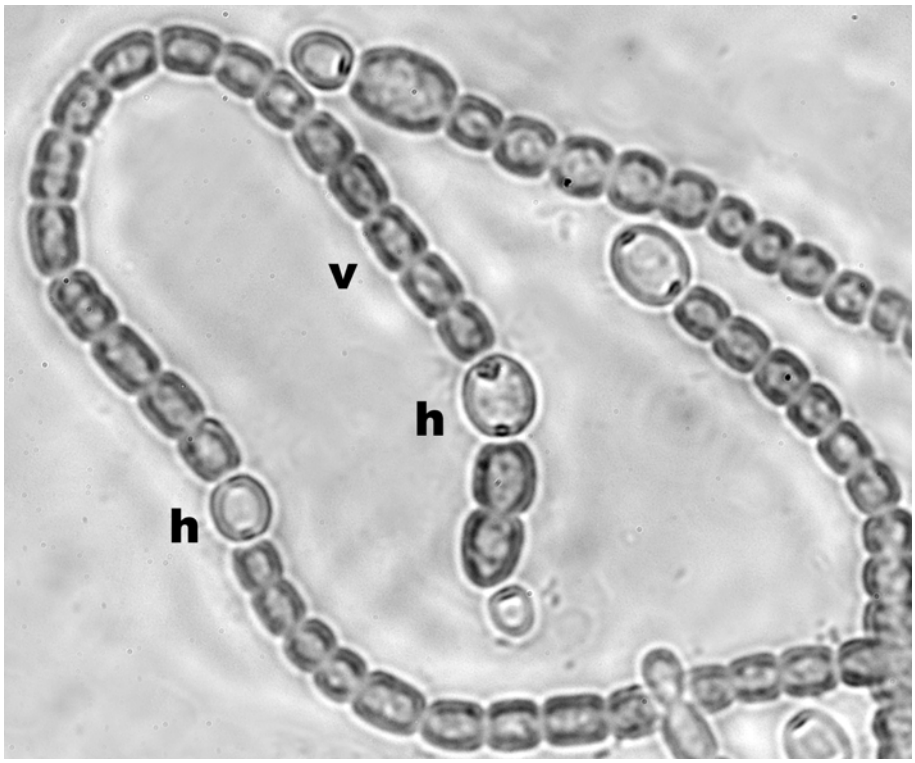
Method implementation and technique development for studies with cyanobacteria



- Fernando A. Lopes Pinto -

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Obrigado Mamã. Obrigado Papá.

Biohydrogen

The increase of global pollution and the depleting of the fossil fuel reserves make the research concerning alternative energy resources a priority. One environmentally acceptable alternative energy carrier, which could initially supplement or even substitute fossil fuel, is molecular hydrogen, H_2 [1].

Hydrogen produced by microalgae and bacteria is biohydrogen [2]. Diverse microorganisms are capable of hydrogen evolution: from light dependent photosynthetic microorganisms to non-photosynthetic microorganisms that depend on organic and inorganic compounds. These are classified into anaerobic bacteria, fermentation bacteria, aerobic bacteria, photosynthetic bacteria, and algae [3] (see Table 1).

Table 1. Classification of hydrogen producing bacteria. Taken from [3].

Available Energy Form	Enzyme of H_2 Evolution	Class of Bacteria		Genus of Bacteria	Electron Donor
Photosynthesis		Green Algae		*Chlamydomonas*	Water
	Hydrogenase	Cyanobacteria	Heterocyst	*Chlorella*	"
				Anabaena	"
			Non-Heterocyst	*Oscillatoria*	"
	Nitrogenase	Photosynthetic Bacteria	Non-sulfur Bacteria	*Rhodopseudomonas*	Organic Matters (Organic Acids)
				Rhodobacter	"
				Rhodospirillum	"
			Sulfur Bacteria	*Chromatium*	Sulfates
				Thiocapsa	"
Non-Photosynthesis	Hydrogenase	Obligate Anaerobes		*Clostridium*	Organic Matters (Sugers)
				Methanobacterium	"
		Facultative Anaerobes		*Escherichia*	"
	Nitrogenase	Nitrogen Fixing Bacteria	Facultative Aerobes	*Azotobacter*	"
				Clostridium	"
			Facultative Anaerobes	*Klebsiella*	"

Production of hydrogen by direct splitting of water using solar radiation is seen as the preferable solution. Three ways to achieve this can be considered: a) the use of photochemical fuel cells, b) by applying photovoltaics, or c) by promoting production of hydrogen by photosynthetic microorganisms [4].

Cyanobacteria

Cyanobacteria have a long evolutionary history thought to extend to at least 3500 million years ago [5]. This group includes many different physiological and morphological types that can be found in many different habitats – from cold seawater to hot springs. Cyanobacteria show a remarkable morphological diversity. They may be unicellular or filamentous, occurring singly or grouped in colonies [6] - an example of their morphology is shown in Figure 1.

All cyanobacteria are characterized as eubacteria that grow as autotrophs with CO_2 as the carbon source, utilizing an oxygen-producing photosynthetic mechanism for the generation of ATP and reductant.

Cyanobacteria are gram-negative prokaryotes with the ability to synthesize chlorophyll *a* and carry out photosynthetic metabolism. Water is typically the electron donor during photosynthesis – that results in oxygen evolution and reduction of carbon dioxide. In fact, this photosynthetic activity is thought to have been the main source of atmospheric oxygen during the first million years of our planets life. But at the same time oxygen inactivates the nitrogenase used to convert atmospheric nitrogen to ammonium. One successful way to circumvent the detrimental influence of oxygen [7, 8] was to restrict the process of nitrogen fixation to a differentiated, non vegetative, cell type whose interior is microaerobic. These cells are called heterocysts (Figure 1).

Hydrogen evolution by cyanobacteria

For photobiological hydrogen production cyanobacteria are among the ideal candidates since they are capable of hydrogen evolution, and have simple nutritional

requirements - they can grow in air (N_2 and CO_2), water and mineral salts, with light as the only energy source [1].

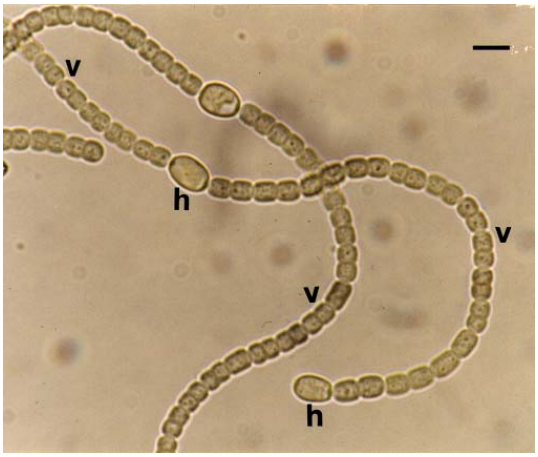

Figure 1 – The filamentous cyanobacterium *Nostoc punctiforme*, with vegetative cells (v) and heterocysts (h).

When analyzing the H_2 metabolism in nitrogen-fixing cyanobacteria in detail, three enzymes should be taken into consideration: a) nitrogenase, evolving hydrogen during nitrogen fixation, b) an uptake hydrogenase, recycling the hydrogen produced, and c) a bi-directional hydrogenase that catalyses both hydrogen production and consumption [9] (see Figure 2 and Equations 1, 2, 3, and 4).

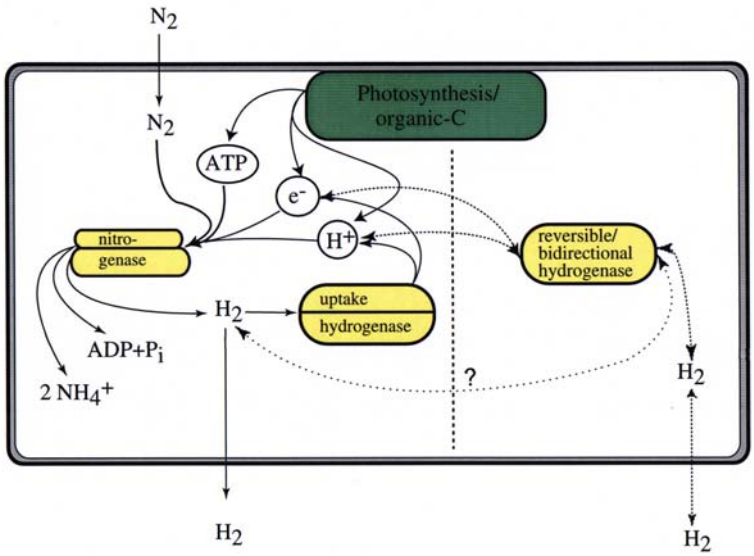

Figure 2 - Schematic representation of hydrogen metabolism in cyanobacteria. The enzymes directly involved in either evolution or uptake of hydrogen are represented. Taken from [10].

Cyanobacteria split water directly into protons and oxygen using light as energy source. This hydrolysis (Equation 1) has very simple requirements – water and light – making it environmentally important [11].

Equation 1. $H_20 \rightarrow 2H^+ + 1/2O_2 \quad (\Delta G^0 = +237kJ)$

Protons obtained from water splitting are then used by nitrogenase to oxidize ferrodoxin, using ATP in the process (Equation 2) [11]. At the same time, the oxygen sensitive nitrogenases promote the reduction of nitrogen to nitrate, in an ATP dependent reaction [1] (Equation 3).

Equation 2. $2H^+ + 2Fd_{red} + 4ATP \rightarrow H_2 + 2Fd_{ox} + 4ADP + 4Pi$

Equation 3. $N_2 + 8H^+ + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16\ ADP + 16Pi$

The hydrogen evolved by the nitrogenase can be further metabolized by a group of enzymes known as hydrogenases. This group of enzymes catalyzes the inter-conversion between hydrogen gas and its elementary particle constituents (Equation 4).

Equation 4. $H_2 \longleftrightarrow 2H^+ + 2e^-$

Uptake hydrogenases, catalyzing the consumption of hydrogen, have been found in all nitrogen-fixing cyanobacteria examined so far [12-14]. The catalyzed reaction, thought to be advantageous to the organisms, is undesired from a hydrogen evolution point of view.

Bidirectional hydrogenase is able to either produce or oxidize hydrogen. Its role in cyanobacterial metabolism is still unclear. It has been suggested that this enzyme acts as a mediator in the release of excess of reducing power in anaerobic environments [15]. Another hypothesis states that bidirectional hydrogenase in *Synechocystis* sp. PCC 6803 works as an electron valve for the disposal of low potential electrons generated at the onset of illumination [16]. Even considering the fact that the bidirectional enzyme is not a universal cyanobacterial enzyme [14, 15, 17, 18], its impact on hydrogen evolution should

not be overseen. The role of this enzyme should be more thoroughly investigated and its potential for hydrogen evolution explored.

The use of cyanobacteria is regarded as promising not only as a putative hydrogen producer but also as a tool to further understand the physiology and genetics of biological hydrogen metabolism. The last 30 years of research gave the field a good level of basic knowledge that should be the base of future research [19]. Initial work focused on the heterocystous filamentous cyanobacterium *Anabaena cylindrica* B-629 - light intensity, oxygen, uptake hydrogenase, and gas phase composition were immediately underlined as key factors in the success of hydrogen evolution (Table 2).

Table 2. Maximum hydrogen evolution by *Anabaena cylindrica* B-629 when grown and assayed under different conditions. Adapted from [19].

Maximum hydrogen evolution	**Growth conditions**	**H_2 evolution assay conditions**	**Reference**
0.486 μmol/mg dry wt/h	air + CO_2 (3%); total incident light was 2 x 10^4 erg/cm^2/s	Ar (100%); total incident light was 6 x 10^4 erg/cm^2/s	[20]
32 μL/mg dry wt/h (1.33 μmol/mg dry wt/h)	air + CO_2 (0.3%); total incident light was 1.8 x 10^4 erg/cm^2/s	Ar + CO_2 (3%); total incident light was 4 x 10^5 erg/cm^2/s	[21]
0.17 μmol/mg dry wt/h	air + CO_2 (0.3%); 7000 lux at the surface of the culture vessels	Ar + CO_2 (3%); 7000 lux at the surface of the culture vessels	[22]
0.11 μmol/mg dry wt/h	air + CO_2 (0.3%); 7000 lux at the surface of the culture vessels	air + CO_2 (3%) + CO (2%) + C_2H_2 (10%); 7000 lux at the surface of the culture vessels	[22]
0.103 μmol/mg dry wt/h	air + CO_2 (5%); 7000 lux at the surface of the culture vessels	Ar + CO_2 (3%); 4000 lux at the surface of the culture vessels	[23]
29 μL/mg dry wt/h (1.21 μmol/mg dry wt/h)	Ar + CO_2 (0.5%); ammonium chloride (0.2 mM) added every 2 days	Ar	[24]
about 0.16 μmol/mg dry wt/h	air + CO_2 (0.3%); 7000 lux at the surface of the culture vessels	Ar + CO_2 (3%); 4000 lux at the surface of the culture vessels	[25]
about 0.22 μmol/mg dry wt/h	air + CO_2 (5%); 7000 lux at the surface of the culture vessels	N_2 + CO (0.2%) + C_2H_2 (5%) + CO_2 (5%); 4000 lux at the surface of the culture vessels	[26]

The cultivation of cyanobacteria in nitrate-free media under air and CO_2, followed by incubation in light under argon and CO_2 atmosphere, rapidly became standard, since it resulted in immediate hydrogen production [20, 21]. It was also established that light saturated, nitrogen-fixing cultures had a good and stable supply of reductant, underlining the relationship between photosynthesis and nitrogen fixation. These conditions were considered vital for optimal nitrogen fixation and biomass production. Filament integrity was also discussed and established as a factor in the duration of hydrogen production, since filament breakage leads to loss of nitrogenase activity [21]. The need for strategies in order to minimize mechanical breakage of filaments and general improvement in experimental conditions became evident.

Recently, research was presented analyzing hydrogen evolution in different cyanobacterial strains in an effort to find a suitable strain for cyanobacterial biohydrogen production [27] (Tables 3 and 4).

Table 3. Comparative study of hydrogen production in some heterocystous cyanobacteria. All organisms were grown under air and 20μE/m^2/s, and H_2 evolution assays were performed under Argon atmosphere and 60 μE/m^2/s. Adapted from [19].

Organism	**Maximum hydrogen evolution (μmol/mg chl a/h)**
Anabaena sp. PCC 7120	2.6
Anabaena cylindrica IAM M-1	2.1
Anabaena variabilis IAM M-58	4.2
Anabaenopsis circularis IAM M-4	0.31
Nostoc muscorum IAM M-14	0.60
Nostoc linckia IAM M-30	0.17
Nostoc commune IAM M-13	0.25
Anabaena cylindrica UTEX B 629	0.91
Anabaena flos-aquae UTEX 1444	1.7
Anabaena flos-aquae UTEX LB 2558	3.2

Table 4. Maximum hydrogen evolution by different *Anabaena* strains. Adapted from [19].

Organism	Maximum hydrogen evolution	Growth conditions	H_2 evolution assay conditions	Reference
Anabaena sp. N-7363	36 μmol/mg chl *a*/h	--	Ar	[28]
Anabaena variabilis ATCC 29413	about 1.3 μL/mg dry wt/h (0.05 μmol/mg dry wt/h)	5000 lux at the surface of the culture vessels	Ar + CO_2 (5%);5000 lux; addition off Tween 85 (77 mM)	[29]
Anabaena variabilis 1403/4B	20 μL/mg dry wt/h (0.83 μmol/mg dry wt/h)	air; 15 μE/m^2/s	no gas phase; cells immobilized in hollow fibers; 25 μE/m^2/s on top surface and 13 μE/m^2/s on bottom surface of reactor	[30]
Anabaena variabilis ATCC 29413	45.16 μmol/mg chl *a*/h	Ar (73%) + N_2 (25%) + CO_2 (2%); 90 μE/m^2/s	Ar (93%) + N_2 (5%) + CO_2 (2%); 90 μE/m^2/s	[31]
Anabaena variabilis PK84*	167.60 μmol/mg chl *a*/h	Ar (73%) + N_2 (25%) + CO_2 (2%); 90 μE/m^2/s	Ar (93%) + N_2 (5%) + CO_2 (2%); 90 μE/m^2/s	[31]
	2.63 μL/mg chl *a*/h (0.11 μmol/mg chl *a*/h)	air + CO_2 (2%); outdoor conditions	air + CO_2 (2%); outdoor conditions (about 400 W/m^2)	[32]
Anabaena variabilis PK17R*	59.18 μmol/mg chl *a*/h	Ar (73%) + N_2 (25%) + CO_2 (2%); 90 μE/m^2/s	Ar (93%) + N_2 (5%) + CO_2 (2%); 90 μE/m^2/s	[31]
Anabaena azollae	38.5 μmol/mg chl *a*/h	air + CO_2 (2%); continuous turbidostat mode; 113 μE/m^2/s	Ar	[33]
Anabaena variabilis ATCC 29413	39.4 μmol/mg chl *a*/h	air + CO_2 (2%); continuous turbidostat mode; 113 μE/m^2/s	Ar	
Anabaena variabilis PK84*	32.3 μmol/mg chl *a*/h	air + CO_2 (2%); continuous turbidostat mode; 113 μE/m^2/s	Ar	
Anabaena variabilis AVM13*	68 μmol/mg chl *a*/h	air + CO_2 (1%); 100 mE/m^2/s	--	[18]
Anabaena sp. PCC 7120	2.6 μmol/mg chl *a*/h	air; 20 μE/m^2/s	Ar; 60 μE/m^2/s	[27]
Anabaena cylindrica IAM M-1	2.1 μmol/mg chl *a*/h	air; 20 μE/m^2/s	Ar; 60 μE/m^2/s	
Anabaena variabilis IAM M-58	4.2 μmol/mg chl *a*/h	air; 20 μE/m^2/s	Ar; 60 μE/m^2/s	
Anabaena cylindrica UTEX B 629	0.91 μmol/mg chl *a*/h	air; 20 μE/m^2/s	Ar; 60 μE/m^2/s	
Anabaena flos-aquae UTEX 1444	1.7 μmol/mg chl *a*/h	air; 20 μE/m^2/s	Ar; 60 μE/m^2/s	
Anabaena flos-aquae UTEX LB 2558	3.2 μmol/mg chl *a*/h	air; 20 μE/m^2/s	Ar; 60 μE/m^2/s	

* mutant strains

Several studies, directly comparing the hydrogen evolution by different cyanobacterial strains, have shown to be extremely useful. In the seventies, a period when developing a new energy production was a major priority, some studies started to diverge from using the initial *Anabaena cylindrica* B-629.

In Table 4 several *Anabaena* strains, together with maximum hydrogen evolution rates, are presented. A significant number of *Anabaena* strains have been, for quite some time, the object of intensive studies. *Nostoc punctiforme*, a very close relative to *Anabaena* strains, is the model organism used in Peter Lindblad's laboratory and the one used in initial testing of the new bioreactor and the techniques developed. This cyanobacteria has a relatively large genome [34], with about 70% of similarity to other cyanobacteria.

Nostoc punctiforme ATCC 29133 and the NHM5 mutant

Nostoc punctiforme is a nitrogen-fixing cyanobacterium belonging to the family Nostocaceae in the order Nostocales [35]. Members of the order Nostocales are broadly characterized by growth as unbranched filaments and the production of up to four kinds of differentiated cells.

Nostoc punctiforme can be obtained from at least two collections: ATCC (strain number 29133), and PCC (strain number 73102). The strain was first isolated from a symbiotic association with the cycad *Macrozamia* sp. It was deposited in the Pasteur Culture Collection in 1973 as *Nostoc* sp. PCC 73102 [36]. For practical reasons, the used strain will be simply referred throughout the text as *Nostoc punctiforme*.

Obtaining mutants and studying their hydrogen evolution capabilities has driven research concerning hydrogen production a step forward - higher levels of cyanobacterial hydrogen production were the result (some examples are given in Table 4). Genetic engineering was used to create mutants lacking uptake hydrogenase in both *Anabaena variabilis* [18, 31], and more recently in *Nostoc punctiforme* [37]. These mutants show that improved production of molecular hydrogen is possible in oxygen evolving photosynthetic microorganisms.

A *hup*L$^-$ mutant strain of *Nostoc punctiforme* was constructed by replacing the *hup*L gene, encoding for the uptake hydrogenase large subunit, with an inactivated copy via

homologous recombination (Figure 3). Therefore, this mutant completely lacks hydrogenase activity, since it does not possess a bidirectional hydrogenase.

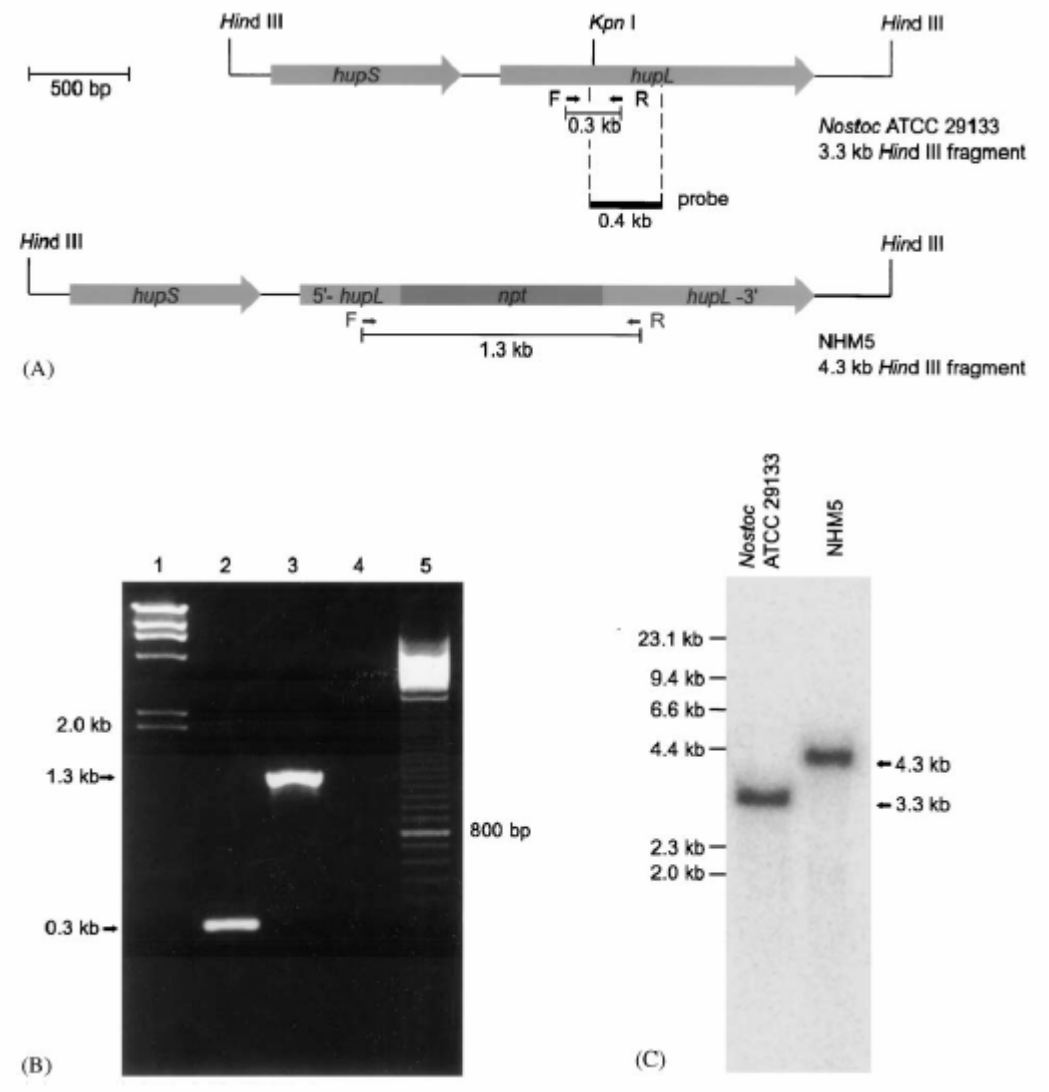

Figure 3 - (A) Physical map of wildtype and inactivated *hup*SL. F and R represent primers used for PCR analysis. HindIII sites and probe used in Southern blot are indicated. (B) Gel with PCR analysis products, with genomic DNA from the wildtype *Nostoc* ATCC 29133 and the mutant NHM5 as templates. Lane 1: Size marker (HindIII fragments), lane 2: wildtype, lane 3: NHM5, lane 4: PCR negative control (dH2O), lane 5: 100 bp ladder. (C) Southern blot of genomic DNA from wildtype *Nostoc* ATCC 29133 and NHM5 digested with HindIII and probed with a fragment from within the wildtype gene (see A). Taken from [37].

The gene was interrupted by inserting a neomycin resistance cassette into a unique KpnI site within *hup*L [37]. The completely segregated recombinant strain NHM5 (Nostoc Hup Mutant 5) was selected for analysis of hydrogen evolution.

Initial proposed work

Cyanobacterial cultures were initially grown in a laboratory-scale helical tubular photobioreactor, made of polyvinyl chloride tubing (Figure 4) [38]. Specific hardware and software allowed the real-time monitoring of several parameters (pH, optical density, temperature, oxygen concentration, and light) and their variations during cyanobacterial growth. The cyanobacterial suspension was kept in a turbulent state by gas injection (active air-lifting).

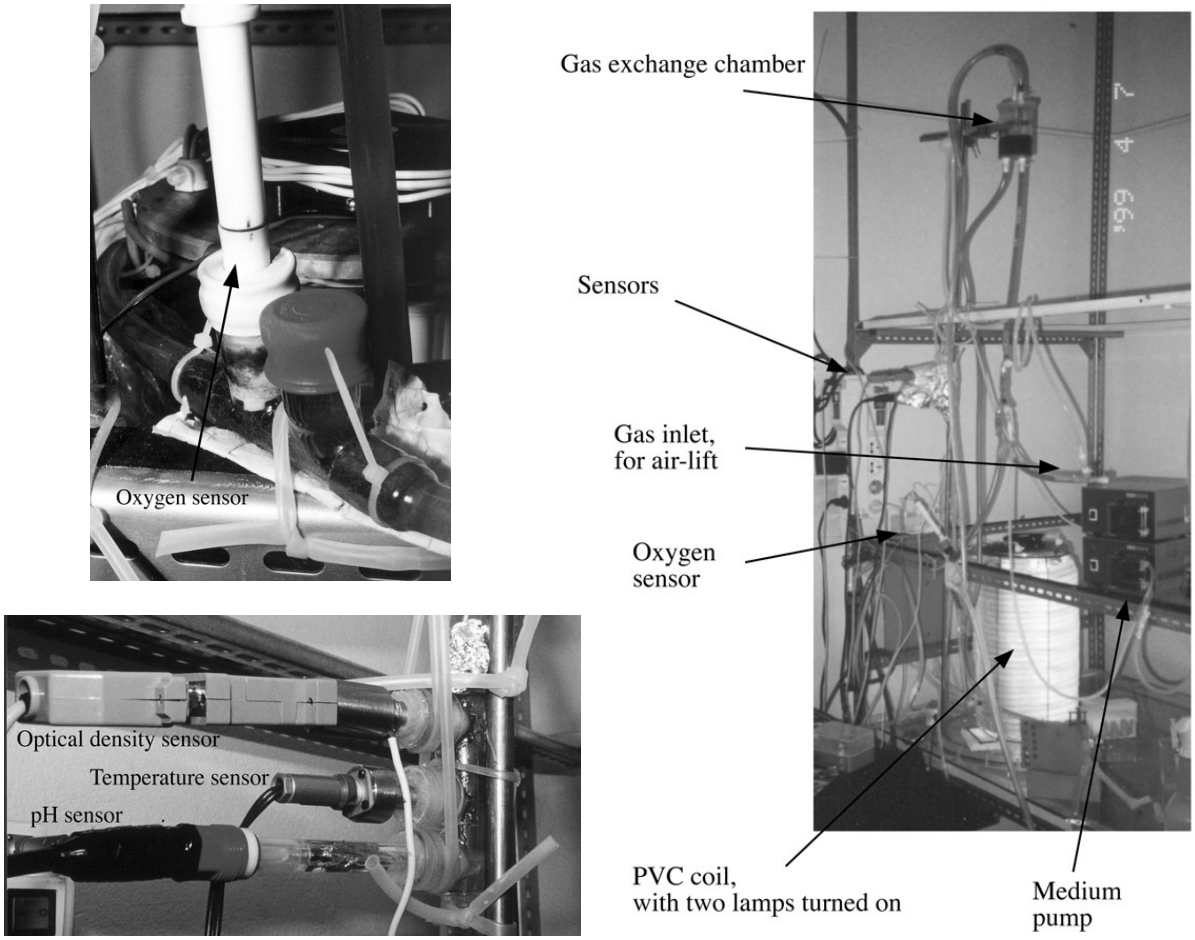

Figure 4 – Right - photobioreactor initially used.
Lower left - optical, temperature and pH sensors. Upper left - oxygen sensor.

The "nature of the biological material" sometimes is the cause of unexpected changes in research planning. Independently of how organized and carefully constructed an experimental procedure is, it will ultimately be dependent on the behavior of the cells. In this particular case, using the equipment described, the cells did not show any sign of growth. The peculiarities of growing *Nostoc* were already known [39], but growth was still expected. Faced with this problem, along with the age and deteriorated state of sensors and signal acquisition of existing equipment, it was decided to make methods development a priority.

Selecting, establishing and developing methods for physiological studies

The main obstacles in the development of H_2 production technologies using cyanobacteria are: a) inhibition of enzymes directly involved in hydrogen production by oxygen, b) H_2 consumption by an uptake hydrogenase, and c) an overall low productivity [40]. Therefore, improved hydrogen production over a long period demands that: a) the strains used must be selected taking in consideration their specific hydrogen metabolism, b) the most suitable strain(s) should be genetically modified in order to improve the H_2 evolution, and c) the overall conditions for cultivation in bioreactors be studied and improved [9].

The bubble column reactor is a bioreactor, in the shape of a column, in which the reaction medium is kept mixed and aerated by introduction of air into the bottom [41]. The use of bubble column reactors is growing specially due to their uncomplicated construction and low cost, associated with high surface area to volume ratio [42] – making them well suited for photosynthetic bacterial growth. The use of this type of bioreactor also facilitates cleaning and maintenance procedures reducing downtime and overall handling demands – making it an interesting solution in a work environment where efforts are focused on physiological problems and not engineering aspects. Even the expected low mixing intensities when compared to stirred tank reactors [43] can be considered as "positive" due to the disrupting effects of filament breakage on metabolism [21].

Research aim and context

This work was done taking in consideration that future research should include: a) screening for wild-type strains possessing high nitrogenase activity and/or very active hydrogen evolving hydrogenase, b) specific genetic modification of these strains (aiming for uptake hydrogenase deficient mutants with higher heterocyst content, increased level of bidirectional hydrogenase and decreased growth/cell division), and c) optimization of cultivation conditions for hydrogen evolution - improvement of parameters like light intensity, CO_2 content, temperature, pH or micronutrient content.

This type of research requires establishment of procedures and techniques that are flexible and time effective. With this in mind, the work focused on: a) selecting, adapting and use of a simple and practical bioreactor model, b) developing strategies for short and long-term hydrogen production measurements, c) improving previously existing and explore new techniques to study competition between wild-type and mutant cells, d) selecting monitoring sensors and acquisition hardware, and e) develop software for culture monitoring and control. This project main objective was to build an adequate and easily operated photobioreactor that permitted the study of the metabolism and hydrogen evolution in cyanobacteria.

Initial photobioreactor

When first essays were attempted a previously existing photobioreactor was used, very similar to a previously described system [38]. The reactor had a total volume of 1.85 liters. Inside the cylinder formed by the coil of polyvinyl chloride tube four 55 W fluorescent lamps were installed (Figure 4). Culture motion was induced by gas injection (6 L/hour) during the experiments.

Light intensity

The installed fluorescent lamps (Figure 4) permitted manipulation of total irradiation, going from 40 μmoles/m^2/s (with room lights only) to 446 μmoles/m2/s. During cell growth two internal lamps were constantly on (228 μmoles/m2/s).

Sensors

To monitor cultivation parameters four sensors were used. The oxygen sensor was placed at the end of the PVC coil, since this should be the point of higher O_2 concentration, resulting from cyanobacterial photosynthesis. The remaining three (for optical density, temperature and pH, respectively) were placed in a vertical section of PVC tube, along with a pneumatic shaker - this way not only the culture passing through the sensors was more homogenized, it also was less prone to cell deposition.

Cleaning of tube walls

Cell deposition on the tube walls is unavoidable due to the laminar nature of the culture flow in the PVC tubes [38]. Biofilm occurs on the walls preventing light from penetrating the tube. To minimize this problem, polyurethane foam cylinders were manufactured and regularly introduced into the photobioreactor.

High-level and low-level control of bioreactor

Data monitoring and control of the bioreactor was executed at two levels. Low-level control was performed by a device for automation of photobioreactors (CODAPH), comprising of one digital measuring and one control system based on a standard AT motherboard, with an 80286 microprocessor. A Pentium II class PC performed high level control, in order to: a) execute calibration of sensors and pumps, b) alter operational settings, c) allow visual data presentation, and d) quickly store data as tables of values or graphics. High and low-level hardware were connected thru RS232 ports. Both low-level and high level systems used specific software.

Sterilization

Before utilization the photobioreactor was sterilized. First, pure water was pumped in and left to circulate for about 30 minutes, with airflow of 12 L/hour. After discarding the water, using the bottom appendix, 1.5% solution of sodium hypochlorite was inserted and left to circulate for about 2 hours. After this solution was discarded, autoclaved pure water was inserted continuously until pH reached a value between 7.0 and 8.0. At this point, the reactor was considered prepared to receive the medium - $BG11_0$ [44] was used in all experiments.

Initiating culture

Medium was inserted into the reactor, and once the temperature stabilized, the oxygen sensor and optical density sensors were calibrated. Some medium was discarded, in order to introduce the cell batch cultures. These cultures, both *Nostoc punctiforme* and the mutant NHM5, were obtained by inoculating 600 ml of autoclaved $BG11_0$ medium which was grown for 14 days, in continuous light (40 μmoles/m^2/s; Thorn Polylux 4000 and Osram Warmton Warm White (400-700 nm)), at 23ºC.

Measuring chlorophyll *a*

To determine chlorophyll *a* content 1 mL samples of culture were taken from the reactor. The samples were spinned (Heraeus Sepatech Biofuge A, 60 seconds, at 13000 rpm), the supernatant discarded and the cells ressuspended in 1 mL methanol. This mixture was vortexed for 60 seconds and stored, at 4ºC, for 24 hours. Afterwards, the cells were centrifuged (15 seconds), the supernatant used to analyze the absorbance at 663 nm (Jasco 7800 UV/VIS spectrophotometer) and thereby be able to calculate the chlorophyll concentration by applying the simplified formula:

Chlorophyll a (μg/ml) = Abs663 * 12.7

Extraction of genomic DNA

Frozen samples were resuspended in 0.5 ml of Resuspension Buffer (10mM EDTA, 50 mM Tris HCl, pH 8.0) in 2.0 ml microfuge tubes, before, 0.6 g of Glass Beads (Sigma type V), 25 ml of SDS 10% and 500 μl phenol/chloroform 1:1 were added. The tubes were vortexed at high speed for 30 seconds followed by a 60 seconds pause on ice (this procedure was repeated four times). After centrifugation (Eppendorf centrifuge 5402, 14000 rpm, 4º C, during 10 minutes), the upper aqueous phase was transferred to a 1.5 ml eppendorf tube. From this solution genomic DNA was extracted by adding 500 μL of chloroform and, after a short vortex, centrifuged (Eppendorf centrifuge 5402, 14000 rpm, 4º C, for 3 minutes) saving the upper phase. Precipitation followed by adding, in fresh tubes, 50 μL 2M of NaAc and 1250 μL of 100% ethanol. After mixing, the tubes were placed at –20º C for 30 minutes, and then centrifuged (Eppendorf centrifuge 5402, 14000 rpm, 4º C, during 10 minutes). After drying using, a speedvac (Savant speedvac), the precipitated DNA was resuspended in 100 μL of TE (0.1 mM EDTA, 10 mM Tris HCl, pH 8.0).

Photobioreactor development

The initial setup used, and described above, showed to be inadequate for cell growth. As an attempted alternative, two bubble column reactors were manufactured. Adapted from old separation columns these units shared all characteristics (see Figure 5). Each reactor had a volume of about 1.7 L and circa 60 cm height. Modified 1.5 mL microtubes were used as gas injectors, placed in the bottom of the column. The gas mixture inserted was filtered using 0.2 μm circular membranes. Inlet of autoclaved media was performed via an orifice created in the main tube just above the bottom screw-on lid – one 4 mm id tube connector was split and used as a support for the tube carrying media into the reactor. A similar procedure resulted in a second inlet/outlet connection – about half the height of the column, used for culture sampling. The main system outlet was a connector opposite to the injector. This outlet in the upper lid had 10 mm id and directed the gas and overflowing culture into an autoclave resistant PVC container.

Figure 5 – The former separation columns adapted to act as bioreactors.

This setup was used for trial growth, in order to assert optimal light and gas flow conditions. The initial phase of growth can be problematic due mostly to light conditions – too high or too low intensities will affect growth. Usually lower light intensities are favored, and in batch cultures it is normal to cover the flasks with some paper during the first 24 to 48 hours of cell cultivating. An intensity of about 250 lux was found to be

suitable for all growth stages (intensity is average of incident light taken from 12 points in the reactor wall). Growth was then possible and surprisingly fast (data not shown) – taking in consideration not only the previous growth problems but also what is generally known about growing *Nostoc punctiforme*. After some planning and literature search the final design for the reactor was decided and a prototype built.

Detection and quantification of Hydrogen, Oxygen and Nitrogen

For numerous reasons, debated in Introduction, the knowledge of gas concentrations for hydrogen, oxygen and nitrogen is vital. The best alternative to quantify these gases is the use of a gas chromatograph. For that purpose, after analyzing the available units in the market, one Perkin Elmer Clarus 500 GC was acquired. Equipped with one Packed Column Injector and one Thermal Conductivity Detector, and after proper calibration and setting, this unit allows for the detection of these three gases in about 1.6 minutes per sample. Each 100 ml gas phase of sample was transferred from the reactor or test vials using a Hamilton 81156 Gastight syringe.

Experiments to measure gas phase variation due to metabolic processes in cyanobacteria were performed using 8 mL Chromacol tubes, with teflon coated rubber septa fixed with plastic lids. The tubes, with 2 mL of cell culture and one 4 mm glass bead, were rotated at 20 revolutions per minute and illuminated with strong light (about 600 lux). This setup should provide maximum hydrogen evolution rate values for every tested strain.

Strain detection and quantification

One possible way of detecting and quantifying different strains of cyanobacteria is the use of PCR. Assuming that different initial concentrations of a DNA template will result in different product concentrations, comparative concentrations between strains can be ascertained. Genomic DNA was isolated from wildtype *Nostoc punctiforme* and mutant strains using the method described in Materials and methods. PCR was initially performed according to standard protocols. The primers used were:

forward primer - 5'-[CGCCATTATGAGGAAGCTGT]-3' and
reverse primer - 5'-[CGGTCTTCATCCAACCAATC]-3'.

These primers yield a 308 bp PCR product from the wildtype *hup*L, and a 1.3 kb product from the inactivated gene (when containing the *npt* gene and part of the vector used for insertion - see Figures 3 and 6) [37]. The first obstacle to be overtaken was the difficulty of simultaneously amplifying two differently sized products using the same pair of primers. Ideally, the two fragments would be correctly amplified so that one pair of primers would be enough to produce gels with two distinct bands per lane, whose intensities could be quantified. This was not possible and an alternative approach was designed (described later in "Results" and analyzed in "Discussion and Conclusions").

ATGACAATTCAATCATTAGACATTTCGCCCGTCGGTAGAGTTGAAGGCGATTTAGATGTCCGAGTTGATA
TTGAACATGGAAGAGTAGTCAACGCCTGGACACACGCTGAATTATTCCGTGGATTTGAAGTTATTCTTCG
TGGTAAAGACCCCCAAGCCGGATTAATTGTCACACCTCGCATTTGCGGAATTTGCGGCGGTTCTCACCTG
ACATCTGCATCTTGGGCATTAGATACAGCTTGGGAAACAGAAGTTCCCCGCAATGCAATTTTAGCGAGAA
ATCTTGGTCAAATTGTCGAGACAATTCAAAGCATTCCCCGCTATTTTTATGGTTTGTTCGCCATTGATTT
AACCAATAAAAAATACCGTAATAGTCGCCATTATGAGGAAGCTGTTAGACGCTTTGCCGCCTTCACTGGC
AAATCTTACGAACTAGGCATAACAATTTCTGCTAAACCCGTAGAAATTTATGCACTGTTGGGCGGACAAT
GGCCTCATTCTAGCTACATGGTACCTGGTGGCGTGATGTGCGCCCCCACTTTAACAGACATTACTCGCGC
TTGGGCGATTCTAGAATATTTCCGCACCAATTGGTTAGAACCAGTGTGGTTAGGTTGTTCATTAGAACGC
TACGAACAAATCCAAACTTATGATGACTTCAGAGATTGGTTGGATGAAGACCGAAATCATCGAGATTCCG
ACTTAGGTTTTTATTGGCGCATGGGTTTAGACATCGGTTTAGATAGATATGGCGCTGGTGTTGGTAAATA
TGTGACTTGGGGATATTTAACCCATGAAGATAAATACCAAAAGCCGACTATCGAAGGACGAAATGCGGCG
ATGATTATGAAAAGTGGAGTGTACGACAGCTTCGCAGACACTCACGTTTTAATGGATCAGTCATTTACCC
GCGAGAATACAACTCACTCTTGGTACGATGAAGGGACAGAGGATATTCACCCTAGCGATCGCACCACTAA
ACCCACTGCAATCAATACCAAAGACTTCGATAACGCCTACTCTTGGTCAAGTGCAGTCCTTCACAAAGAC
TTCGGACGCTTGGAAACCGGCCCTTTAGCGCGGCAATTAGTAGCTGGTGGTCAGCATGGCGAATCTTGGC
AACATTACGACGGTTTTATCTTGGATGCTTTCCAAAAAATGGGTGGTGCTAGTATTCATTTACGTCAACT
TGCACGAGTTCACGAAATTGTCAGGTTATATCGCCAAGCGGAAAGATGTCTGCGTGAGTTTGTCTTAAAT
GACCCTTGGTATATCAAACCCAAAGAAAAAGACGGACGCGGTTGGGGTGCAACGGAAGCCTCACGGGGTT
CTTTGTGTCACTGGATAGATATAGAAGGTGGCAAGATTAAGAATTATCAGGTGATTGCTGCGACTACTTG
GAATATCGCCCTTCGTGACGGCGAAGGAATCCGCGGCCCAATTGAAGAAGCGTTAATTGGGACACCCATT
TACGATTCTAGCGATCCGGTGGAAGTTGGTCATGTGGCGCGATCGTTTGATTCATGTTTGGTGTGTACAG
TCCACGCCCATGATGCGAAGACTGGTGAAGAGTTGGCGCGTTTTCGGACTGCTTAA

Figure 6 – *Nostoc punctiforme* *hup*L homolog gene, complete cds. Marked areas are: forward primer region in red, reverse primer region in green, and blue defines the remaining of the product obtained by PCR from *Nostoc* ATCC 29133 wildtype DNA.

The new strategy involved: a) finding the ideal PCR conditions that favored the amplification of the smaller fragment using the above described primers (in the case of wiltype and mutant mixtures), and b) the use of a second pair of primers, specific for the *npt* gene. The chosen primers were:

forward primer - 5'-[AGGCTATTCGGCTATGACTGGG]-3' and
reverse primer - 5'-[TGGATACTTTCTCGGCAGGAGC]-3'.

This new second pair of primers, for detection of the *npt* gene resulted in a PCR product of 292 bp (see Figure 7). The two pair primer strategy allows the detection and/or quantification of the relative concentrations of the two strains.

ATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACT
GGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCT
TTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTG
GCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTAT
TGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGC
TGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGC
ATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGG
GGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGAC
CCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGC
CGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCG
GCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTA
TCGCCTTCTTGACGAGTTCTTCTGA

Figure 7 – *npt* gene, complete cds. Marked areas are: forward primer region in red, reverse primer region in green, and blue defines the remaining part of the product obtained by PCR from *Nostoc* ATCC 29133 NHM5 mutant DNA.

Initial experiments

The initial growth experiments were just the beginning of a new set of problems/challenges, and the proof that not getting cyanobacteria to grow can be the start of a good challenge. The solution for the problem involved the construction of a new photobioreactor (Figure 8).

The new reactor

In Figure 8 is shown the new 1.55L bubble column reactor, which as three main parts: one central cylinder (50 cm long), one base cone and a top cone. The central cylinder comprehends most of the reactors volume. It has three input/output points: one small at middle height for sampling and, opposite, two larger for culture circulation (for future culture parameter monitoring, see Figures 8 and 9).

The lower cone supports the gas injection system and the primary inlet/outlet. Gas injection is done using a double membrane system on top of a cylindrical chamber that precedes the gas injection point. The chamber is then fixed to the cone by screwing, with a rubber ring to seal the reactor content (see Figure 10). Four 0.2 μm PTFE membranes (Acro 50 Vent Device, Gelman Laboratory) filter all incoming gas, and the pressure is constantly regulated to 1 atm.

The upper cone provides the system with an exit for both gas and liquid. This is then connected to a trap that allows for gas recirculation, with liquid/solid elements being drained to a container for posterior decontamination (see Figure 11).

Recirculation is made possible using a KNF NMP 05 S micro pump (see Figure 11). This feature and the fact that all tubing is high quality PVC (Tygon by Saint-Gobain) allows for the analysis of hydrogen gas content variation for an extended time.

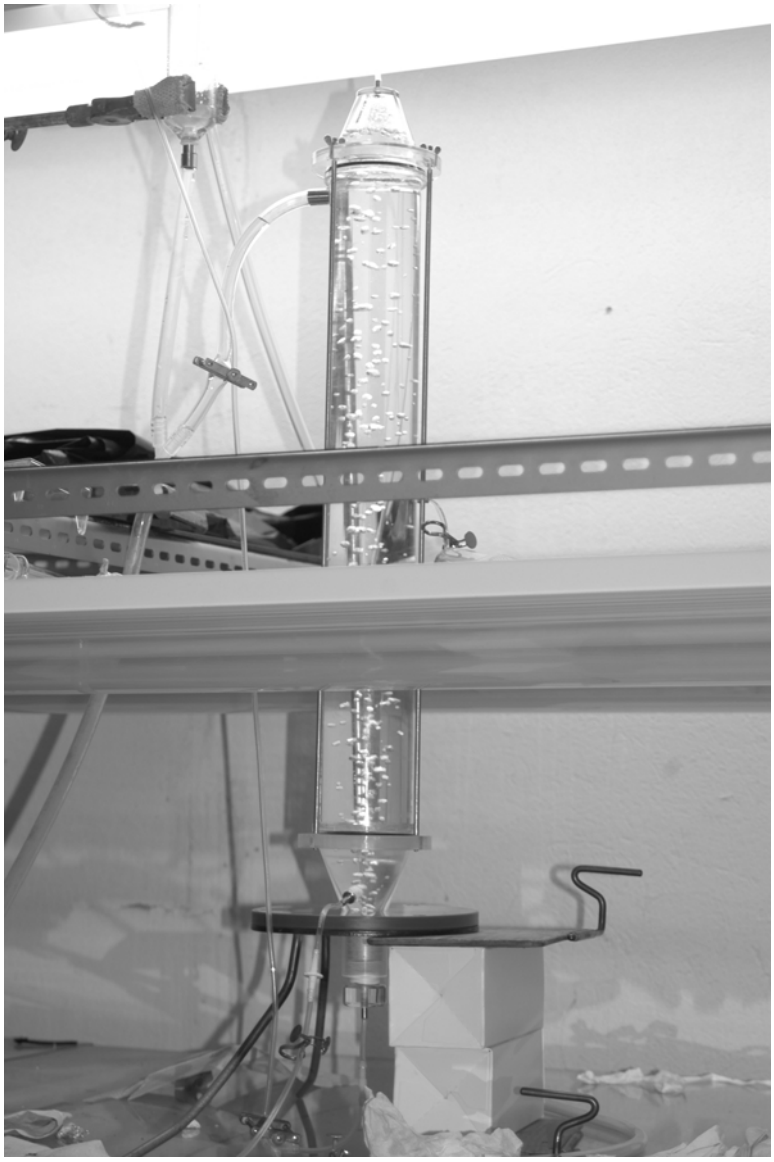

Figure 8– General view of the prototype bubble column reactor built.

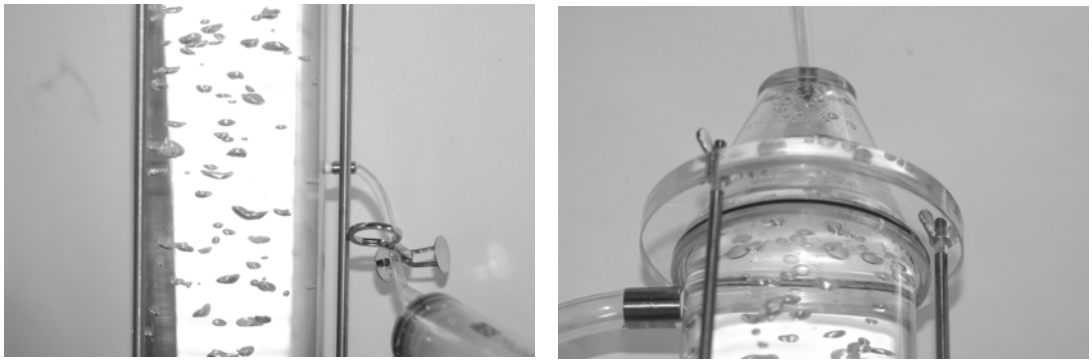

Figure 9 – Left image: the sampling point about half height of the main cylinder; right image: the top cone and recirculation exit immediately below it.

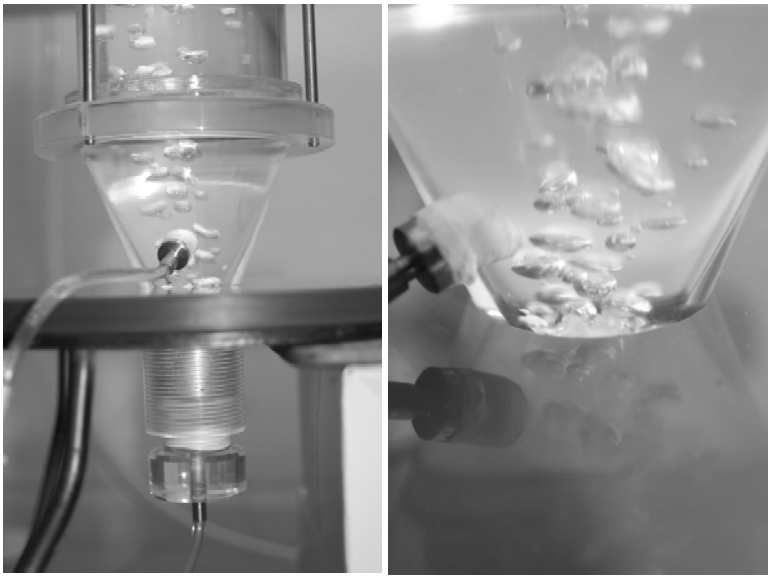

Figure 10 – Left image: the lower cone with primary inlet/outlet; right image: detail of gas injection.

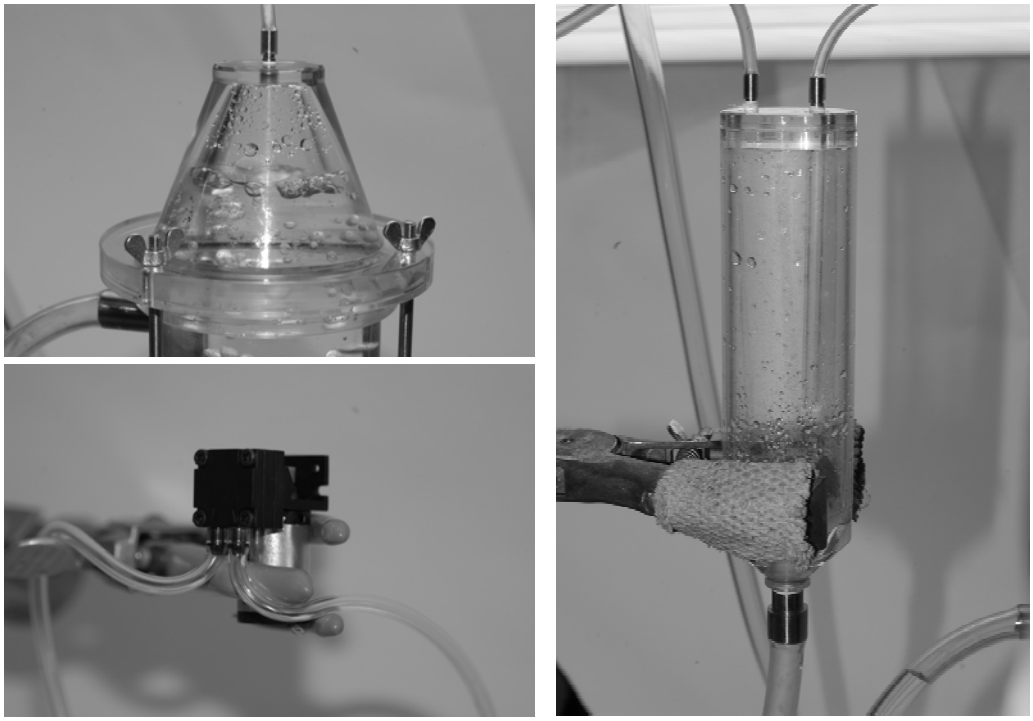

Figure 11 – Left higher image: upper general view; left lower corner: the micro pump for gas recirculation; right image: trap to ensure only the gas phase is recirculated and liquid phase is rejected.

Growth kinetics

After installation and first detail modifications, several culture cycles were performed in fed-batch to calculate the basic growth kinetics of *Nostoc punctiforme*. All data taken was compiled and the resulting growth curve is shown in Figure 12.

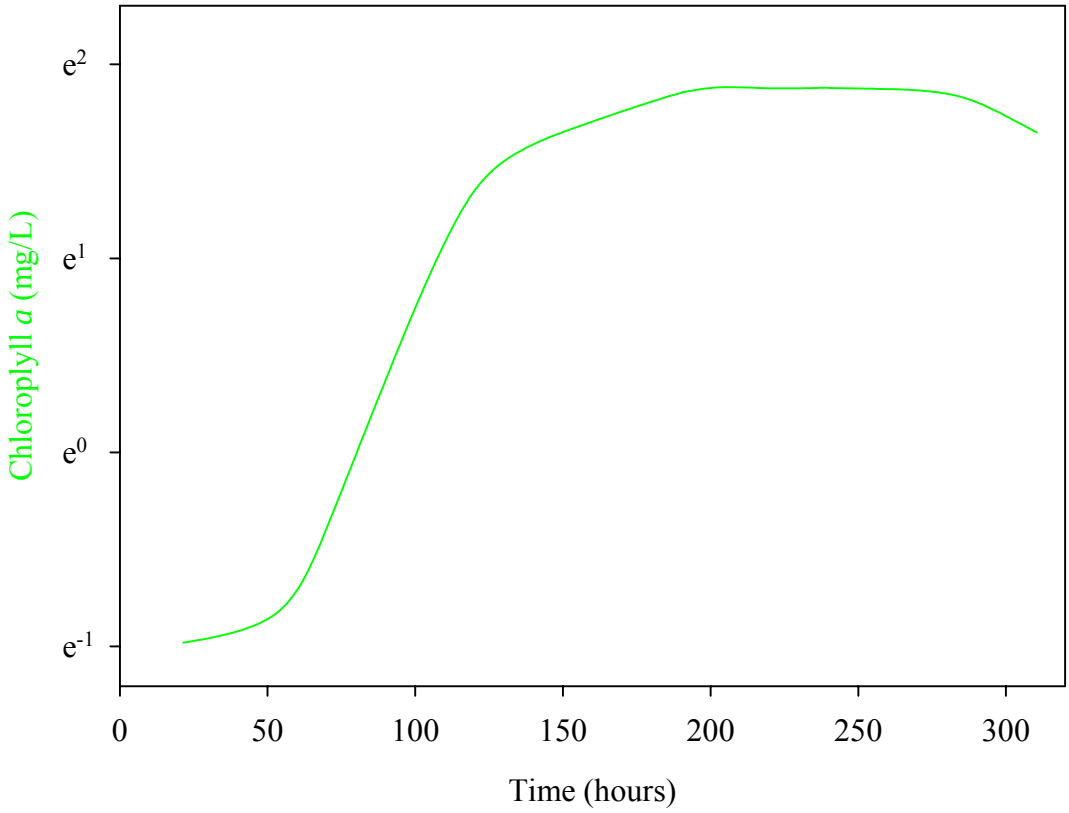

Figure 12 – Variation in chlorophyll *a* concentration during *Nostoc punctiforme*, under constant light, air flow rate mixture and temperature conditions.

The growth curve is the result of several cell growth cycles, in order to get a curve as accurate and informative as possible. From this curve the values of K, doubling time (dt) and specific growth rate (μ) were calculated applying the following equations.

Equation 5. $$K = \frac{\ln(n2) - \ln(n1)}{t(n2) - t(n1)}$$

Equation 6. $dt = \frac{\ln(2)}{K}$

Equation 7. $\mu = \frac{1}{dt}$

From the growth curve shown above in Figure 12, the values calculated concerning growth kinetics were: 0.038 for K, 18.17 hours for doubling time and 0.055 for μ. Previous to this work, tests run in the lab have shown that in batch culture the doubling time is about 24 hours.

Gas content variation in short term experiments

In Figure 13 the variation in gas composition during H_2 production is shown. This graphical representation is the results of the use of 8 mL Chromacol vials described in "New methods - selection and development". For this particular experiment, 4 such vials were used all containing: 2 mL of the same cell culture, one 4 mm glass bead in 21% oxygen and 79% nitrogen atmosphere. The glass bead allows for improved mixing of the populated culture medium and better gas exchange between the liquid and the gas phase.

The initial variation for the three gases monitored was quite fast, but declined between 50 and 60 minutes the start of the experiment. Adaptation to the new environment (more precisely the higher light intensity) might help explain the reduction in hydrogen evolution, after such a promising start. It is know that light intensity variations can have implications in the growth of cyanobacteria, both wildtype and mutants [45].

During the initial period the maximum hydrogen evolution rate was 72 nmol/μg chl *a*/h. This result is higher than the initially expected (14 nmol/μg chl *a*/h), taking in consideration previous tests performed [37]. This is evidence that growth and hydrogen evolution testing conditions are vital to optimize hydrogen production.

The variation in hydrogen concentration observed between minutes 80 and 210 is interesting and will be the subject of further analysis in "Discussion and Conclusions".

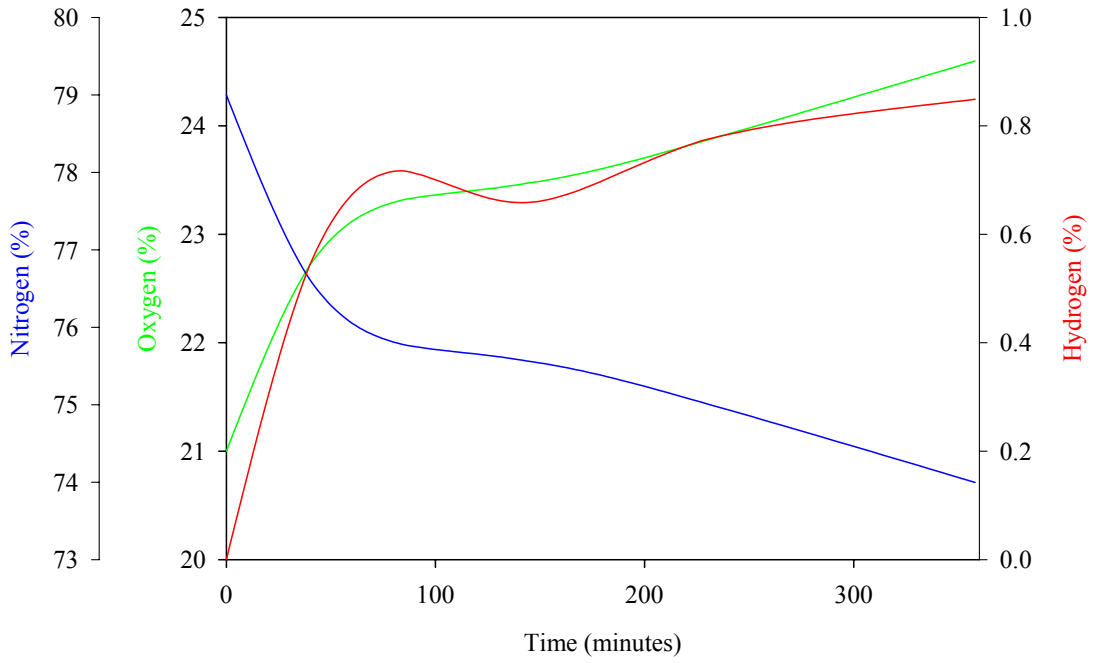

Figure 13 – Variation in gas phase composition during short-term experiment.

Use of PCR for strain quantification

The initial set of tests with PCR was performed in order to overcome the problem posed by simultaneous amplification of two different sized products (Figure 3). The primers specific for the *hup*L gene were used having a 99% mutant/1% wildtype DNA mixture as target. Several gradient PCR, associated with different $MgCl_2$ concentrations, were performed – in Figure 14 some results of those tests are shown. The aim of the test was to find such PCR conditions that allowed for single amplification of the smaller fragment (wildtype). The manipulation of $MgCl_2$ was enough to create such condition that, even a relatively small amount of wildtype DNA in a given sample would still result in the amplification of the smaller fragment. In section 3 of Figure 14 the success to avoid amplification of the largest fragment is clear. Therefore, the final PCR conditions included an annealing temperature of 60ºC and 3 mM $MgCl_2$ (final concentration).

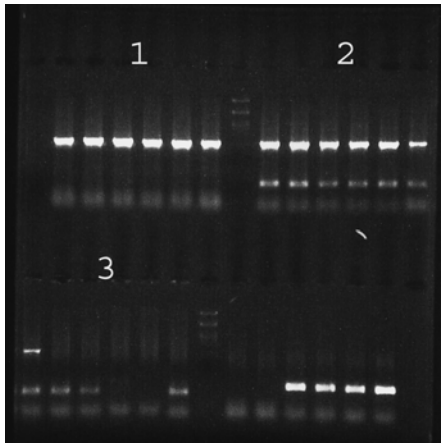

Figure 14 – Analysis of PCR products from a DNA mixture of both wildtype and mutant. In 1, 2, and 3 gradient temperature for PCR was between 62 and 57ºC (from left to right). The concentration of $MgCl_2$ increased from situation 1 to situation 3.

The second pair of PCR primers, specific for the *npt* gene and previously described, was tested and the results of those tests are shown in Figure 15. The product resulting from amplification of NHM5 DNA containing the *npt* gene (lanes 4 and 5) shows very similar size when compared to the fragments resulting from amplification with the *hup*L specific primers (lanes 2 and 3). Lanes 6 and 7 are the negative controls for both primers. Lanes 1 and 8 contained λ DNA cut with the enzyme *Pst* I for band size assessment.

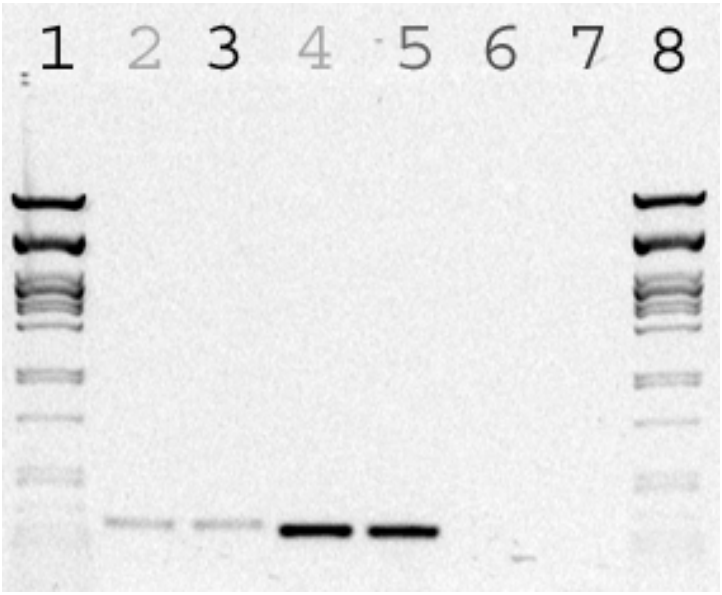

Figure 15 – *Nostoc punctiforme* and NHM5 analysis by PCR. In lanes 2 and 3 - fragments resulting from amplification with the *hup*L specific primer. In lanes 4 and 5 - product resulting from amplification of NHM5 DNA containing the *npt* gene. Lanes 6 and 7 are the negative controls for both primers and lanes 1 and 8 contained λ DNA cut with the enzyme *Pst* I as marker.

To further check for the specificity of the *npt* specific pair of primers, other PCR reactions were performed (Figure 16). In these, the *npt* specific primers were applied in samples containing only wildtype DNA - as expected no amplification at all occurred (lanes 2 and 3). Also, *hup*L specific primers were used with a sample of 100% NHM5 DNA – the resulting bands, shown in lanes 4 and 6, have about 1.3 Kbp and are in accordance with the expected size (see Figure 3, in Introduction). Lanes 7 and 8 contain the controls for both pairs of primers, and lanes 1 and 6 are makers of λ DNA cut with the enzyme *Hind* III.

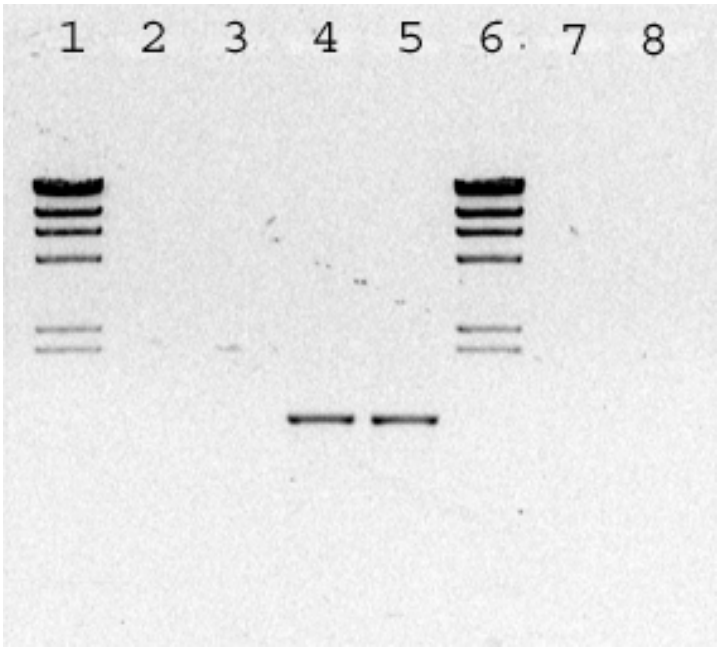

Figure 16 – *Nostoc punctiforme* and NHM5 analysis by PCR, in order to assure *npt* primer pair specificity. In lanes 2 and 3 - *npt* specific primers were applied in samples containing only wildtype DNA. In lanes 4 and 6 - *hup*L specific primers were used with a sample of 100% NHM5 DNA. Lanes 7 and 8 contain the controls for both pairs of primers, and lanes 1 and 6 are makers of λ DNA cut with the enzyme Hind III.

The ability to establish a relation between one specific PCR product and the concentration of a strain in a culture sample was tested (Figures 17 and 18). Using different mixtures of strains (from 0 to 100%, in 20 % intervals), the extracted DNA was used for PCR with *npt* specific primers (Figure 17, from lanes 2 to 7). There is a clear difference in band intensity, which gives a first indication about the feasibility of the test. Lanes 1 contains the maker (λ DNA cut with the enzyme *Hind* III) and lane 8 is the control.

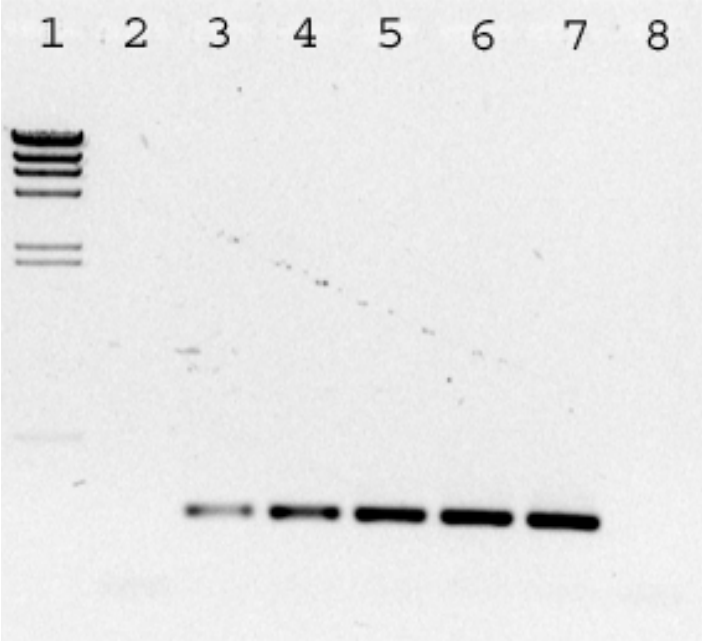

Figure 17 – Strain mixture analysis using PCR. In lanes 2 to 7- mixtures of strains from 0 to 100%, in 20 % intervals). Lane 1 contains λ DNA cut with the enzyme Hind III.

Further analysis of this gel image with appropriate software (LabImage version 2.62a, Kapelan GmbH) supplied the data that made possible the graphic presented in Figure 18. The graphic shows that a linear relation between strain concentration and band intensity is not possible for this PCR conditions. This, however, does not undermine the technique as will be discussed later.

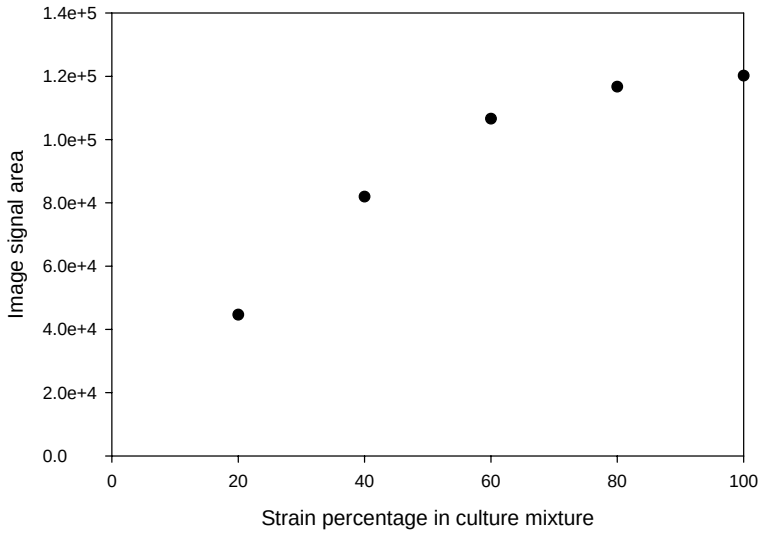

Figure 18 – The relation between strain relative concentration and band intensity.

Studies with *Gloeocapsa alpicola*

After initial testing with *Nostoc punctiforme*, it was suggested that the new setup could be used in the study of *Gloecapsa alpicola.* This work was performed by Prof Taras Antal, currently working in Prof Lindblad's group.

This work was performed using different gas mixtures and flow rates, and varying light intensities. It was possible to obtain valuable data that otherwise, using the traditional methods, would take considerably longer time and resources. The increased culture volume, five to seven times higher than the typical batch culture, allows for more frequent sampling and analysis of a higher number of parameters.

Preliminary results are shown in Figure 19.

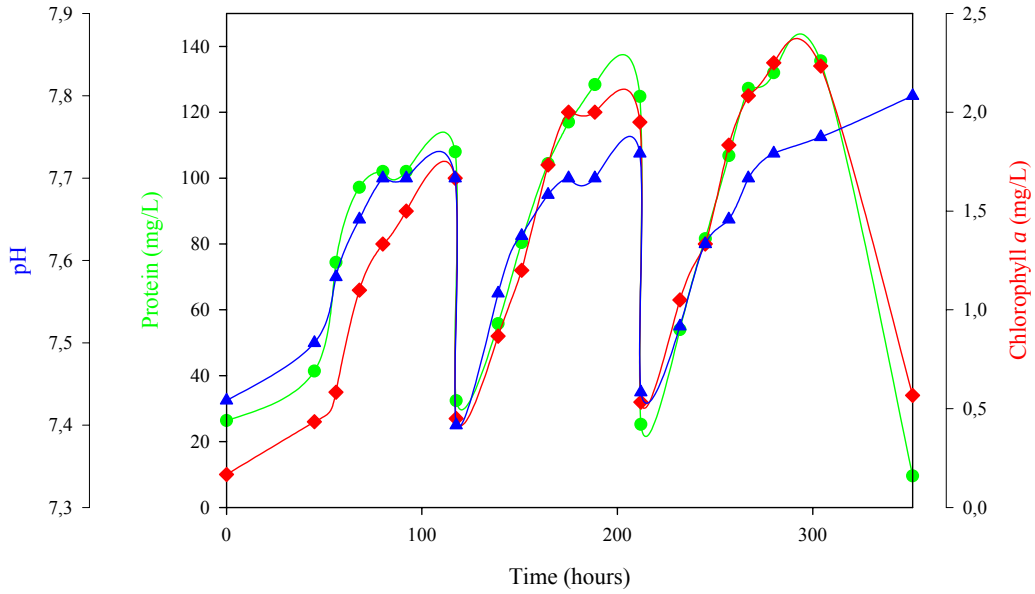

Figure 19 – Variation in pH, protein content and chlorophyll a concentration during three consecutive growth periods.

Even when faced with a seemingly unavoidable contamination, the stability and reproducibility of growth conditions allowed for the replication of the problem. Final data is not available yet. Taking in consideration available information, it is possible that a particular growth environment might have provided the cells with good conditions for exopolysaccharide production [46].

Results from this work will be published in the near future.

Method implementation

The choice of the bubble column reactors has been positive. The growth rates obtained for *Nostoc punctiforme* support this method as a real alternative for improved cell growth, when compared to the previously used method. The use of the new bioreactor also allows for bigger culture volume, easier sampling, and much larger array of environmental variables that can be introduced and allow for better physiological studies.

The new method gained general acceptance, and another two units are being built so that bioreactor use can be more intensive and broader. Hopefully, the coming publications from the group will include several studies made easier, or even possible, due to the use of the bubble column photobioreactor. However, work concerning the design and development is not complete. This system still lacks the control and automation component that will increase its overall value and capabilities. The development has not stopped, and further improvements will be presented in "Future developments".

Gas analysis was made much easier and faster with the introduction of the Perkin Elmer Clarus 500 GC. The user friendly interface and velocity made this unit a huge success, and its use has expanded from hydrogen quantification only to being used in nitrogen uptake tests in more than one cyanobacterial strain.

The use of Chromacol tubes with teflon/rubber sealing has also proved to be a good method for hydrogen production assays. This very simple procedure, combined with the GC velocity, allows for fast testing and very good hydrogen evolution.

The peak hydrogen evolution found, 72 nmol/μg chl *a*/h, was surprisingly high and the subject of discussion. The overall conclusion was that not only the analysis method might be more effective, but the physiological state of the cells might have played a key role. The previous test, in witch maximum production was 14 nmol/μg chl *a*/h, was done when the batch culture used was already entering stationary phase. In contrast, the latest tests were performed with cultures in the initial logarithmic growth phase.

In Figure 13, the unexpected drop in H_2 concentration does not have a clear justification. NHM5 does not possess any hydrogenase, so an answer has to how could there have been H_2 uptake still remains. The hypothesis of contamination with wildtype was raised. In this scenario, a decrease in hydrogen evolution has a late response to the high light intensity could have taken the net hydrogen evolution to negative figures, due

uptake activity by the contaminating wildtype community. After adaptation by NHM5 cells, around minute 120, H_2 evolution returned to positive figures and concentration in the gas phase increased. This situation occurred systematically, so an effort to maintain strain purity of cultures is an essential requirement for future testing.

Technique development

The use of quantitative PCR for strain relative concentration assertion is still not fully developed, but has already shown great potential. It was important to refine PCR conditions in order to eliminate the amplification of the bigger band when using the *hup*L specific primers. This allows for exclusive amplification of wiltype DNA – these settings emulate exclusivity of these primers for the intact *hup*L gene. This opens the possibility of band intensity comparation, since the final products for both pairs of primers have similar size. Having similar size implicates that under equal PCR conditions the probability of primer/template annealing and extension will have approximate values. The option for a second pair of primers was necessary and gave good results.

Even the non-linear relation between band intensities and relative concentration reported is of low relevance. There are numerous ways to reduce or even eliminate this, since the basic problem will ultimately be the quantity of the fragment DNA producing the band in the gel. One way is to reduce the number of PCR cycles – 30 cycles were used, and a decrease to 28 cycles alone would result in a theoretical reduction of 75% of produced DNA fragment. Another way, for instance, is to dilute all samples to the same degree just before applying in the electrophoresis gel.

Basically a relation must be established between: a) total genomic DNA after extraction, b) number of PCR cycles and c) quantity of DNA to be used for electrophoresis. Establishing this relation is still not done, but getting this task completed is more time consuming then technically challenging. This method has all the potential to be, in the short term, established as the *de facto* method for strain quantification for all competition experiments to be performed in the future. An important part of the planned work, and to be presented in short next, will require such experiments and a solid technique to reliably provide strain relative concentration data.

Overall conclusions

The focus points of this work were: a) selecting, adapting and use of a simple and practical bioreactor model, b) developing strategies for short and long-term hydrogen production measurements, c) improving previously existing and explore new techniques to study competition between wild-type and mutant cells, d) selecting monitoring sensors and acquisition hardware, and e) develop software for culture monitoring and control. Of these, only d) and e) were not extensively researched. They were not forgotten, and will the referenced again in “Future developments”.

The main objective of this project was achieved: an adequate and easily operated photobioreactor was produced, its use proved fruitful already, and the basis for future successful work established.

Molecular basis for growth and cell division in *Nostoc punctiforme*

Typically the aim in traditional cyanobacterial growth engineering is to maximize growth rates in order to obtain higher biomass. When obtaining specific products from cyanobacteria, like specific polysaccharides for instance, high growth rates become secondary or even an obstacle. When it comes to hydrogen evolution by cyanobacteria, it is known that in batch cultures H_2 output is higher when cells are not in exponential growth. With these facts in mind, a strategy to theoretically improve overall productivity would be obtaining cyanobacteria with altered growth rate.

Cell division in bacteria occurs by the coordinated invagination of the cell envelope layers that make up the cell wall. At least nine proteins have been identified that are required for the process of dividing a cell to compartmentalize intracellular constituents (cytokinesis) - FtsA, FtsI, FtsK, FtsL, FtsN, FtsQ, FtsW, FtsZ, and ZipA [47]. It is also known that MinC, MinD, and MinE coordinate action is required for proper placement of the division septum [48] - in prokaryotes cell division is mediated by the septal ring. In *Escherichia coli*, this organelle consists of several essential division proteins, including FtsZ, FtsA, and ZipA [49]. FtsZ and FtsA are essential for cell division in *Escherichia coli* and are located by the septal ring [50]. In the current model for bacterial cell division, FtsZ protein forms a ring that marks the division plane, creating a cytoskeletal framework for the subsequent action of other proteins such as FtsA. This putative protein complex ultimately generates the division septum [51].

The work concerning cyanobacteria has mostly focused in the presence and role of FtsZ and *Anabaena* PCC 7120 [52-54]. It has been found that a mutant lacking the ability to produce a functional FtsZ [53] is not viable – implying that septation is required for cell viability. In *Anabaena* PCC 7120, and according the fully sequenced genome available at Cyanobase, the following Fts proteins and genes can are present: FtsW (in all0154), FtsY (in all1759), FtsH (in all3642, all4776, all4936, and alr1261), FtsE (in alr1706), and FtsZ (in alr3858). Table 5 presents a brief summary of the presence of putative division related genes in cyanobacteria, including *Anabaena* PCC 7120 - the translated sequenced taken from these different genes can be useful in determining the presence of important genes in the, so far non complete*, genome of *Nostoc punctiforme*.

Table 5 – Presence of different cell division related proteins in cyanobacteria.
Summary of search using SRS over the SpTrEMBL database.

Organism	Gene name	Description	SpTrEMBL accession
***Anabaena* PCC 7120**	FTSK OR ALL7666	FtsK protein	Q8ZS46
	FTSE OR ALR1706	Cell-division ATP-binding protein	Q8YWA9
	FTSH OR ALR1261	Cell division protein	Q8YXF2
	FTSH OR ALL4776	Cell division protein	Q8YMZ8
	FTSH OR ALL3642	Cell division protein	Q8YR16
	FTSH OR ALL4936	Cell division protein	Q8YMJ7
	FTSW OR ALL0154	Cell division protein	Q8Z0E4
	FTSY OR ALL1759	Cell division protein	Q8YW57
	ALR0446	Hypothetical protein Alr0446	Q8YZL1
	ALR0452	Hypothetical protein Alr0452	Q8YZK5
	ALR0653	Hypothetical protein Alr0653	Q8YZ34
	ALL0808	Hypothetical protein All0808	Q8YYN8
	ALL0906	Hypothetical protein All0906	Q8YYE4
	ALR1506	Hypothetical protein Alr1506	Q8YWT8
	ALL1757	Cell-division protein	Q8YW59
	ALR3648	Hypothetical protein Alr3648	Q8YR10
	ALR3857	Hypothetical protein Alr3857	Q8YQH2
	ALR4281	Hypothetical protein Alr4281	Q8YPB7
	ALR4974	Hypothetical protein Alr4974	Q8YMG0
	ALL5346	Hypothetical protein All5346	Q8YLF2
	ALL0473	Hypothetical protein All0473	Q8YZI7
	MINC OR ALR3455	Septum site-determining protein	Q8YRJ1
	MIND OR ALL2033	Cell division inhibitor	Q8YVE5
	MIND OR ALL2797	Cell division inhibitor	Q8YTC5
	MIND OR ALR3456	Septum site-determining protein	Q8YRJ0
	MINE OR ASR3457	Septum site-determining protein	Q8YRI9
***Synechococcus* PCC 6301**	FTSW	FtsW	Q9R6T2
***Prochlorococcus* PCC 9511**	FTSZ	Cell division protein FtsZ	Q9RLE3
***Synechococcus* PCC 7942**	FTSZ	Cell division protein FtsZ	O85785
	FTSY	FtsY	Q8GIR8
Prochlorococcus marinus	FTSZ	FtsZ protein	Q93JX6
	FTSQ	FtsQ protein	Q93JX7
***Synechocystis* PCC 6803**	SLR0594	Hypothetical protein slr0594	P74738
	SLL1632	Hypothetical protein sll1632	P73457
	SLR0950	Hypothetical protein slr0950	P74319
	SLL1482	Hypothetical protein sll1482	P74616
Synechococcus elongatus	FTSH OR TLL0131	Cell division protein	Q8DMI5
	FTSW OR TLR0440	Cell division protein	Q8DLN8
	TLL0452	Tll0452 protein	Q8DLN1
	FTSH OR TLR0528	Cell division protein	Q8DLG5
	FTSH OR TLL0734	Cell division protein	Q8DKW7
	FTSY OR TLR0928	Cell division protein	Q8DKD1
	TLR1583	Tlr1583 protein	Q8DIK2
	FTSH OR TLL1832	Cell division protein	Q8DHW1
	FTSZ OR TLL2382	Cell division protein	Q8DGD6
	MINC OR TLR2016	Septum site-determining protein	Q8DHE3
	MIND OR TLR2017	Septum site-determining protein	Q8DHE2
	MINE OR TLR2018	Septum site-determining protein	Q8DHE1

Nostoc punctiforme has been used for several years as a model organism at Peter Lindblad's laboratory and the future work will focus in this organism. The available genomic data for this organism has already been searched, and genes like ftsZ (already annotated) and ftsA (not annotated at the time of the search) were found (see Table 6).

The overall goal of this project is to obtain the basic genomic and physiological knowledge in order to create mutants with restricted and/or controlled growth rates. The genomic/proteonomic data allows for either direct searches (using available databases, see Table 2, or indirect searches (using molecular tools like PCR or RT-PCR). This search should be the first step in this project.

The cell growth will be performed in photobioreactors that allow for continuous cell growth (either in fed-batch or chemostat). The presently developed and here presented growth methods are flexible and adequate for the fulfillment of the overall objective of this coming project.

* (submitted by the DOE Joint Genome Institute, in November the 7th 2002)

Table 6 – Results of *Anabaena* PCC 7120 aminoacid sequence BLAST [55] against the available information for *Nostoc punctiforme*.

Search base	Definition for N. punctiforme	Accession	Score	E value
FtsW / all0154	hypothetical protein	ZP_00111434	570	e-163
FtsY / all1759	hypothetical protein	ZP_00112012	699	0.0
	hypothetical protein	ZP_00110921	133	4e-32
FtsH / all3642	hypothetical protein	ZP_00105811	1146	0.0
	hypothetical protein	ZP_00108866	832	0.0
	hypothetical protein	ZP_00111391	665	0.0
	hypothetical protein	ZP_00106389	500	e-142
FtsH / all4776	hypothetical protein	ZP_00108866	1019	0.0
	hypothetical protein	ZP_00105811	817	0.0
	hypothetical protein	ZP_00111391	624	e-179
	hypothetical protein	ZP_00106389	498	e-141
FtsH / all4936	hypothetical protein	ZP_00106389	692	0.0
	hypothetical protein	ZP_00111391	513	e-146
	hypothetical protein	ZP_00108866	471	e-133
	hypothetical protein	ZP_00105811	453	e-128
FtsH / alr1261	hypothetical protein	ZP_00111391	1089	0.0
	hypothetical protein	ZP_00108866	624	e-180
	hypothetical protein	ZP_00105811	619	e-178
	hypothetical protein	ZP_00106389	529	e-151
FtsE / alr1706	hypothetical protein	ZP_00110062	323	1e-89
FtsZ / alr3858	hypothetical protein	ZP_00111461	647	0.0

Hardware and software development for photobioreactor

The development of hardware should include the integration of sensors for pH, oxygen, temperature and optical density. All this parameters could then be at least partially controlled. For instance, a pH value could be set and maintained, or a culture could be kept within certain density values. This work could also contemplate the installation of a scale that could monitor in real time the total weight of the reactor. Knowing this value would allow for automatic compensation of medium due to sampling, for instance.

The possibilities are numerous and will rely not only on proper hardware, but also in well designed software. The construction of the reactor already had in mind the installation of some devices, but all the sensors, accessory electronics and software must be designed and assembled by a specialized team. Since it is not easy to acquire a full prepared solution, the cooperation with an institution or a company is the most effective way to achieve this goal. A cooperative work with a team of engineers would be ideal.

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The initial, preparatory, tasks for this project included a very extensive search of the literature produced in the last decades, concerning hydrogen production by cyanobacteria. The results of that research was presented has a poster in the international symposium "Biohydrogen 2002" (held in Ede, The Netherlands, April 21–24, 2002), and published as a review in the International Journal of Hydrogen Energy. The following pages contain both the poster and the paper.

A brief look at three decades of research on cyanobacterial hydrogen evolution

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Summary.

Hydrogen evolution by cyanobacteria is a potential way of biohydrogen production for the future. The basic and early applied research over the last 30 years has established the basis of present knowledge in the field and is a platform for future R&D directions. This work briefly surveys some of the progress made in the field of cyanobacterial hydrogen evolution during this time period. Data surveys, as the one currently performed, present researchers with problems such as:

a) knowledge of and access to all related literature is sometimes difficult,

b) results are presented in a heterogeneous manner,

c) experimental procedures usually present a high degree of variation, and

d) the nature of the biological material itself makes comparisons a difficult task.

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Table 1. Comparative study of hydrogen production in some heterocystous cyanobacteria [1].

Organism	Maximum hydrogen evolution	Growth conditions	H_2 evolution assay conditions
Anabaena sp. PCC 7120	2.6 μmol/mg chl *a*/h	air; 20 μE/m²/s	Ar; 60 μE/m²/s
Anabaena cylindrica IAM M-1	2.1 μmol/mg chl *a*/h	air; 20 μE/m²/s	Ar; 60 μE/m²/s
Anabaena variabilis IAM M-58	4.2 μmol/mg chl *a*/h	air; 20 μE/m²/s	Ar; 60 μE/m²/s
Anabaenopsis circularis IAM M-4	0.31 μmol/mg chl *a*/h	air; 20 μE/m²/s	Ar; 60 μE/m²/s
Nostoc muscorum IAM M-14	0.60 μmol/mg chl *a*/h	air; 20 μE/m²/s	Ar; 60 μE/m²/s
Nostoc commune IAM M-13	0.25 μmol/mg chl *a*/h	air; 20 μE/m²/s	Ar; 60 μE/m²/s
Anabaena cylindrica UTEX B 629	0.91 μmol/mg chl *a*/h	air; 20 μE/m²/s	Ar; 60 μE/m²/s
Anabaena flos-aquae UTEX 1444	1.7 μmol/mg chl *a*/h	air; 20 μE/m²/s	Ar; 60 μE/m²/s
Anabaena flos-aquae UTEX LB 2558	3.2 μmol/mg chl *a*/h	air; 20 μE/m²/s	Ar; 60 μE/m²/s

Table 2. Comparative study of hydrogen production in some non-nitrogen-fixing cyanobacteria [2].

Organism	Maximum hydrogen evolution	Growth conditions	H_2 evolution assay conditions
Synechococcus PCC 602	0.66 μmol/mg chl *a*/h	air; photon fluence rate was 20 μE/m²/s	Ar + CO (13.4μmol); photon fluence rate was 20-30 μE/m²/s; or Ar + CO (13.4 μmol) + C_2H_2 (1.34 mmol); darkness
Microcystis PCC 7820	0.16 μmol/mg chl *a*/h	air; photon fluence rate was 20 μE/m²/s	Ar + CO (13.4 μmol) + C_2H_2 (1.34 mmol); photon fluence rate was 20 to 30 μE/m²/s
Gloebacter PCC 7421	1.38 μmol/mg chl *a*/h	air; photon fluence rate was 20 μE/m²/s	Ar + CO (13.4μmol) + C_2H_2 (1.34 mmol); photon fluence rate was 20 to 30 μE/m²/s
Synechocystis PCC 6308	0.13 μmol/mg chl *a*/h	air; photon fluence rate was 20 μE/m²/s	Ar + CO (13.4μmol) + C_2H_2 (1.34 mmol); photon fluence rate was 20 to 30 μE/m²/s
Aphanocapsa montana	0.40 μmol/mg chl *a*/h	air; photon fluence rate was 20 μE/m²/s	Ar (100%); photon fluence rate was 20 to 30 μE/m²/s

Table 3. Hydrogen evolution during cyanobacterial fermentation.

Organism	Maximum hydrogen evolution	Growth conditions	Reference
Oscillatoria limosa	about 0.83 μmol/mg chl *a*/h	air; incubation in 16h light, 8h darkness cycles	[3]
Cyanothece PCC 7822	0.92 μmol/mg chl *a*/h	N_2 + CO_2 (5%)	[4]
Microcystis PCC 7806	about 11.3 nmol/mg prot/h	air; incubation in 16h light, 8h darkness cycles	[5]
Microcoleus chtonoplastes	about 1.7 nmol/mg prot/h	air; ferric ammonium citrate added (46 μM); 30 μE/m²/s	[6]
Anabaena variabilis SPU 003	5.58 nmol/mg dry weight/h	air; incubation in 16h light (3000 lux light intensity), 8h darkness cycles; mannose used as carbon source	[7]

Table 4. Maximum hydrogen evolution by different *Anabaena* mutant strains.

Organism	Maximum hydrogen evolution	Growth conditions	H_2 evolution assay conditions	Reference
Anabaena variabilis 1403/4B	20 μL/mg dry weight/h	air; 15 μE/m²/s	no gas phase; cells immobilized in hollow fibers; 25 μE/m²/s on top surface and 13 μE/m²/s on bottom surface of reactor	[8]
Anabaena variabilis PK84	167.60 μmol/mg chl *a*/h	Ar (73%) + N_2 (25%) + CO_2 (2%); 90 μE/m²/s	Ar (93%) + N_2 (5%) + CO_2 (2%); 90 μE/m²/s	[9]
	2.63 μL/mg chl *a*/h	air + CO_2 (2%); outdoor conditions	air + CO_2 (2%); outdoor conditions (about 400 W/m²)	[10]
Anabaena variabilis PK17R	59.18 μmol/mg chl *a*/h	Ar (73%) + N_2 (25%) + CO_2 (2%); 90 μE/m²/s	Ar (93%) + N_2 (5%) + CO_2 (2%); 90 μE/m²/s	[9]
Anabaena variabilis AVM13	68 μmol/mg chl *a*/h	air + CO_2 (1%); 100 mE/m²/s	---	[11]

Keynote. Further studies should address the issue of increasing observed hydrogen production by applying different biochemical and genetic methods.

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A brief look at three decades of research on cyanobacterial hydrogen evolution

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Abstract

Hydrogen evolution by cyanobacteria is a potential way of biohydrogen production for the future. The basic and early applied research over the last 30 years has established the basis of present knowledge in the field and is a platform for future R& D directions. This work briefly surveys some of the progress made in the field of cyanobacterial hydrogen evolution during this time period.

Keywords: Cyanobacteria; Hydrogen production; Nitrogenase; Hydrogenase

1. Introduction

The increase of global pollution and the depletion of fossil fuel reserves make research concerning alternative energy resources a priority. One environmentally acceptable, alternative energy carrier, which could initially supplement or even substitute fossil fuel, is molecular hydrogen, H_2 [1].

Photoconversion of water to hydrogen is seen as the preferable solution. Three ways to achieve this can be considered: (a) the use of photochemical fuel cells, (b) by applying photovoltaics, or (c) by promoting production of hydrogen by photosynthetic microorganisms, either prokaryotic phototrophic anoxygenic bacteria and cyanobacteria or eukaryotic green algae [2–4]. For photobiological H_2 production cyanobacteria are among the ideal candidates since they: (a) are capable of H_2 evolution, and (b) have simple nutritional requirements—they can grow in air (N_2 and CO_2), water and mineral salts, with light as the only energy source [1].

A broad range of approaches to examine cyanobacteria and their metabolism of hydrogen has been performed [3]. Nevertheless, no practical biohydrogen production process is a reality [4], but its development should be the base of future research.

The main obstacles in the development of H_2 production technologies using cyanobacteria are: (a) inhibition of enzymes directly involved in hydrogen production by oxygen, (b) H_2 consumption by an uptake hydrogenase, and (c) an overall low productivity [5].

When analyzing the hydrogen metabolism in nitrogen-fixing cyanobacteria in detail, three enzymes should be considered: nitrogenase, evolving hydrogen during nitrogen fixation, an uptake hydrogenase, recycling the hydrogen produced by nitrogenase, and a bidirectional hydrogenase that catalyses both hydrogen production and consumption [1–6].

The oxygen sensitive nitrogenases, of capital importance for the maintenance of the nitrogen cycle, are catalysts for hydrogen production—evolving H_2 concomitantly with the fixation of N_2 to NH_3 [1].

$$N_2 + 8\ H^+ + 8\ e^- + 16\mathrm{ATP} \rightarrow 2\ NH_3 + H_2 + 16\mathrm{ADP} + 16\ \mathrm{Pi}.$$

Uptake hydrogenases, catalysing the consumption of hydrogen, have been found in all nitrogen-fixing cyanobacteria examined so far [7–9]. The catalysed reaction, thought to be advantageous to the organisms, is undesired from a hydrogen evolution point of view. A solution to this

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Table 1
Maximum hydrogen evolution by *Anabaena cylindrica* B-629 when grown and assayed under different conditions

Maximum hydrogen evolution	Growth conditions	H_2 evolution assay conditions	Reference
0.486 μmol/mg dry wt/h	Air + CO_2 (3%); total incident light was 2×10^4 erg/cm^2/s	Ar (100%); total incident light was 6×10^4 erg/cm^2/s	[15]
32 μl/mg dry wt/h (1.33 μmol/mg dry wt/h)	Air+CO_2 (0.3%); total incident light was 1.8×10^4 erg/cm^2/s	Ar + CO_2 (3%); total incident light was 4×10^5 erg/cm^2/s	[16]
0.17 μmol/mg dry wt/h	Air+CO_2 (0.3%); 7000 lx at the surface of the culture vessels	Ar+CO_2 (3%); 7000 lx at the surface of the culture vessels	[17]
0.11 μmol/mg dry wt/h	Air+CO_2 (0.3%); 7000 lx at the surface of the culture vessels	Air+CO_2 (3%) + CO (2%) + C_2H_2 (10%); 7000 lx at the surface of the culture vessels	[17]
0.103 μmol/mg dry wt/h	Air + CO_2 (5%); 7000 lx at the surface of the culture vessels	Ar+CO_2 (3%); 4000 lx at the surface of the culture vessels	[18]
29 μl/mg dry wt/h (1.21 μmol/mg dry wt/h)	Ar + CO_2 (0.5%); ammonium chloride (0.2 mM) added every 2 days	Ar	[19]
About 0.16 μmol/mg dry wt/h	Air+CO_2 (0.3%); 7000 lx at the surface of the culture vessels	Ar+CO_2 (3%); 4000 lx at the surface of the culture vessels	[20]
About 0.22 μmol/mg dry wt/h	Air + CO_2 (5%); 7000 lx at the surface of the culture vessels	N_2 +CO(0.2%)+C_2H_2 (5%) + CO_2 (5%); 4000 lx at the surface of the culture vessels	[21]

problem has actively being searched and the use of mutants lacking uptake hydrogenase activity [10,11] is currently a reality.

Bidirectional hydrogenase is able to either produce or oxidize hydrogen. Its role in cyanobacterial metabolism is still unclear. It has been suggested that this enzyme acts as a mediator in the release of excess of reducing power in anaerobic environments [12]. Another hypothesis states that bidirectional hydrogenase in *Synechocystis* sp. PCC 6803 works as an electron valve for the disposal of low potential electrons generated at the onset of illumination [13]. Even considering the fact that bidirectional enzyme is not a universal cyanobacterial enzyme [9,14], its impact on hydrogen evolution should not be overseen.

Data surveys, as the one currently performed, present researchers with problems such as: (a) knowledge of and access to all related literature is sometimes difficult, (b) results are presented in a heterogeneous manner, (c) experimental procedures usually present an high degree of variation, and (d) the nature of the biological material itself makes comparison a difficult task.

2. Hydrogen evolution synopsis

It is accepted that efficient photoconversion is influenced by many factors. The research on the metabolism of hydrogen in cyanobacteria over the last three decades clearly reflects that multitude of factors.

Initial work by several authors focused on the heterocystous filamentous cyanobacterium *Anabaena cylindrica* B-629. The role of light intensity, oxygen, uptake hydrogenase and gas phase composition was immediately underlined as key factors in the success of hydrogen evolution.

The need of light for hydrogen evolution, nitrogenase oxygen sensitivity, and lower hydrogen evolution versus acetylene reduction were shown and discussed [15]. The cultivation of cyanobacteria in nitrate-free media under air and CO_2, followed by incubation in light under argon and CO_2 atmosphere, rapidly became standard, since it resulted in immediate hydrogen production [15,16]. The highest hydrogen evolution observed under these conditions was 32 μl/mg dry wt/h (1.33 μmol/mg dry wt/h), see Table 1 [16].

It was also established that light saturated, nitrogen-fixing cultures had a good and stable supply of reductant, underlining the relationship between photosynthesis and nitrogen fixation. These conditions were considered vital for optimal nitrogen fixation and biomass production. Filament integrity was also discussed and established as a factor in the duration of hydrogen production—filament breakage leads to loss of nitrogenase activity [16]. The need for strategies in order to minimize mechanical breakage of filaments and general improvement in experimental conditions became evident.

Localization of nitrogenase in heterocysts provides an oxygen-free environment and the ability of heterocystous cyanobacteria to fix nitrogen in air. Hydrogenase and nitrogenase inhibitors were used in an attempt to screen for aerobic hydrogen evolution potential—the conclusion was that these inhibitors allowed for hydrogen to be released from aerobic cultures in amounts similar to those in argon, see Table 1 [17].

The use of non-argon gaseous atmospheres was further studied and it was concluded that suitable air and nitrogen based mixtures with carbon monoxide and acetylene were almost as efficient as argon for rate and duration of hydrogen evolution [21]. Under these conditions maximum hydrogen evolution was 0.22 μmol/mg dry wt/h, see Table 1.

Table 2
Maximum hydrogen evolution comparing the non-marine *Anabaena cylindrica* B-629 with three marine cyanobacterial strains [18]

Organism	Maximum hydrogen evolution (μmol/mg dry wt/h)	Growth conditions	H_2 evolution assay conditions
Anabaena cylindrica B-629	0.103	Air + CO_2 (5%); 7000 lx at the surface of the culture vessels	Ar + CO_2 (3%); 4000 lx at the surface of the culture vessels
Oscillatoria brevis B-1567	0.168	Air + CO_2 (5%); 7000 lx at the surface of the culture vessels	Ar + CO_2 (3%); 4000 lx at the surface of the culture vessels
Calothrix scopulorum 1410/5	0.128	Air + CO_2 (5%); 7000 lx at the surface of the culture vessels	Ar + CO_2 (3%); 4000 lx at the surface of the culture vessels
Calothrix membranacea B-379	0.108	Air + CO_2 (5%); 7000 lx at the surface of the culture vessels	Ar + CO_2 (3%); 4000 lx at the surface of the culture vessels

Table 3
Maximum hydrogen evolution by *Oscillatoria* sp. Miami BG7 [22]

Organism	Maximum hydrogen evolution	Growth conditions	H_2 evolution assay conditions
Oscillatoria sp. Miami BG7	About 6.0 μl/mg dry wt/h (0.25 μmol/mg dry wt/h)	Air; 100 μE/m^2/s; NH_4Cl(25 mg/l) used as combined nitrogen source	Ar (100%); 90 μE/m^2; 11-day-old cells; 37°C

Table 4
Comparative study of hydrogen production in some heterocystous cyanobacteria [23]

Organism	Maximum hydrogen evolution (μmol/mg chl *a*/h)	Growth conditions	H_2 evolution assay conditions
Anabaena sp. PCC 7120	2.6	Air; 20 μE/m^2/s	Ar; 60 μE/m^2/s
Anabaena cylindrica IAM M-1	2.1	Air; 20 μE/m^2/s	Ar; 60 μE/m^2/s
Anabaena variabilis IAM M-58	4.2	Air; 20 μE/m^2/s	Ar; 60 μE/m^2/s
Anabaenopsis circularis IAM M-4	0.31	Air; 20 μE/m^2/s	Ar; 60 μE/m^2/s
Nostoc muscorum IAM M-14	0.60	Air; 20 μE/m^2/s	Ar; 60 μE/m^2/s
Nostoc linckia IAM M-30	0.17	Air; 20 μE/m^2/s	Ar; 60 μE/m^2/s
Nostoc commune IAM M-13	0.25	Air; 20 μE/m^2/s	Ar; 60 μE/m^2/s
Anabaena cylindrica UTEX B 629	0.91	Air; 20 μE/m^2/s	Ar; 60 μE/m^2/s
Anabaena flos-aquae UTEX 1444	1.7	Air; 20 μE/m^2/s	Ar; 60 μE/m^2/s
Anabaena flos-aquae UTEX LB 2558	3.2	Air; 20 μE/m^2/s	Ar; 60 μE/m^2/s

Several studies, directly comparing the hydrogen evolution by different cyanobacterial strains have shown to be extremely useful. In the seventies, a period when developing a new energy production was a severe priority, some studies started to diverge from using the initial *A. cylindrica* B-629. Work performed by Lambert and Smith, summarized in Table 2, confirmed *A. cylindrica* B-629's potential for hydrogen evolution (see Table 1), and in addition showed the potential of marine cyanobacteria such as *Oscillatoria brevis*, *Calothrix scopulorum*, and *C. membranacea* [18]. The tested four strains showed maximum hydrogen evolution between 0.103 and 0.168 μmol/mg dry wt/h of hydrogen under Ar and CO_2 (3%) atmosphere.

Based on previous knowledge, the role of light intensity and temperature was investigated demonstrating the potential of *Oscillatoria* sp. Miami BG7 as an organism to be used for a photobiological production of hydrogen [22]. Maximum hydrogen evolution under conditions described in Table 3 was about 6.0 μl/mg dry wt/h (0.25 μmol/mg dry wt/h).

Recently, research was presented analyzing different cyanobacterial strains representing an effort in the search for a suitable strain for cyanobacterial biohydrogen production, see Table 4. *A. variabilis* IAM M-58 presented the maximum hydrogen evolution for all tested strains (4.2 μmol/mg chl *a*/h) [23]. One important detail referred to in this study is the fact that each strain required different incubation time in order to attain highest hydrogen evolution, demonstrating the diversity when working with and analysing biological material.

Table 5
Comparative study of hydrogen production in some non-nitrogen-fixing cyanobacteria [24]

Organism	Maximum hydrogen evolution (μmol/mg chl *a*/h)	Growth conditions	H_2 evolution assay conditions
Synechococcus PCC 6830	0.26	Air; photon fluence rate was 20 $\mu E/m^2/s$	Ar + CO(13.4 μmol) + C_2H_2 (1.34 mmol); darkness
Synechococcus PCC 602	0.66	Air; photon fluence rate was 20 $\mu E/m^2/s$	Ar + CO(13.4 μmol); photon fluence rate was 20–30 $\mu E/m^2/s$; or Ar + CO(13.4 μmol) + C_2H_2 (1.34 mmol); darkness
Synechoccus PCC 6301	0.09	Air; photon fluence rate was 20 $\mu E/m^2/s$	Ar + C_2H_2(1.34 mmol); photon fluence rate was 20–30 $\mu E/m^2s$
Synechococcus PCC 6307	0.02	Air; photon fluence rate was 20 $\mu E/m^2/s$	Ar(100%); photon fluence rate was 20–30 $\mu E/m^2/s$
Microcystis PCC 7820	0.16	Air; photon fluence rate was 20 $\mu E/m^2/s$	Ar + CO(13.4 μmol) + C_2H_2(1.34 mmol); photon fluence rate was 20 –30 $\mu E/m^2/s$
Gloebacter PCC 7421	1.38	Air; photon fluence rate was 20 $\mu E/m^2/s$	Ar + CO(13.4 μmol) + C_2H_2(1.34 mmol); photon fluence rate was 20 –30 $\mu E/m^2/s$
Synechocystis PCC 6308	0.13	Air; photon fluence rate was 20 $\mu E/m^2/s$	Ar + CO(13.4 μmol) + C_2H_2(1.34 mmol); photon fluence rate was 20 –30 $\mu E/m^2/s$
Synechocystis PCC 6714	0.07	Air; photon fluence rate was 20 $\mu E/m^2/s$	Ar + CO(13.4 μmol); photon fluence rate was 20–30 $\mu E/m^2/s$ or Ar + CO(13.4 μmol) + C_2H_2(1.34 mmol); darkness
Aphanocapsa montana	0.40	Air; photon fluence rate was 20 $\mu E/m^2/s$	Ar(100%); photon fluence rate was 20–30 $\mu E/m^2/s$

Hydrogen production has also been examined in unicellular non-nitrogen-fixing cyanobacteria, see Table 5 [24].

In the absence of a nitrogenase, all unicellular strains tested showed the presence of hydrogenase evolving hydrogen. The unicellular strain *Gloebacter* PCC 7421 demonstrated highest hydrogen evolution rate (1.38 μmol/mg chl *a*/h) when incubated under low light (20–30 $\mu E/m^2/s$) and CO and C_2H_2, comparable with the values presented for nitrogen-fixing heterocystous cyanobacteria, in Table 4. It was suggested that the capacity to metabolize hydrogen via hydrogenase may be widespread among non-nitrogen-fixing cyanobacteria [24].

In many various cyanobacteria hydrogenase-dependent hydrogen production has been observed [2]. In Table 6, a brief summary of hydrogen evolution by cyanobacteria under dark anoxic conditions is given. The available data showed that hydrogen evolution during cyanobacterial fermentations presently is lower than evolution of hydrogen by nitrogenase.

In Table 7, several *Anabaena* strains together with maximum hydrogen evolution rates are presented. A significant number of *Anabaena* strains have been, for quite some time, the object of intensive studies.

Obtaining mutants and studying their hydrogen evolution capabilities has driven research into new levels of cyanobacterial hydrogen production. Genetic engineering was used to create uptake hydrogenase mutants of both *A. variabilis* [11,33] and more recently, *Nostoc* ATCC 29133 [36] demonstrating the option of a constant production of molecular hydrogen by oxygen evolving photosynthetic microorganisms. *Anabaena variabilis* mutants, in particular AVM13 [11], PK84 and PK17R [33], have shown good results, and differences between wild-type and mutants are significant (Table 7). Maximum hydrogen evolution for *A. variabilis* ATCC 29413 was 45.16 μmol/mg chl *a*/h, while the PK17R mutant evolved 59.18 μmol/mg chl *a*/h and the PK84 mutant evolved 167.6 μmol/mg chl *a*/h [33]. Studies with the PK84 mutant demonstrated the possibility of outdoor

Table 6
Hydrogen evolution during cyanobacterial fermentation

Organism	Maximum hydrogen evolution	Growth conditions	Reference
Oscillatoria limosa	About 0.83 μmol/mg chl *a*/h	Air; incubation in 16 h light, 8 h darkness cycles	[25]
Cyanothece 7822	0.92 μmol/mg chl *a*/h	$N_2 + CO_2$ (5%)	[26]
Microcystis PCC 7806	About 11.3 nmol/mg prot/h	Air; incubation in 16 h light, 8 h darkness cycles	[27]
Microcoleus chtonoplastes	About 1.7 nmol/mg prot/h	Air; ferric ammonium citrate added (46 μM); 30 $\mu E/m^2/s$	[28]
Anabaena variabilis SPU 003	5.58 nmol/mg dry wt/h	Air; incubation in 16 h light (3000 lx light intensity), 8 h darkness cycles; mannose used as carbon source	[29]

Table 7
Maximum hydrogen evolution by different *Anabaena* strains

Organism	Maximum hydrogen evolution	Growth conditions	H_2 evolution assay conditions	Reference
Anabaena sp. N-7363	36 μmol/mg chl *a*/h	—	Ar	[30]
Anabaena variabilis ATCC 29413	About 1.3 μl/mg dry wt/h (0.05 μmol/mg dry wt/h)	5000 lx at the surface of the culture vessels	Ar + CO_2 (5%); 5000 lx; addition of Tween 85 (77 mM)	[31]
Anabaena variabilis 1403/4B	20 μl/mg dry wt/h (0.83 μmol/mg dry wt/h)	Air; 15 $\mu E/m^2/s$	No gas phase; cells immobilized in hollow fibers; 25 $\mu E/m^2/s$ on top surface and 13 $\mu E/m^2/s$ on bottom surface of reactor	[32]
Anabaena variabilis ATCC 29413	45.16 μmol/mg chl *a*/h	Ar(73%) + N_2(25%) + CO_2(2%); 90 $\mu E/m^2/s$	Ar(93%) + N_2(5%) + CO_2(2%); 90 $\mu E/m^2/s$	[33]
Anabaena variabilis PK84	167.60 μmol/mg chl *a*/h	Ar(73%) + N_2(25%) + CO_2(2%); 90 $\mu E/m^2/s$	Ar(93%) + N_2(5%) + CO_2(2%); 90 $\mu E/m^2/s$	[33]
	2.63 μl/mg chl *a*/h (0.11 μmol/mg chl *a*/h)	Air + CO_2(2%); outdoor conditions	Air + CO_2(2%); outdoor conditions (about 400 W/m^2)	[34]
Anabaena variabilis PK17R	59.18 μmol/mg chl *a*/h	Ar(73%) + N_2(25%) + CO_2(2%); 90 $\mu E/m^2/s$	Ar(93%) + N_2(5%) + CO_2(2%); 90 $\mu E/m^2/s$	[33]
Anabaena azollae	38.5 μmol/mg chl *a*/h	Air + CO_2(2%); continuous turbidostat mode; 113 $\mu E/m^2/s$	Ar	
Anabaena variabilis ATCC 29413	39.4 μmol/mg chl *a*/h	Air + CO_2(2%); continuous turbidostat mode; 113 $\mu E/m^2/s$	Ar	[35]
Anabaena variabilis PK84	32.3 μmol/mg chl *a*/h	Air + CO_2(2%); continuous turbidostat mode; 113 $\mu E/m^2/s$	Ar	
Anabaena variabilis AVM13	68 μmol/mg chl *a*/h	Air + CO_2(1%); 100 $mE/m^2/s$	—	[11]
Anabaena sp. PCC 7120	2.6 μmol/mg chl *a*/h	Air; 20 $\mu E/m^2/s$	Ar; 60 $\mu E/m^2/s$	
Anabaena cylindrica IAM M-1	2.1 μmol/mg chl *a*/h	Air; 20 $\mu E/m^2/s$	Ar; 60 $\mu E/m^2/s$	
Anabaena variabilis IAM M-58	4.2 μmol/mg chl *a*/h	Air; 20 $\mu E/m^2/s$	Ar; 60 $\mu E/m^2/s$	[23]
Anabaena cylindrica UTEX B 629	0.91 μmol/mg chl *a*/h	Air; 20 $\mu E/m^2/s$	Ar; 60 $\mu E/m^2/s$	
Anabaena flos-aquae UTEX 1444	1.7 μmol/mg chl *a*/h	Air; 20 $\mu E/m^2/s$	Ar; 60 $\mu E/m^2/s$	
Anabaena flos-aquae UTEX LB 2558	3.2 μmol/mg chl *a*/h	Air; 20 $\mu E/m^2/s$	Ar; 60 $\mu E/m^2/s$	

photobioreactor hydrogen production [34]. During continuous growth in outdoor photobioreactor the maximum hydrogen evolution registered was 2.63 μl/mg chl *a*/h (0.11 μmol/mg chl *a*/h).

Future research should include: (a) screening for wild-type strains possessing high nitrogenase activity and/or very active hydrogen evolving hydrogenases, (b) specific genetic modification of these strains (aiming for e.g. uptake hydrogenase deficient mutants with higher heterocyst frequency, increased level of active bidirectional hydrogenase enzyme and decreased growth/cell division), and (c) optimization of cultivation conditions for hydrogen evolution—improvement of parameters like light intensity, CO_2 content, temperature, pH or micronutrient content.

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