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Hydrogen Production by the Cyanobacterium *Plectonema boryanum*: Effects of Initial Nitrate Concentration, Light Intensity, and Inhibition of Photosystem II by DCMU

Blaine Carter and Michael Huesemann

ABSTRACT

The alarming rate at which atmospheric carbon dioxide levels are increasing due to the burning of fossil fuels will have incalculable consequences if disregarded. Fuel cells, a source of energy that does not add to carbon dioxide emissions, have become an important topic of study. Although significant advances have been made related to fuel cells, the problem of cheap and renewable hydrogen production still remains. The cyanobacterium *Plectonema boryanum* has demonstrated potential as a resolution to this problem by producing hydrogen under nitrogen deficient growing conditions. *Plectonema boryanum* cultures were tested in a series of experiments to determine the effects of light intensity, initial nitrate concentration, and photosystem II inhibitor DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) upon hydrogen production. Cultures were grown in sterile Chu. No. 10 medium within photobioreactors constantly illuminated by halogen lights. Because the enzyme responsible for hydrogen production is sensitive to oxygen, the medium was continuously sparged with argon/CO_2 (99.7%/0.3% vol/vol) by gas dispersion tubes immersed in the culture. Hydrogen production was monitored by using a gas chromatograph equipped with a thermal conductivity detector. In the initial experiment, the effects of initial nitrate concentration were tested and results revealed cumulative hydrogen production was maximum at an initial nitrate concentration of 1 mM. A second experiment was then conducted at an initial nitrate concentration of 1 mM to determine the effects of light intensity at 50, 100, and 200 μmole m^{-2} s^{-1}. Cumulative hydrogen production increased with increasing light intensity. A final experiment, conducted at an initial nitrate concentration of 2 mM, tested the effects of high light intensity at 200 and 400 μmole m^{-2} s^{-1}. Excessive light at 400 μmole m^{-2} s^{-1} decreased cumulative hydrogen production. Based upon all experiments, cumulative hydrogen production rates were optimal at an initial nitrate concentration of 1 mM and a light intensity of 100 μmole m^{-2} s^{-1}. DCMU was shown in all experiments to severely decrease hydrogen production as time progressed. With the information acquired so far, future experiments with reducing substances could determine maximum rates of hydrogen production. If maximum hydrogen production rates proved to be large enough, *Plectonema boryanum* could be grown on an industrial scale to provide hydrogen gas as a renewable fuel.

Introduction

While greenhouse gases are responsible for making Earth's surface temperature supportable for life, the rate at which carbon dioxide is being emitted into the atmosphere is increasing. Even with the most stringent mitigation practices, the Intergovernmental Panel on Climate Change predicts a 1.4° to 5.8°C increase in global average surface temperature by the year 2100 [1]. While temperature changes of this magnitude have both foreseeable and unforeseen consequences, it could take hundreds of years for the full consequences of these carbon dioxide levels to take effect. Even if carbon dioxide emissions were significantly reduced and atmospheric carbon dioxide levels were allowed time to stabilize, increases in temperature and sea level would be seen for approximately 1,000 years [1]. Since energy demands will only continue to increase with time, renewable energy has become an important area of research.

Increasing atmospheric carbon dioxide levels are largely due to the burning of fossil fuels, therefore technology that aims at finding sources of renewable energy directly address this problem. Virtually all sources of renewable energy are directly or indirectly driven by

the sun, making them fundamentally different than other sources of energy such as fossil fuels or nuclear power. Because of this, renewable sources of energy produce far less pollution and release fewer greenhouse gases. Some of the major renewable technologies include wind power, hydro power, solar energy, geothermal energy, and biofuels. Although biofuels are most commonly associated with the conversion of biomass to liquid biofuels, another valuable source of renewable energy is hydrogen produced by photobiological systems. Hydrogen, with an extremely high energy density, has the potential to positively impact fossil fuel reduction and global warming by providing a means of sustainable energy generation. Major discoveries have been made with fuel cell related technologies, but a large problem still remains: cheap hydrogen production.

There are many different ways to produce hydrogen, such as natural gas reformation, renewable electrolysis, gasification, renewable liquid reforming, nuclear high temperature electrolysis, and high temperature thermochemical water splitting [2]. All of these methods have potential, but only a few are efficient enough at this point to meet demands. Natural gas reformation and electrolysis, methods which can currently meet production demands, do not solve any problems because they require large amounts of energy, which is inevitably generated by the burning of fossil fuels. A resolution to this problem is hydrogen production from a renewable source, in this case photobiological systems.

Photobiological H_2 production by microalgae has been investigated since the early 1970s [3]. The fundamental concept is to use microalgae to catalyze the conversion of solar energy and water into H_2, with O_2 as by-product. Most current research is focusing on the direct biophotolysis approach, in which water is split into H_2 and O_2 without intermediate CO_2 fixation [3]. As shown in Figure 1, during direct biophotolysis, electrons flow "directly" from water via photosystem II (PSII) and photosystem I (PSI) to the hydrogenase enzyme, which transfer these electrons to protons, thereby causing the release of hydrogen gas. However, this reaction is limited by the strong inhibition of the process by the simultaneously produced O_2. Searching for, or protein engineering of, hydrogenases that exhibit an O_2 resistant H_2 evolution reaction has been, and continues to be, a major field of research, but has not yet been successful. Indeed, achieving the levels of oxygen resistance required in a practical process may well be problematic, with perhaps a million-fold improvement required. The alternative approach, to use irreversible (e.g., glucose-glucose oxidase) or reversible (e.g. hemoglobin) O_2 absorbers, does allow sustained direct biophotolysis. However, the irreversible O_2 absorbers contain, and thus dissipate, as much energy as contained in the H_2, resulting in essentially zero net energy gain. Even if O_2 inhibition were somehow overcome, direct biophotolysis would still suffer from the practical problems of generating explosive H_2:O_2 mixtures as well as requiring very expensive photobioreactors to contain such a reaction.

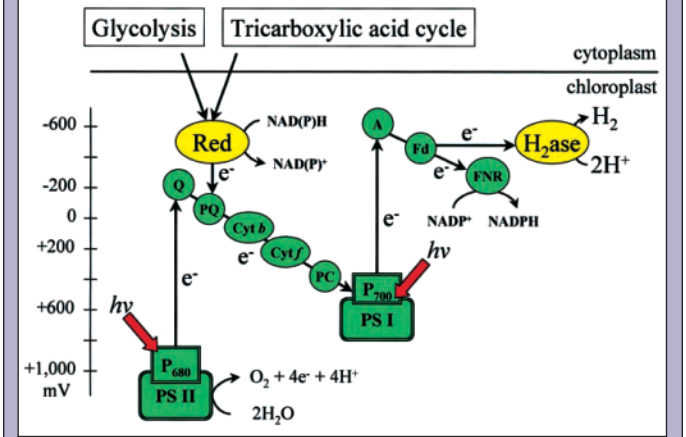

Figure 1. Electron transport in the light reactions of photosynthesis [4].

These various problems of direct biophotolysis led to proposals for indirect, two-stage light-driven processes, with CO_2 fixation and O_2 release occurring in the first stage followed by H_2 production reactions in the second stage [5]. In the indirect biophotolysis process (see Figure 1), reduced substrates (i.e., carbohydrates, such as starch or glycogen) accumulate during the photosynthetic O_2 production and CO_2 fixation stage, and these are then used in a second stage for H_2 production. By temporarily separating the photosynthetic oxygen production phase from the hydrogen production phase, indirect biophotolysis overcomes the two major problems associated with direct biophotolysis, oxygen inhibition of the hydrogenase and the generation of highly explosive H_2:O_2 mixtures.

In green microalgae such as *Chlamydomonas reinhardtii*, starch accumulates within the cells under nutrient-limited culture conditions such as sulfur or nitrogen deficiency [6]. As shown in Figure 1, during indirect biophotolysis, the electrons from the accumulated starch are donated to plastoquinone (PQ). Then they flow down-gradient through the electron transport chain to photosystem I (PSI) to be energized again and transferred to ferredoxin (Fd). The hydrogenase enzyme then accepts the electrons from ferredoxin and combines them with protons from the medium to generate hydrogen gas. The relative contributions of hydrogen production from direct and indirect biophotolysis can be quantified by challenging the culture with DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea), which shuts down the water splitting, oxygen-generation activity of photosystem II (PSI II) by inhibiting the transfer of electrons between electron carriers Q and PQ (see Figure 1). For example, the addition of DCMU will have no effect on hydrogen production rates if hydrogen is generated solely by indirect biophotolysis but will completely inhibit any hydrogen production by direct biophotolysis.

The non-heterocystous nitrogen-fixing blue-green microalga (cyanobacterium) *Plectonema boryanum* is capable of producing hydrogen only by indirect biophotolysis. During the daytime, this organism carries out photosynthetic oxygen production and CO_2 fixation. The depletion of nitrogen in the culture results in glycogen-accumulation within the cells. At night, the cells respire the remaining dissolved oxygen and the medium becomes anaerobic. If the culture is both nitrogen-limited and anaerobic, the nitrogenase enzyme is induced to fix nitrogen from the atmosphere according to the following reaction [7]:

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

It can be seen that one mole of hydrogen is produced as a byproduct of the fixation of one mole of nitrogen into two moles of

ammonia [8]. It is also important to note that this reaction is very energy intensive since it requires 8 moles of high-energy electrons, which are donated from the accumulated glycogen, per mole of hydrogen generated [7]. While this nitrogen-fixation reaction occurs in the dark, it is enhanced in the light because electrons are energized at photosystem I, resulting also in cyclic ATP generation. Because of its extreme sensitivity to oxygen, the nitrogenase, as the hydrogenase, is only active under strict anaerobic conditions.

The hydrogen yield of this reaction can be increased by eliminating dinitrogen gas from the medium and replacing it with an inert gas such as argon. The nitrogenase will then use the high energy electrons that are donated from the accumulated glycogen to produce hydrogen (instead of ammonia), as long as the medium remains anaerobic and nitrogen-deficient. Challenging the culture with DCMU should have no effect on the rates of hydrogen generation since it proceeds entirely by indirect biophotolysis. However, since DCMU inhibits photosynthetic CO_2 fixation, the intracellular glycogen reserves cannot be restored after their depletion during the hydrogen production phase.

In addition to hydrogen generation by the nitrogenase enzyme, cyanobacteria also have the ability to generate hydrogen via hydrogenase-catalyzed reactions. Hydrogenases occur as two distinct types in cyanobacteria. One of them, the uptake hydrogenase, has the ability to oxidize hydrogen to generate energy (ATP) and reducing equivalent (NAD(P)H) while at the same time removing oxygen via respiration, thereby protecting the oxygen-sensitive nitrogenase enzyme from inhibition [7]. The other type of hydrogenase is the reversible or bidirectional hydrogenase and it can take up or produce hydrogen, depending on the redox status of the culture [9]. Generally, the contribution to hydrogen evolution by the reversible hydrogenase is neglible [9]. Therefore, the net hydrogen generation rate in cyanobacteria is the sum of the hydrogen production rate by nitrogenase and the hydrogen consumption rate by the uptake hydrogenase.

Cyanobacterium *Plectonema boryanum* has been shown by Weave and Benemann to produce hydrogen under nitrogen deficient conditions [10]. In their study, oxygen was found to be a strong and irreversible inhibitor of the enzyme responsible for hydrogen production at very low concentration (>0.5%) [10]. This problem was overcome by continually flushing cultures with an argon/carbon dioxide gas mixture, or by adding reducing substances that encourage hydrogen production.

It is the objective of this experiment to determine the effects of initial nitrate concentration, light intensity, and photosystem II inhibitor DCMU upon hydrogen production by the cyanobacterium *Plectonema boryanum*.

Materials And Methods

Strain Selection and Medium Composition

Cyanobacterium *Plectonema boryanum*, acquired from American Type Culture Collection (Catalog #18200), was used in a series of experiments to determine the effects of nitrogen concentration, light intensity, and photosystem II inhibitor DCMU upon hydrogen production. Cultures were grown in sterile Chu No. 10 medium.

Compound	Mass (mg/L)
$Ca(NO_3)_2 \cdot 4H_2O$	Variable (Growth Medium = 232)
K_2HPO_4	10
$MgSO_4 \cdot 7H_2O$	25
Na_2CO_3	20
Ferric Citrate	3.5
Sodium Citrate	5.4
$Na_2SiO_3 \cdot 9H_2O$	58.9

Table 1. Chu No. 10 medium ingredients.

To prepare sterile Chu No. 10 media, concentrated stock solutions containing all of the medium ingredients except calcium nitrate tetrahydrate were created (See Table 1). Separate concentrated calcium nitrate tetrahydrate solutions were prepared as well, and were always made fresh or frozen, in order to prevent degradation. The stock solutions were mixed together in appropriate ratios for the specific experiment and diluted to one liter. After the appropriately mixed solutions were adjusted to a pH of 7.5 with hydrochloric acid, solutions were sterile filtered using 0.2 µm nitrocellulose filters. After filtration, the sterile solutions were ready for inoculation.

Bioreactor Setup

Roux bottles were used for carrying out all experiments and were continuously sparged with argon/CO_2 (99.7%/0.3% vol/vol) at 60 mL/min by gas dispersion tubes immersed in the culture. The culture medium within the Roux bottle was constantly stirred by a magnetic stir bar. Continuous illumination was maintained by 300 or 500 W halogen bulbs in a Regent PN PQS45 light housing.

Measurement of Light Intensity

Whenever the light intensity illuminating the Roux bottles was measured, lightmeter readings were taken at five evenly distributed points at the face of the Roux bottle. The average of these values approximated the overall light intensity selected for the specific experiment. Whenever photon absorbance was measured, readings at the five points were taken both in front of and behind the bottle, relative to the light source. All readings were taken using a Hansatech Quantitherm Lightmeter.

Measurement of Optical Density (OD) and Nitrate Concentration

During the initial active growth phases, nitrate concentration and optical density (OD) were frequently measured. Nitrate concentration was determined via cadmium reduction using Hach NitraVer packets. A 10 mL culture sample was taken from the Roux bottle and spun in a centrifuge at 2500 rpm for three minutes. The supernatant was then poured off into a sealable test tube. A single Hach NitraVer packet was added to the supernatant and was mixed for one minute by continuous inversion. After mixing, the solution was allowed to react for precisely five minutes. The cuvette was rinsed with the sample solution twice and then an absorbance reading

was taken at 500 nm using a UNICO 1100 spectrophotometer. Based upon a previously constructed calibration curve, the nitrate concentration was determined by the absorbance. Because this method is technique sensitive, results were merely looked upon in a qualitative manner. The optical density of the culture samples was measured at 595 nm. Culture samples were diluted to ensure that observed readings were within the linear range of acquisition ($0 < OD_{590} < .5$), in accordance with Beer's law.

Measurement of AFDW

Ash free dry weight (AFDW), another measurement of cell biomass, was determined by vacuum filtering a measured volume of culture through a Whatman, 55mm, GF/F glass microfibre filter. The filter was dried overnight in a 105°C oven and the mass was measured. At this point the filter was combusted in a 550°C furnace for 30 minutes and then weighed. The difference in these weights represents the combustible organic cellular materials. AFDW, with units of mg/L, is calculated by dividing the difference of the dried and combusted weights by the measured volume of the sample. Filters were pre-vacuum rinsed with 25ml of de-ionized water and pre-ashed in a 550°C furnace for 15 minutes to ensure accuracy.

Gas Composition Analysis

Although all culture bottles were continuously sparged with argon/CO_2 (99.7%/0.3% vol/vol) at 60 mL/min for the duration of all experiments, the gas flow was reduced to 10 mL/min to allow for hydrogen measurements. Once flow rates were reduced, a minimum of three hours was allocated to allow hydrogen concentration within the headspace of the Roux bottle to stabilize (as seen in Figure 2). After this time period, the gas composition was analyzed using an SRI 8610C gas chromatograph equipped with a thermal conductivity detector. The carrier gas used in all measurements was argon. The cumulative hydrogen production (V_{H2}) was calculated using the following equation:

$$V_{H2} = \Sigma f_{H2} \bullet F_G \bullet \Delta T$$

where f_{H2} is the measured fraction of hydrogen, F_G is the flow of the sparge gas, and ΔT is the time interval between measurements.

Glycogen Assay

In order to monitor glycogen concentration within the cells, glycogen measured as glucose was determined by the method described by Gfeller and Gibbs [6]. One mL samples were drawn from the culture and spun down at 13,000 rpm for two minutes. The supernatant was discarded and the pellet was then washed two times with 1 mL of methanol and two times with 1 mL of sodium acetate (pH 4.5). The samples were sonicated and spun down for each stage of the wash. These wash cycles broke apart the cells and removed components that could interfere with future absorbance readings. After completing the two wash cycles, the pellet was resuspended in 1.7 mL acetate buffer and 0.02 mL of α-amylase (Sigma Aldrich Catalog # A4582) was added to solution. The samples were autoclaved for 10 minutes to solubilize the glycogen. After autoclaving, 0.05 mL of amyloglucosidase (Sigma Aldrich Catalog # S9144) was added to solution and the samples were incubated at 55°C for 14 hours to convert glycogen to glucose. Sample volumes were then readjusted to 2.0 mL with distilled water. At time zero, a reaction period was initiated by adding 2.0 mL of glucose assay reagent (Sigma Aldrich Catalog # G3666 and # D2679) followed by mixing. Sixty second time intervals were allocated between the additions of glucose assay reagent to each subsequent tube. Immediately after mixing, each tube was incubated for thirty minutes at 37°C. The reaction was stopped at the end of the thirty minute incubation by adding 2.0 mL of 6 M H_2SO_4. Each tube was mixed thoroughly and the absorbance was measured at 540 nm. Chemically, when glucose oxidase contained within the glucose assay reagent reacts with glucose, hydrogen peroxide and gluconic acid are formed. The hydrogen peroxide then reduces dianisidine, which turns a brown color. The strength of the absorbance is directly related to the glucose concentration. In order to normalize glycogen concentrations to biomass concentrations, AFDW was always taken the same time glycogen samples were withdrawn. Based upon a calibration curve, glucose concentration was calculated based upon the absorbance.

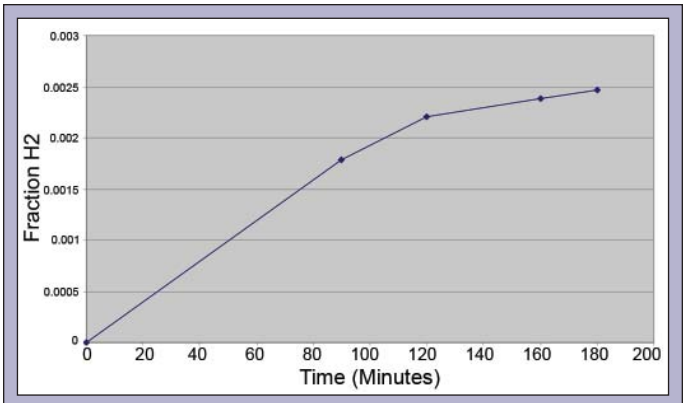

Figure 2. The graph above shows the fraction of hydrogen changing within the Roux bottle headspace as a function of time. At an initial sparge rate of 60 mL/min, the fraction of hydrogen within the headspace is below the detection limit of the gas chromatograph. When the gas flow is reduced to 10 mL/min, the fraction of hydrogen increases to a detectable level, but as seen above, it takes approximately three hours for the value to stabilize. During the stabilization period, equilibrium is being established between the amount of hydrogen produced by the algae and the amount of hydrogen being expelled from the headspace due to the sparge gas.

Chlorophyll Concentration

Chlorophyll concentration was measured spectrophotometrically in experiments to observe possible changes with varying nitrate concentration or light intensity. Chlorophyll was extracted from 1 mL of cells in boiling methanol following the procedure described by Tett [11]. One mL of algae culture was transferred to an amber glass centrifuge tube and 0.2 mL of 1% (w/v) $MgCO_3$ was added. A boiling chip was placed in the tube along with 9 mL of 90% (v/v) methanol/water. The sample was vortexed and then boiled in a water bath at 90°C for two minutes. In this process the cell walls of the algae were broken apart and the chlorophyll was extracted into solution. The tubes were then spun down at 1,500 rpm for four minutes to remove particulates from the solution. Sample volume was readjusted to 10 mL using 90% (v/v) methanol/water. Blanks of

90% (v/v) methanol/water were measured at 663 nm and 750 nm, followed by the chlorophyll samples. The chlorophyll concentration was then calculated in mg/L from the following equation:

$$Chla = DF*(1/77)*((C_{663}-B_{663})-(C_{750}-B_{750}))*1000$$

where DF is the dilution factor (10), C_{663} and C_{750} are the absorbance of the extract at 663 and 750 nm respectively, and B_{663} and B_{750} are the absorbance of the blank at 663 and 750 nm, respectively.

Results and Discussion

Effects of Nitrogen Concentration and Inhibition of Photosystem II by DCMU

In the first experiment, the effects of nitrogen concentration and DCMU upon *Plectonema boryanum* hydrogen production were observed. Four cultures within Roux bottles were grown at a light intensity of 100 µmole m^{-2} s^{-1} in the following initial medium nitrate concentrations: Bottle A=0.5 mM NO_3, Bottle B=1.0 mM NO_3, Bottle C=1.0 mM NO_3, and Bottle D=2.0 mM NO_3. When nitrate concentrations approached zero in culture C, 10 µmol of DCMU was added to inhibit photosynthetic oxygen production.

As seen in Figure 3, after approximately 70 hours, bottles A, B, and C reached a stationary growth phase. At this point, DCMU was added to bottle C. Bottle D reached a stationary growth phase at approximately 100 hours of growth. Nitrate concentrations were estimated to approach zero when the optical density no longer increased, because readings obtained with the Orion nitrate probe proved to be inaccurate and imprecise. It was observed that the maximum optical density, which was reached during stationary phase, was directly related to the initial nitrate concentration. Since nutrients available within the media decrease as the cells continue to grow, a maximum biomass concentration is reached when the nutrients are depleted. For this experiment, the maximum biomass concentration, measured as optical density, is directly related to the initial nitrate concentration.

As each respective bottle reached the stationary growth phase, the sparge gas flow was reduced to 10 mL/min to allow for gas composition analysis. For an unknown reason, the sparge gas flow rates had an occasional problem of slowly approaching and then reaching zero. When this occurred, the average flow rate could not be accurately calculated and hydrogen production values became uncertain. While most of the bottles did not have this problem most of the time, bottle C had the particular problem of reaching zero sparge gas flow overnight. Daytime measurements, where the flow was closely monitored, resulted in a different rate of hydrogen production than the overnight readings. A new system was devised to sparge the cultures at high flow rates overnight to keep them anaerobic, accurately set the flow during the day, and then analyze the gas composition once with the accurately calculated flow rates. This method eliminated the degree of uncertainty and provided a more accurate and precise measurement of hydrogen production. After reviewing the data, a revised curve for bottle C was constructed to estimate the hydrogen production values based upon the rates obtained during daytime measurements (Figure 4).

From Figure 4 it is evident that at a light intensity of 100 µmole m^{-2} s^{-1}, an initial nitrate concentration of 1.0 mM is optimal for hydrogen production. After 500 hours, culture B (1.0 mM nitrate) produced 395 mL of hydrogen, followed by cultures A and D, which produced 298 and 165 mL of hydrogen, respectively. Although hydrogen production should be normalized to ash free dry weight (biomass), the only time an AFDW measurement was taken was at the end of the experiment (Table 2). The cultures had undergone considerable lysis and decomposition by this point, so hydrogen production rates normalized to biomass were not

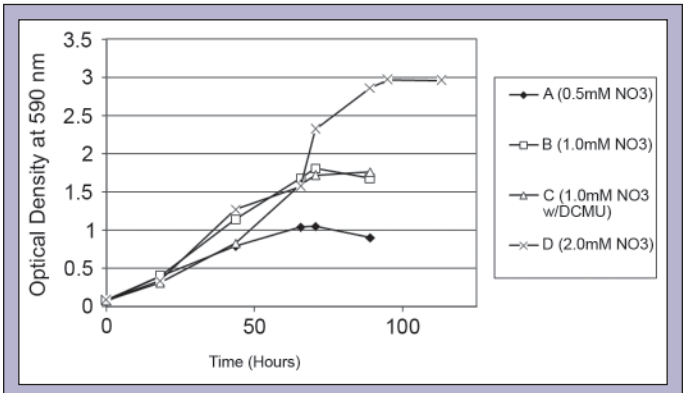

Figure 3. Optical Density measured at 590 nm as a function of time in cultures with varying initial nitrate concentrations.

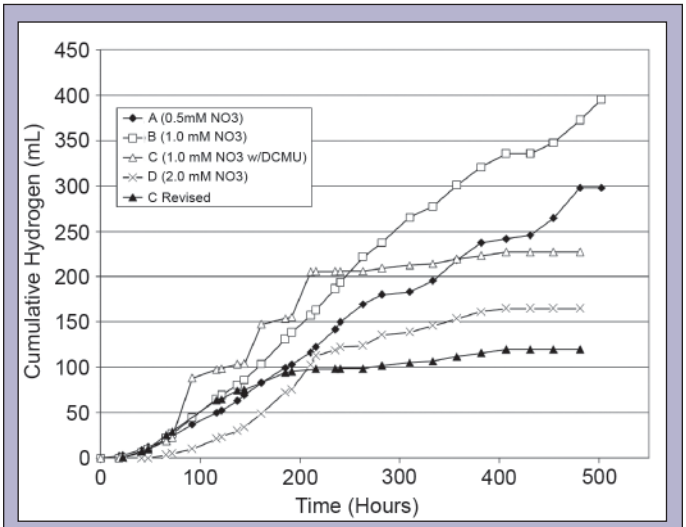

Figure 4. Cumulative hydrogen production in cultures with varying initial nitrate concentrations as a function of time.

Culture	AFDW (mg/L)
A (0.5 mM NO_3)	145
B (1.0 mM NO_3)	240
C (1.0 mM NO_3 w/DCMU)	30
D (2.0 mM NO_3)	165

Table 2. AFDW measurements taken at the end of the experiment.

calculated. Cumulative hydrogen production should therefore be looked upon only in a comparative manner.

It is also apparent from Figure 4 that DCMU has a latent effect of severely decreasing hydrogen production. Once glycogen energy stores are used up by all the cells, the cells begin to deteriorate and DCMU inhibits new culture growth within the medium. This means that as time progresses, the DCMU culture continues to deteriorate and produces very little hydrogen. Before this occurs however, hydrogen production rates are very similar in the culture with the same initial nitrate concentration not inhibited by DCMU. Another important observation was a cyclic pattern of hydrogen production in bottle A after approximately 250 hours. Culture A, at an initial nitrate concentration of 0.5 mM, the most nitrogen deficient, shows alternating periods of fast and slow hydrogen production. It is possible these cycles indicate a synchronized culture, where periods of photosynthesis for glycogen synthesis alternate with nitrogenase based hydrogen production.

Effects of Light Intensity and Inhibition of Photosystem II by DCMU

In a second experiment, the effects of light intensity and DCMU upon hydrogen production by *Plectonema boryanum* were observed. Four cultures were grown in Roux bottles at an initial nitrate concentration of 1.0 mM with the following light intensities: Bottle A = 50 µmole m^{-2} s^{-1}, Bottle B = 100 µmole m^{-2} s^{-1}, Bottle C = 100 µmole m^{-2} s^{-1}, and Bottle D = 200 µmole m^{-2} s^{-1}. When nitrate concentrations approached zero in culture C, 10 µmol of DCMU was added to inhibit photosynthetic oxygen production.

As seen in Figure 5, after approximately 48 hours, all nitrate had been removed from the media in bottles B (100 µmole m^{-2} s^{-1}), C (100 µmole m^{-2} s^{-1} w/DCMU), and D (200 µmole m^{-2} s^{-1}). It was not until 70 hours that all nitrate had been removed from the medium in bottle A (50 µmole m^{-2} s^{-1}). Even when all nitrate was removed from the media, the cultures continued to grow for a short time (Figure 6). Bottle D (200 µmole m^{-2} s^{-1}) reached a stationary growth phase at 67 hours, followed by bottles B (100 µmole m^{-2} s^{-1}) and C (100 µmole m^{-2} s^{-1} w/DCMU), reaching stationary phases at 86 hours. At this point DCMU was added to bottle C. As seen from figures 6 and 7, bottle A (50 µmole $m^{-}2$ s^{-1}) never reached a stationary phase before entering hydrogen production.

As shown in Figure 7, cumulative hydrogen production increased with increasing light intensity. After approximately 470 hours, bottle D (200 µmole m^{-2} s^{-1}) produced 589 mL of hydrogen, the highest cumulative amount. Bottle B (100 µmole m^{-2} $s^{-}1$) produced 491 mL of hydrogen by 470 hours, followed by bottle A (50 µmole m^{-2} s^{-1}), which produced 271 mL of hydrogen. When hydrogen production values were normalized to biomass, bottle B (100 µmole m^{-2} s^{-1}) had the greatest rate of hydrogen production. All of the hydrogen production rates were calculated based upon a line of best fit for the cumulative hydrogen production curves as a function of time and were standardized to biomass concentration based upon the AFDW taken at the onset of hydrogen production.

As shown in Figure 8, the cumulative volume of hydrogen produced was also plotted as a function of the total number of photons absorbed. In this regard, bottle A (50 µmole m^{-2} s^{-1}) had the greatest rate of hydrogen production in terms of photon absorption, followed by bottles B (100 µmole m^{-2} s^{-1}), D (200 µmole m^{-2} s^{-1}), and C (100 µmole m^{-2} s^{-1} w/DCMU), respectively.

Light measurements taken periodically throughout the experiment revealed an overall decrease in photon absorption as the experiment progressed (Figure 9). The major contributor to this decrease was the considerable cell lysis and decomposition that occurred. At the end of the experiment, when chlorophyll

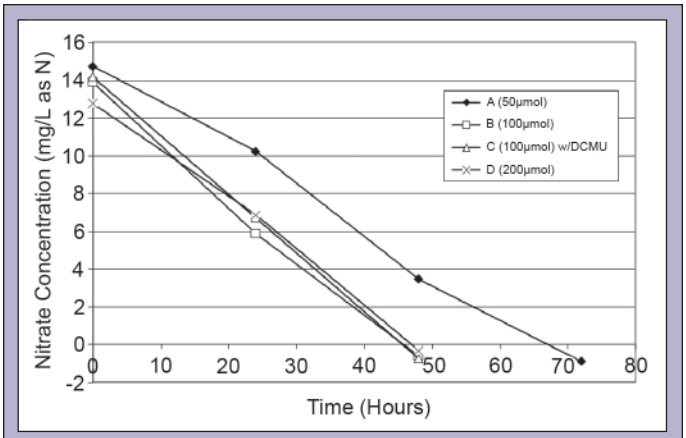

Figure 5. Nitrate concentration as a function of time in cultures grown at varying light intensities.

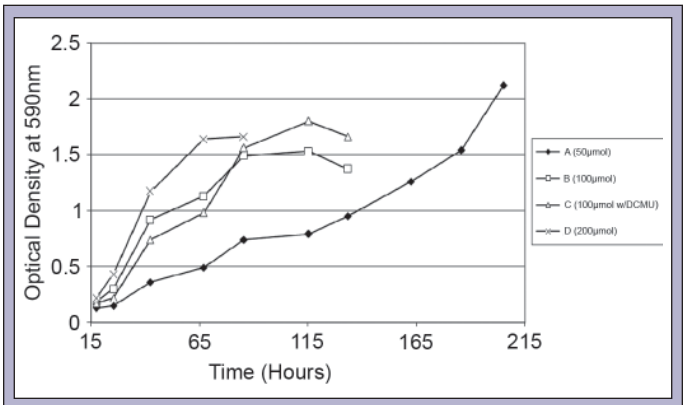

Figure 6. Optical Density at 590 nm as a function of time in cultures grown at varying light intensities.

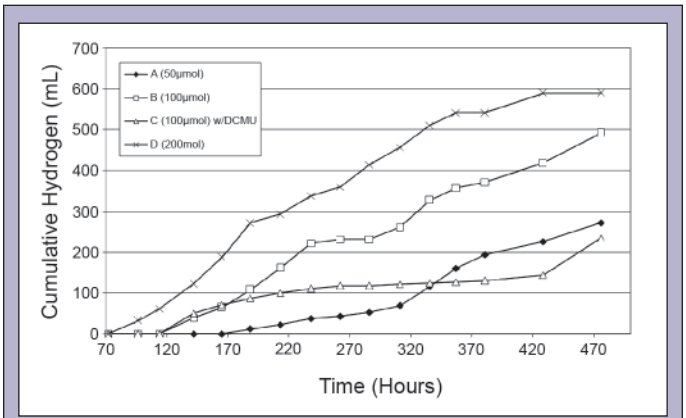

Figure 7. Cumulative hydrogen production in cultures at varying light intensities as a function of time.

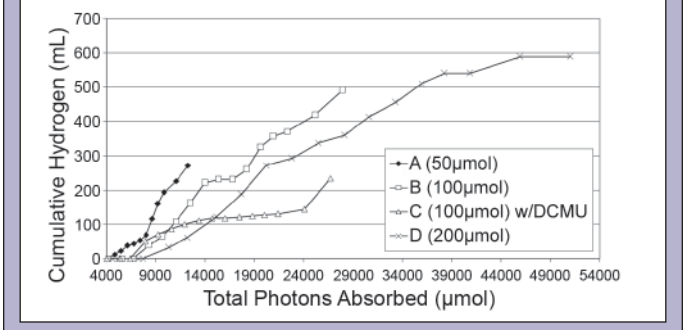

Figure 8. Hydrogen production as a function of total photons absorbed in cultures at varying light intensities.

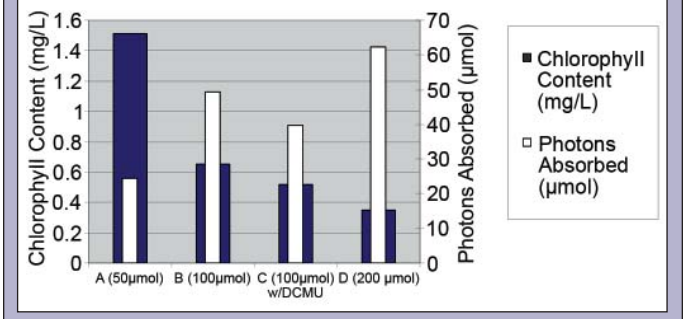

Figure 10. Chlorophyll concentration and photons absorbed at varying light intensities.

concentration was measured, a direct correlation could be seen between the light intensity and the chlorophyll concentration (Figure 10). At higher light intensities, the chlorophyll content was lowest. At low light intensities, the chlorophyll content was highest. From an efficiency perspective, it would make sense that chlorophyll content would decrease as light intensity increases. If large numbers of photons are being projected, less chlorophyll is needed to effectively utilize the light. At low light intensities however, more chlorophyll is synthesized to capture light and adequately drive photosynthesis. Although culture A has the highest chlorophyll content, the number of photons absorbed is the lowest because it is only being grown at a light intensity of 50 µmole m^{-2} s^{-1} (Figure 10). Bottle D however, with the lowest chlorophyll content, is still absorbing the greatest number of photons, for it is being illuminated with a light intensity of 200 µmole m^{-2} s^{-1}. These observations also relate back to hydrogen production, which shows that the larger number of photons a culture absorbs, the higher their cumulative hydrogen production, the exception being cultures inhibited by DCMU (Figure 8).

It is evident in this experiment, just as in the previous one, that DCMU has a severe effect on decreasing hydrogen production as time progresses. Bottles B (100 µmole m^{-2} s^{-1}) and C (100 µmole m^{-2} s^{-1} w/DCMU) showed similar rates of hydrogen production until approximately 190 hours, at which point bottle C (100 µmole m^{-2} s^{-1}) with DCMU showed dramatic decreases in hydrogen production. Because DCMU inhibits photosystem II and thus prevents any further accumulation of glycogen within the cell, once glycogen stores are depleted in the DCMU culture, there is no source of electrons remaining for hydrogen production. Cultures not inhibited by DCMU can continue photosynthesis and accumulate glycogen as necessary. This is why bottle B (100 µmole m^{-2} s^{-1}), not inhibited by DCMU, continued to produce hydrogen at the initial rate. After 240 hours however, bottle B (100 µmole m^{-2} s^{-1}) showed the cyclic pattern of hydrogen production that was also seen in the previous experiment.

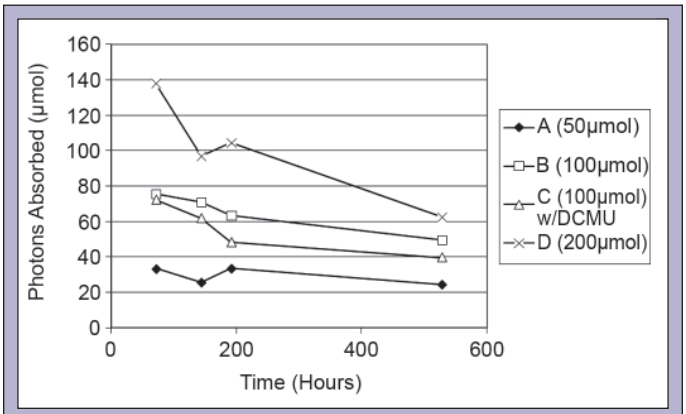

Figure 9. Photons absorbed by culture as a function of time.

Glycogen content (measured as glucose) was measured for all cultures at the onset of hydrogen production and at the end of the experiment. Results revealed an overall decrease in glucose concentration for all cultures during this time period, especially in culture C inhibited by DCMU (Figure 11). When glucose concentrations were normalized to biomass concentrations, it appears that the glycogen content of the cells also decreases. During the initial growth phase, cells accumulate stores of glycogen. When nutrients are depleted within the medium, the cells then use glycogen to produce hydrogen. Cultures not inhibited by DCMU can continue to accumulate glycogen via photosynthesis and carbon fixation, but cultures inhibited by DCMU can no longer synthesize any glycogen stores. This may be why culture C, inhibited by DCMU, decreased in glycogen fraction by 20 percent (Figure 12). Both cultures A and B, not inhibited by DCMU, showed a glycogen fraction decrease of only 10%. Culture D, which produced the largest cumulative amount of hydrogen, appeared not to change its glycogen fraction. An important observation to note is that at the point this final glycogen measurement was taken, bottle D is possibly entering a cyclic phase of hydrogen production. The cyclic patterns of hydrogen production observed suggest the entire culture is entering a growth phase, which is then followed by a hydrogen production phase. The cycle continues as cells accumulate glycogen for a given period of time and then enter a hydrogen production phase until glycogen stores are depleted. No hydrogen production was observed in bottle D the day the glycogen sample was taken, so it is possible bottle D was entering a growth phase. Future experiments could attempt to monitor glycogen concentration during these cyclic phases of hydrogen production.

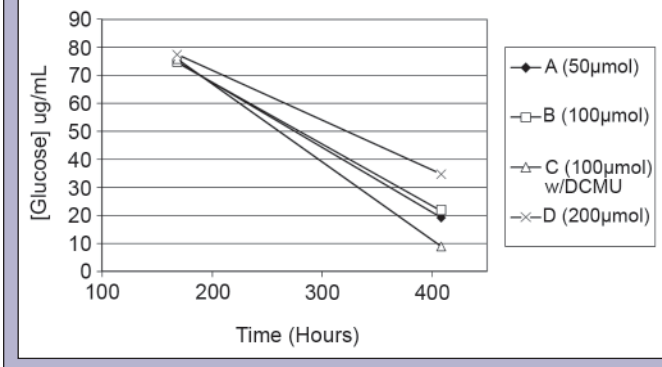

Figure 11. Glucose Concentration as a function of time in cultures grown at varying light intensities.

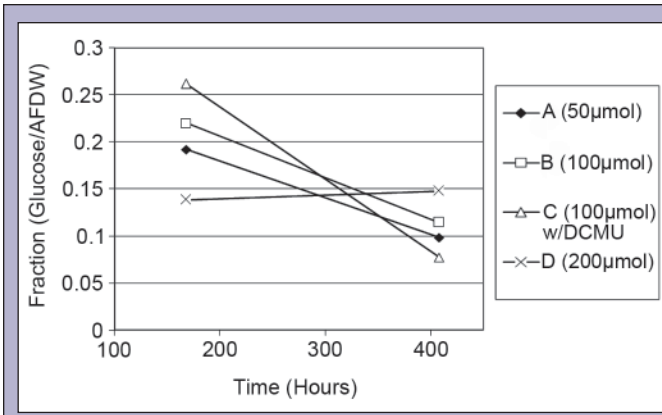

Figure 12. Fraction of Glucose/AFDW as a function of time in cultures grown at varying light intensities.

Effects of High Light Intensity and Inhibition of Photosystem II by DCMU

In a final experiment, the effects of high light intensity and DCMU upon hydrogen production by *Plectonema boryanum* were observed. Four cultures were grown at an initial nitrate concentration of 2.0 mM and were grown at two different light intensities. Cultures A and B were grown at a light intensity of 200 µmole m^{-2} s^{-1}, while cultures C and D were grown at a light intensity of 400 µmole m^{-2} s^{-1}. When nitrate concentrations approached zero in cultures B and D, 10 µmol of DCMU was added to inhibit photosynthetic oxygen production.

As seen in Figure 13, the nitrate concentration approached zero in all of the cultures after approximately 68 hours of growth. All of the cultures continued to grow for another 48 hours until the stationary phase was reached, as shown in Figure 14. Based upon the nitrate concentration and optical density curves, increasing the light intensity above 200 µmole m^{-2} s^{-1} does not increase the growth rate of the cultures at initial nitrate concentration of 2 mM.

As seen in Figure 15, cumulative hydrogen production was greatest in culture A, which was grown at light intensity of 200 µmole m^{-2} s^{-1} and not inhibited by DCMU. While culture A produced over a total of 300 mL of hydrogen, all other cultures failed to produce more than 110 mL of hydrogen. It can be concluded that excessive light intensity decreases cumulative hydrogen production, possibly by photoinhibition. This is confirmed by hydrogen production rates normalized to biomass. (These rates were calculated based upon a line of best fit for cumulative hydrogen production as a function of time and the AFDW taken at the onset of hydrogen production). Both cultures grown at a light intensity of 200 µmole m^{-2} s^{-1} had higher rates of hydrogen production than any of the cultures grown at a light intensity of 400 µmole m^{-2} s^{-1}.

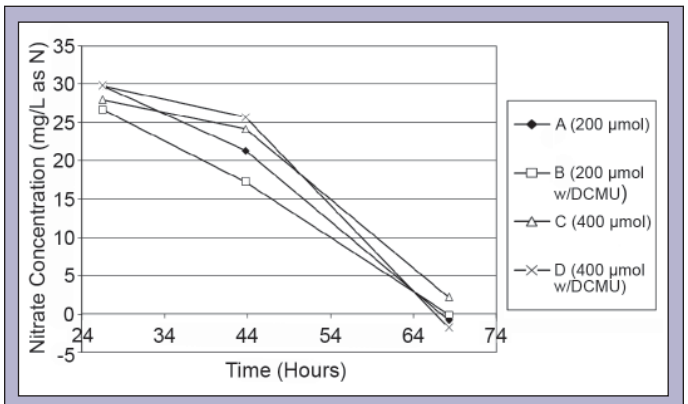

Figure 13. Nitrate Concentration as a function of time with cultures grown at two different light intensities.

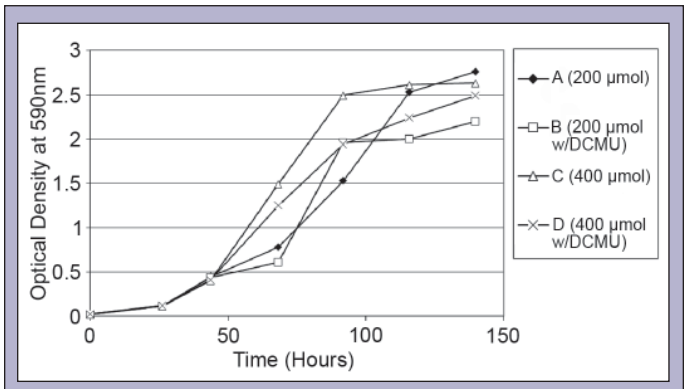

Figure 14. Optical Density at 590 nm as a function of time with cultures grown at two different light intensities, with and without DCMU, respectively.

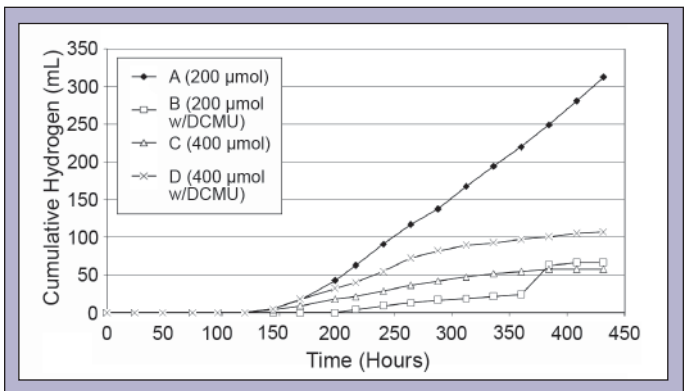

Figure 15. Cumulative Hydrogen Production as a function of time in cultures grown at two different light intensities, with and without DCMU, respectively.

As seen in Figure 16, glycogen concentration decreased in all of the cultures as a function of time. It is also apparent that the final glycogen concentration very closely correlates to cumulative hydrogen production. Culture A, which produced the largest amount of cumulative hydrogen, ended with the highest glucose concentration. Culture D, which produced the second largest amount of cumulative hydrogen, had the second highest final glucose concentration. Cultures B and C, which were very close in producing the lowest amount of cumulative hydrogen, ended with the lowest glucose concentrations.

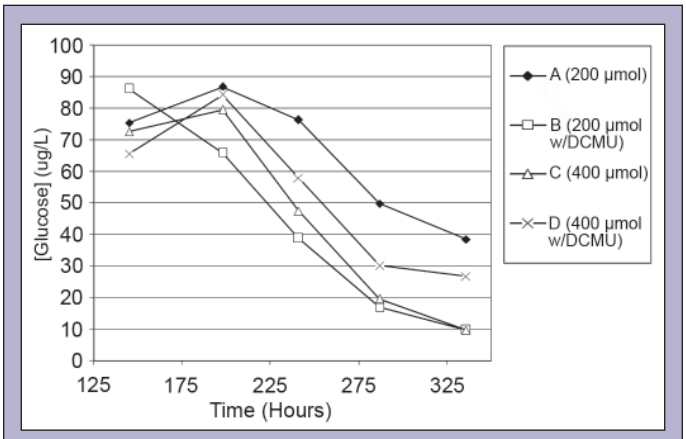

Figure 16. Glycogen measured as glucose as a function of time in cultures grown at two different light intensities, with and without DCMU, respectively.

Conclusion

After testing the effects of initial nitrate concentration, light intensity, and photosystem II inhibitor DCMU upon hydrogen production by *Plectonema boryanum*, it can be concluded that *Plectonema boryanum* is a suitable candidate for future studies on renewable hydrogen production. In the first experiment, where the effects of initial nitrate concentration were tested, results showed cumulative hydrogen production was maximum at an initial nitrate concentration of 1 mM. With this information, a second experiment was conducted at 1 mM initial nitrate concentration to test the effects of light intensity at 50, 100, and 200 µmole m^{-2} s^{-1}. Cumulative hydrogen production was shown to increase with increasing light intensity. A final experiment was then performed at high light intensity with an initial nitrate concentration of 2 mM. Excessive light at 400 µmole m^{-2} s^{-1} decreased cumulative hydrogen production, possibly because of photoinhibition, thus optimal cumulative hydrogen production occurred at an initial nitrate concentration of 1 mM and a light intensity of 200 µmole m^{-2} s^{-1}. Hydrogen production rates, normalized to biomass, were shown to be optimal at an initial nitrate concentration of 1 mM with a light intensity of 100 µmole m^2 s^{-1}. When compared to values reported in literature [12], hydrogen production rates by *Plectonema boryanum* were measured to be at most 0.12 µmole H_2/hr/mg without reducing substances. In this experiment however, *Plectonema boryanum* was shown to produce 0.16 µmole H_2/hr/mg without reducing substances. When reducing substances are added, hydrogen production rates can be increased by up to six times [12]. If other variables such as temperature, pH, and salinity were optimized, hydrogen production by *Plectonema boryanum* could prove to be a viable source of renewable hydrogen. Results from all DCMU challenged experiments support the idea that glycogen is the source of electrons for hydrogen production within *Plectonema boryanum*.

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References

[1] Intergovernmental Panel on Climate Change (IPCC): 2001a, *Third Assessment Report – Climate Change 2001: The Scientific Basis, A Report of Working Group I – Summary for Policymakers*, www.ippcc.ch.

[2] McHugh, Kathleen, "Hydrogen Production Methods", *MPR Associates*, 2005. http://www.mpr.com/pubs/hydroprod.pdf

[3] Prince, R.C., and H.S. Kheshgi, "The Photobiological Production of Hydrogen: Potential Efficiency and Effectiveness as a Renewable Fuel", *Critical Reviews in Microbiology*, **31(1)**:19–31, 2005.

[4] Melis A., and T. Happe, "Hydrogen Production: Green Algae as a Source of Energy", *Plant Physiology*, **127**:740–748, 2001

[5] Hallenbeck, P.C., and J.R. Benemann, "Biological Hydrogen Production: Fundamentals and Limiting Processes", *International Journal of Hydrogen Energy*, **27**:1185–1193, 2002.

[6] Gfeller, R., and M. Gibbs, "Fermentative Metabolism of *Chlamydomonas reinhardtii*", *Plant Physiology*, **7(75)**:212–218, 1984.

[7] Madamwar, D., N. Garg, and V. Shah, "Cyanobacterial hydrogen production", *World Journal of Microbiology and Biotechnology*, **16(1)**:757–767, 2000.

[8] Dutta, D., D. De, S. Chaudhuri, and S.K. Bhattacharya, "Hydrogen Production by Cyanobacteria", *Microbial Cell Factories*, **4**:36, 2005.

[9] Rao, K., and D. Hall, “Hydrogen production by cyanobacteria: Potential, problems and prospects”, *Journal of Marine Biotechnology*, **4(1)**: 10–15, 1995.

[10] Weare, N.M., and J.R. Benemann, “Nitrogenase Activity and Photosynthesis in Plectonema boryanum”, *Journal of Bacteriology*, **119(1)**:258–265, 1974.

[11] Tett, P., M.G. Kelly, and G.M. Hornberger. “A Method for the Spectrophotometric Measurement of Chlorophyll a and Pheophytin a in Benthic Microalgae”, *Limnology and Oceanography*, **20(5)**:887–896, 1975.

[12] Kashyak, A.K., K.D. Pandey, and S. Sarkar, “Enhanced Hydrogen Photoproduction by Non-Heterocystous Cyanobacterium Plectonema Boryanum”, *International Journal of Hydrogen Energy*, **21(2)**:107–109, 1996.