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Research paper

Optimization of biomass and hydrogen production by *Anabaena* sp. (UTEX 1448) in nitrogen-deprived cultures

Sarah Regina Vargas*, Paulo Vagner dos Santos, Marcelo Zaiat, Maria do Carmo Calijuri

Department of Hydraulics and Sanitation, São Carlos School of Engineering, University of São Paulo, São Carlos, SP, Brazil

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A B S T R A C T

Hydrogen is a non-polluting source of energy, which is renewable and very abundant in the universe. Microalgae and cyanobacteria produce hydrogen by breaking down water and organic compounds. The aim of this research was to develop hydrogen production using cyanobacterium *Anabaena* sp. by nitrogen deprivation in two experimental phases of cultivation, optimizing its biomass from physicochemical variables. The experiment was carried out with axenic culture, in BG-11 medium, in triplicate, in two steps and under continuous illumination: in the first step, cultures were maintained in an aerobic condition until the first half of growth phase under nitrogen limitation; in the second step, the biomass was transferred, by centrifugation and cell wash, to anaerobic photobioreactors under nitrogen deprivation and the atmosphere was changed by argon for hydrogen production. Hydrogen was detected by gas chromatography and the hydrogen production parameters were tested using the Gompertz model and the volume by the general ideal gas equation. The optimization of biomass in the first step of cultivation increased its yield by 18.3% and heterocysts formation was 3.4 times higher in nitrogen privation conditions. Therefore, hydrogen production by cell increased 55.2% and the productivity 57.6% when compared to the culture of *Anabaena* sp. in control condition. The method and the strain were effective for hydrogen production and the pH, temperature and light intensity affected the improvement of this production and increased the biomass yield.

1. Introduction

The hydrogen production by microalgae and cyanobacteria is an alternative of production of lower cost because they use environmental resources [1,2]. It is also easy to cultivate and can be grown in closed systems enabling the capture of gas [3]. Three fundamental enzymes can be found in these microorganisms involved in hydrogen production and metabolism: bi-directional hydrogenase, membrane-bound uptake hydrogenases and nitrogenases. These enzymes can be active simultaneously, and there are alterations in their activities with small changes in growth conditions. There are also various well-known pathways and structural variations of these enzymes which are sensitive to oxygen [3–7]. Consequently, efforts in genetic engineering have been to improve the tolerance of these enzymes to oxygen and hence increase the production of biohydrogen [8].

Hydrogen production by bidirectional hydrogenase occurs associated with the electron transfer chain of photosynthesis and uses the electron flux from ferredoxin to promote reduction of H^+ to H_2 [9]. Bidirectional hydrogenase reduces to molecular hydrogen the protons from the breakage of water in photosynthesis and from organic compounds in fermentation [10,11]. However, both bidirectional hydrogenase and uptake hydrogenase can oxidize molecular hydrogen back to protons [7,12] decreasing the efficiency of H_2 production.

Nitrogenase is the enzyme involved in nitrogen fixation and may be present in bacteria and cyanobacteria [7,12]. In cyanobacteria nitrogenase can occur in vegetative cells (non-heterocytous species) or in differentiated cells called heterocysts in which nitrogen fixation develops in time and space other than photosynthesis and respiration [13–15]. *Anabaena* is a filamentous cyanobacteria in which nitrogen fixation take place in heterocysts.

Nitrogenase reduces atmospheric N_2 to ammonia (NH_3) using six electrons and two more are used to reduce H^+ to H_2. In anaerobic conditions and in nitrogen absence electrons carried by ferredoxin are transferred to nitrogenase which will reduce protons to hydrogen by means of an ATP dependent reaction ($2e^- + 2H^+ + 4ATP \rightarrow H_2 + 4\,ADP + Pi$) [7,16].

There are results of hydrogen production by cyanobacteria when they are grown under nitrogen deprivation and with argon in headspace, which provides a microaerobic environment. The lack of nitrogen in the environment reduces photosynthetic rates, as it is a

* Corresponding author.
E-mail address: sarahvargas@alumni.usp.br (S.R. Vargas).

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structural element of photosynthetic pigments, which helps to decrease levels of oxygen produced by the system, and its lack stimulates the formation of heterocysts and, consequently, nitrogenases, which will produce hydrogen through indirect biophotolysis [9,12,17,18]. The lack of nitrogen in the culture medium and in the atmosphere causes the electrons carried by the nitrogenase to be directed only to hydrogen formation from two electrons, thus nitrogen fixation and ammonia formation do not occur, and there is lower energy consumption [7].

However, in order for effective hydrogen production to take place, the cultivation of organisms should be carried out in two stages. In the first step, the algal culture remains until the first half of the growth phase in an aerobic condition, so that energy reserves (e.g. carbohydrates) and oxygen can be produced by photosynthesis. In the second phase (microaerobic or anaerobic), nitrogen deprivation reduces photosynthetic rates, which is effective for hydrogen production. In this stage, the algal cells use light energy to break down water molecules and carbohydrates and, consequently release hydrogen without interference of oxygen [6].

Researches has been developed using microorganisms to optimize biohydrogen production as an alternative fuel, such as techniques to improve their cultivation, by manipulating physicochemical variables in direct and indirect biophotolysis processes [19,20]. Cyanobacteria and microalgae grow quickly when adapted to the environment. Furthermore, when they have ideal conditions to grow in, they can double their density within hours, even in closed photobioreactors [21]. The growth improvement of these microorganisms benefits hydrogen production, as high biomass culture is needed so that efficient production can take place [6,9,22].

Some factors that change the growth of these organisms and can increase the yield of biomass and the production of hydrogen are pH, temperature, light intensity and source of organic carbon [6,20,23–25]. Glucose supplementation in cyanobacteria cultures lead to mixotrophic growth which contributes to higher biomass yields and growth rates [26]. Furthermore, uptake hydrogenases can be inhibited [19,27] and breakdown of organic carbon provides energy to hydrogen production [18,19].

Several strains of *Anabaena* sp. have the potential of hydrogen production according to Masukawa et al. [28] and Allahverdiyeva et al. [29]. Berberoglu et al. [30], Sveshnikov et al. [31], Vyas and Kumar [32], Tsygankov et al. [33] and Markov et al. [34] also demonstrated hydrogen production by strains of *A. variabilis* and Yeager et al. [18] by strains of *A. cylindrica*. According to these studies the best hydrogen production was obtained in anaerobic conditions, with nitrogen deprivation, with argon in the headspace and under light presence. Cyanobacteria have advantages concerning hydrogen production as they can carry out direct biophotolysis using bidirectional hydrogenase when in anoxic environments, as well as indirect biophotolysis from nitrogenases [4,7,27,35].

Therefore, the aim of this research was to improve the biomass yielding cultivation of *Anabaena* sp. (UTEX 1448) using physicochemical variables, as well as to verify the influence of this optimization on hydrogen production efficiency by this cyanobacteria since there are no studies with this strain of *Anabaena*.

2. Material and methods

2.1. Strain and inoculum maintenance

Anabaena sp. (UTEX 1448) was obtained from the Culture Collection of Algae at the University of Texas at Austin. It was maintained in an axenic culture, BG-11 medium [36], at pH 9.2, 24 °C, 12 h light/dark, light intensity of 4440 lux obtained with cool white fluorescent tubular lamp, and 1.05 kg m^{-3} of glucose.

Table 1
Conditions of the factorial design for the cultivation of *Anabaena* sp.

Temperature (°C)	pH	Light (lux)	Glucose Supplementation (kg m^{-3})	Condition
24	8.2	2220	1.05	1
			2.10	2
		6660	1.05	3
			2.10	4
	10.2	2220	1.05	5
			2.10	6
		6660	1.05	7
			2.10	8
28	9.2	4440	1.525	9
32	8.2	2220	1.05	10
			2.10	11
		6660	1.05	12
			2.10	13
	10.2	2220	1.05	14
			2.10	15
		6660	1.05	16
			2.10	17

2.2. Experimental design

The experiments were carried out in triplicate using Erlenmeyers flasks or Duran glass bottles as photobioreactors (500 cm^3) with 300 cm^3 of culture. The strain was cultivated in a BG-11 growth medium (pH 9.2) in two steps.

In the first step (biomass production phase), the culture was maintained in an aerobic condition until the first half of growth phase under continuous illumination in 500 cm^3 Erlenmeyers flasks, with 300 cm^3 of culture, under nitrogen limitation (10 g m^{-3} of sodium nitrate) to form heterocysts in order to improve the hydrogen production via nitrogenase.

In the second step (hydrogen production phase), the biomass was centrifuged in 33.3 Hz for 10 min, washed twice and suspended in the BG-11 medium with nitrogen deprivation, in 500 cm^3 Duran glass bottles with 300 cm^3 of culture and sealed with a butyl cover. Nitrogen deprivation was maintained aiming to improve hydrogen production by nitrogenase in heterocystis. The atmospheric conditions of the bottles were changed by adding argon gas for 10 min, previously sterilized using a filter with 0.2 μm of porosity. The cultures were maintained under continuous illumination (lamp cool white) as specified in each experiment. Experiments were carried out until stabilization of H_2 production.

The gas production was measured by gas chromatography (Shimadzu GC-2010) using a thermal conductivity detector (TCD) and argon as a carrier gas. 0.5 cm^3 of headspace gas were collected every 12 h using a syringe and valve (push button valve 22285). The results were obtained in moles of hydrogen based on the regression method with calibration curves ($r^2 = 0.996$) and were shown curves of hydrogen production in pmol by cell. The hydrogen volume was calculated by the ideal gas equation.

The biomass was measured by weighing the suspended solids [37] and the yield was calculated based on the difference between the final and initial biomass values both in steps one and two. To measure cell density, in the early phase of the second step of cultivation, samples of 0.5 cm^3 were taken from each photobioreactor. The samples were fixed with acetic Lugol's solution and stored in the dark until counting with Fuchs Rosenthal chamber and Olympus BX51 microscopy [37].

2.2.1. Experimental assays for optimization of biomass and hydrogen production

A control assay of *Anabaena* sp. cultivation was carried out using the same physicochemical conditions in which it is kept in the microorganism bank, i.e. 24 °C, at pH 9.2, 1.05 kg m^{-3} of glucose, with constant light intensity of 4440 lux, in the first step experimental.

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