

External Factors Influencing Light-Induced Hydrogen Evolution by the Blue-Green Alga, *Nostoc muscorum*

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This study was conducted to determine some physiological conditions which influence light-induced hydrogen evolution by intact cells of the blue-green alga *Nostoc muscorum*.

This activity was found to be dependent on the age of the culture, cultivation temperature, ageing of the cells, and on gassing the cultures with nitrogen or air. Nitrogenase is particularly active after cultivation at 22 °C but not at 32 °C although growth is about the same. The molar ratio of acetylene reduction to hydrogen evolution – both gas exchanges obviously catalyzed by nitrogenase – is also dependent on age and temperature of the culture. A hydrogen-consuming („uptake“) hydrogenase activity is present and affected by prolonged microaerobic, but not by aerobic growth conditions, and stimulated by light.

External factors during growth apparently influence differently the activities of nitrogenase and uptake hydrogenase. Consequently – besides the electron donor supply – net light-induced hydrogen evolution by intact cells is dependent on these two variable activities.

Introduction

Light-induced hydrogen evolution by intact blue-green algae is influenced by environmental factors. For instance, hydrogen production of *Anabaena cylindrica* was reported to attain a maximum after a 2-day nitrogen starvation [1], it is increased by optimum iron supply and arrested by ammonia [2]. Further, hydrogen evolution is increased and stabilized by inhibitors of linear photosynthetic electron transport in *Nostoc muscorum* [3]. The latter treatment lowers the oxygen level in the cells, thereby decreasing the activity of the hydrogen-consuming („uptake“) hydrogenase (see also [4]).

In order to further increase hydrogen production, the role of some external factors affecting the cells during cultivation must be known. Therefore, after growth either under air or nitrogen, and at different temperatures the nitrogenase and hydrogenase activities of intact cells of *Nostoc muscorum* were followed during growth.

Materials and Methods

Nostoc muscorum (strain 7119), now proposed to be denoted as *Anabaena* 7119 [5], was grown in 1300 ml batches in 2 l Fernbach flasks as previously described, at 21–24 °C in a temperature-controlled room, and illuminated continuously by white fluorescent lamps composed equally of Osram type 65 W/25 and 65 W/32 [3]. Light intensity as indicated was adjusted by appropriate layers of filter paper. Cultures were gassed with air or nitrogen, both enriched with 5% CO₂ (v/v). The nitrate-free cultures were inoculated with a 100 ml suspension from nitrogen-grown cultures (which contained 3–4 percent of heterocysts) with a packed cell volume (pcv) of approx. 14 µl/ml. For sufficient heterocyst formation slow gassing is necessary when using air/CO₂ (95% : 5% CO₂, v/v) which is about 0.3–0.4 ml gas/second. Culturing of algae was always done in duplicate. – The temperature experiments of Table I were carried out in a Pilot Shake (type Kühner, Braun, Melsungen, Germany) at 90 rev/min and 4 W/m² continuous white light supplied by 5 fluorescent tubes (Osram 30 W/25-2). Temperature was constant to ±0.1 °C.

Gas exchange was assayed in 37 ml cylindrical all-glass reaction vessels closed by Suba Seal rubber stoppers (Freeman, Barnsley, England) and filled with 10 ml of cell suspension concentrated before-

Abbreviations: Chl, chlorophyll a; DCMU (= diuron), N-(3,4-dichlorophenyl)-N'-N'-dimethylurea; pcv, packed cell volume.

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hand by sedimentation to yield a packed cell volume of 8 to 10 $\mu\text{l}/\text{ml}$ suspension. Pretreatment of samples included a series of 1 min waterpump evacuation and a 15 min flushing period with purified argon or nitrogen. To avoid over-pressure, aliquots of the gas phase were removed before injection of other gases except for acetylene which, due to its high water solubility, was directly injected in the same way as liquid samples. Assays were performed at 25 °C under 5 W/m^2 white light in a thermostated shaker bath at 100 rev/min for 4–5 h unless indicated otherwise. Light intensity was measured with the Radiometer 65 A (Yellow Springs, Ohio). Unless mentioned otherwise, photosynthetic oxygen evolution was measured in separate experiments with the oxygen electrode (Yellow Springs, Ohio) under saturating red light (RG 610 cut-off filter+KG1 heat filter from Schott, Mainz) at 24 °C. Ethylene and hydrogen were determined by gas chromatography as previously described [3]. For hydrogen uptake the gas phase of the assay consisted of 97% N_2 , 1% H_2 , and 2% O_2 , v/v; the first measurement was taken 2–3 h after incubation.

DCMU was purchased from Riedel-de Haen (Hannover), Cl-CCP from Sigma (München). All gases were obtained from Linde (München); high-quality argon and specially purified nitrogen were used. 2 ml graduated micro-centrifuge tubes (Assistant, made by Hecht, Bernhausen, Germany) were used to determine a packed cell volume up to 80 μl .

Results

1. Hydrogen gas exchange, acetylene-reducing activity during growth

Transfer of cells to a medium lacking combined nitrogen leads to an increase of heterocyst frequency to 7–9% of total cell number during the first 3 or 4 days of cultivation.

Oxygen production on a chlorophyll basis starts at high initial level, and gradually declines with cultivation time as the bulk chlorophyll is greatly increased due to mutual shading of the cells (Fig. 1). During the first 3 days of cultivation, the photosynthetic oxygen evolution was lowest when based on packed cell volume (data not shown). This is the period of nitrogenase induction, and heterocysts increase to 7–9% of cell number, then declining again. The early cultivation period marks the time of oxy-

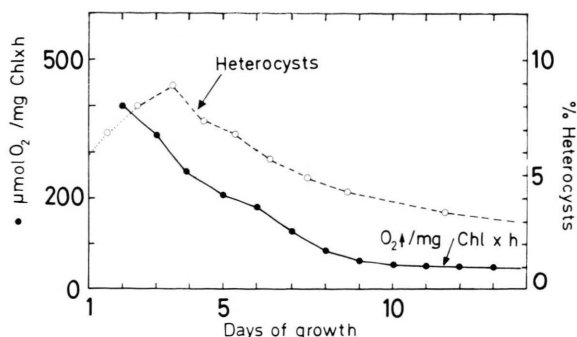


Fig. 1. Photosynthetic activity and heterocyst frequency during cultivation of *Nostoc muscorum* grown under nitrogen + CO_2 , 5% (v/v). Growth was started with a 1300 ml batch having a total pcv of 750 μl at start. Light intensity was 5 W/m^2 for 3 days, then 8 W/m^2 . ●, Oxygen evolved as $\mu\text{mol O}_2/\text{mg Chl} \times \text{h}$. ○---○, Heterocysts in % of total cell number.

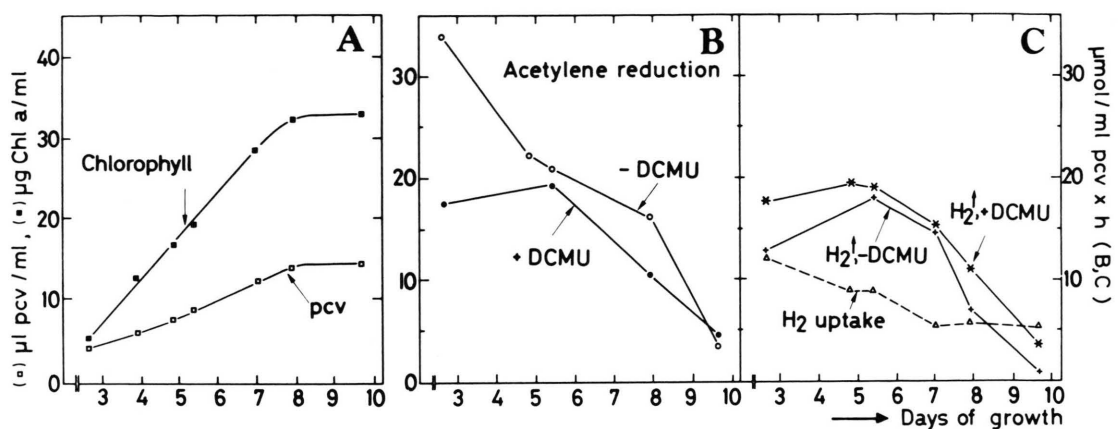
gen sensitivity, since either increased light or stronger bubbling with air result in bleaching and irreversible inhibition of growth.

Since hydrogen formation apparently is mediated by nitrogenase but also severely counteracted by hydrogen uptake, we studied hydrogen evolution and uptake as well as acetylene reduction of cultures grown under aerobic (air + CO_2) and microaerobic (nitrogen + CO_2) conditions of Figs 2 A, B. The latter cultures contain minute oxygen in the gas phase (about 1% v/v). They attain a cell density of 15 μl pcv/ml suspension after 10 days, similarly to cultures grown under air with nitrate as their nitrogen source. Presence of DCMU in the assays arrested the activity of the uptake hydrogenase due to lack of oxygen present [3]. This allows for determination of the full hydrogen-evolving capacity of the cells.

Acetylene reduction is steadily declining in both aerobic and microaerobic cultures during the cultivation time. This roughly parallels the decreasing percentage number of heterocysts. The sensitivity towards DCMU (4 μM , a concentration sufficient to suppress oxygen evolution to 95%) decreases with age in both cultures and is at its maximum at the start of cultivation.

Hydrogen gas exchange, on the other hand, exhibits marked differences between both types of cultures. Aerobic cultures produce 2 to 3fold more hydrogen in the presence of DCMU with a maximum level attained after 5 cultivation days (Fig. 2 II, C); thereafter activities decline. In cultures grown under nitrogen the basal rate of the sample minus DCMU is

I. Under nitrogen



II. Under air

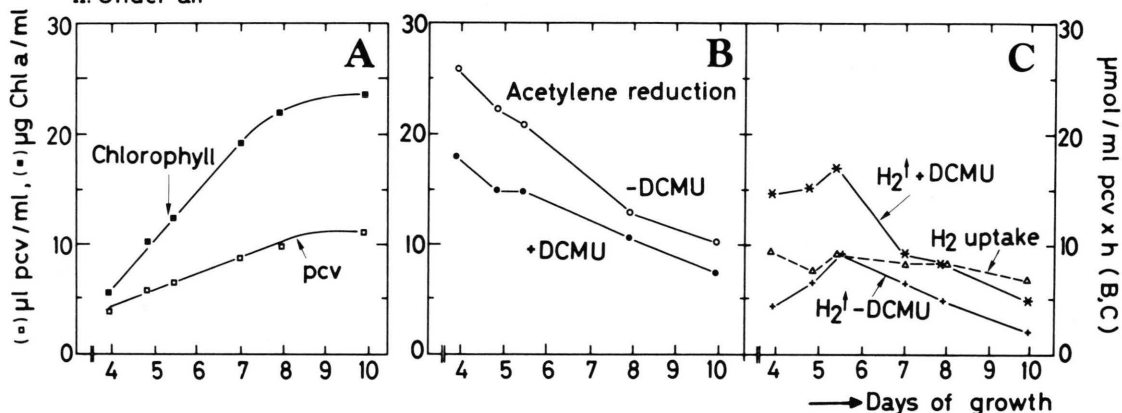


Fig. 2. Growth kinetics of *Nostoc muscorum* grown under [I]: $\text{N}_2 + \text{CO}_2$, 5% (v/v); [II]: air + CO_2 , 5% (v/v). Continuous cultivation was carried out for 3 days under 5 W/m^2 , then, after the culture had reached the maximum number of heterocysts per cells, under 8 W/m^2 . Inoculum: see Fig. 1. A: chlorophyll, pcv; B: acetylene reduction, assay performed with or without $4 \mu\text{M}$ DCMU; C: hydrogen evolution (\uparrow) assay (—) or (+) $4 \mu\text{M}$ DCMU (solid lines); hydrogen uptake (broken lines) was assayed without DCMU.

smaller at the beginning only. Thereafter, in contrast to aerobic cultures, a high level of hydrogen evolution is maintained for about 4 days regardless of the presence of an electron transport inhibitor. Acetylene reduction of nitrogen-grown cultures generally is higher than in air-grown cultures, and hydrogen evolution in the presence of DCMU reaches up to 60% of that rate, e. g. $15\text{--}20 \mu\text{mol H}_2/\text{ml pcv} \times \text{h}$ for both types of cultures.

Hydrogen uptake ranges between $5\text{--}11 \mu\text{mol H}_2/\text{ml pcv}$ during the growth period and surprisingly is lower than hydrogen evolution. The hydrogen-consuming activities of both cultures are rather similar, although in nitrogen-grown cultures hydrogen up-

take is lower towards the end of the growth period. Indeed, not all of the hydrogen produced can be consumed again by the uptake hydrogenase activity.

In the stationary phase, heterocyst content, acetylene reduction, hydrogen evolution and uptake decrease to a minimum.

II. Influence of cultivation temperature

We studied the dependence of nitrogenase and hydrogenase activity on the cultivation temperature covering a range of 10°C . After 3 days, aliquots of each culture were withdrawn, concentrated 3- to 4fold and assayed at 25°C . Data of Table I demonstrate that photosynthetic oxygen evolution is

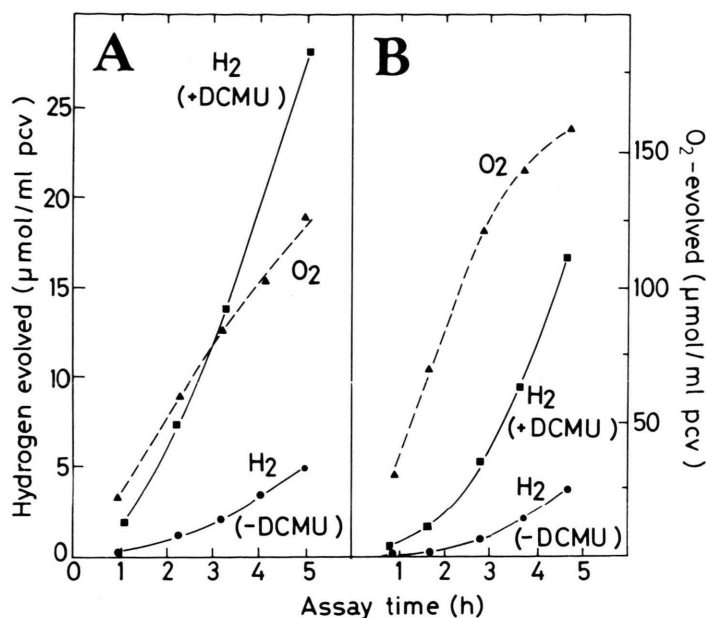


Fig. 3. Effect of ageing on hydrogen and oxygen evolution by *Nostoc muscorum*. Cultures were grown under air for 3 days under 5 W/m² light intensity, further growth was then arrested by placing them in the dark for 7 h (A) and 12 h (B). DCMU was 4 μM when indicated. Incubation conditions for assay: 25 °C; gas phase Ar; light intensity, (A) 5 W/m², (B) 15 W/m². All assays were supplemented with 5 mM bicarbonate, pH 7.9. Oxygen was determined by gas chromatography.

somewhat decreased in nitrogen-fixing cultures grown under air/CO₂ at 25 °C and 32 °C vs. those cultured at 22 °C (line 1). Chlorophyll formation is smaller in the 32 °C cultures as compared to that of 22 °C and 25 °C, while growth is about the same in all samples during the period assayed here (lines 2, 3), being at optimum around 25 °C. Cultures grown at 22 °C and 25 °C exhibit a substantially increased hydrogen uptake in the light vs. the samples assayed in the dark (lines 4, 5). Also in the absence of CO₂ or bicarbonate this effect is observed (data not shown). Noteworthy, ethylene formation of cultures grown at 22 °C is doubled as compared to those grown at 32 °C and considerably higher than that of cells grown at 25 °C (line 7). Consequently, net hydrogen formation shows a similar dependence on growth temperature (line 6).

III. Ageing effects; oxygen sensitivity

A young culture of *N. muscorum* responds rather quickly to changes of external conditions. Fig. 3 demonstrates cellular activities observed after placing air-grown algae (containing almost the maximum percentage of heterocysts) in a closed culture vessel in the dark for 7 and 12 hours. Whereas oxygen evolution is unaffected, hydrogen evolution (in the presence of DCMU) is more decreased after 12 h of darkness (Fig. 3 B) as compared to the sample kept in

Table I. The influence of the temperature of cultivation on hydrogen evolution and associated cellular activities of *Nostoc muscorum*.

Activity and conditions	Temperature of cultivation		
	22 °C	25 °C	32 °C
(1) O ₂ evolution	335	250	250
(2) Chl, μg/ml	4.0	5.0	3.5
(3) pcv, μl/ml suspension	2.0	2.7	2.0
(4) H ₂ uptake, dark	3.2	3.3	3
(5) H ₂ uptake, light	5.0	4.0	4.0
(6) H ₂ evolution, light	5.0	2.0	<0.5
+ 4 μM DCMU			
(7) C ₂ H ₄ formation, light	24.0	17.0	12.0

For assay, samples (air-grown cells) were harvested on the 3rd cultivation day (see Fig. 1) and assayed at 25 °C for 4–6 h, Nos. 5–7, under 5 W/m². Data Nos. 1, 4 to 7 are rates expressed as μmol/mg Chl × h.

Table II. Effect of oxygen on hydrogen evolution and ethylene formation in the presence of DCMU in *Nostoc muscorum*.

Gas evolved *	Oxygen in the gase phase in % (v/v)			
	0	2	5	10
Hydrogen	1.7	0.4	0.1	–
Ethylene	7.9	6.3	6.2	6.2

* Rates are expressed as μmol/ml pcv × h. Samples were from air-grown algae, assayed in the presence of 4 μM DCMU.

the dark for only 7 h (Fig. 3 A) even when higher light intensity is employed. Light-induced hydrogen evolution without DCMU – being low anyway – is little affected. A distinct lag phase in hydrogen evolution becomes apparent after 12 h darkness (Fig. 3 B).

Data for the influence of oxygen on hydrogen or ethylene generated are given for *Nostoc* in Table II. Hydrogen is consumed with increasing amounts of oxygen present in the assay, decreasing light-induced net hydrogen evolution to almost zero at 2% oxygen present or more. Inhibition of acetylene reduction with intact cell material, however, is only 20% in the presence of 10% oxygen.

Discussion

From our findings reported earlier [3] we propose that maximum hydrogen evolution can be achieved by the suitable choice of photosynthetic electron transport inhibitors and illumination intensity vs. a CO/acetylene gas mixture to inhibit the uptake hydrogenase [4]. Even though the former method leads to high increases over the control in air-grown cultures highest rates in absolute values are always obtained with material grown under nitrogen (+CO₂), *i. e.* microaerobic conditions (Fig. 2).

For at least 6 to 7 cultivation days, acetylene reduction is inhibited by DCMU which implies that the donor supply (either water or an organic donor) can be decreased down to 50%. It appears that the nitrogenase reaction is dependent on a donor pool which is possibly small and obviously built up during concurrent photosynthesis as suggested by others (see *e. g.* [6]). Hydrogen evolution in the presence of DCMU is accelerated by a factor up to 3 with air-grown cell material in the initial growth phase (Fig. 2). After 6 cultivation days, however, DCMU induces no further change vs. the control minus DCMU. Apparently reflecting a lower cellular oxygen tension present (in the cells) due to smaller photosynthetic oxygen evolution of older cultures.

Sensitivity of DCMU-stimulated hydrogen evolution towards ageing (Fig. 3) of algal material with high heterocyst content can therefore be explained by gradual exhaustion of organic electron donor(s) upon standing. This is further expressed in the pronounced lag phase of hydrogen production after a 12 h ageing, which is hardly seen with cells from physiologically older cultures.

It should be mentioned that both ethylene and hydrogen evolution could be abolished at all cultivation stages by *m*-chlorocarbonyl-cyanide-phenylhydrazine (20 µM). Although hydrogenase activity *evolving* hydrogen in the assay has been found in blue-green algae ([7–9], and own unpubl. results) only a few percent of hydrogen evolution activity is possibly due to such a hydrogenase under the conditions described here. It was shown previously that heterocyst formation parallels nitrogenase development in *Anabaena* [10–12]. So, as evidenced by other authors (*e. g.* [4, 13, 14]) and our own findings, hydrogen evolution is mainly catalyzed by nitrogenase.

The time courses of hydrogen and ethylene production, however, are distinctly different throughout the cultivation time (Fig. 2). It follows that the nitrogenase-catalyzed hydrogen production is not invariably coupled to the counteracting activity of the uptake hydrogenase. Interestingly, the molar ratios of ethylene formed to hydrogen evolved exhibit a strong variation during growth and are dependent on temperature. Whereas after 4 days of cultivation a ratio of 0.6 µmol H₂ : 1 µmol C₂H₄ formed is found in parallel assays carried out at 25 °C, a ratio of even 1 : 1 can be found under optimum growth. The maximum evolution of 20 µmol H₂/mg Chl × h (\triangleq 20 µmol H₂/µl pcv × h; see Fig. 2 I, C) achieved under the conditions described here is again a substantial improvement as against our earlier report [3].

Oxygen decreases hydrogen evolution of intact cells with little influence on nitrogenase activity (Table II). Similar results were reported for *Gloeocapsa* and *Anabaena flos-aquae* [15] but are in contrast to findings of others [16]. – Generally, nitrogenase activity increases with assay temperature (for refs. see [17, 18]). The striking enhancement of hydrogen production by lower growth temperature (22 °C) observed with *Nostoc* is seemingly due to increased nitrogenase and not to decreased uptake hydrogenase activity (Table I). Little work has been done on this type of temperature experiments and more data should be gathered. The data of Rodgers obtained from the endophytic *Nostoc* of *Blasia pusilla* also exhibit an optimum of nitrogenase activity due to pre-cultivation temperature ([19] and pers. commun.). Future research has to answer whether this enhancement is caused by changed nitrogenase activity alone or/and by its donor supply. The almost undimin-

ished photosynthesis after growth at temperatures $>22^{\circ}\text{C}$ renders unlikely the latter possibility.

This study gives evidence that the capacity of light-induced hydrogen evolution in *Nostoc* is influenced by many variables. Growth temperature affects the hydrogen-evolving ability of the nitrogenase. Hydrogen generation on the other hand is counter-acted by the activity of the uptake hydrogenase. Light contributes substantially to the activity of the latter (Table I). Since this effect is also observed when CO_2 is excluded (p. 7) and blue-green algae apparently do not exhibit photoreduction of CO_2

with hydrogen [20] we may tentatively assume that hydrogen is activated via (partial) photosynthetic electron transport. In addition, a variable electron donor supply for proton (and nitrogen) reduction is evident. The mechanism and interaction of these parameters have to await further investigation.

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