C_{a+b} = (6.1 \times A_{665}) + (20.04 \times A_{649})$$

The value obtained was multiplied by 5, the dilution factor used.

For the other algal strains, DMSO (dimethyl sulfoxide) was used as an extractive solvent [155]. One mL of culture sample was taken and centrifuged for 5 minutes at 14000

g. One mL of DMSO was added to the obtained pellet and incubated for 10 minutes at 70 °C.

The samples were then centrifuged at 7000 g for 4 minutes. This led to bleaching of the pellet and the recovered supernatant was measured at the following wavelengths: 649 nm and 665 nm. The concentrations (µg/mL) of chlorophyll a (C_a) and b (C_b) were calculated with the following formulas, obtained by Wellburn *et al*., 1994 [155]:

$$C_a = 12.19 \times A_{665} - 3.45 \times A_{649}$$
$$C_b = 21.99 \times A_{649} - 5.32 \times A_{665}$$

5.6 Hydrogen production

After a preliminary growth in TAP medium to mid-log phase (10-12 × 10^6 cells/mL), the cultures were diluted with fresh medium to reach a chlorophyll concentration of 10 µg/mL (around, 3-4 × 10^6 cells/mL) and then transferred to the hermetically sealed bioreactors (Media Bottle 125 mL clear graduated, with 33-430 Black phenolic cap butyl septa, WHEATON) where they remained until the analysis was completed. The space of each bioreactor was divided between 100 mL of liquid culture and 55 mL of headspace [106,146].

For the isolated strains, different approaches were used to detect biohydrogen production pathways. The used strategies were: dark incubation, low light exposition (12 PAR) and sulfur deprivation (in high light, 100 PAR). Moreover, the same conditions were tested with different carbon source: acetate, glucose, sodium bicarbonate and sucrose. Except replete acetate media, the cultures were subjected to different centrifugations and washing steps to remove the traces of the elements not to be included.

Regarding *Chlamydomonas* single and double mutants and related control strains in TAP media, different light conditions were tested: 12, 50, 100 and 450 PAR.

The bioreactors were placed in a containing growth chamber LED panels (AlgaeTron AG 230, Photon System Instruments) at 25 °C with 200 rpm continuous agitation.

5.6.1 Head-space gas analysis

Daily, a 250 µL sample from the bioreactor headspace was withdrawn using a 1 mL Hamilton SampleLock™ syringe. The sample was manually injected into a gas chromatograph, GC (Agilent 7820A, Agilent Technologies).

H_2, O_2 and N_2 were separated using a packed column (60/80 Molecular Sieve 5A, Ref. 13133-U, Supelco) at 75 °C and detected using a Thermal Conductivity Detector (TCD), using Argon as a carrier gas.

The integration of the chromatographic peaks and the analysis of the results were performed with EZ-Chrome software (Agilent technologies) [106,146].

5.7 Statistical analysis

The statistical validity of the results was assessed using Microsoft Excel 2016 (Microsoft, Redmond, USA) and GraphPad Prism 8.0.2 (San Diego, USA). The experiments were performed at least three times and each measurement was acquired at least in duplicate.

To estimate the statistical significance between all the groups, an analysis was carried out using one way ANOVA test. A *p value* of less than 0.05 was considered significant (indicated with an asterisk in the figures).

CHAPTER 6 – RESULTS AND DISCUSSION

6.1 Isolation

Freshwater samples collected in the Basilicata region represented the starting material for microalgal isolation.

Small aliquots of water (10 µL) were placed on Petri dishes. Initially, Minimal Medium was chosen as a nutrient supplier, in which acetic acid is not present as a carbon source. The plates were placed under cold light at 25 °C. In these conditions, the growth of algal microorganisms is favoured over fungal or bacterial contaminants (Figure 6.1). Only photosynthetic microorganisms can jointly exploit light and atmospheric CO_2 through the photosynthetic process to grow efficiently. To further disadvantage other contaminants, the broad-spectrum antibiotic ampicillin (100 µg/mL) was used.

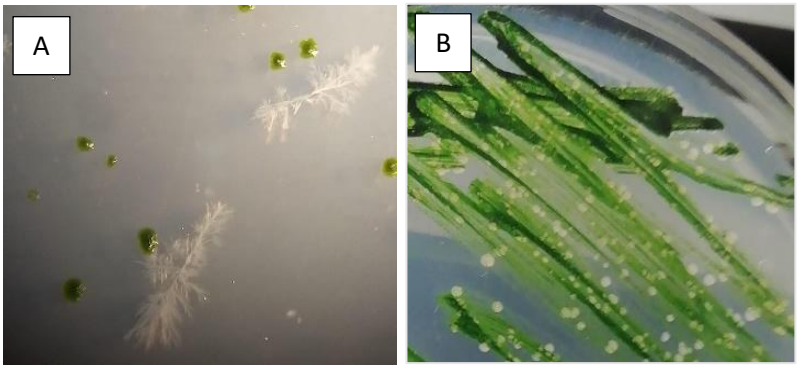


Figure 6.1 - Example of typical a) fungal (white arboreal elements) and b) bacterial (white dots) contaminants found on the plates during the purification process of algal colonies (green spots or smears in both pictures, details of full-size plates) derived from freshwater samples

After repeated streak plating steps, fungal or bacterial colonies were reduced and eliminated: the algal colonies were transferred to plates with TAP as nutrient media (Figure 6.2). The streak plating process was also repeated to obtain pure colonies consisting of a single algal morphology.

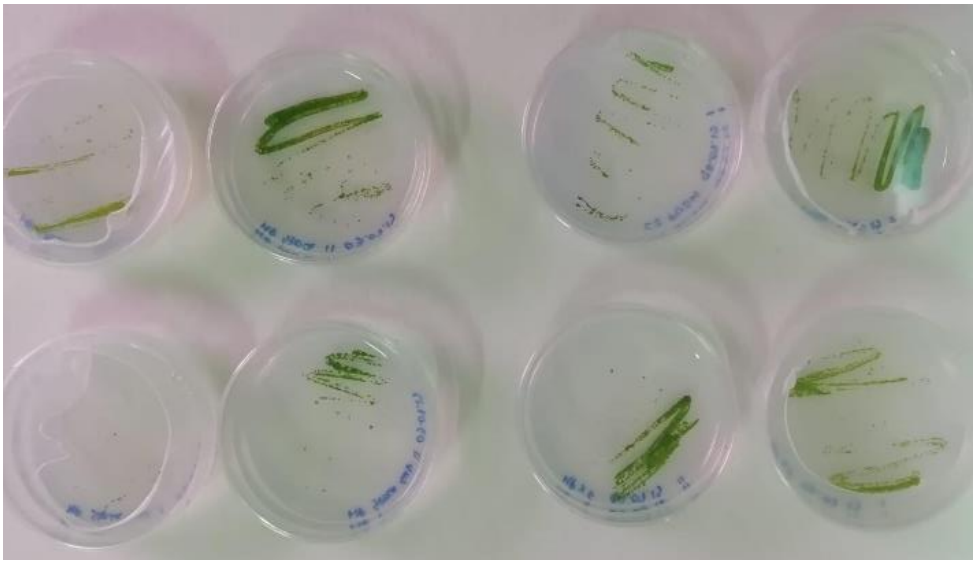


Figure 6.2 - Petri dishes with algal strains

Liquid medium cultures were subsequently started from the isolated colonies. This step was used as a further screening aimed at selecting algal strains able to adapt and grow in *in vitro* conditions. Indeed, it is necessary for an algal strain to be adequately studied, that its growth times are not excessively dilated. Furthermore, this aspect is also relevant for downstream applications, linked to the overall productivity of algal culture.

For this reason, the next step consisted in monitoring the rate of microalgal growth phases, as described in the section 6.3.

6.2 Microalgae identification

Identification of the strains began once perfectly axenic cultures were obtained from the colonies on the plates. In particular, the magnifications used (10X, 20X, 40X) made it possible to observe the typical morphology of microalgal eukaryotic cells with the organelles of the chloroplasts well evident and widespread in the cell cytoplasm. The observation confirmed the absence of contaminants originally present in the starting environmental samples and the presence of uniform cell morphology.

In the observed conditions, it was not possible to appreciate different cellular stages: however, it should be remembered that these often occur only following stressful situations which have not been specifically applied. Figure 6.3 and Figure 6.4 show the microscopic images of two among the genera identified with next molecular confirmation.

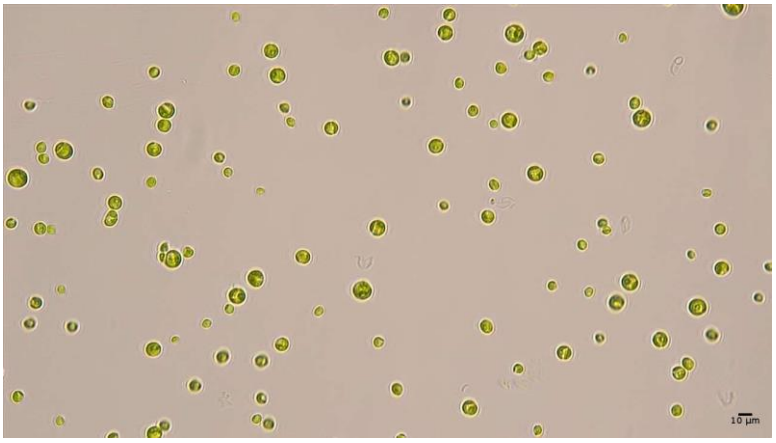


Figure 6.3 - *Desmodesmus* sp. microalgae 40 X

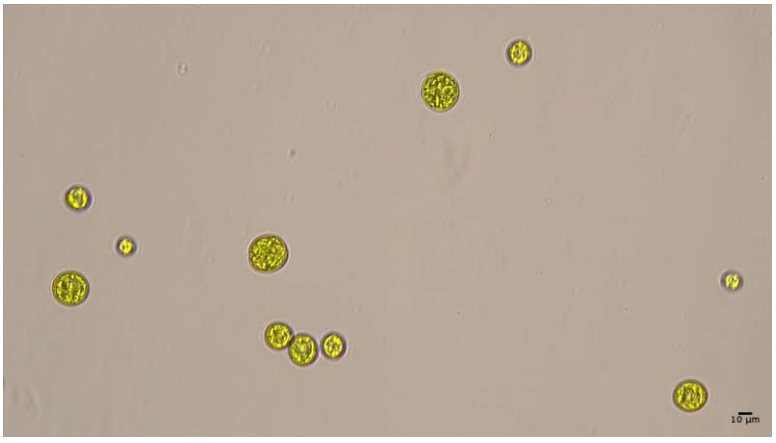


Figure 6.4 - *Haematococcus* sp. microalgae 40 X

Amplification of the rRNA 18S gene yielded unique and specific products of the expected weight (Figure 6.5). Samples were prepared for sequencing. The sequences obtained were subjected to similarity analysis using the Blast algorithm.

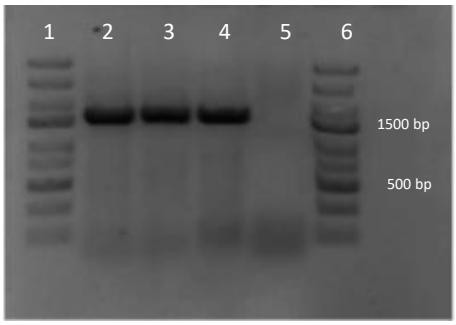
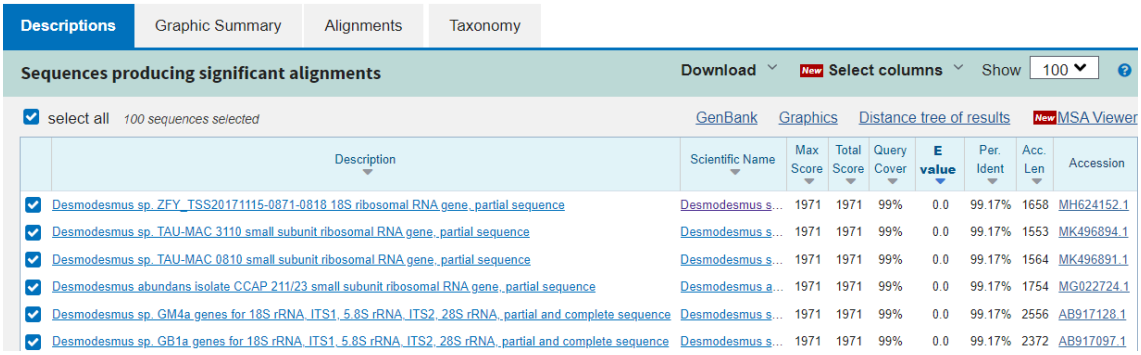


Figure 6.5 - Amplification of rRNA 18 S gene
(1% agarose gel marker 1,6; sample 2,3,4; negative control 5)

It is here reported one of the obtained sequences in FASTA format of the sample identified as belonging to the genus *Desmodesmus* sp. (Figure 6.6):

>RAP18S/1-1091

ACTGCTTATACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATAGTTTATTTGGTGGTACCTTCTTACTC
GGAATAACCGTAAGAAAATTAGAGCTAATACGTGCGTAAATCCCGACTTCTGGAAGGGACGTATATATTA
GATAAAAGGCCGACCGGACTTTGTCCGACCCGCGGTGAATCATGATATCTTCACGAAGCGCATGGCCTTG
TGCCGGCGCTGTTCCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATAGAGGCCTACCATGGTGG
TAACGGGTGACGGAGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGG
AAGGCAGCAGGCGCGCAAATTACCCAATCCTGATACGGGGAGGTAGTGACAATAAATAACAATACCGGG
CATTTTATGTCTGGTAATTGGAATGAGTACAATCTAAATCCCTTAACGAGGATCCATTGGAGGGCAAGTCT
GGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTTAAGTTGTTGCAGTTAAAAAGCTCGTAG
TTGGATTTCGGGTGGGTTTCAGCGGTCCGCCTATGGTGAGCACTGCTGTGGCCTTCCTTACTGTCGGGGAC
CTGCTTCTGGGCTTCATTGTCCGGGACAGGGATTCGGCATGGTTACTTTGAGTAAATTAGAGTGTTCAAAG
CAGGCTTACGCCGTGAATACTTTAGCATGGAATAACACGATAGGACTCTGCCCTATTCTGTTGGCCTGTAG
GAGTGGAGTAATGATTAAGAGGAACAGTCGGGGGCATTCGTATTTCATTGTCAGAGGTGAAATTCTTGGA
TTTATGAAAGACGAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACRAAAGTTGGG
GGCTCGAAGACGATAAGATACCGTCGTAGTCTCAACCATAAACGATGCCGACTAGGGATTGGCGGACGTT
TTTGCATGACTCCGTCAGCACCTTGRRAGAAATCAAAGTTTTTGGGTTCCGGGGGGAWWWTGTTCSCAA
GGCTGAAACTTAAAGGAATTGACGGAAGGGCAC



Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments — Download — New Select columns — Show 100

select all 100 sequences selected — GenBank | Graphics | Distance tree of results | New MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Desmodesmus sp. ZFY_TSS20171115-0871-0818 18S ribosomal RNA gene, partial sequence	Desmodesmus s...	1971	1971	99%	0.0	99.17%	1658	MH624152.1
Desmodesmus sp. TAU-MAC 3110 small subunit ribosomal RNA gene, partial sequence	Desmodesmus s...	1971	1971	99%	0.0	99.17%	1553	MK496894.1
Desmodesmus sp. TAU-MAC 0810 small subunit ribosomal RNA gene, partial sequence	Desmodesmus s...	1971	1971	99%	0.0	99.17%	1564	MK496891.1
Desmodesmus abundans isolate CCAP 211/23 small subunit ribosomal RNA gene, partial sequence	Desmodesmus a...	1971	1971	99%	0.0	99.17%	1754	MG022724.1
Desmodesmus sp. GM4a genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence	Desmodesmus s...	1971	1971	99%	0.0	99.17%	2556	AB917128.1
Desmodesmus sp. GB1a genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequence	Desmodesmus s...	1971	1971	99%	0.0	99.17%	2372	AB917097.1

Figure 6.6 - Highest scored results obtained between *Desmodesmus* sp. 18S rRNA gene sequence and the comparison in the database

In particular, three strains were isolated and suitable for *in vitro* cultivation which allowed the subsequent analyses to be carried out. The identified strains belong to the following genera:

- *Chlorella* sp. (from water sample collected in Potenza)*;*
- *Desmodesmus* sp. (from water sample collected in Rapone)*;*
- *Haematococcus* sp. (from water sample collected in Potenza).

The identified genera are normally found in the environmental contexts in which the water samples were collected. They are all freshwater microalgae that colonize geographic areas in latitudes compatible with those of the Basilicata region [156].

6.3 Microalgal growth

A mixotrophic growth condition with TAP media was chosen for microalgae cultivation [157,158], after visual and qualitative testing of the best growth conditions using different carbon sources. In order to monitor growth, after several tests, it was decided to opt for measurements of optical density and chlorophyll content. Both optical density and chlorophyll content measurements were performed considering three isolated strains qualitatively well adapted to in *in vitro* condition: *Chlorella* sp., *Desmodesmus* sp. and *Haematococcus* sp.

Wet biomass measurement and cell counting were more time-consuming procedures and often did not provide reliable representations of growth trends, therefore they were not taken into consideration.

6.3.1 Optical density

Before carrying out the measurements, a pre-inoculation of a smear of colonies from a plate was performed in 50 ml of TAP medium inside a flask covered with aluminium foil to keep sterility. After three days, this pre-culture was used as a basis to carry out a homogeneous liquid inoculum for each sample to be subjected to OD measurements: 1 ml was added in flasks containing 50 ml of TAP medium. Measurements were performed approximately every two days (Figure 6.7).

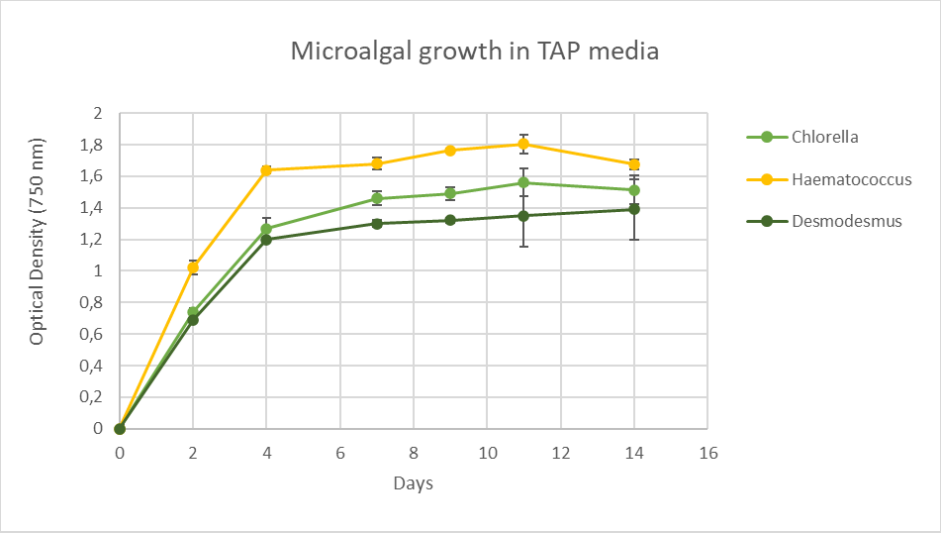


Figure 6.7 - Microalgal growth trend obtained through OD measures. The data comes from three independent experiments. Error bars represent standard error of the mean (SEM)

The three strains of green microalgae showed a growth trend reaching the stationary phase within 4-6 days, in line with the values reported in the literature [159,160].

6.3.2 Chlorophyll contents

As regards the extraction of chlorophylls, it was possible to perform the procedure using ethanol as a solvent only in *Chlamydomonas* strains. For none of the other strains this method was effective. Probably a thicker cell wall is behind this failure. Considering the evolution of microalgae, it is not surprising that some may have evolved a particularly resistant wall as a border with the external environment [161]. In particular, invasive treatments for cell wall disruption are commonly reported in *Chlorella* species [162,163].

The procedure which uses DMSO as an extractive solvent was effective with all the remaining strains. As shown in Figure 6.8, the cell pellet resulted completely whitened by this treatment.

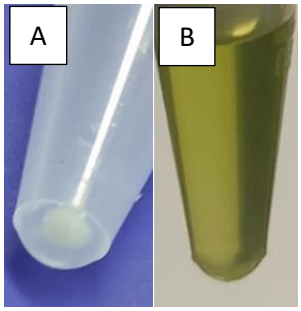


Figure 6.8- Chlorophyll extraction with DMSO in *Chlorella* sp. A) Whitened pellet, B) Supernatant with extracted chlorophylls

As previously described for OD measurement, 1 ml was added from the pre-inoculum into flasks containing 50 ml of TAP medium. Measurements were performed approximately every day.

The amount of chlorophyll measured in the isolated strains is reported below in function of days of growth (Figure 6.9). The data obtained follow the growth trends obtained with the measurement of optical density with a good approximation.

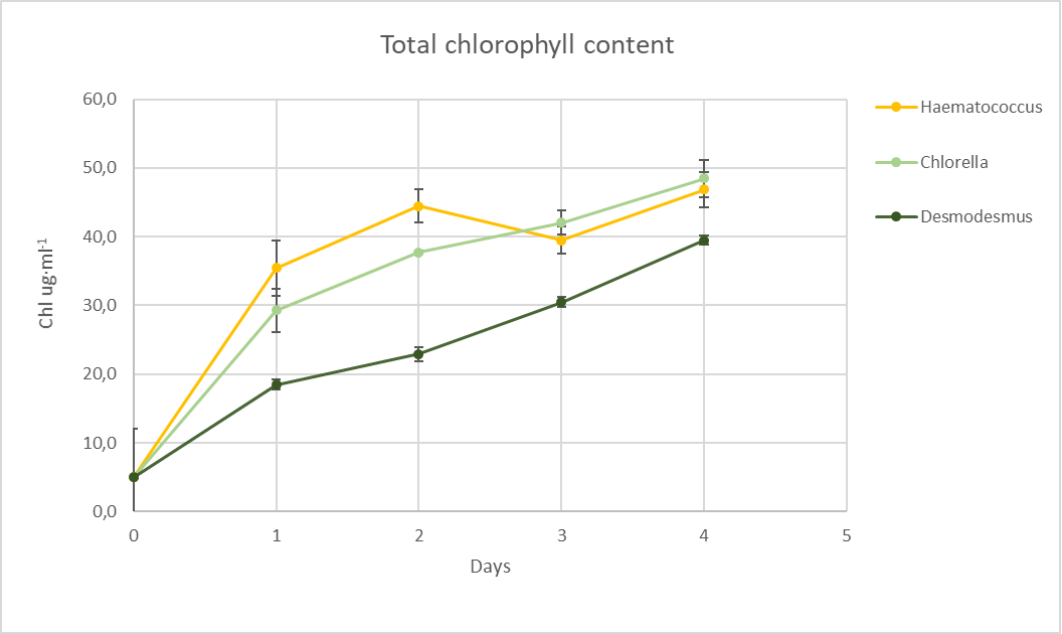


Figure 6.9 - Microalgal growth trend obtained through total chlorophylls content measures. The data comes from three independent experiments. Error bars represent SEM

6.4 Hydrogen production

6.4.1 Hydrogen production in microalgae isolated in Basilicata

The strains of *Chlorella* sp., *Desmodesmus* sp. and *Haematococcus* sp. were tested for H_2 production. In particular, three different strategies have been implemented to ascertain its production:

- incubation in the dark to highlight the fermentation process;
- low light incubation (12 PAR) and S deprivation (in high light, 100 PAR) to highlight the photosynthetic H_2 production pathways through the photosystems activities.

Different carbon sources were tested for each strategy: acetate, glucose, sucrose and bicarbonate. Although the strains showed appreciable growth under the conditions of mixotrophic cultures in aerobic conditions, incubation in hermetically sealed bioreactors showed different behaviour.

Among the carbon sources, only two, acetate and glucose, showed detectable hydrogen production. The two carbon sources allowed the progressive establishment of an anaerobic environment. This is the ideal condition for the expression of hydrogenases that can catalyse H_2 production (Figure 6.10).

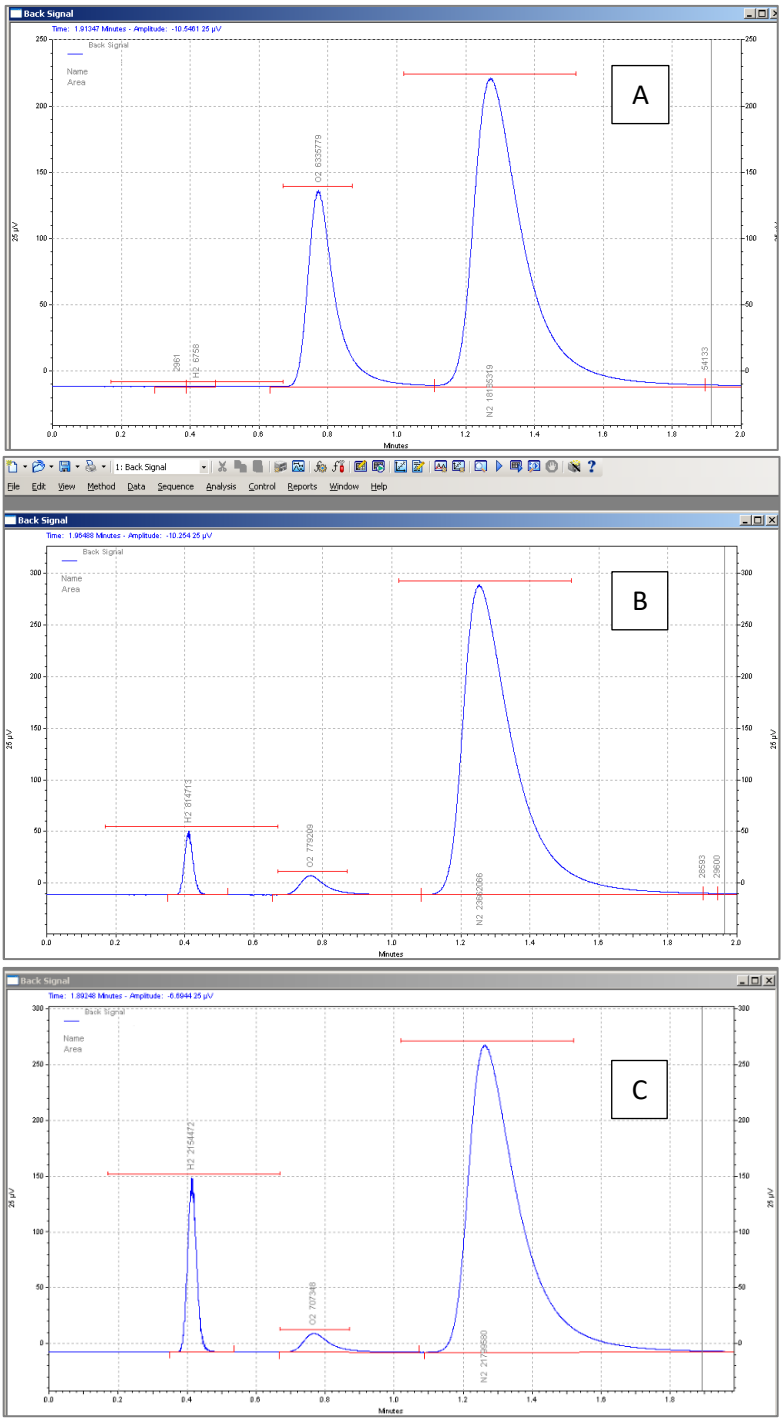


Figure 6.10 - Typical chromatographic profile of the conditions that lead to H2 production in microalgae.
A progressive decrease of the oxygen peak (the central one) is observed.
The first peak of hydrogen is progressively more intense
(The third peak represents the nitrogen that does not undergo significant change).
Specifically, the profiles reported is related to the *Haematococcus* sp. microalgae in glucose
after 24 (A), 48 (B), 72 hours (C) from anaerobic incubation, in dark conditions.

6.4.1.1 *Haematococcus* sp.

In *Haematococcus* cultures, H_2 production was measured using acetate or glucose as source of carbon. Different yields were obtained as reported below.

In particular, in acetate, the production was marginally detectable after 2-3 days in all the conditions tested (Figure 6.11). The production using glucose as a heterotrophic source of carbon was different: a higher production was observed in the dark condition (~5 mL/L). In low light conditions the production is about half while it is undetected in sulfur deprivation (Figure 6.13).

The levels of O_2 were monitored in both cases (Figure 6.12 and Figure 6.14): it is evident that in a condition of S deprivation the establishment of an anaerobic environment is not favoured. Conversely, this occurs with the dark and low light conditions, albeit with different effects on the H_2 production. It might be interesting to clarify why these differences occur in particular when using glucose instead of acetate.

As mentioned, no H_2 production was displayed in bicarbonate and sucrose. In particular, in these conditions, the culture appeared visibly stressed as reported by the culture spots in Table 6.1. Indeed, the colour tends to take on a brownish tone, indicative of low biomass production and a depletion of chlorophylls. Conversely, in acetate and glucose low light conditions an intense green is indicative of a greater quantity of biomass and chlorophylls.

Furthermore, to the best of our knowledge, there are no other cases reported in the scientific literature relating to the H_2 production in the genus *Haematococcus.*

Table 6.1 - Spot of *Haematococcus* sp. cultures in the different conditions tested

Haematococcus sp.			
Before conditioning			
			
After 5 days	Dark	Sulphur deprivation	Low light
Acetate			
Sucrose			
Sodium Bicarbonate			
Glucose			

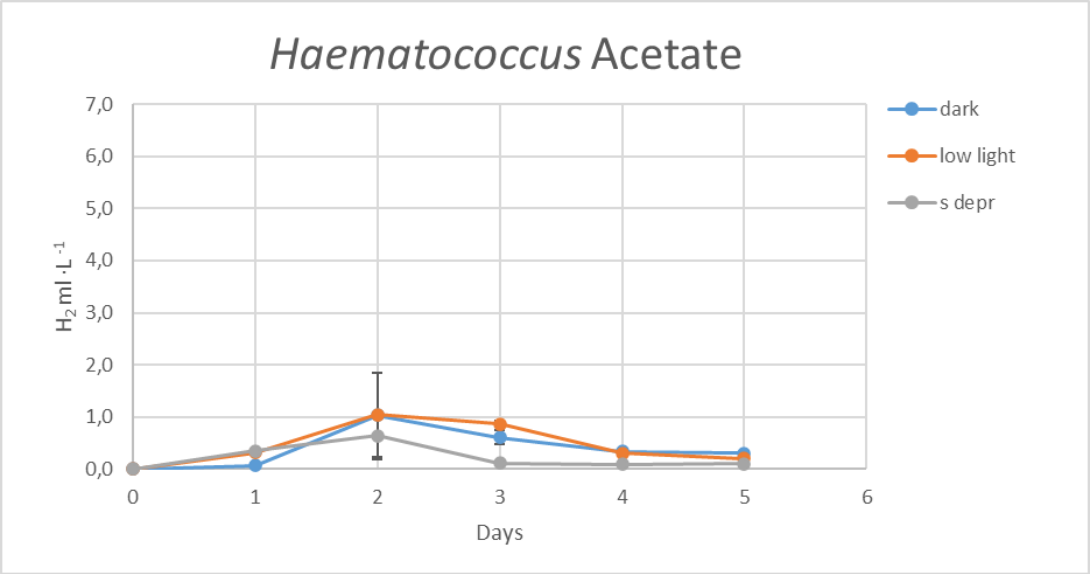


Figure 6.11 - H_2 produced by *Haematococcus* sp. in acetate. The data comes from three independent experiments. Error bars represent SEM

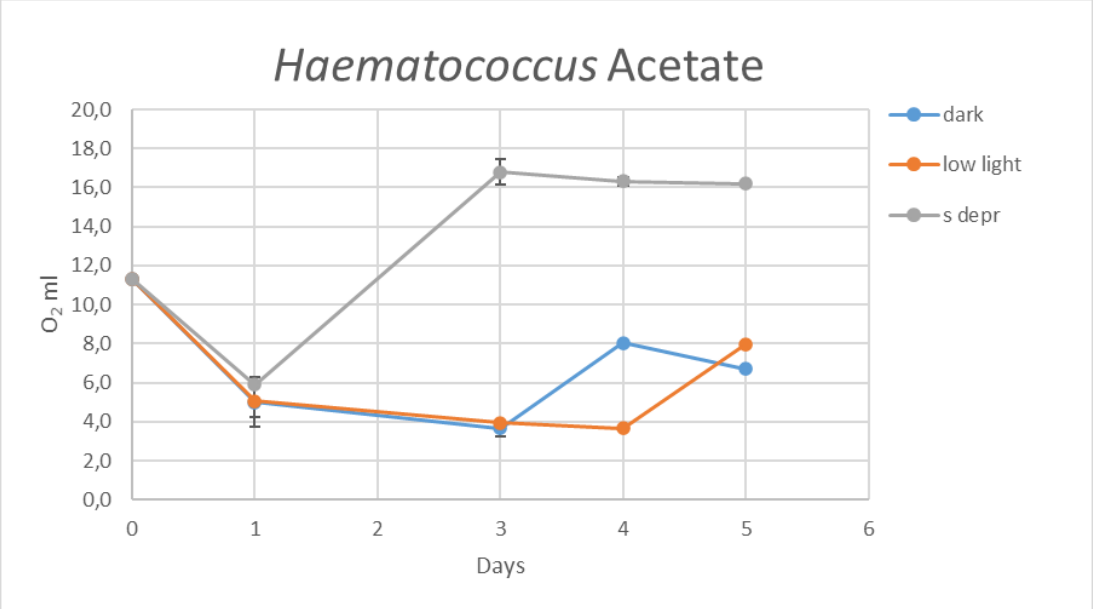


Figure 6.12 - O_2 levels in *Haematococcus* sp. culture in acetate. The data comes from three independent experiments. Error bars represent SEM

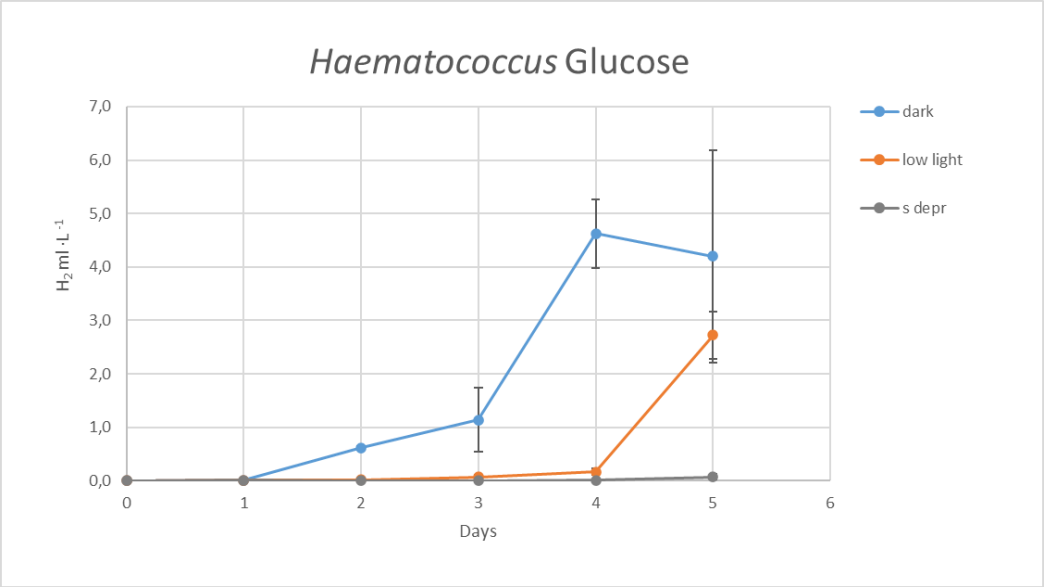


Figure 6.13 - H_2 produced by *Haematococcus* sp. in glucose. The data comes from three independent experiments. Error bars represent SEM

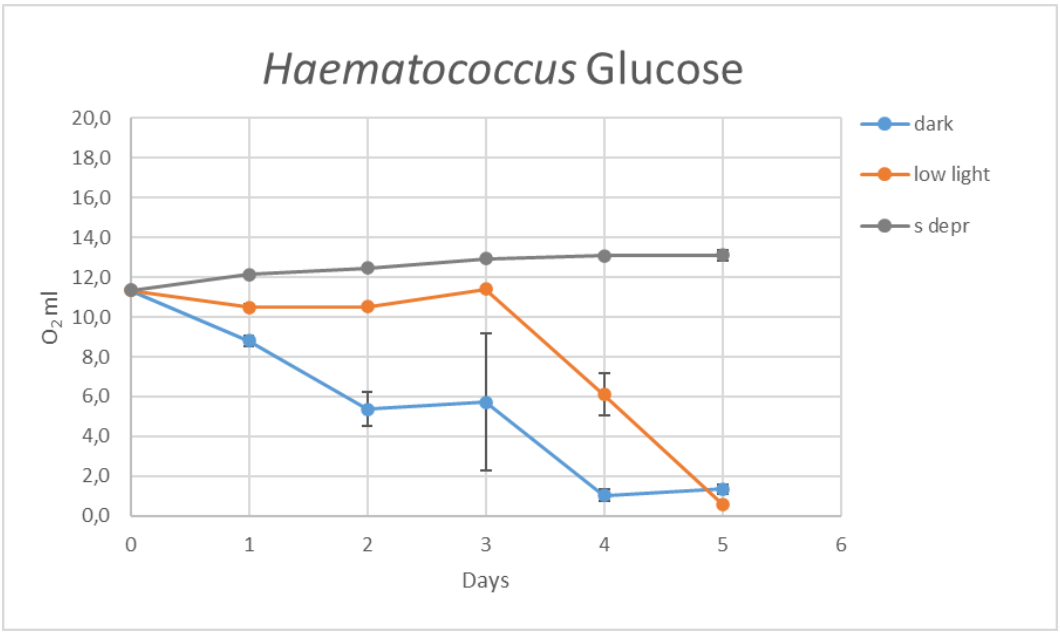


Figure 6.14 - O_2 levels in *Haematococcus* sp. culture in glucose. The data comes from three independent experiments. Error bars represent SEM

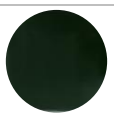
6.4.1.2 *Chlorella* sp.

In any tested conditions, H_2 production was not observed in *Chlorella* sp. culture, not even in dark conditions where an anaerobic condition was rapidly established (data not shown).

Also in this case, during the experiment, an evident state of suffering was noted in the cultures tested with sucrose and bicarbonate (Table 6.2).

There are several studies regarding H_2 production in species afferent to the genus *Chlorella*. However, an important heterogeneity among the species is reported: a *Chlorella* sp. KLSc59 strain was reported to produce H_2 in complete media containing acetate or glucose [109], while, a *Chlorella pyrenoidosa* strain was reported to produce H_2 after nitrogen deprivation [164].

Table 6.2 - Spot of *Chlorella* sp. cultures in the different conditions tested

Chlorella sp.			
Before conditioning			
			
After 5 days	Dark	Sulphur deprivation	Low light
Acetate			
Sucrose			
Sodium Bicarbonate			
Glucose			

6.4.1.3 *Desmodesmus* sp.

In the cultures of *Desmodesmus* sp., H_2 production in acetate and glucose was observed, albeit with different yields.

In particular, in acetate, the production was detectable, but not abundant, and mainly in low light conditions (Figure 6.15). The production using glucose as a heterotrophic source of carbon showed a better trend: a three times higher production, ~6 mL/L, was observed in the low light condition (Figure 6.17). In dark and sulphur deprivation conditions little increases were detected day by day with an overall scarce yield.

O_2 levels were monitored in both cases (Figure 6.16 and Figure 6.18): in dark and low light conditions, there was a rapid drop in oxygen levels which allowed for H_2 production. While in the case of S deprivation a similar pattern of O_2 consumption was observed but it was delayed: marked reduction of O_2 at day 3 instead of day 1. In fact, after 5 days a slight production of H_2 seems to start. This strategy probably takes longer to get more noticeable yields. It could be interesting to evaluate with subsequent analyses whether the volumes of H_2 produced over a longer time span are to be considered more advantageous.

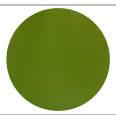
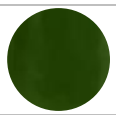
As mentioned, no H_2 production in bicarbonate and sucrose was shown. In these conditions, compared to the previous cases, the culture appeared qualitatively less stressed as reported by the culture spots in Table 6.3.

Furthermore, to the best of our knowledge, other cases relating to the production of H_2 in the genus *Desmodesmus* sp. are not reported in the literature.

The similar behaviour between *Chlamydomonas* 704 strain and *Desmodesmus* sp. in H_2 production trend may reflect the presence of a catalytically analogue enzyme [106]. Indeed, in *Scenedesmus* genus, the taxonomically closest algae to *Desmodesmus*, evidence has been collected regarding the presence of a Fe-hydrogenase, structurally similar to that of *Chlamydomonas* [75,76].

However, it would be interesting to evaluate how these species can produce H_2 similarly by exploiting different sources of carbon, acetate in *Chlamydomonas* and glucose in *Desmodesmus*.

Table 6.3 - Spot of *Desmodesmus* sp. cultures in the different conditions tested

Desmodesmus sp.			
Before conditioning			
			
After 5 days	Dark	Sulphur deprivation	Low light
Acetate			
Sucrose			
Sodium Bicarbonate			
Glucose			

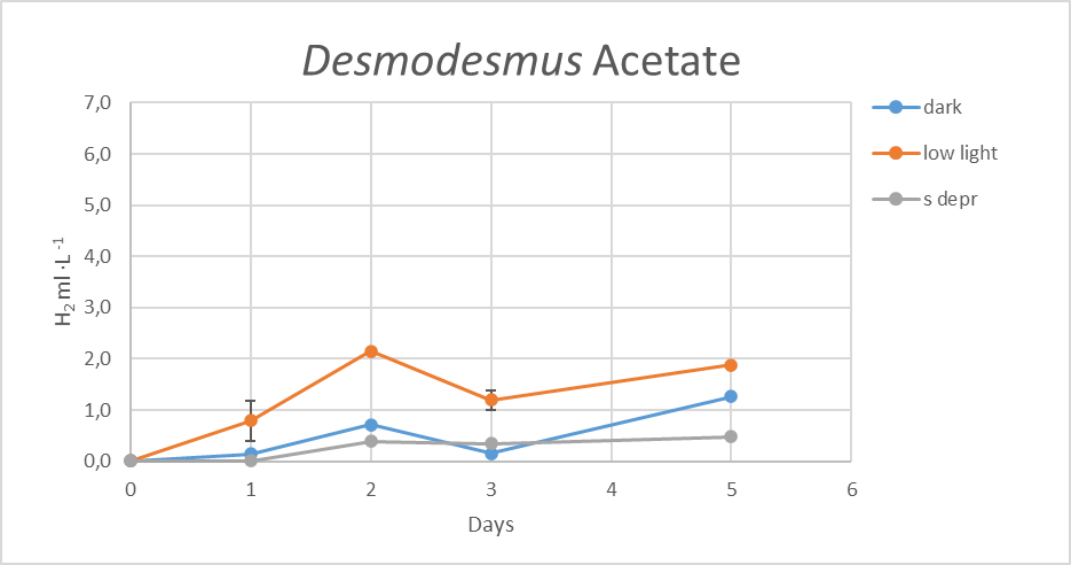


Figure 6.15 - H_2 produced by *Desmodesmus* sp. in acetate. The data comes from three independent experiments. Error bars represent SEM

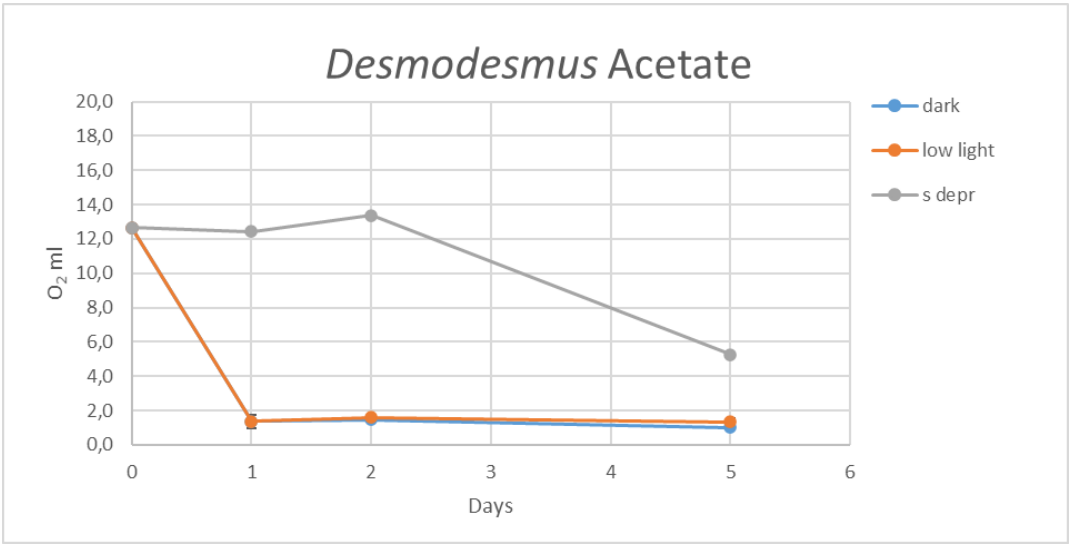


Figure 6.16 - O_2 levels in *Desmodesmus* sp. culture in acetate. The data comes from three independent experiments. Error bars represent SEM

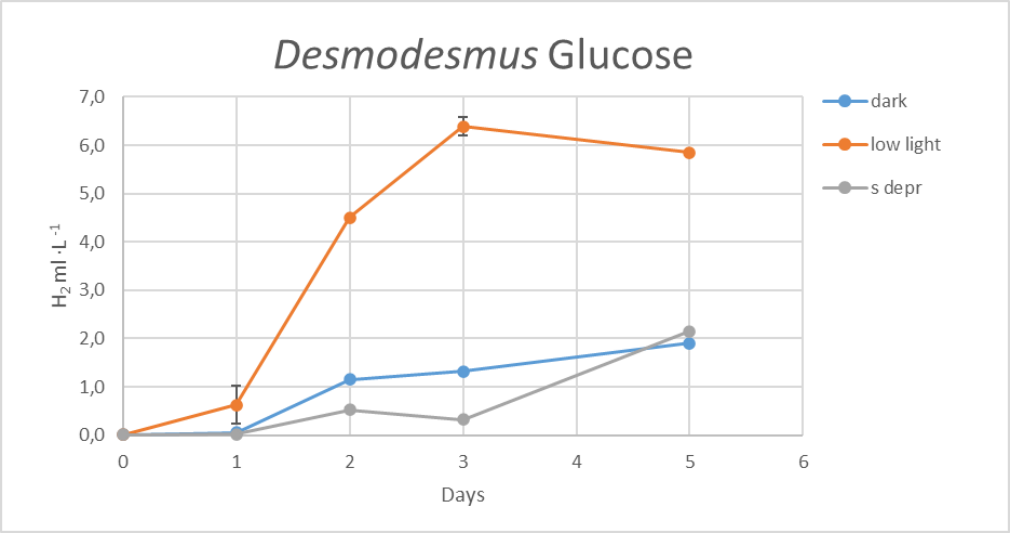


Figure 6.17 - H_2 produced by *Desmodesmus* sp.in glucose. The data comes from three independent experiments. Error bars represent SEM

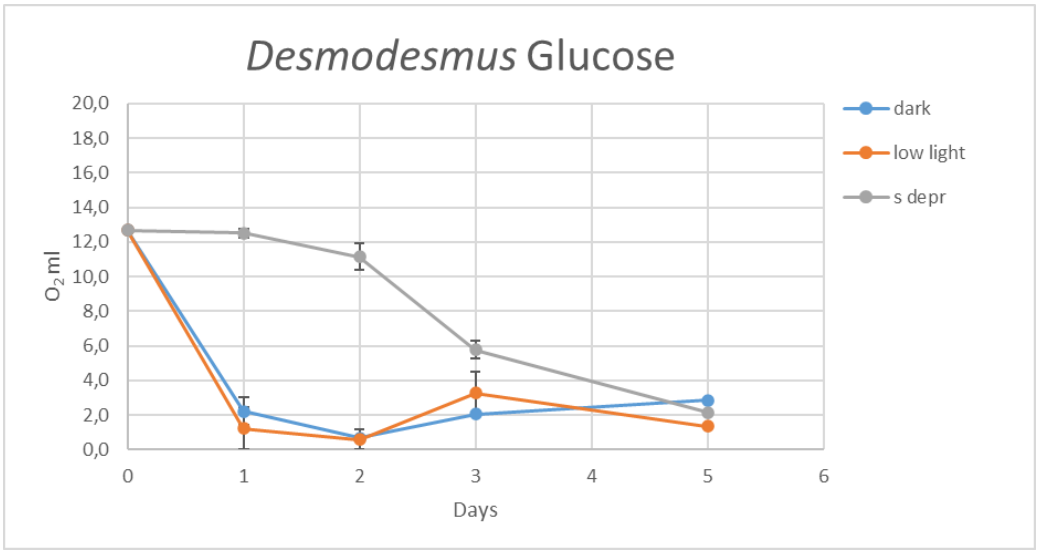


Figure 6.18 - O_2 levels in *Desmodesmus* sp. culture in glucose. The data comes from three independent experiments. Error bars represent SEM

6.4.*2* *Chlamydomonas* mutants and hydrogen production by light modulation in replete acetate media

Among the microorganisms capable of H_2 production, the *Chlamydomonas* model is the one that most of all has allowed the accumulation of great knowledge in the microalgal world. This species lends itself to optimal laboratory cultivation favouring a considerable collection of biochemical and metabolic findings regarding the biological processes involved. At the same time, advances in bioinformatics have led to an in-depth understanding of nuclear, mitochondrial, and chloroplast genomes. This is the most important requirement to be able to apply genetic and metabolic engineering approaches to condition the behaviour of the alga in an optimal way [165].

In physiological conditions, microalgal H_2 production does not occur continuously with great yield. Several approaches have prolonged this production but with collateral effects. In particular, S deprivation from the media leads to significant increases but also induces cellular stress or death. Furthermore, to apply similar approaches is necessary to subject the cellular biomass to various centrifuge-washing steps that inevitably entail further stress and represent a technical obstacle, especially with higher cultivation volumes or in mutant strains [166].

This section discusses the data relating to some already characterized *Chlamydomonas* mutants, but with no data regarding H_2 production in non-stressful conditions. Recent studies have highlighted new strategies to create an optimal environment for H_2 production in culture without stress induction in TAP medium at a low light intensity [106]. Considering the different light sensitivity in mutants, also other ranges were investigated (Figure 6.19).

In particular, low light intensity, such as 12 PAR, was tested as it was considered suitable for the production of H_2 without excluding the simultaneous photosynthetic process and biomass production. Values of 50 PAR represent an intermediate light condition that pushes the cellular metabolism towards the photosynthetic process and the consequent production of biomass. Values at the level of 100 PAR are conditions that further stimulate the cellular photosynthetic systems, rapidly pushing towards cell growth. In wild-type strains, the H_2 production is hindered at these light intensities, considering the oxygen levels reached with a sustained photosynthetic process.

Four hundred and fifty PAR instead represents a limit value, in which wild-type strains are normally unable to grow due to the excessive light stress on the cellular photosynthetic structures which does not translate into optimal physiological growth.

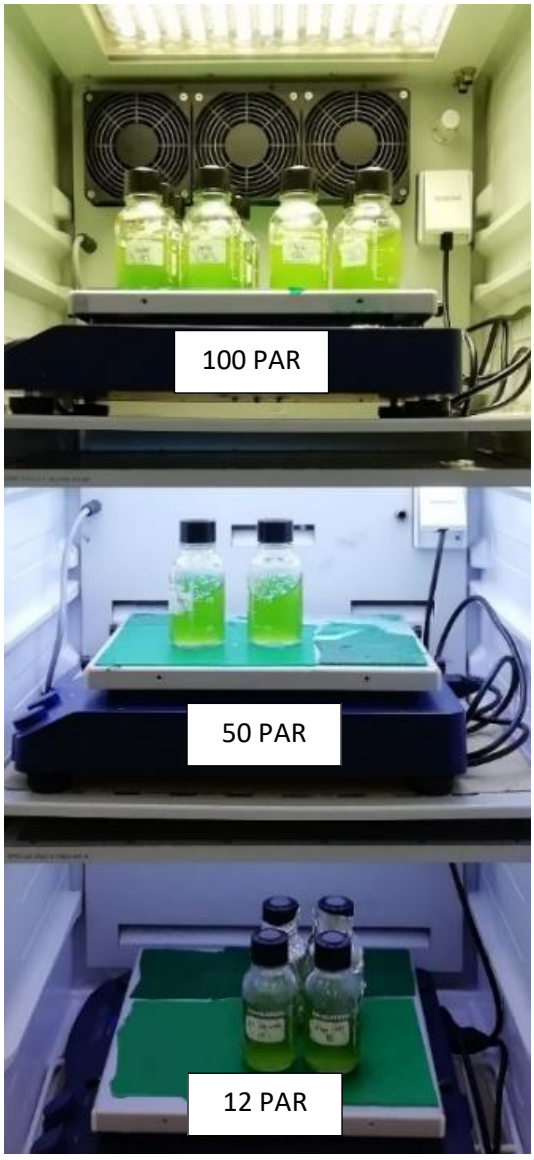


Figure 6.19 - *Chlamydomonas* strains during the tests in different light intensity as described in the pictures

6.4.2.1 *tla3* and *pgrl1* mutants

In this first experimental set, strain 137c was the control strain *Chlamydomonas*. *Tla3* and *pgrl1* were the single mutants while the "p + t" strain was the double mutant, obtained by the genetic cross of the two previous mutants. The double mutant allowed us to evaluate the joint effect of the two mutations.

In particular, the *tla3* mutants were selected to be less subject to saturation and photo-inhibition phenomena. While *pgrl1* mutants steal electrons from one of the competitive hydrogenase pathways, the CEF around PSI. Mutants such as those of the *tla3* series in the same condition of the other *Chlamydomonas* strains showed a qualitatively less intense green. This is indicative of a lower total chlorophylls content. For this reason, the data collected on the H_2 production were related to the total chlorophylls for all the strains. In particular, the double mutants p + t showed greater versatility in the tested light conditions regarding the H_2 production.

The best production trend was recorded in 12 PAR, but a similar trend occurred also at 50 and 100 PAR (Figure 6.20). Compared to the single mutants, the double mutant exhibited behaviour that seems to be affected by the cumulative effects of the two different mutations.

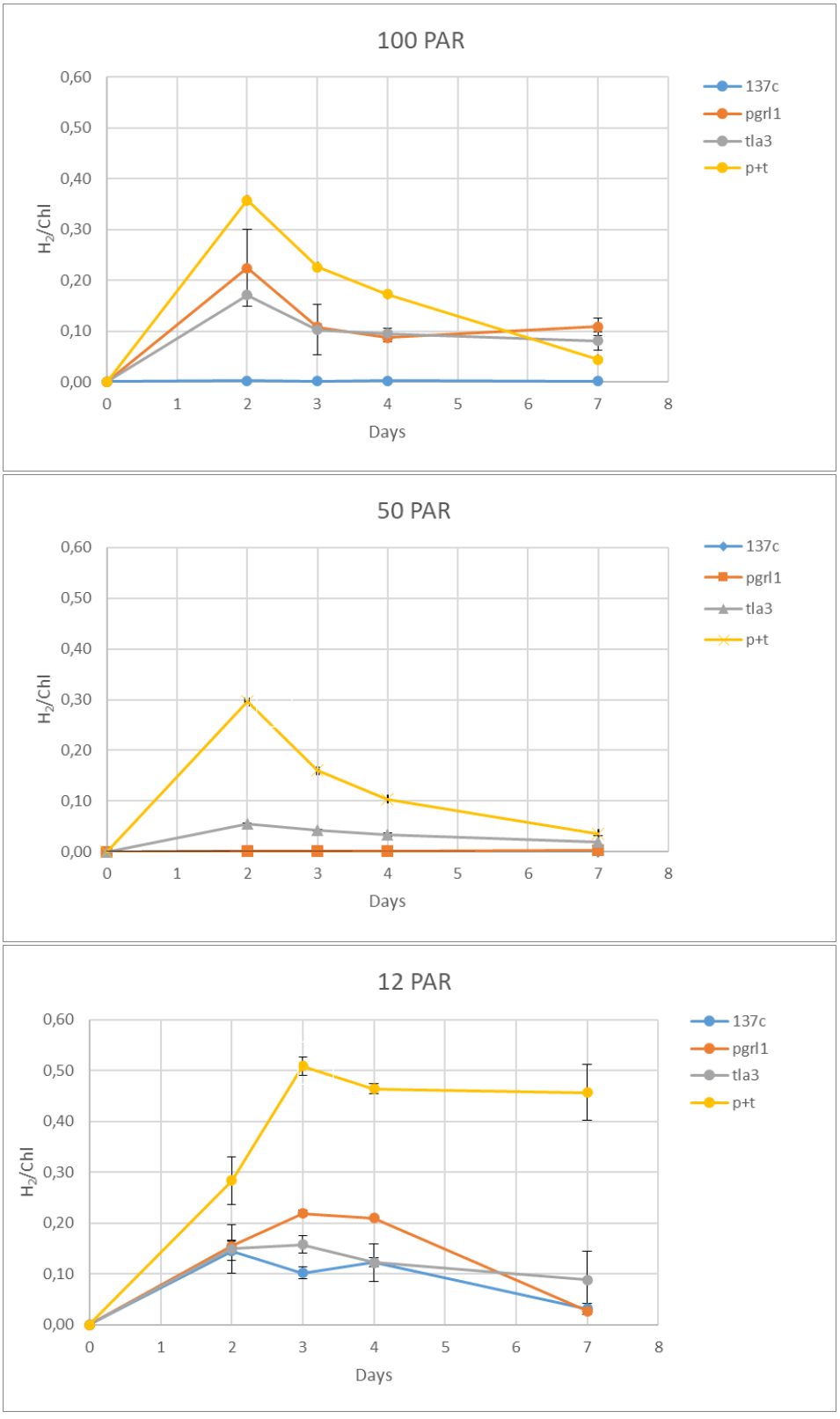


Figure 6.20 - *tla3* and *pgrl1* mutant strains in 12, 50 and 100 PAR. The data comes from three independent experiments. Error bars represent SEM

6.4.2.2 *Clostridium* and *pgrl1* mutants

In this second experimental set, strain 137c was the control strain *Chlamydomonas*. *Clostridium* (clostr) and *pgrl1* were the single mutants, while the "p + c" strain was the double mutant, obtained from the genetic crossing of the two previous mutants. The double mutant allowed us to evaluate the effect of the two mutations together. In particular, the *Chlamydomonas* strain *Clostridium* has a heterologous *Clostridium acetobutylicum* [FeFe]-hydrogenase, less sensitive to O_2.

Also, in this case, qualitative differences related to the different content of total chlorophyll were observed. For this reason, the data collected on H_2 production were related to total chlorophylls for all strains.

Compared to single mutants, the double mutant, in this case, showed no amplified effects (Figure 6.21). The single mutant *Clostridium* showed instead a fast hydrogenase activity in a replete TAP medium. Although this behaviour was predictable given the lower sensitivity of bacterial hydrogenase to oxygen, the inverse trend to that of algal hydrogenases should be underlined. It is noted a production that increases proportionally with the increase of light. This effect might reflect the different characteristics of bacterial hydrogenase.

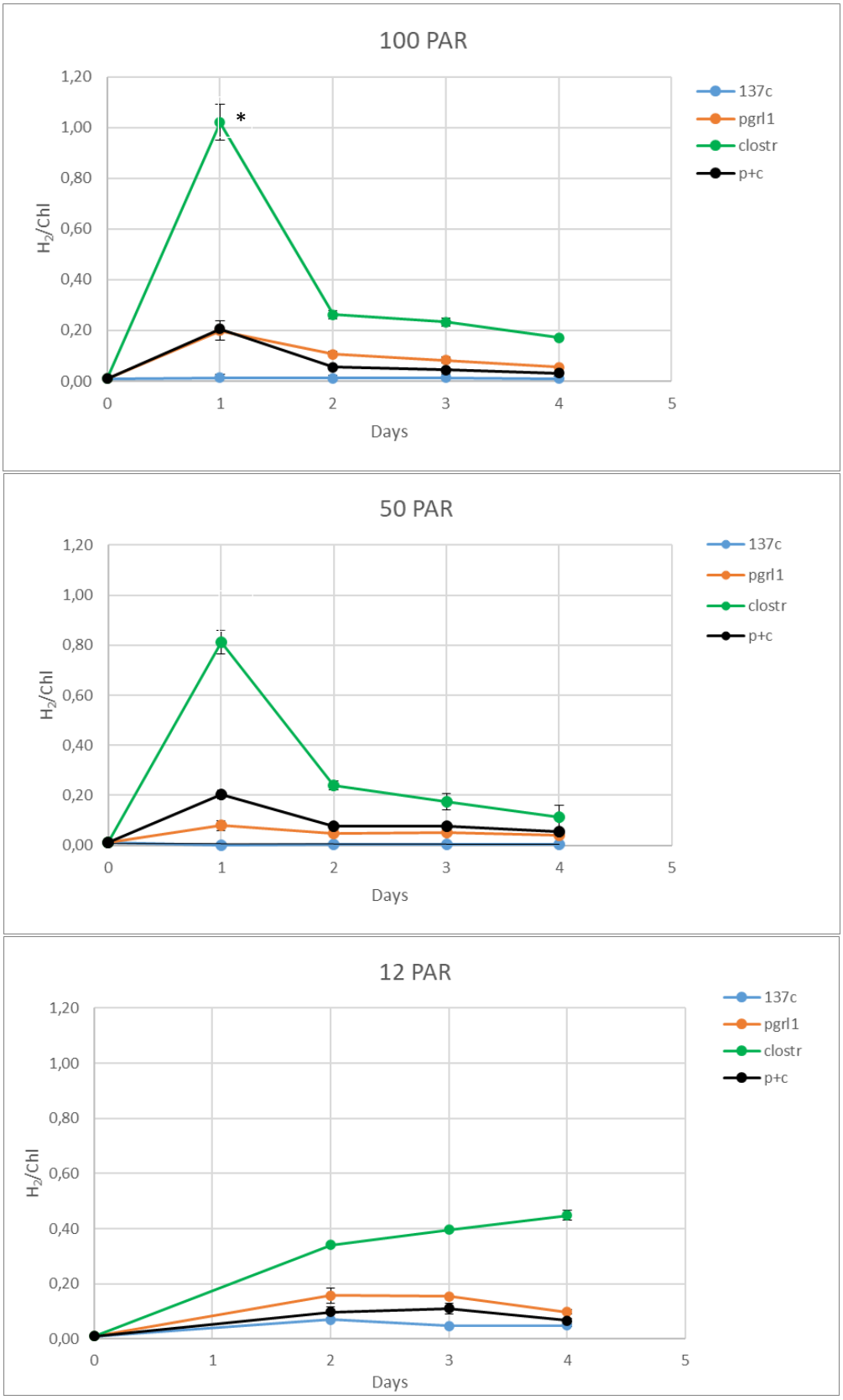


Figure 6.21 - *clostr* and *pgrl1* mutant strains in 12, 50 and 100 PAR. The data comes from three independent experiments. Error bars represent SEM. Statistical difference * ($P<0.05$) in H_2 yields (day 2, 100 PAR) was determined using one-way ANOVA.

6.4.2.3 Mutants exposition at 450 PAR

Considering the marked tolerance to higher light intensities of the *tla3* mutants and the lower oxygen sensitivity of *clostr* mutants, all the mutants involved in the previous experiments were also tested at a light with a high intensity of 450 PAR (Figure 6.22).

The ability to face a possible greater electron photosynthetic flow and the effects on the hydrogen production pathways were evaluated. However, none of the mutants showed significant hydrogen production. The cultures in the bioreactors clearly showed signs of distress.

In this case, no differential growth was evident in any of the cultures of the mutants. Therefore, the data are not related to the chlorophyll content.

In particular, mutations in *tla3* mutants are not sufficient to mitigate the 450 PAR exposition effects or use efficiently high light for hydrogen production.

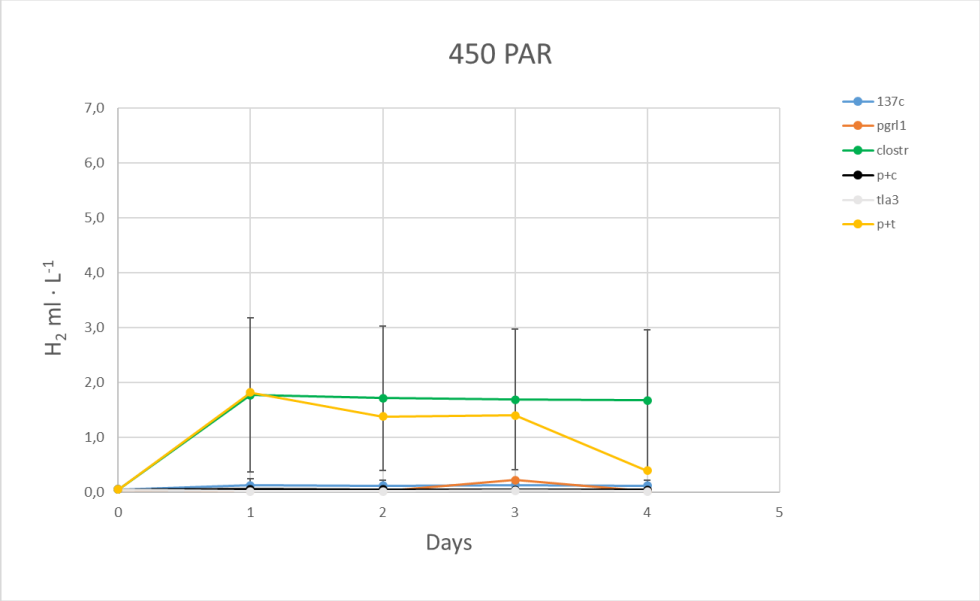


Figure 6.22 - H_2 production of *Chlamydomonas* mutant strains in extremely high light condition. Error bars represent SEM

CHAPTER 7 – CONCLUSIONS

During this PhD project, important, albeit preliminary, evidence emerged regarding new microalgal species that can be considered for hydrogen bio-production.

In particular, two species isolated and characterized in the Basilicata region, *Haematococcus* sp. and *Desmodesmus* sp, have highlighted the presence of metabolic and enzymatic structures capable of producing hydrogen.

Although much of the evidence in the scientific literature reports hydrogen production under heterotrophic growth conditions in acetate, the isolated species *Haematococcus* sp. and *Desmodesmus* sp. have shown versatility from this point of view. The hydrogen production was recorded, albeit with different intensities, using acetate and glucose. To the best of our knowledge, in the literature, there is no evidence relating to hydrogen production in any species belonging to these genera.

This leads to the need to carry out a more in-depth investigation of the mechanisms underlying the metabolism of these microalgae both from a physiological and a molecular point of view. In particular, information on the genetics of the algal species could be collected to structure genetic engineering approaches. These strategies could be used to improve the performance of the enzymes involved in the process or act indirectly on competitive pathways.

New data have been acquired regarding a more optimal hydrogen production as it is not linked to potentially harmful stress conditions and affecting yields.

In particular, regarding single and double *Chlamydomonas* mutants, knowledge about their behaviour in different light conditions and the feasibility of hydrogen production has been expanded. Interestingly, the combination *tla3 + pgrl* proved to be more effective than the respective single mutants being able to produce H_2 even at light intensities that are generally not very tolerated. The single mutant *Clostr* showed instead a fast hydrogenase activity in a replete media also proportionally with the increase of light.

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ANNEX

Publications related to the doctoral project

applied sciences

MDPI

Review

Biohydrogen from Microalgae: Production and Applications

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Abstract: The need to safeguard our planet by reducing carbon dioxide emissions has led to a significant development of research in the field of alternative energy sources. Hydrogen has proved to be the most promising molecule, as a fuel, due to its low environmental impact. Even if various methods already exist for producing hydrogen, most of them are not sustainable. Thus, research focuses on the biological sector, studying microalgae, and other microorganisms' ability to produce this precious molecule in a natural way. In this review, we provide a description of the biochemical and molecular processes for the production of biohydrogen and give a general overview of one of the most interesting technologies in which hydrogen finds application for electricity production: fuel cells.

Keywords: green fuel; biohydrogen; microalgae; *Chlamydomonas reinhardtii*; hydrogenase; fuel cell

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1. Introduction

Nowadays, searching for renewable energy represents one of the major challenges for the global scientific community. Population growth and industrial activities, with consequential high-energy demand, require solutions and proposals in the short-term. It is assumed that the world population could reach 8–10 billion in 2040, while the increase in global energy demand, within the same year, is estimated between 100 and 170 qBtu. However, models cannot accurately predict sudden changes in the global energy situation, as different socio-economic scenarios and political choices modulate each country's response [1,2]. Considering the slow oil, coal, natural gas formation processes, the excessive exploitation of fossil fuels has triggered a drastic and irreversible reserve reduction. Another side aspect of fossil fuel use concerns greenhouse gas (GHG) emissions, particularly carbon dioxide (CO_2), during the combustion reaction [3]. In particular, in the years between 1998 and 2018, CO_2 emissions increased by 48%, making it necessary to implement carbon capture and storage (CCS) technology to limit serious and detrimental effects on climate change [2]. In this worrying scenario, fossil fuels still provide more than 80% of primary energy needs, although the interest in renewable sources, together with their consumption, are steadily increasing [4].

Among renewable options, hydrogen shows the important advantage of CO_2-free combustion, with water as a by-product. Thermodynamic properties, compared to traditional fuels, confirm the interest in its investments in research, although several aspects related to production and storage are still to be mastered. Today, several industrial applications depend on hydrogen, but the most is obtained by techniques, such as steam reforming or electrolysis, not entirely free from the involvement of fossil fuels [5]. Some living organisms, such as microalgae and bacteria, are the basis of processes capable of producing hydrogen in a completely eco-sustainable way. Microalgal hydrogen production is made possible

by biological processes directly or indirectly, depending on sunlight, or by fermentation processes and thermochemical technologies for biomass conversion (Figure 1).

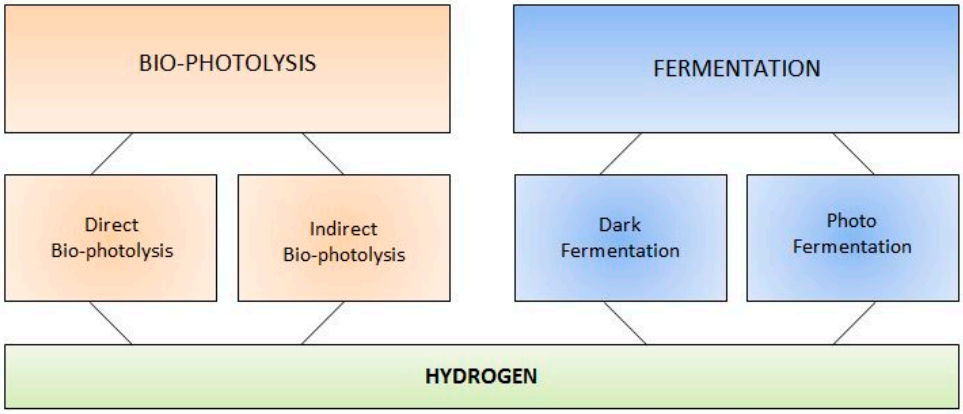

Figure 1. Hydrogen production processes in microalgae.

In recent years, many green systems have focused on algal biomass to obtain energy from living matter. Microalgae store and exploit light energy thanks to the photosynthetic process. To support cell growth and metabolic activities, they use resources widely available in nature, such as water and CO_2. These organisms can concentrate considerable CO_2 amounts and obtain nutrients necessary for growth, even from substrates or waters deriving from industrial waste. Algal cultivation systems, which are simple and relatively inexpensive, ensure advantageous growth rates. Furthermore, they can be set up on infertile territories without competing with agricultural areas that can be exploited for food resources. In particular, the general interest in microalgae has increased significantly in recent decades due to the variety and versatility of the metabolites present in various and numerous species [6].

The present review article is a collection of the most recent evidence on hydrogen production in green microalgae and the efforts to understand and improve the above-mentioned processes in the most widely used species.

2. Hydrogen Metabolism in Green Microalgae: Biophotolysis

Hydrogen metabolism was firstly observed in eukaryotic microalgae in 1939 by German physiologist Hans Gaffron [7]. Due to the oxygen incompatibility, it occurs only transiently in photosynthetic organisms.

2.1. Photosynthetic Electrons and Hydrogenase

The pivotal process of microalgal metabolism consists of oxygenic photosynthesis and complex redox reactions that take place at the level of the thylakoid membranes in chloroplasts through two successive phases. During the first light-depending reactions, ATP and reduced NADH, and NADPH, are generated to be involved in the next dark reactions where the atmospheric CO_2 is fixed by a RuBiSco (ribulose-1,5- bisphosphate carboxylase/oxygenase) enzyme to ultimately generate energy rich-carbohydrate stores. Specifically, during the light phase, an electron transport chain is generated along with photosystems II (PSII) via the plastoquinone (PQ) pool, cytochrome b6f complex (Cyt b6f), and photosystems I (PSI) due to the light energy received as photosystems are associated with light-harvesting complexes I and II (LHC I and LHCII), consisting of numerous photoreceptive pigments. These electrons through PSI leave the electron transport chain and reach the final acceptor ferredoxin (Fd) [8,9].

In anoxic conditions, Fd is able to address electrons to the hydrogenase enzyme. This kind of enzyme catalyzes a reversible reaction in which hydrogen can also be split into electrons and protons:

$$2H^+ + e^- \leftrightarrow H_2 \quad (1)$$

Hydrogenases from the green algae *Chlamydomonas reinhardtii* are the most well-characterized among microalgae. This model organism expresses two different Fe-hydrogenases genes: *HydA1*, the isoform mainly involved under anoxic conditions, and *HydA2* [10,11]. Hydrogenase activity is part of the various responses that microalgal organisms carry out in response to anoxic or stress conditions, and is highly regulated on several levels. After the ribosomal translation in the cytoplasm, the protein is driven to chloroplasts through a transit peptide at the N-terminal end [12]. Assembling of the protein and the catalytic center, H cluster, are required to obtain the functional 47–48 kDa enzyme. *HydEF* and *HydG* genes encode for maturation proteins, crucial for its activation [13]. H cluster is composed of [4Fe4S] unit cysteine-linked to a di-iron unit able to receive electrons via Fd, but easily reversibly inactivated by oxygen [14,15]. Evidence of hydrogen metabolism is reported also in less investigated species, such as *Scenedesmus obliquus* [16,17], *Chlorella fusca* [18], *Chlamydomonas moewusii*, *Lobochlamys culleus* [19], *Chlorococcum littorale* [20], *Tetraselmis subcordiformis* [21].

2.2. Direct and Indirect Biophotolysis

Hydrogenase can receive electrons from different metabolic sources deriving upstream from the biophotolysis of water. At the PSII level, the splitting of water simultaneously produces O_2 and electrons, which, in lighting conditions, reach the Fd and are processed for carbon fixation through the ferredoxin-$NADP^+$ reductase. Under anoxic conditions, oxygen depletion generates a favorable environment for the expression of hydrogenases that start to receive photosynthetic electrons. This process is the direct biophotolysis.

Biophotolysis can also indirectly feed the hydrogen evolving process through the electrons deriving from the breakdown of complex stored carbohydrates, such as starch, which reach Fd and, finally, the hydrogenase. Moreover, the electron load is transferred to the PQ pool via a specific type-II calcium-dependent NADH dehydrogenase (Nda2), and then is transferred to Fd, bypassing PSII without oxygen cogeneration. This process is called indirect biophotolysis [22,23]. Hydrogen evolution is triggered and promoted by modulating different growth strategies, largely experimented on *Chlamydomonas reinhardtii* strains. The most commonly adopted strategy is sulfur deprivation that forces cells to reduce protein synthesis and recover sulfur (S) from protein degradation, such as PSII linked protein D1, impairing the photosystem functions. Exposure to light after dark incubation causes the formation of electrons, but also reactive oxygen species near the defective photosystems with potentially harmful consequences on cell structures. In this context, hydrogenase activity works as a safety valve to preserve structures from oxidative stress, combining electrons with protons to produce hydrogen [24].

3. The Role of Growth Parameters in Hydrogen Production

Acting on cultivation parameters is the simplest way to produce immediate effects on the growth or the specific metabolite production.

Regarding media components, the aforementioned S deprivation gives the expected effects only if preceded by a photosynthetic growth phase, providing all of the necessary elements for growth. More recently, alongside this strategy, which envisages separate growth and a deprivation step with several operational issues, a different modality that involves minimal quantities of S to simulate a condition of starvation has also been tested [25,26]. Moreover, particular attention should be paid to the carbon source used: it has been observed that an increase in starch reserves during the phase preceding S deprivation can be crucial in supporting production. Mixotrophic conditions involving both inorganic and organic carbon sources appear to be preferred. [27,28].

The less studied condition of nitrogen (N) deprivation induces hydrogen production in *Chlamydomonas reinhardtii,* albeit with a delayed effect and lower yields, suggesting a different mechanism in establishing process [29]. In *Scenedesmus obliquus,* N deprivation modulates metabolism towards lipids accumulation to be used as an energy reserve, suggesting that this strategy is not advantageous for the purposes of hydrogen production [30]. In ma-

rine *Chlorella* spp., where S deprivation is inapplicable due to the sulfates-rich presence in seawater, the effect of phosphorus (P) deficiency was evaluated. Similarly, P deprivation was able to establish anaerobic metabolism with a sustained hydrogen photoproduction, even if low-density culture were required to reduce the effect linked to cellular P reservoirs [31]. Less common element deprivations, such as magnesium (Mg), were also tested. Mg-deprived *Chlamydomonas reinhardtii* cells exhibited a longer hydrogen production over time than the same cells in the case of S deprivation. This response may depend on the lesser Mg importance for cellular activities compared to S [32].

Various chemical compounds added in the cell media can affect protons and electrons mobilization inside the cell and, consequently, hydrogen metabolism. The protonophores carbonyl cyanide m-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol (DNF) increase hydrogen production, owing to proton mobilization [33]. The action of other similar decoupling agents, such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DMCU) and carbonyl cyanide p-trifluoromethoxy phenylhydrazone (FCCP) allow to stimulate hydrogen production through the PS II independent pathway, although the underlying mechanisms are not deeply understood [34].

Parameter changes to produce hydrogen require several operational steps, especially during cell transfer upon different media. To handle culture more easily, some attempts have demonstrated the feasibility of cell cultivation through different immobilization systems [35]. Most of the attempts have been applied in *Chlamydomonas reinhardtii* through the use of fiberglass matrix, thin alginate films, or, more recently, sodium alginate beads. In particular, the latter study selected beads of few millimiters diameter to further investigate the process in the classical photobioreactors, bringing out the need to adapt bioreactors to new immobilization systems [36–38].

Indeed, photobioreactor design must take into account downstream applications and process needs. Closed systems are used in lab-scale hydrogen production experiments and represent a better alternative to open systems. Growth parameters, especially temperature and pH, are strictly monitored and, at the same time, the collection of the gas produced is less subject to dispersions. Particular importance is attached to the agitation mode and the intensity and quality of the light radiation. Vertical column, tubular, and flat panel, are the most investigated photobioreactor modalities for hydrogen production. The latter seems to be preferred as it provides the highest surface/volume ratio, although it is not a solution to all technical issues, and this area of research still faces numerous challenges [9,39].

4. Genetic Engineering Approaches

4.1. Random Mutagenesis

The approaches described in the previous paragraph act in a transitory way, since it is not possible to manage microalgal culture by applying stress conditions permanently. Indeed, depriving a cell culture of the optimal growth conditions for a long time leads to cellular suffering that can culminate in cell death. In this way, only a discontinuous hydrogen production is possible, alternating stress and recovery phases to avoid permanent damage to the cells. One strategy to overcome this limitation concerns the selection of organisms with a higher aptitude to withstand these stress conditions; another one could be the selection of organisms which are less susceptible to the conditions that usually hinder hydrogen evolution in photosynthetic green microalgae.

Physical and chemical treatments induce transmissible genomic mutations that favor the appearance of new traits in an organism. These treatments are not oriented towards a specific target and it is necessary to take into account an expensive screening phase among the mutants produced to select the ones with characteristics of interest. However, a recent study demonstrated that *Chlamydomonas reinhardtii* mutants obtained via atmospheric and room temperature plasma (ARTP) showed a lighter green coloration, compared to wild types, indicative of lower chlorophyll content. The lower chlorophyll content is associated with better photosynthetic performance (probably due to the improved light transmit-

tance and the consequent increased solar energy conversion efficiency), as confirmed by transcriptomic analyses, with consequent benefits in hydrogen production [40].

4.2. Targeted Mutagenesis

On the other hand, targeted approaches turn out to be more advantageous and several proposals have been provided by genetic engineering tools to increase yields and overcome current limitations.

As already mentioned, Fd receives electrons from the electron transport chain to address them to hydrogenase. Even under optimal conditions for enzyme expression, other assimilatory pathways compete for the reductants impairing the overall yield. Ferredoxin-$NAD(P)^+$ reductase (FNR) represents the main competitor shuttling electrons from Fd towards CO_2 fixation. To bypass this limitation, fusion protein has been designed. Among the most recent attempts, a fusion complex between Fd and hydrogenase in an in vivo culture of *Chlamydomonas reinhardtii* was evaluated. The complex demonstrated higher production rates and greater oxygen tolerance than the sole enzyme [41]. Similar behavior was also observed in the *Chlamydomonas reinhardtii* protein D1 mutant. A double amino acid substitution (L159I – N230Y) gave the mutant several new characteristics, including greater oxygen tolerance than the wild strain [42]. Similarly, *Chlamydomonas reinhardtii* mutants knock out for flavodiiron protein (FDP) showed higher photoproduction of hydrogen than wild types. In this way, it is again demonstrated how by eliminating a competing pathway (for example the FDP-mediated O_2 photoreduction pathway), the electrons are preferentially conveyed towards the production of hydrogen. Furthermore, it has been seen that, even exposure to prolonged light pulses in these mutants do not direct the metabolism towards CO_2 fixation. It is, therefore, demonstrated how genetic engineering approaches together with actions on growth parameters are jointly useful to increase production [43].

LHC complexes exhibit a poor light energy conversion with more than 80% absorbed light energy wasted as fluorescence or heat and not addressed towards hydrogen production. Conversely, a truncated light-harvesting antenna has demonstrated improvements in terms of photoinhibition and light saturation phenomena. Afterward, the same approach has also shown encouraging results for hydrogen production: tla1 CC-4169 *Chlamydomonas reinhardtii* mutant has exhibited to produce until six-time more hydrogen compared to the wild type strain with a light intensity of 350 $\mu E\ m^{-2}\ s^{-1}$ [44,45].

Non-coding RNA molecules, such as microRNA (miRNA) or long non-coding RNA (lncRNA), with regulatory function, are part of the most recent discoveries in microalgae and several studies have already exploited them for innovative approaches. In particular, miRNAs exhibit a regulatory function on the translation process by binding or degrading the messenger RNA and avoiding the corresponding protein synthesis. Transcriptomic studies showed that stressful situations in microalgae lead to an increase in these molecules which reflects the need to obtain immediate responses by the cell [46]. In *Chlamydomonas reinhardtii*, some endogenous miRNAs have overexpressed in S deprivation conditions. These observations led to the design of several artificial miRNAs (amiRNAs) to increase hydrogen yields by stimulating a faster oxygen consumption or repressing *psbA* gene expression that encodes for PSII linked D1 protein [47,48]. Similarly, optogenetic systems have also developed using properly blue light-inducible expression amiRNAs. This gene control system has enhanced hydrogen production, confirming as a most promising tool [49]. Approaches related to genetic engineering require a fine upstream design and considerable resources. Certainly, specific approaches compared to random ones allow for better management of resources. Genome-scale metabolic reconstructions have already been used to direct choices in this sense for many species of prokaryotes and eukaryotes. For algal organisms, a similar tool has been developed using information from the literature. In particular, the AlgaGEM software is configured as a tool capable of defining the primary metabolism of Chlamydomonas reinhardtii and allowing the in silico prediction of any changes in the growth parameters or the engineering of specific metabolic pathways [50].

Engineering strategies generally allow the establishment and introduction of new traits that are advantageous for different research fields. Although they are not easy to implement techniques and require huge resources, new bioinformatics tools to support this area seem to push further to consider this strategy as one of the most promising for the energy sector.

5. Fermentation Processes and Biomass-Applied Technologies

Direct and indirect biophotolysis processes are intrinsically linked to the photosynthetic process and the connected electron transport chain. Together with these two photosynthetic pathways that contribute to the production of hydrogen, another one linked to fermentative metabolism has also been identified. In dark conditions, the enzymatic activity of pyruvate:ferredoxin oxidoreductase (PFR) in *Chlamydomonas reinhardtii* is responsible for the reduction of Fd and the passage of electrons towards hydrogenase. Overall, this pyruvate-dependent hydrogen production acts in ways similar to those observed in bacteria, and though the yield is low, its contribution is not negligible [51]. The accumulation of complex carbohydrates, such as starch, or endogenous substrates, is positively associated with the production of hydrogen, while, it was also observed that exogenous carbon-rich media further stimulate its production in the early anaerobic stages. Several fermentative bacteria use anaerobic processes to transform carbon sources into various by-products, including hydrogen. Processes, such as photo- and dark- fermentation, are commonly exploited and widely investigated in bacteria species, such as *Escherichia coli*, *Clostridium* spp., *Thermococcales* spp., *Rhodobacter* spp., and *Rhodopseudomonas* spp. [52,53]. The consumption of organic substrates, also deriving from waste, by photofermentation, include the transformation into organic acids, alcohols, CO_2,, and H_2 in presence of light, but with a low overall yield of solar energy conversion. In a similar way, but without light, dark fermentation uses various substrates and waste too, leading to the release of different components and gaseous mixtures in which hydrogen is present [46,54]. It has recently been observed that hydrogen production can be increased by up to 60% compared to *Chlamydomonas reinhardtii* monoculture systems, by using co-culture systems with *Escherichia coli*. Growth media glucose-rich are exploited by bacteria that produce acetic acid, which can be used in algal metabolism [55]. Synergistically, different photobiological and fermentative microbial metabolisms may interact and cooperate increasing the hydrogen yields [56].

Microalgae show an enormous biodiversity being present in different habitats, even extreme and hostile to most living organisms, suitably adapting their metabolism. It is therefore possible to modulate the growth conditions to obtain biomass of the desired composition, based on the requirements of the downstream process also using industrial and agricultural processing water and waste. In this perspective, various strategies have been applied by combining bioenergy production and bioremediation approaches [57,58]. Unicellular green alga *Scenedesmus obliquus* managed to biodegrade the phenolic content present in the olive oil mill wastewater. This strategy makes it possible to remedy a problem particularly encountered in the Mediterranean area and, since the biotransformation carried out consumes oxygen, favorable conditions to trigger a concomitant production of hydrogen are also generated [59]. A consortium of microalgae, mainly composed of *Scenedesmus* and *Chlorella* species, grown in pig manure showed good growth, without the addition of external nutrients, and significant fermentative hydrogen production [60,61].

One of the main problems associated to these approaches is the elevated costs in terms of management and purification of the components obtained, which include the presence of several by-products, also toxic. Moreover, although the biomass of the microalgae contains a reduced lignin content compared to other lignocellulosic feedstock previously used for energy purposes, preliminary treatments are often necessary to facilitate the extraction and conversion of the microalgae content. Mechanical, thermal, chemical, or biological treatments are often applied to biomass separately or in combination as a preliminary step [57,58].

6. Fuel Cell

Hydrogen is an energy carrier with a high calorific value of 122 kJ/g, which is about 2.75 folds than fossil fuels, and it is also an environmentally friendly molecule since it only gives water as a by-product of its combustion [62]. For this reason, hydrogen environmental damage ratio has been estimated as 1, compared to that of several hydrocarbon fuels about 20 times higher [8,63]. Hence, hydrogen is considered as the best alternative to fossil fuels, which usage is supposed to be drastically reduced by 2050, according to the 2015 Paris Climate Agreement. Several technologies are currently available for hydrogen production (Figure 2), which can be classified according to the starting material: on the one hand, from fossil fuels, hydrogen can be obtained by thermochemical conversions, such as partial oxidation, autothermal reforming, or steam reforming. On the other hand, exploiting renewable sources, hydrogen is made by water electrolysis or biomass thermochemical conversion [64].

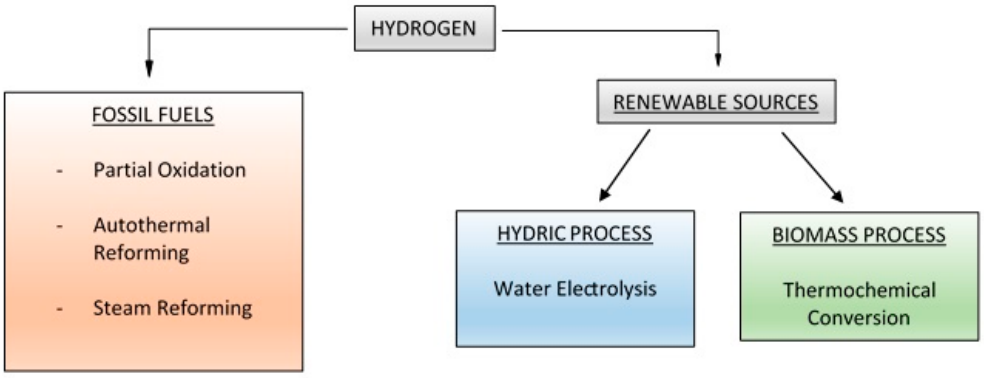

Figure 2. Major hydrogen production technologies.

However, none of these processes is sustainable: they all are energy-intensive process, usually requiring high temperatures. For example, water electrolysis requires temperatures ranging from 20 °C to 100 °C [65], while thermochemical processes can reach 2000 °C [66]. Moreover, fossil thermochemical conversions release high amounts of CO_2 [62]. Theoretically, it could be possible to convert these technologies into green ones by pairing them to mechanism that prevent CO_2 releasing into the atmosphere. This is the CSS approach, according to whom CO_2 can be stored into adequate geological sites, or reutilized for the chemical synthesis of useful products such as CO, urea, methanol, polymer, and carbonates [67]. Nevertheless, CSS approach has high design and operational costs that make his application unfeasible on the long term [68]. To implement a completely green sustainable economy, it is necessary to switch to biohydrogen, the biological hydrogen produced by microorganisms, including microalgae, according to the methods described in the previous paragraphs.

An interesting technology that has raised attention in recent years is that of fuel cells, in which hydrogen is often used as fuel. A fuel cell is an electrochemical technology capable of energy conversion. It is indeed capable to transform the chemical energy of a fuel into electricity [69]. A fuel cell may vary for it architecture, for the kind of fuel or for its catalyst; but it always consists of a few simple main parts (Figure 3): electrodes (an anode and a cathode), electrolytes, and an external circuit [70].

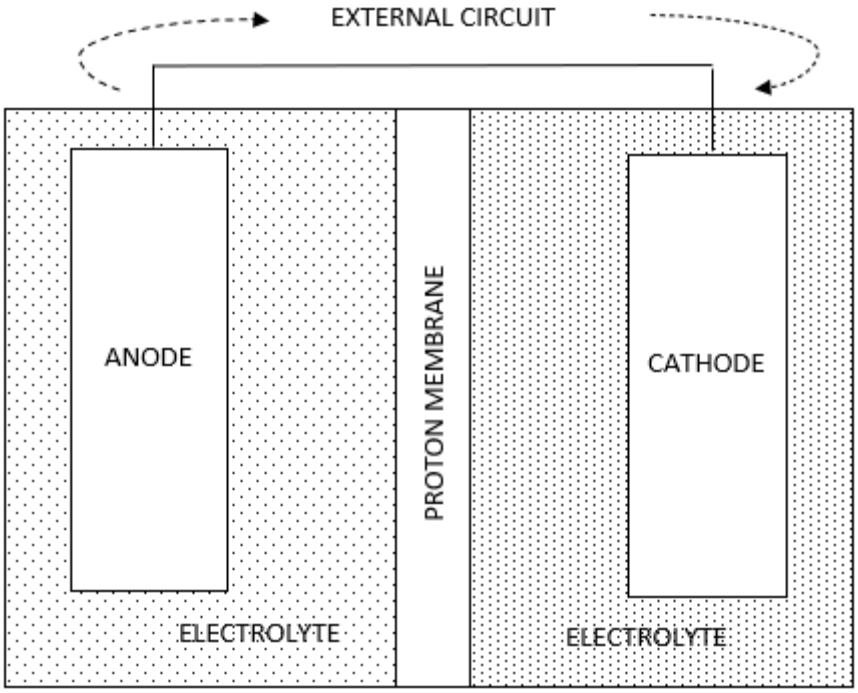

Figure 3. A basic fuel cell.

The reactions that happen in a fuel cell are simple: the fuel (usually hydrogen) at the anode is oxidized; then the electrons, through the circuit, reach the cathode, where (usually) oxygen is reduced to water [71]. Despite the simplicity of the processes, fuel cells currently present numerous criticalities that limit their use on a large scale. Their main problems are durability of the materials and high costs, often both related to the catalyst used. The most commonly used catalyst is platinum, both pure and alloyed, due to its maximum activity and chemical stability. In some cases, platinum group metals (PGMs) have even been used, including palladium, ruthenium, rhodium, iridium, and osmium. However, all of these materials have high costs due to their global scarcity [72]. The second issue of this technology concerns how to get hydrogen to the fuel cell itself. Storage is, at the moment, the main limitation to the development of an effective hydrogen economy. Many technologies are being studied in order to reach the highest volumetric density possible: physical methods, such as high-pressure cylinders for gaseous hydrogen or cryogenic tanks for liquid hydrogen, chemical reaction with metal and alloys, new materials, such as carbonaceous nanostructures for hydrogen absorption [73–75].

For this reason, technological research continues unabated. Based on the various innovative solutions that are proposed, it is possible to give a classification, to the different types of fuel cells currently under development. There are six of them [72]:

1. Proton Exchange Membrane Fuel Cell (PEMFC);
2. Alkaline Fuel Cell (AFC);
3. Phosphoric Acid Fuel Cell (PAFC);
4. Molten Carbonate Fuel Cell (MCFC);
5. Solid Oxide Fuel Cell (SOFC);
6. Microbial Fuel Cell (MFC).

6.1. Proton Exchange Membrane Fuel Cell

In a PEMFC, platinum is substituted by an ion exchange membrane that facilitates ion migration. This membrane is a polymer that usually has negatively charged group in order to let protons to flow toward cathode; but there also exists anion exchange membranes that hold positively charged groups so that the anion can be transported [76]. The most commonly used membrane is that made of perfluorosulfonic acid polymers—commonly

known as Nafion®. It was first commercialized by the DuPont company in the 1960s and it soon received wide acceptance because of its qualities. Nafion® is a robust polymer with high chemical and mechanical resistance, good conductivity, and little water or fuel crossover [70,77]. However, it is a costly material, covering about 20% of the cost of a fuel cell [77], and it is also thermolabile since it works only at a low temperature (50–80 °C) [78]. Alternative polymers are under development, mainly to allow the fuel cell to operate at high temperatures in order to optimize its yield. The most promising materials are currently aromatic-based membranes consisting of aryl rings and polybenzimidazole linkages [77].

6.2. Alkaline Fuel Cell

AFCs work at high pH using anion exchange membranes generally based on poly(olefine), poly(arylene ether), poly(phenylene oxide), poly(phenylene), polysulfone, and poly(ether imide). These fuel cells have lower costs than PEMFCs, and they are more resistant to high temperature. However, their main weak point is intrinsic due to the lower conductivity of OH- compared with protons [79].

6.3. Phosphoric Acid Fuel Cell

Phosphoric acid as an electrolyte in fuel cell consent to elevate the working temperature to high temperature around 220 °C. Therefore, it is possible to connect the PAFC directly to a steam reformer, to easily take up hydrogen from the source. The main flaws of this type of fuel cell are the necessity to use a metal catalyst on the electrode, and the hydrogen source that is not sustainable [80]. Recent studies have evaluated how the yield of PEMFCs improve by doping the membrane with phosphoric acid [81].

6.4. Molten Carbonate Fuel Cell

The electrolyte of this fuel cell is a molten carbonate salt solubilized in a lithium aluminate matrix. It can reach very high working temperatures (650 °C); thus, it is not necessary to connect it to an external hydrogen source because it self-reforms gases, also functioning with different hydrocarbon fuels [82,83].

6.5. Solid Oxide Fuel Cell

It is a high-temperature fuel cell and exists in two different types, the oxygen ion conducting fuel cell, and the proton-conducting one. Both have high-energy conversion efficiency and fuel flexibility, but the high fabrication cost makes them commercially not competitive [84].

6.6. Microbial Fuel Cell

These are the greenest and most sustainable types of fuel cells, and undoubtedly represent the future of energy production. MFCs can be double-chambered, with separated anodes and cathodes, or single-chambered, having the electrodes in the same container [85]. In both cases, they exploit microorganisms and their metabolism to produce the fuel necessary for the fuel cell to function. Most MFCs are mixed, using anode bacterial cultures for hydrogen production and cathode microalgae strains for oxygen supply [86–88]. However, prototypes of fuel cells that work only with algal strains are in development [89,90]. MFCs have significant environmental benefits. Thanks to the biological processes underlying their functioning, they can combine energy production with other functions, such as bioremediation activities [91–93]. It also bypasses the hydrogen storage limitation, since hydrogen is produced and utilized almost at the same time in the anodic chamber. However, this technology is not yet widely applied due to high costs and ineffective yields, which require further study for improvement [86,94–97].

7. Conclusions

In this review, we offered a general summary of the current status of biohydrogen applications. Hydrogen is undoubtedly the future fuel for its green and environmentally

friendly properties. However, its production technology is still based on fossil fuel; thus, carbon releasing. Scientific research is working hard to improve new strategies to reach green and sustainable hydrogen production and exploitation technologies.

Microalgae seem to be an attractive solution to this problem. As previously described, they can produce biological hydrogen without carbon emissions; rather, by fixing it during the process. The limiting factor for large-scale applications of this ability is that of low production yield, and, therefore, scientific research must focus in this direction. Solutions described in this review represent the most promising developments for implementing hydrogen yield.

The extreme versatility of microalgae also consents to combine several applications; thus, multiplying the benefits. The use of microalgae in dedicated fuel cells allows the development of an ecological energy production system, which can be associated with bioremediation advantages. In fact, microalgae can grow even in wastewater, purifying them from heavy metals and other dissolved substances.

Since current wastewater treatment plants present some critical issues concerning GHG emissions [98–100], developing an integrated purification and energy production facility based on microalgae could represent a promising ecological technology for the future.

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