Hydrogen Photo-Production by Mixotrophic Cultivation of *Chlamydomonas Reinhardtii*: Interaction between Organic Carbon and Nitrogen

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Hydrogen photo-production by a wild type and two engineered strains of *Chlamydomonas reinhardtii* was investigated. Growth rate values and hydrogen yields attained as the concentration of acetate and nitrogen vary were compared. In the analysis of microalgal growth, the interaction between organic carbon (acetate) and nitrogen (nitrate) was investigated by recourse to an experimental factorial design. This analysis evidenced the existence of a statistically significant interaction between organic carbon and nitrate. Hydrogen production was attained by cultivating microalgae previously grown in mixotrophic regime with sulphur deprived medium. The influence of varying the photobioreactor headspace on hydrogen production was investigated. This analysis revealed an increase in the hydrogen produced per unit volume of culture of about one order of magnitude when the headspace volume is modified from 100 to 350 mL. This result provides valuable indications on how to design and operate photobioreactors for hydrogen production optimization and was thoroughly discussed in terms of the metabolic pathways activated by sulphur depletion.

1. Introduction

Hydrogen represents a clean form of energy because it does not produce green house emissions. Moreover, it configures a competitive energy vector because characterized by an energy content per weight unit larger than any other known fuel. These characteristics have motivated over the past decade an increasing interest towards the development of efficient technologies for hydrogen production. The largest fraction of commercially available hydrogen is however produced by industrial processes employing fossil fuels as, for example, steam reforming of natural gas and gasification of coal. These processes are energy intensive and invariably cause the formation of green-house gas emissions.

A promising technological alternative to the large scale production of hydrogen is represented by biological processes. These allow for the conversion of renewable resources to hydrogen and are controlled by photosynthetic and/or fermentative routes. Examples of biological processes aimed at hydrogen production include the bio-photolysis of water by unicellular green algae and cyanobacteria, photodecomposition of organic compounds by photosynthetic bacteria and fermentation of organic compounds. The photosynthetic production of hydrogen by microalgae represents in this framework an attractive technological alternative because based on the application of largely available and inexpensive resources: sunlight, water and carbon dioxide.

The approach originally implemented to perform hydrogen photo-production was to impose anaerobic conditions by either purging the photobioreactor with inert gases or incubation in dark, and then expositing the culture to light. This could however ensure low hydrogen productivity during a short period of time. Larger productivity values were in contrast attained during numerous days by a procedure involving a preliminary growth phase followed by the suspension of microalgae in sulphur depleted medium. The depletion of sulphur decreases the evolution of oxygen making the anaerobic pathways becoming active. Sulphur deprivation is in particular evidenced to cause the partial and reversible inhibition of photo-system

II (PSII) water-oxidation activity in algae (Wykoff et al., 1998) resulting in culture transition from an aerobic to an anaerobic state (Melis et al., 2000; Ghirardi et al., 2000; Kosourov et al., 2002).

The establishment of anaerobiosis induces the expression of a reversible hydrogenase, an enzyme mediating the photo-production of hydrogen (Kosourov et al., 2002). This enzyme redirects the flow of electrons generated through the photosynthetic electron-transport chain from carbon fixation towards proton reduction. Consequently, sulphur deprived microalgae can produce hydrogen during numerous days (Melis et al., 2000; Ghirardi et al., 2000). Following transfer of the microalgae to a sulphur depleted medium, progress through the following five phases is observed: aerobic, oxygen consumption, anaerobic, hydrogen production, and termination phases (Kosourov et al., 2002).

Several strategies were proposed to enhance the production of hydrogen including the optimization of pH and light regime, the addition of sulphur traces back to the re-suspended culture, cells synchronization and optimization of medium composition. In this framework, the possibility to enhance hydrogen production by addition of organic carbon was investigated. Experiments reported for *Chlamydomonas r.* have, for example, evidenced the need of acetate to ensure the achievement of satisfactory hydrogen productivity values. The addition of acetate can stimulate photorespiration and thus help to maintain anoxia needed to activate the hydrogenase. Moreover, the presence of acetate can increase growth rate leading to the accumulation of carbohydrates whose successive degradation can contribute to the formation of hydrogen. The addition of acetate increases however the cost of the produced hydrogen and can thus compromise economic sustainability. Moreover, it can allow for the rapid development of heterotrophic bacteria competing with microalgae for nutrients. The employed amount of acetate must therefore be minimized. The achievement of this latter objective and of the production of satisfactory amount of hydrogen imposes to thoroughly analyze the influence of acetate on microalgae growth rate and hydrogen production. The interaction of organic carbon with other nutrients must in particular be analyzed (Pagnanelli et al., 2013).

Despite the promising preliminary results attained in this area, hydrogen photo-production appears still far from being optimized. The identification of an optimal strain, the influence of the cultivation parameters, the formulation of an effective control strategy are some fundamental issues to be thoroughly investigated. In this contribution, hydrogen photo-production by three different strains of *Chlamydomonas r.* is investigated. Growth rate values and hydrogen yields attained under autotrophic and mixotrophic conditions are compared. In the analysis of growth, the interaction between organic carbon (acetate) and nitrogen (nitrate) is investigated by recourse to an experimental factorial design. Statistical analysis of the collected data demonstrates the existence of a significant interaction between organic carbon and nitrate. Hydrogen production in sulphur depleted medium is moreover analyzed as the acetate concentration and the headspace volume of the employed photobioreactor are varied. The illustrated results provide valuable indications on how to design and operate photobioreactors for hydrogen production maximization.

2. Materials and methods

2.1 Strains and medium
A wild type (CC-125) and two genetically modified strains (CC-4169, CC-4170) of the microalga *Chlamydomonas r.* were used in the study. The strains were purchased by Chlamydomonas Resource Center at University of Minnesota (http://chlamycollection.org/). The two genetically modified strains differ from the wild-type one because they are characterized by a truncated light-harvesting chlorophyll antenna enhancing the efficiency of solar energy conversion.

Stock cultures of *C. reinhardtii* were grown on a standard Tris–acetate–phosphate (TAP) medium (Harris, 1989) in 300 mL Erlenmeyer flasks at room temperature under cool-white fluorescent light (200 μE m^{-2} s^{-1}).

2.2 Growth conditions
The cells were grown under batch conditions at room temperature in 300 mL Erlenmeyer flasks. Experimental runs were performed with magnetic agitation, under constant illumination (200 μmol·m^{-2}·s^{-1}). The air, and consequently the carbon dioxide contained in it, was fed continuously to the cultures by pumps after passing through traps for particulate and humidity. The initial concentrations of nitrate C_{N0} and of acetate C_{A0} were varied according to a 2^2 experimental design. Four experimental tests, denoted as *1*, *n*, *a* and *na* were developed starting from two levels of C_{N0} and of acetate C_{A0} (Table 1). Two independent runs were performed for any test by using a culture adapted under mixotrophic conditions and cultivated with the standard TAP medium. The culture obtained from each run was used as inoculum to start a second run. For the second run, the inoculum to medium ratio was selected to ensure that the initial cell concentration was equal to that found for the first run.

Table 1. Initial acetate and nitrate concentrations adopted in experiments

	$C_{A0} = 10$ mM	$C_{A0} = 20$ mM
$C_{N0} = 3$ mM	n	na
$C_{N0} = 2$ mM	1	a

The evolution of the cell concentration during two successive generations of microalgae was monitored for each test in duplicate. For any set of initial concentration, no statistical significant difference was found between the evolutions of cell concentration recorded during two successive generations. These evolutions were therefore considered as replicates. Overall, four replicates were available for each treatment in Table 1.

2.3 Sulphur deprivation strategy

After reaching the exponential growth phase, microalgae were separated from the medium by centrifugation (2000 rpm) and rinsed three times with distilled water. The microalgae were then suspended in a sulphur depleted medium. This latter medium was identical to the TAP medium employed during the preliminary growth stage with the exception that it was deprived of compounds containing sulphur. The microalgae suspended in the sulphur depleted medium were transferred to a round flask with volume equal to about 600 mL. Following purging with nitrogen, the flask was sealed to allow for the achievement of anaerobic conditions. The flasks were exposed to light (200 μmol·m^{-2}·s^{-1}) under magnetic agitation during the following five days.

Two different approaches were explored to analyze hydrogen production under sulphur depleted conditions. A first series of experimental runs was performed by transferring 250 mL of the sulphur depleted culture to the 600 mL flask. After sealing the flask, the head volume available to the gas phase was therefore about 350 mL. 500 mL of the sulphur depleted culture were employed in second series of experimental runs leading to a head volume within the flask equal to about 100 mL.

2.4 Cell counting

The cell concentration was determined by direct counting (Leitz, Laborlux 12 optical microscope) of the number of cells contained in the culture volume collected in a 10^{-4} mL Thoma chamber.

2.5 Analysis of gas composition

The composition of the gas phase generated during the period of cultivation in sulphur depleted medium was determined by a gas chromatograph (model 3400, Varian) using a thermal conductivity detector and a 5Å molecular sieve column. The temperatures of injector, detector and column were 260, 236, and 280 °C, respectively. Helium gas was used as carrier gas at a flow rate of 18 mL min^{-1}.

3. Results

3.1 Influence of acetate and nitrate concentration on microalgal growth

The influence of nitrate and acetate on microalgal growth is analyzed in Fig.1. For any considered strain, the temporal evolution of cell concentration observed as initial nitrate and acetate concentration C_{N0} and C_{A0} vary are displayed in Fig.1(a),(c),(d). The effect of these two latter factors and their interaction computed by analysis of variance are reported in Fig.1(b),(d),(f). The effect of a factor is defined as the variation of the dependent variable (cell concentration) determined by changing the factor from the low to the high level. The estimate of the effects for each day was compared with the 95% confidence interval due to random errors determined from the six replicates: effects exceeding such intervals are significant.

Negative effect of nitrate is found for any strain. Increasing C_{N0} from 2 to 3 mM causes a reduction in the growth rate and in the attained maximum cell concentration at any considered C_{A0} value. Displayed results show in contrast the possibility to increase both growth rate and maximum cell concentration by increasing C_{A0}. It is in this framework important to note that negative interaction between nitrate and acetate is observed for any strain even though it results statistically significant only for CC-4169. This indicates the need to tightly control the nitrate to acetate ratio in order to maximize biomass productivity, or minimize the amount of acetate needed to attain a prescribed productivity value.
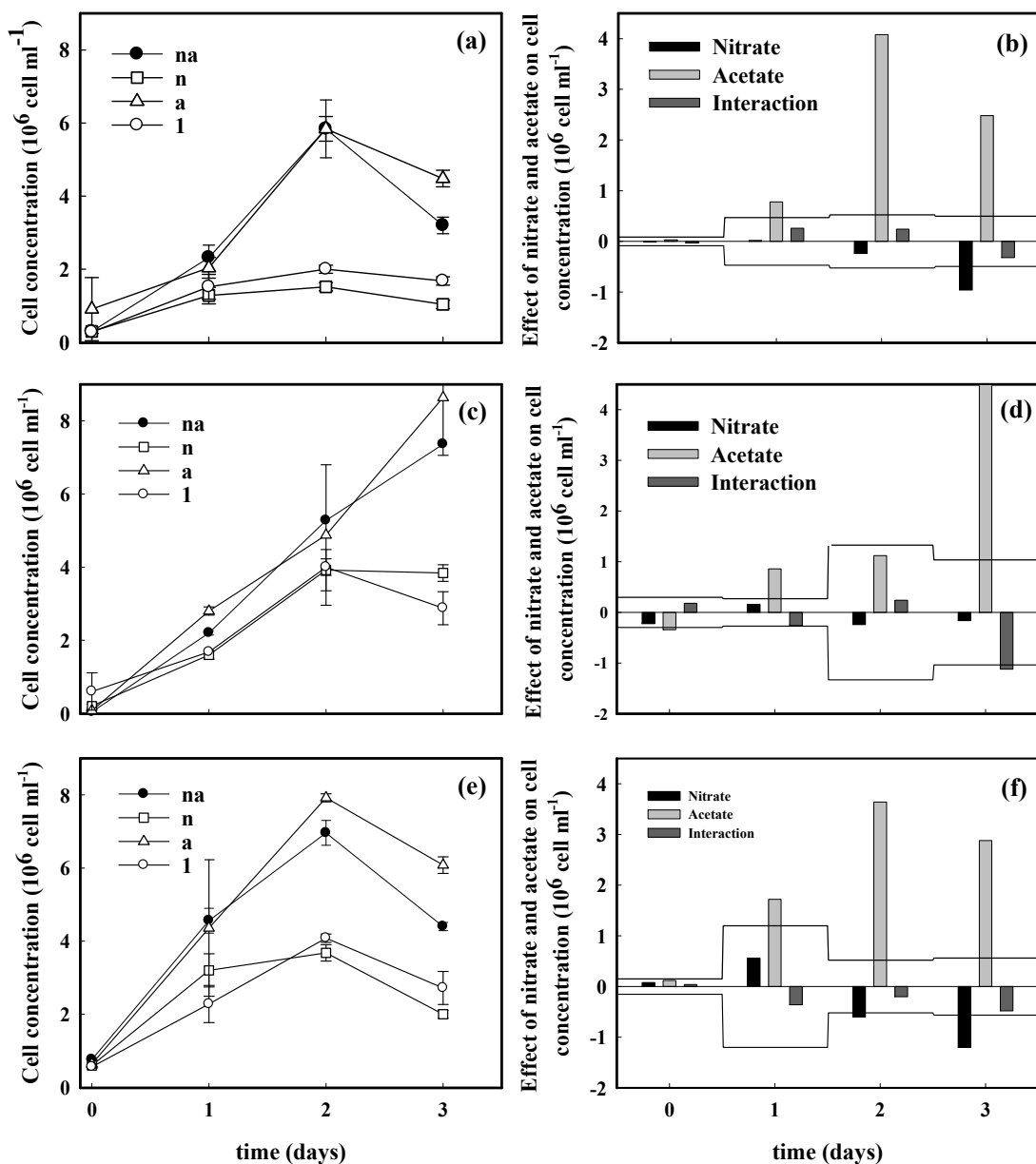
Figure 1. Influence of acetate and nitrate on microalgal growth. Temporal evolutions of the cell concentration and results of the analysis of variance are reported on the left and right respectively. (a),(b) CC-125; (c),(d) CC-4169; (e),(f) CC-4170.

3.2 Analysis of the hydrogen photo-production

Initial acetate and nitrate concentrations corresponding to treatment *a* ensured for any strain the largest growth rate and cell concentration values (Fig.1). Since larger cell concentration values are in general found to increase the amount of produced hydrogen, a preliminary analysis of hydrogen photo-production was performed by transferring to the sulphur depleted medium microalgal cultures previously grown with C_{N0} and C_{A0} values corresponding to treatment a.

The amount of hydrogen generated with this strategy was compared to that obtained from cultures previously grown in the standard TAP medium (corresponding to treatment 1). For both treatments 1 and a, the effect of varying the fraction of the photobioreactor volume during the sulphur depletion stage was also analyzed. Two series of experiments were in particular performed by transferring to a 600 mL round flask 250 and 500 mL of culture (corresponding to 350 and 100 mL of headspace respectively). Results of this analysis are displayed in Fig.2.
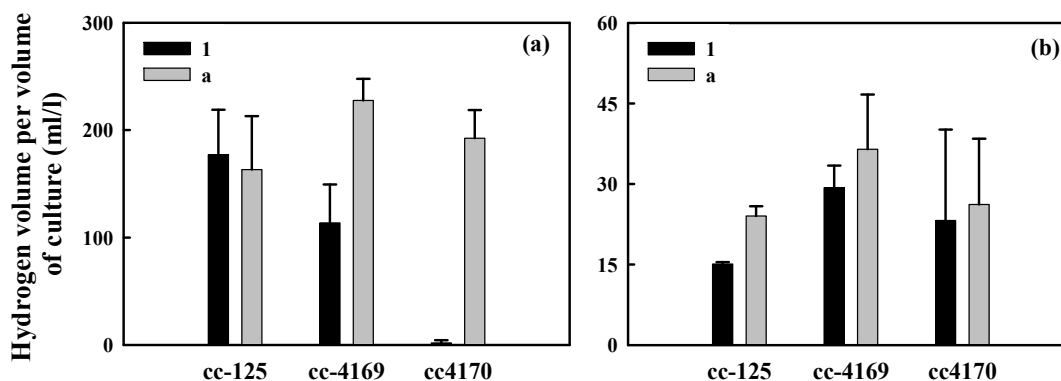
Figure 2. Hydrogen photo-production from microalgae grown with acetate and nitrate concentrations corresponding to treatments 1 and a; (a) culture volume equal to 250 mL (corresponding to headspace volume equal to 350 mL); (b) culture volume equal to 500 mL (corresponding to headspace volume equal to 100 mL). Reported volumes refer to standard temperature and pressure conditions.

With 250 mL culture volume and strain CC-125, no statistically significant difference was found between hydrogen produced per volume of culture attained with treatments *1* and *a*. With identical culture volume, statistically significant larger hydrogen production per volume of culture was in contrast determined and for the strain CC-4169 with treatment *a*. The strain CC-4170 also exhibited with 250 mL culture and treatment *a* larger hydrogen production. In this latter case, hydrogen was however almost absent in the gas phase for treatment *1*. With culture volume equal to 500 mL, treatment *a* was found for any strain to generate larger average hydrogen production per volume of culture. Only for strain CC-4170, the difference between the values attained with the two considered treatments was not statistically significant.

The most remarkable result displayed in Fig.2 is the considerable increase (of about one order of magnitude) in the amount of hydrogen generated per volume of culture when the headspace of the 600 mL photobioreactor employed in sulphur depletion stage is modified from 100 to 350 mL (corresponding to 500 and 250 mL of culture).

The analysis of this result imposes a more detailed description of the cell metabolic pathways activated by sulphur depletion and regulating the reduction of protons to hydrogen.

The lack of sulphur causes the deactivation of the photo-system II and thus partially inhibits the oxidative water activity. This determines in turn a drop in cellular oxygen level and ensures the achievement of anaerobic conditions. In these conditions, the oxidative phosphorylation and associated reactions (ATP formation, NADH oxidation) are inhibited. Alternatively to oxidative phosphorylation fermentation of endogenous carbon can determine the generation of ATP, the oxidation of NADH and hydrogen production by proton reduction. The reduction of protons to hydrogen offers in particular the cell a fundamental valve to increase the production of ATP and balance the decrease in the level of the cofactor NAD^+. The formation of hydrogen can indeed determine a gradient in the proton concentration and thus produce ATP via ATP synthase.

The analysis of gas composition revealed that the only species present in the gas phase were hydrogen, nitrogen, carbon dioxide and negligible traces of methane (experimental results not reported here). No statistical significant difference was found between the total gas concentration values (determined by pressure measurement and application of gas state law equation) as the headspace volume was varied from 350 to 100 mL. Significantly different concentrations of carbon dioxide were in contrast detected as the headspace volume was varied. The concentration of carbon dioxide was, with 350 mL headspace volume, about ten times lower than that found with 100 mL headspace volume. For example, with the strain CC-125 and treatment *a*, the carbon dioxide changed from 2.13 to 0.23 mmol/L as the headspace volume was decreased from 350 to 100 mL. This result can be explained by the difference between culture and headspace volumes found in the two experiments. The initial gas phase oxygen concentration is independent of the headspace volume and equal to that found in air. As the headspace volume is increased, larger amounts of oxygen are consumed by lower culture volumes at the beginning of the sulphur depletion stage. Oxygen is converted to carbon dioxide by photorespiration. The fulfilment of equilibrium conditions and mass balance imposes that larger concentration of carbon dioxide is found in both liquid and gas phases in the experiment with larger headspace.

It is remarkable to note that previous studies have conjectured that an increase in the level of carbon dioxide can limit hydrogen production (Matthew et al., 2009). An increase in carbon dioxide concentration can indeed enhance the flow through the Calvin cycle and thus convert NADPH to NADP$^+$. This reduces the need of hydrogen formation whose main role is to work as an electron valve and to generate ATP by generation of a proton gradient. Such analysis is however in contrast with the results observed in the present study where larger hydrogen productions were found in the experiments where larger carbon dioxide concentrations were attained.

4. Conclusions

Hydrogen photo-production by a wild type and two engineered strains of *Chlamydomonas r.* was investigated. The influence of organic carbon (acetate) and nitrogen (nitrate) on microalgal growth was examined. The analysis evidenced a negative interaction between acetate and nitrate in determining microalgal growth pattern. This result indicates that the acetate to nitrate ratio must be tightly controlled to minimize the consumption of nutrients while ensuring satisfactory growth rate and maximum cell concentration values.

The cultivation of microalgae in sulphur depleted medium was conducted to induce the production of hydrogen. The dependence of hydrogen produced per volume of culture on the acetate concentration adopted in the preliminary growth phase was investigated. Moreover, the effect of changing the headspace volume of the photobioreactor employed during the sulphur depletion stage was analyzed. Hydrogen per volume of culture and carbon dioxide concentration increased with larger headspace volume. This result is in contrast with previous studies on hydrogen photo-production and may provide important indications for the development of novel operating strategies aimed at hydrogen production optimization. For this purpose, a more detailed analysis of the evolution of gas composition and metabolite concentration during sulphur depletion stage is needed.

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