Appl Microbiol Biotechnol (1997) 47: 701–707

ORIGINAL PAPER

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The biotechnology of hydrogen production by *Nostoc flagelliforme* grown under chemostat conditions

Received: 14 October 1996 / Received revision: 18 February 1997 / Accepted: 22 February 1997

Abstract The potential of using N_2-fixing cyanobacteria to produce hydrogen photobiologically has stimulated research on the physiology and biotechnology of species exhibiting high H_2 production rates over long periods of time. In this work *Nostoc flagelliforme*, a terrestrial N_2-fixing cyanobacterium, has been examined to establish its physiology and potential for H_2 production under controlled conditions. Cell filaments of *N. flagelliforme* were purified and grown in liquid culture to optimize its H_2 metabolism. In batch-grown cultures the activity of nitrogenase, the key enzyme for H_2 production in N_2-fixing organisms, was found to be high only during a short phase of exponential growth. A chemostat system was thus constructed for long-term experiments using continuous cultures, with the aim of exploiting the exponential growth phase. The dilution rate (D) and environmental factors, such as N_2 concentration in the gas phase and temperature, significantly influenced H_2 production. Cells grown continuously under the optimized conditions of $D = 0.022\ h^{-1}$, 34 °C and 5.1 kPa N_2 in the gas phase exhibited H_2 production rates that were more than four times higher than the maximal rates under standard batch growth conditions.

Introduction

The concept of using solar energy to drive the conversion of water into hydrogen and oxygen by technologies using photobiological systems has been reviewed extensively (Benemann 1994; Hall et al. 1995; Bolton 1996). Cyanobacteria have been regarded for many years as offering interesting prospects for photobiological hydrogen production (Hall and Rao 1989; Bothe and Kentemich 1990; Markov et al. 1995). These organisms are very flexible in their metabolism and adaptive to different environmental conditions (Tandeau de Marsac and Houmard 1993).

In nitrogen-fixing cyanobacteria, nitrogenase is the key enzyme for hydrogen production. This oxygen-sensitive enzyme catalyses the reduction of molecular nitrogen to ammonia with the concomitant, wasteful evolution of hydrogen (Houchins 1984). Some species are capable of fixing nitrogen aerobically since they produce specialized cells, the heterocysts, in which nitrogenase is efficiently protected against the damaging effects of oxygen (Gallon 1992). The activity of nitrogenase varies considerably under different growth conditions and, in the absence of any substrates, its reducing power can be channelled entirely into the production of hydrogen. In the hydrogen metabolism of heterocystous cyanobacteria two different hydrogenases are involved, the genes of which have only recently been identified and sequenced (Carrasco et al. 1995; Schmitz et al. 1995). First, the so-called uptake hydrogenase, which preferentially consumes hydrogen and appears to be associated with nitrogen fixation in the heterocysts; part of its physiological function is recycling hydrogen produced by nitrogenase and thus regaining some of the reductant and energy lost during the process of nitrogen fixation. Second, the reversible hydrogenase is capable of catalysing both hydrogen uptake and production, but it is postulated that this enzyme operates generally in the direction of hydrogen uptake in vivo (Houchins 1984; Schmitz et al. 1995).

Although considerable research has been undertaken for more than 20 years, the hydrogen production rates and solar-energy conversion efficiencies of cyanobacteria are still relatively low (Benemann 1994; Bolton 1996). Problems such as low hydrogen production rates and low metabolic stabilities of the hydrogen production process have yet to be resolved. The molecular biology of cyanobacteria has progressed considerably in the last

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decade (Golden et al. 1987; Houmard 1994); however, little work has so far been done in the field of hydrogen production because of the complex gene regulation of nitrogenase (Arnold et al. 1988; Gallon 1992; Carrasco and Golden 1995) and hydrogenases (Carrasco et al. 1995; Schmitz et al. 1995).

Only a limited number of studies have been reported on the use of continuous microbial cultures for hydrogen production, mainly by purple photosynthetic bacteria (Zürrer and Bachofen 1982; Kim et al. 1987). In the case of cyanobacterial hydrogen production, chemostat techniques have not been utilized so far. Here we describe the physiological optimization of hydrogen production by the nitrogen-fixing, heterocystous cyanobacterium *Nostoc flagelliforme* in continuous cultures. *N. flagelliforme* (Geitler and Pascher 1925) is a terrestrial subspecies of the commonly known *Nostoc commune*, which for this study has been grown in its free-living form in liquid-culture suspension.

We show that *N. flagelliforme* grown under controlled and optimized chemostat conditions is capable of producing hydrogen at greatly enhanced rates on a stable and continuous basis.

Materials and methods

Gases and light intensity measurements

Partial gas pressures are expressed in kilopascals (kPa; 1.013×10^5 Pa = 1 atmosphere = 1.013 bar). Incident light intensities were measured as photosynthetically active radiation (PAR) on the surface of the cultivation vessels, using a quantum meter (LI-COR, model LI 189, Lambda Instrument Corp., Nebraska, USA), and were expressed as the photosynthetic photon flux density (400–700 nm) in μmol photons m^{-2} s^{-1}.

Organism and culture conditions

Terrestrial material of the cyanobacterium *Nostoc flagelliforme* was initially obtained as desiccated macrofilaments from Dr. Ding-Ji Shi (Academia Sinica, Institute of Botany, Beijing, China). In the present experiments liquid-culture suspensions of *N. flagelliforme* (deposited with the Culture Collection of Algae and Protozoa, CCAP 1453/33, Ambleside, Cumbria LA22 0LP, UK) were used, which were isolated and purified by the authors from the terrestrial material. Desiccated macrofilaments were first rewetted and then incubated with growth medium (Allen and Arnon 1955) in 250-ml conical flasks under standard conditions (30 °C, continuous illumination with white fluorescent light at 30 μmol m^{-2} s^{-1} PAR). Short cell filaments that were released into the liquid supernatant were separated from the terrestrial material by filtration through sterile cheesecloth. Free-living cells were purified by phenol treatment after the method described by Carmichael and Gorham (1974). Purified N_2-fixing cell filaments of *N. flagelliforme* were grown under sterile conditions in liquid-culture suspension with growth medium (Allen and Arnon 1955, without fixed nitrogen), modified to 5.2 mM phosphate. Batch cultures of *N. flagelliforme* were grown in 250-ml and 500-ml conical flasks under the above-mentioned standard growth conditions. The flasks were kept agitated on a rotary shaker at 100 rpm at 30 °C, and the atmosphere inside the incubator was supplied with a constant stream of air plus 1 kPa CO_2 (2 l min^{-1}). Continuous cultures were grown in a chemostat unit (LH Fermentation, Inceltech Ltd., UK) in a cylindrical 2-l glass vessel with a constant culture volume of 1.5 l. The pH of the culture was monitored and controlled (LH Engineering, pH control 505 D) using 1 M HCl and 1 M NaOH solutions. Constant illumination was provided by two circular warm white fluorescent lamps (Sylvania, 32 Watt) at an intensity of 70 μmol m^{-2} s^{-1} PAR. The temperature of the culture suspension was kept constant by means of a cooling rod that was connected to a thermostat-controlled waterbath. The culture was constantly agitated by a stirring impeller at 100 rpm and was gassed at a flow rate of 700 ml min^{-1} (using an air compressor) with either air supplied with 1 kPa CO_2 or other gas mixtures as described. Carbon dioxide, argon and nitrogen were mixed into the gas stream from gas cylinders using flow meters (Cole-Palmer Instrument Company Ltd., UK). Before entering the growth vessel, the gas stream was passed through a humidifier and through sets of bacterial gas filters with 0.3 μm pore size (Hepa-Vent, Whatman, UK). During experimentation, samples of the culture suspension were withdrawn aseptically from the growth vessel into an attached 70-ml flask using a syringe.

Heterocyst frequencies of N_2-fixing cultures were determined as means (± standard errors) of at least five microscopic countings of about 500 cells each.

Analytical assays

Chlorophyll *a* of free-living culture suspensions of *N. flagelliforme* was extracted in 90% methanol in the dark at 4 °C for at least 6 h or at 60 °C for 5 min. The concentration of chlorophyll *a* in the centrifuged extracts was measured after Tandeau de Marsac and Houmard (1988). Biomass was determined as cell dry weight by filtration of the culture suspensions (Whatman no.1 filters), washing the cell mass twice with distilled water and drying it at 90 °C to constant weight. In order to estimate the growth rates of the free-living cultures the absorbance of the undiluted cell suspensions was measured at 750 nm (turbidity measurements).

Nitrogenase activity and H_2 production in vivo

The activity of nitrogenase in whole cells of *N. flagelliforme* was determined by C_2H_2 reduction (Dilworth 1966). Cultures (4 ml) with chlorophyll *a* concentrations of 6–9 μg ml^{-1} were transferred to 13-ml glass vials and sealed with Suba seals (Freeman and Co., UK). The sealed vials were evacuated and flushed with argon twice and then sparged with argon for 10–15 min. The samples (triplicates) were incubated with 10% C_2H_2 at 30–38 °C (according to experiment) for 1–3 h. Incubation was carried out under magnetic stirring and illumination of 70 μmol m^{-2} s^{-1} PAR white fluorescent light. C_2H_4 was measured by gas chromatography (HP 5890A, Hewlett Packard Ltd., UK), using a Porapak S column and a flame ionisation detector. Nitrogenase activity was calculated as the C_2H_2 reduction rate on the basis of chlorophyll (as μmol C_2H_4 mg chlorophyll a^{-1} h^{-1}). For hydrogen production, culture samples (triplicates) were prepared and incubated as described above but with argon in the incubation gas phase only. Hydrogen was measured by gas chromatography (HP 5890A, Hewlett Packard Ltd., UK), using a molecular sieve column (60–80 mesh) and a thermal conductivity detector. H_2 production rates were expressed on the basis of chlorophyll (as μmol H_2 mg chlorophyll a^{-1} h^{-1}).

Results

Nitrogenase activities and H_2 production rates in batch cultures

C_2H_2 reduction and H_2 production rates of *N. flagelliforme* in vivo were measured during aerobic batch growth (Fig. 1). A distinct maximum of nitrogenase activity (C_2H_2 reduction) was found 1 day after inoculation, which correlated with the early exponential

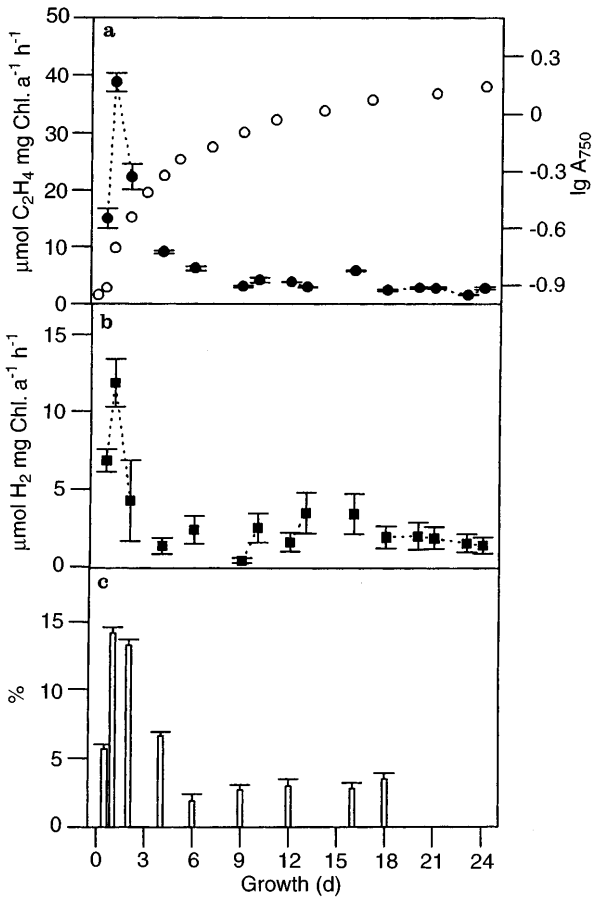


Fig. 1 **a** C_2H_2 reduction (●), turbidity (○). **b** H_2 production rates (■). **c** Heterocyst frequency (*bars*) of *Nostoc flagelliforme* during aerobic batch growth under N_2-fixing conditions. The growth rate in the exponential phase was 0.017 h^{-1}. *chl. a* equals chlorophyll *a*

growth phase of the culture (Fig. 1a). With further batch growth, C_2H_2 reduction decreased substantially and levelled-off at less than 10% of its maximal rate. H_2 production rates measured during batch growth were similar to C_2H_2 reduction, showing a distinct maximum at day 1 (Fig. 1b). The decrease in C_2H_2 reduction and H_2 production rate in cultures older than 1 day was accompanied by a rapid decline in the heterocyst frequency (Fig. 1c).

Nitrogenase activity and H_2 production rates in continuously grown cultures

In order to carry out long-term experiments with continuous light-limited cultures a chemostat was assembled similar to the continuous-culture apparatus found especially suitable by Thomas (1973) for filamentous cyanobacteria such as *Anabaena*. The following experiments investigated whether or not continuous culture techniques have a potential for improving the activity of nitrogenase and the resulting H_2 production rates of *N. flagelliforme*.

Effect of dilution rate (D)

Since the activity of nitrogenase varies with the different growth phases in batch culture, it was of particular interest to know if controlling and varying the dilution rates would lead to enhanced rates. Figure 2 shows C_2H_2 reduction and H_2 production rates of *N. flagelliforme* growing continuously under light-limited conditions, during which the dilution rate was increased in steps from 0.014 h^{-1} to 0.030 h^{-1}. The steady-state rates are also shown in Fig. 2. A substantial increase in steady-state C_2H_2 reduction and H_2 production was noted upon the increase from $D = 0.014$ h^{-1} to 0.022 h^{-1}. With the further increase of the dilution rate, the steady-state C_2H_2 reduction varied rather insignificantly and the H_2 production rates decreased slightly. The maximal growth rate μ_{max} under the applied growth conditions, determined by the wash-out method (Pirt 1975; data not shown) was 0.032 h^{-1}. At $D = 0.014$ h^{-1} the calculated ratio of C_2H_2 reduction: H_2 production rate was 4.2, which was significantly higher than the average ratio of 1.4 at dilution rates of 0.022 h^{-1} and higher.

Effect of temperature

The influence of temperature on nitrogenase activity and H_2 production in continuous cultures of *N. flagelliforme* was investigated independently of the growth rate. This is not possible in batch cultures, where many interrelating changes affect the enzyme activities and the growth rate of the organism is influenced by the temperature (Ratowsky et al. 1982).

As shown in Fig. 3, C_2H_2 reduction and H_2 production rates were clearly influenced by the growth temperature (the assay temperature was kept identical to the growth temperature in the chemostat). The steady-state activities displayed a distinct maximum at 34 °C with rates two to three times higher than at 30 °C. An Arrhenius plot of nitrogenase activity indicated a linear relationship between 25 °C and 34 °C with an activation energy of about 76 kJ mol^{-1} (data not shown).

Effect of N_2 concentration in the gas phase

In aerobically growing, diazotrophic species, the period of nitrogen starvation in the absence of a bound nitrogen source in the growth medium is restricted to the time required for the nitrogenase to be synthesized (Tandeau de Marsac and Houmard, 1993). In order to investigate the effect of nitrogen limitation on H_2 production in continuous cultures of *N. flagelliforme* the N_2 concentration in the chemostat was therefore decreased in steps from atmospheric levels (78.9 kPa) to 2 kPa in the gas phase. C_2H_2 reduction and H_2 production rates did not vary significantly at $D = 0.022$ h^{-1} and partial pressures of 78.9–10.1 kPa N_2 (Fig. 4). A considerable increase in

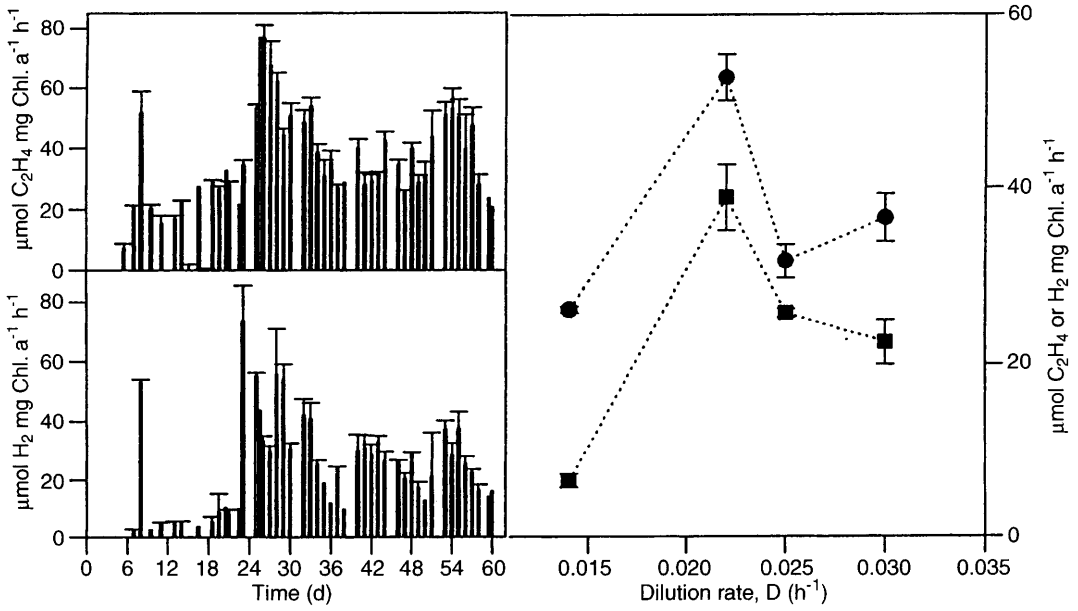
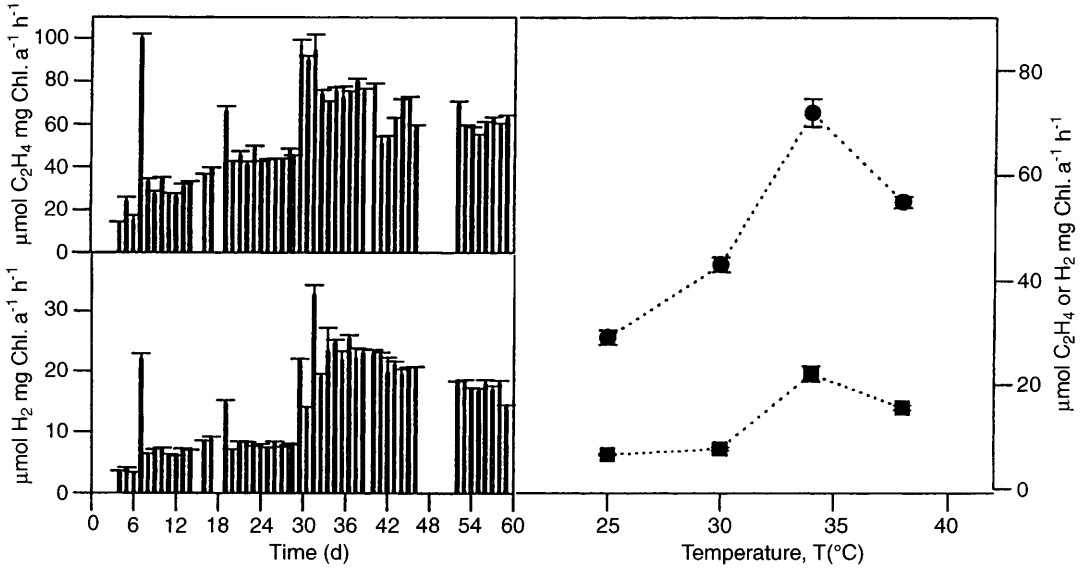


Fig. 2 C_2H_2 reduction (*top left*) and H_2 production rates (*bottom left*) of *N. flagelliforme* during continuous culturing at different dilution rates. The culture was grown continuously in a chemostat at 30 °C, pH 7.1 and a light intensity of 70 µmol m^{-2} s^{-1} photosynthetically active radiation under a gas phase of air plus 1 kPa CO_2. The step changes in dilution rate were as follows: 0.014 h^{-1} (days 4–20), 0.022 h^{-1} (days 21–32), 0.025 h^{-1} (days 33–45), 0.030 h^{-1} (days 46–58). Steady-state activities of nitrogenase (*right*, ●) and H_2 production (*right*, ■) were calculated as means and standard errors for the rates measured on at least 6 successive days during the steady states under each set of growth conditions

both rates occurred upon the decrease to 5.1 kPa N_2, which was accompanied by an increase in the average heterocyst frequency from 7.8% to 13.3%; hence a fairly constant ratio of C_2H_2 reduction : heterocyst frequency of 2.4 was calculated. The further decrease of N_2 to 2.0 kPa led to an irreversible perturbation of the steady state and a subsequent culture wash-out. Since the balance for all reduced-N_2 gas phases was argon (supplied with 1.0 kPa CO_2), these culture conditions were regarded as microaerobic. However, no significant changes in the

Fig. 3 C_2H_2 reduction (*top left*) and H_2 production rates (*bottom left*) of *N. flagelliforme* during continuous culturing at different temperatures. The culture was grown in a chemostat at $D = 0.022$ h^{-1}, pH 7.1, and a light intensity of 70 µmol m^{-2} s^{-1} photosynthetically active radiation under a gas phase of air plus 1 kPa CO_2. The step changes in temperatures were as follows: 25 °C (days 1–14), 30 °C (days 15–28), 34 °C (days 29–39), 38 °C (days 40–58). Steady-state activities of C_2H_2 reduction (*right*, ●) and H_2 production (*right*, ■) were calculated as means and standard errors for the rates measured on at least 6 successive days during the steady states under each set of growth conditions



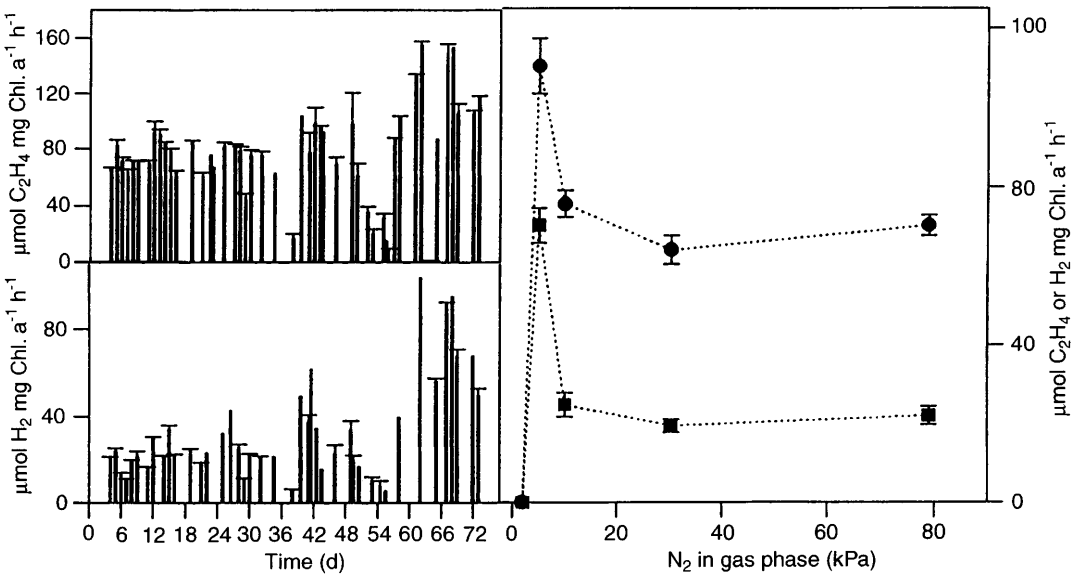


Fig. 4 C_2H_2 reduction (*top left*) and H_2 production rates (*bottom left*) of *N. flagelliforme* during continuous culturing at different N_2 concentrations in the gas phase. The culture was grown in a chemostat at $D = 0.022$ h^{-1}, 34 °C, pH 7.1, and a light intensity of 70 µmol m^{-2} s^{-1} photosynthetically active radiation. The step changes in N_2 concentrations were as follows: 78.9 kPa (days 1–15), 30.4 kPa (days 16–31), 10.1 kPa (days 32–47), 5.1 kPa (days 48–66), 2.0 kPa (days 67–75). All gas phases were supplied with 1 kPa CO_2, and the balance for all N_2-reduced gas phases was argon. Steady-state activities of C_2H_2 reduction (*right*, ●) and H_2 production (*right*, ■) were calculated as means and standard errors for the rates measured on at least 6 successive days during the steady states under each set of growth conditions

growth behaviour (pigment concentration, biomass and heterocyst frequency) was observed between aerobic and microaerobic conditions.

A further evaluation of the effect of limiting concentrations of N_2 under continuous growth conditions was carried out at different dilution rates in the chemostat system (Table 1). The aim was to determine optimal values for both variables (dilution rate D and partial pressure of N_2) for the production of H_2 under continuous growth conditions. It was found that, at an increased dilution rate ($D = 0.024$ h^{-1}), the culture required N_2 concentrations higher than 5.1 kPa in order to maintain steady-state growth. At the lower dilution rate $D = 0.015$ h^{-1}, the culture reached steady state even at 3.0 kPa N_2 but with greatly decreased H_2 production rates of about 8.9 µmol H_2 mg chlorophyll a^{-1} h^{-1} (Table 1).

Discussion

H_2 production rates of *N. flagelliforme* showed a pattern very similar to nitrogenase activity (C_2H_2 reduction) with both varying together significantly during batch growth. The rates were highest in the early exponential growth phase of the culture, declining significantly thereafter. This has also been observed for other cyanobacteria (Weare and Benemann 1973; Benemann et al. 1980; Yakunin et al. 1995) and seems to be to a great extent due to the effects of the limitation of light and nutrients as a result of the rapid increase of cell density during batch growth. The rapid decline in heterocyst frequency observed in parallel to the decrease in nitrogenase activity also indicates a decrease in nitrogenase synthesis, since in heterocystous cyanobacteria these processes seem to be closely linked (Elhai and Wolk 1990; Carrasco and Golden 1995). Yakunin et al. (1995) showed that the nitrogenase activity of *Anabaena variabilis* during batch growth follows a pattern similar

Table 1 Steady-state C_2H_2 reduction (µmol C_2H_4 mg chlorophyll a^{-1} h^{-1}) and H_2 production rates (µmol H_2 mg chlorophyll $a^{-1}h^{-1}$) of *N. flagelliforme* grown continuously at different dilution rates and with different N_2 concentrations in the gas phase

Reaction	Activity (µmol mg chlorophyll a^{-1} $h^{-1}1$ ± SE)							
	78.9 kPa (air) $D = 0.022$ h^{-1}	30.4 kPa $D = 0.022$ h^{-1}	10.1 kPa $D = 0.022$ h^{-1}	10.1 kPa $D = 0.024$ h^{-1}	5.1 kPa $D = 0.024$ h^{-1}	5.1 kPa $D = 0.022$ h^{-1}	3.0 kPa $D = 0.022$ h^{-1}	3.0 kPa $D = 0.015$ h^{-1}
Nitrogenase	70.4 ± 2.6	64.3 ± 3.5	75.8 ± 3.3	104.4 ± 1.9	Not steady	110.4 ± 7.0	Not steady	94.0 ± 9.9
H_2 production	22.1 ± 2.4	19.5 ± 1.7	24.9 ± 3.1	71.8 ± 5.8	Not steady	84.2 ± 2.3	Not steady	8.9 ± 3.2

to the changes in the C:N ratio of the cells, which is known to regulate expression of nitrogenase in *Klebsiella pneumoniae* (Gallon 1992). In chemostat cultures of *Rhodobacter capsulatus*, light intensity had a direct influence on the C:N ratio, which in turn controlled cellular nitrogenase activity (Steinborn et al. 1991).

By applying chemostat techniques to exponentially growing cultures of *N. flagelliforme* it has been possible to monitor and control the growth rate of the organism. Only in some reported studies on biological H_2 production by photosynthetic bacteria have continuous cultures been used, and little information is available on the effect of the dilution rate in such systems (Zürrer and Bachofen 1982; Kim et al. 1987; Fascetti and Todini 1995). Here the dilution rate was found to influence the steady-state C_2H_2 reduction and H_2 production rate significantly (Fig. 2). A two-to threefold increase of C_2H_2 reduction upon the increase of D from 0.014 h^{-1} to 0.022 h^{-1} was accompanied by a seven-to ninefold increase in the H_2 production rate. The fact that the increase in H_2 production was significantly greater than the increase in C_2H_2 reduction could be an indication that, at lower growth rates, hydrogenase-catalysed H_2 uptake activities were greater than at higher growth rates. This was also evident from the high ratio of C_2H_2 reduction:H_2 production of 4.2 at $D = 0.014$ h^{-1}. H_2 uptake with rates of 5–35 μmol H_2 mg chlorophyll a^{-1} h^{-1} was identified in this organism under comparable circumstances (Lichtl 1996) but was not frequently measured during continuous growth.

At $D = 0.022$ h^{-1}, a growth (and assay) temperature of 34 °C was found to be optimal for C_2H_2 reduction and H_2 production. In this steady state the chlorophyll and biomass concentrations of the culture (data not shown) were maximal, which indicated that a temperature of 34 °C was also optimal for the growth of this organism. No comparable data on continuous cultures of cyanobacteria are available in the literature, but an optimum temperature for H_2 production between 30 °C and 33 °C was found for continuous cultures of *Rhodospirillum rubrum* (Zürrer and Bachofen 1982), which was also identical to the optimum growth temperature.

N. flagelliforme was grown continuously under nitrogen limitation by decreasing the partial pressure of N_2 in the gas phase to 5.1 kPa at a dilution rate of 0.022 h^{-1}; this led to a significant increase of steady-state C_2H_2 reduction and H_2 production. Hence, the requirement for nitrogen under this nitrogen-limiting condition was met by increasing the capacity for N_2 fixation. Since an increase in the average heterocyst frequency was detected in parallel (the ratio of C_2H_2 reduction to heterocyst frequency stayed fairly constant), the raised activity of nitrogenase could be due to an enhanced synthesis of the enzyme. The differentiation of heterocysts and the expression of nitrogenase are known to occur in response to the lack of usable bound nitrogen (Tandeau de Marsac and Houmard 1993). A further decrease of the N_2 concentration (at $D = 0.022$ h^{-1}) or increase of the dilution rate (at 5.1 kPa N_2) led to nitrogen starvation and wash-out of the culture (Table 1). A similar effect of the N_2 concentration was found for the photosynthetic bacterium *Rhodobacter capsulatus*, where the growth rate of Mo-grown cultures was maximal and remained constant from 13% to 100% N_2 in the gas phase, thus indicating N_2 saturation (Tsygankov and Laurinavichene 1993); below 13% N_2 a decrease in the growth rate indicated nitrogen limitation.

N. flagelliforme showed maximal H_2 production rates of 71–84 μmol H_2 mg chlorophyll a^{-1} h^{-1} (38–45 ml H_2 g dry weight^{-1} h^{-1}) under the optimized growth conditions of $D = 0.022$ h^{-1}, 34 °C, and 5 kPa N_2 in the culturing gas phase; this is more than four times higher than the maximal rates during batch growth. The maximal H_2 productivity of *N. flagelliforme*, calculated on a daily chemostat basis, was 27–32 mmol H_2 day^{-1} (670–800 ml H_2 day^{-1}). High rates of H_2 production were recently reported for *A. variabilis* under short-term batch growth conditions. However, this cyanobacterium was grown photoheterotrophically with 10 mM fructose in the medium (Reddy et al. 1996).

To our knowledge, the production of H_2 by continuously growing, autotrophic cultures of cyanobacteria has not previously been studied systematically. Greatly increased H_2 production rates could be achieved through controlled and optimized growth chemostat conditions – thus demonstrating the consistency and stability of such a system.

Acknowledgements This work was supported by the Research Institute for Innovative Technology for the Earth (RITE), Tokyo, Japan. We would like to thank Dr. A. Vonshak (Desert Research Institute, Ben Gurion University, Israel) for his excellent advice during this research.

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