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Ultrastructure of the Cyanobacterium *Nostoc muscorum* and Exploitation of the Culture for Hydrogen Production

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ABSTRACT. An effect of various physico-chemical parameters on nitrogenase-catalyzed oxygen-free hydrogen production by *Nostoc muscorum* was demonstrated. More hydrogen was produced in the light than in the dark. Optimum temperature was 40 °C. Various sugars increased hydrogen production whereas on easily metabolized nitrogen sources it was inhibited. The production was sensitive to salinity and Fe^{3+}, Cu^{2+}, Zn^{2+} and Ni^{2+} ions. Ultrastructural study revealed many electron-dense layers outside the cell-wall area that have not been observed earlier.

The present technological development is based on energy production from fossil sources. Acid rain, depleted ozone layer and shortage of fossil fuels are some of the many concerns resulting from this fact. To meet the future demand for energy, research is focused on a wide range of renewable energy sources (*see*, *e.g.*, Madihah *et al*. 2001; Verma and Madamwar 2002). One environmentally acceptable alternative fuel, which could conceivably supplement or even substitute fossil fuel, is molecular hydrogen. Its advantages are numerous: it is clean, efficient, renewable, and generates no toxic by-products as it can be produced by water decomposition (Hansel and Lindblad 1998).

Microbial production of hydrogen would be non-polluting and more cost-effective than non-biological hydrogen production. Of the microorganisms that produce hydrogen, cyanobacteria offer numerous advantages in terms of cheaper inputs and simpler mass cultivation. However, the development of hydrogen production technology by cyanobacteria, is hindered by, *e.g.*, inhibition of the relevant enzymes by oxygen; hydrogen consumption by uptake hydrogenase and low productivity (Rao and Hall 1996). Many attempts have been made to eliminate these obstacles (Madamwar *et al*. 2000) but an ideal organism with continuous production and an optimal set of conditions for maximum production have not been identified.

In the search for hydrogen producing cyanobacteria, *Nostoc* sp., showing ability to produce hydrogen, was found (Dawar *et al.* 1999; Shah *et al.* 2001). Here we examine the cell structure and various physico-chemical factors relevant to maximum hydrogen production.

MATERIALS AND METHODS

Culture conditions. The axenic culture of the filamentous, heterocystous cyanobacterium *Nostoc muscorum* SPU 005 was maintained in Arnon's (AA) medium (Arnon *et al*. 1974) at 25 ± 1 °C, 16-h light (light intensity 3 klx) and 8-h dark cycles, and transferred into fresh medium every fortnight. The culture was isolated from the Hamisar Pond of Bhuj (Gujarat, India; Shah *et al.* 2000). For hydrogen production studies, the culture was initially grown in AA medium for 5 d with KNO_3 as nitrogen source and then in AA combined nitrogen-free medium for 5 d at 30 ± 1 °C, 16-h light (3 klx) and 8-h dark cycles. The cultures were centrifuged, the cells washed with nitrogen-free medium and resuspended in fresh nitrogen-free medium unless otherwise stated. The results presented are means of five replicates.

Scanning electron microscopy. A 5-d-old culture grown in nitrogen-containing medium was collected and fixed with 2 % glutaraldehyde for 1.5 h at 4 °C. The fixed cells were washed with 0.1 mol/L phosphate buffer (pH 7.2) and then post-fixed for 2 h in 1 % osmium tetroxide in the same buffer at 4 °C. After a few washes in 0.1 mol/L phosphate buffer, the culture was dehydrated in graded acetone solutions. Critical-point drying was done using liquid CO_2 (*Polaron* Jumbo apparatus) and gold sputter coating was carried out under reduced pressure in an inert argon atmosphere (*Balzer* SCD 020 sputter device).

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After sputter coating the culture was examined under scanning electron microscope (*Philips* 501B) operated at 15 kV.

Transmission electron microscopy. Filaments were fixed, washed with cold 0.1 mol/L phosphate buffer (pH 7.2) after fixation, post-fixed and were dehydrated as above. They were then embedded in CY 212 araldite. Ultrathin sectioning of the filaments was done on an Ultracut E (*Reichert*, Austria) microtome. The sections were stained in ethanolic uranyl acetate and lead citrate and viewed under a transmission electron microscope (*Philips* CM 10) operated at 60 kV.

Absorption spectra. The filaments were grown in a medium with or without nitrogen for 5 d and pigments were extracted with 80 % acetone. Absorption spectra were measured in a *Hewlett-Packard* diode array UV-vis spectrophotometer (model 8452A).

Hydrogen assay. Vials (13-mL capacity) containing 4 mL bacterial suspension were sealed gas-tight, bubbled with argon for 1 min and incubated under different conditions for 1 d and the gas phase was analyzed on a *Sigma* (*Baroda*, India) gas chromatograph fitted with a thermal conductivity detector and molecular sieve 13X column. The column temperature was maintained at 50 °C while the detector and injector temperature were kept at 75 °C. Argon was used as carrier gas.

Nitrogenase activity. Acetylene reduction was assayed according to Turner and Gibson (1980). From the argon-flushed cultures in vials, 10 % gas phase was withdrawn and replaced with the same quantity of acetylene and the vials were incubated under selected conditions. The reaction was stopped with 0.2 mL of 20 % trichloroacetic acid. The gas phase was analyzed for ethylene using the same *Sigma* gas chromatograph fitted with a flame ionization detector and Porapak Q column with nitrogen as carrier gas. The column, injector and detector temperatures were 60, 65 and 85 °C, respectively.

Dry mass. The cultures were centrifuged (10 000 ***g***, 10 min), the pellet washed with distilled water and transferred to preweighed aluminum cups and dried to constant mass.

RESULTS AND DISCUSSION

Strain morphology and absorption spectra. Fig. 1A,B shows the ultrastructure of the two-week-old organism while Fig. 1C shows ultrastructure of a one-month-old culture. Fig. 2 shows the external morphology of the organism. For exponentially growing cells, the cell wall is four-layered and consists of a sheath, outer membrane, peptidoglycan layer and cell membrane (Fig. 1A). The sheath layer in this organism may be the reason for the shiny appearance of the filaments when observed under SEM (Fig. 2). The cytoplasm of two cells is separated by a single-layered wall (Fig. 1B) but the cells are seen to be connected at a number of points. The thylakoid membrane system radiates from various areas around the cell periphery and in the central region and is closely spaced (Fig. 1A,B). Many dark inclusion bodies are also seen within the cells (Fig. 1A). The photographs of a 1-month-old culture show some changes in the cell structure. The micrograph shows electron-transparent granules surrounded by thylakoids (Fig. 1C). These granules may either be storage granules (Carr 1966) or interthylakoidal spaces (Komárek and Cepák 1998). Also many electron-dense layers are seen outside the cell-wall area (Fig. 1C). The reason for such dense layers is unclear and peculiar as they have not been previously observed on such dense layers around vegetative cells growing in a nitrogen-sufficient medium.

The absorption spectrum of the acetone extract from cells grown in the presence of nitrogen (Fig. 3) is quite similar (except for the peak around 658 nm) to that from cells grown in nitrogen-deficient medium. The A_{620}/A_{680} ratios remained the same in both extracts, indicating that phycocyanin present in phycobilisomes is not degraded during nitrogen starvation (cf. Prasanna *et al*. 2003). Absorbance around 480 nm can be attributed to the presence of carotenoids in the culture (Sinha *et al*. 1995).

Physico-chemical parameters affecting H_2 production. Influence of dark-light regime, temperature and pH. Maximum hydrogen production was observed under continuous light with no dark phase (Table I), and decreased with prolongation of the dark phase. The amount of oxygen produced due to photosynthesis was negligible in all vials incubated in the light irrespective of the duration of exposure. Under total light conditions in argon-flushed vials, maximum H_2 production was seen at 40 °C. There was no significant effect of pH 6–9.

Influence of oxygen. Maximum H_2 production was obtained only in total dark conditions in the absence of O_2 (9 nmol H_2 per h per mg dry mass). The production decreased with increasing O_2 concentration. At 0.5 and 1.0 % O_2, 7.8 and 6.4 nmol H_2 were produced, respectively. With 2 and 2.5 % O_2, the production of H_2 further decreased (5.9 and 4.2 nmol, respectively).

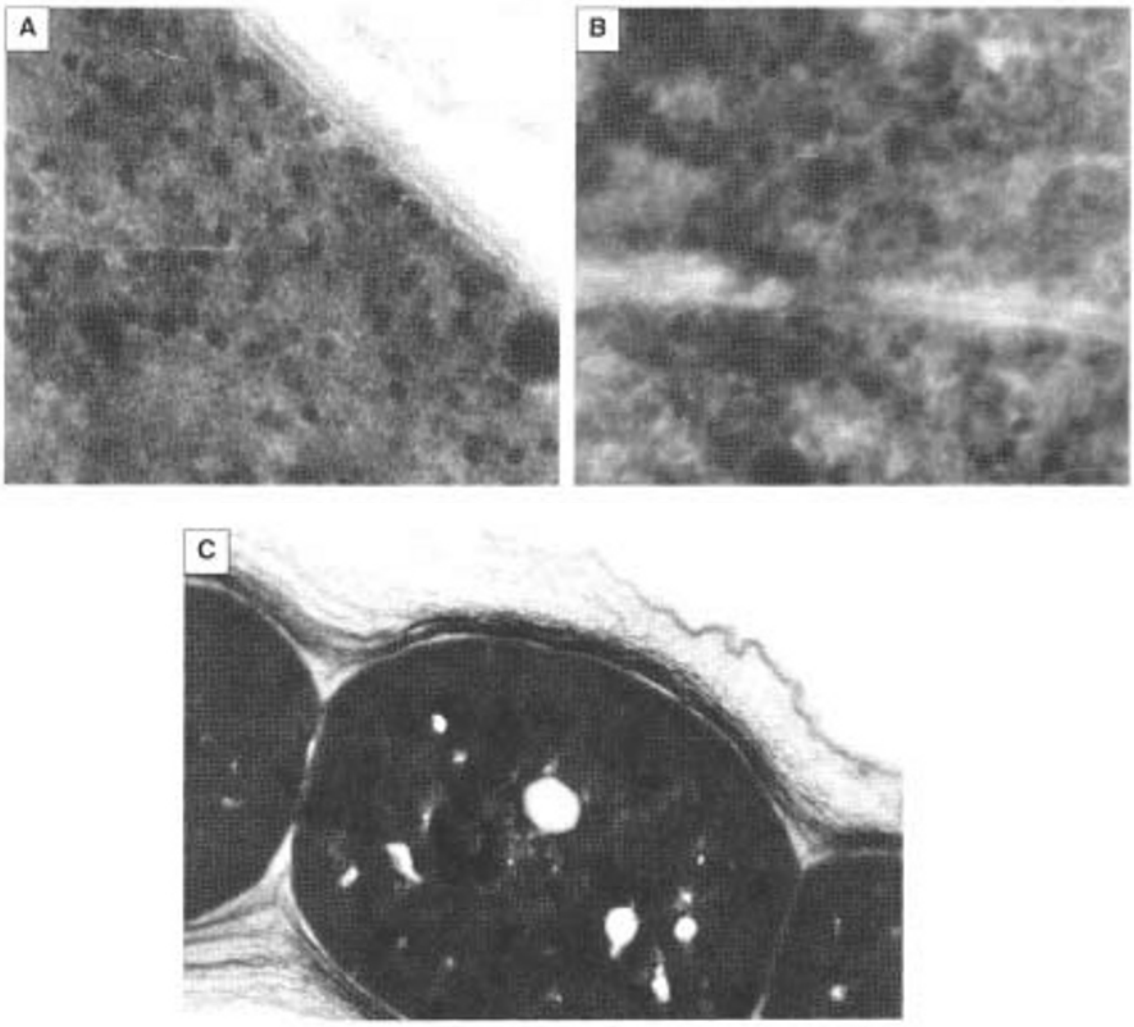

Fig. 1. Transmission electron micrograph of *N. muscorum*. **A**: a 14-d-old culture showing four-layered cell wall, dark inclusion bodies and arrangement of thylakoid membranes in cytoplasm; ×115 000; **B**: a 14-d-old culture showing unilayered wall separating the two cells; ×115 000; **C**: a 30-d-old culture showing the multilayered electron-dense-cell wall and electron-transparent granules in the cell; ×17 000.

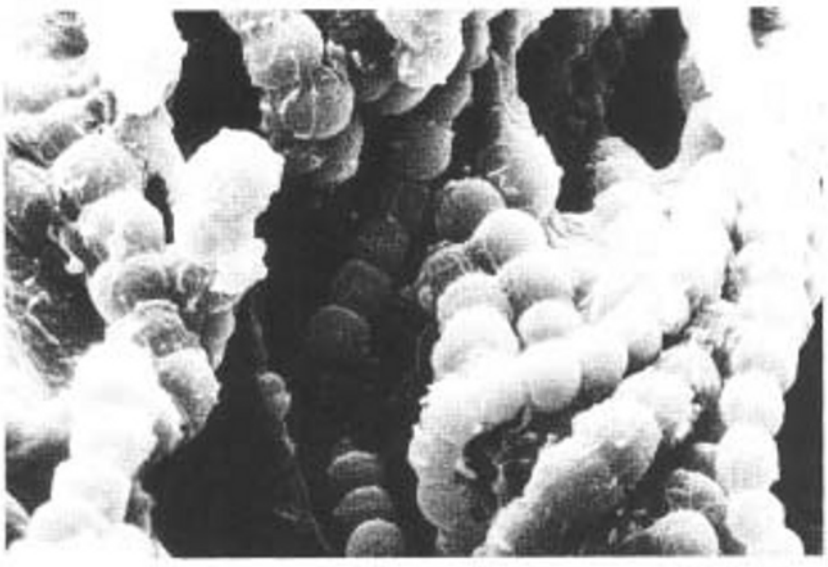

Fig. 2. Scanning electron micrograph of filaments of a fortnight-old *N. muscorum*; ×3000.

Nitrogenase activity. Nitrogenase was found to be active both in the light and in the dark, the activity in the dark being slightly higher (Fig. 4). Although nitrogenase is active similarly in the light and in the dark the reason for a higher production in the light remains unclear. The decrease in hydrogen production with increasing oxygen concentration reflects the oxygen sensitivity of the enzyme.

Influence of carbon source. Hydrogen production was observed with almost all sugars at 10 mmol/L. Glucose increased it by more than five-fold and maltose and sucrose nearly three-fold comparing to cultivation without any carbon source.

Influence of nitrogen source. All amino acids supplemented (10 mmol/L) decreased the hydrogen production with the maximum inhibition by cysteine and glutamic acid (Table II). Amino acids used as nitrogen source in cyanobacteria inhibited also nitrogenase activity in *Anabaena doliolum* (Rai and Abraham 1995). Hydrogen production was also suppressed by nitrite, nitrate and ammonium ions.

Effect of salinity and micronutrients. Hydrogen production decreased with increasing concentration of sodium ions (Table III). Other metal ions (10 mmol/L) inhibited it in the sequence $Fe^{3+} > Cu^{2+} > Zn^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+}$. As a freshwater cyanobacterium, *N. muscorum* is susceptible to sodium concentration in the absence of a nitrogen source. This may be attributed to the diversion of energy and reductants for extrusion of Na^+ from the cells or preventing Na^+ influx (Tel-Or and Melhamed-Harel 1981), increased respiration and cytochrome-oxidase activity (Lefort-Tran *et al.* 1988), or increased membrane permeability of heterocysts (Thomas and Apte 1984). Excessive external Na^+ causes a sharp decline in nitrogenase activity (Moore *et al*. 1985) and the same may hold for the other metal ions.

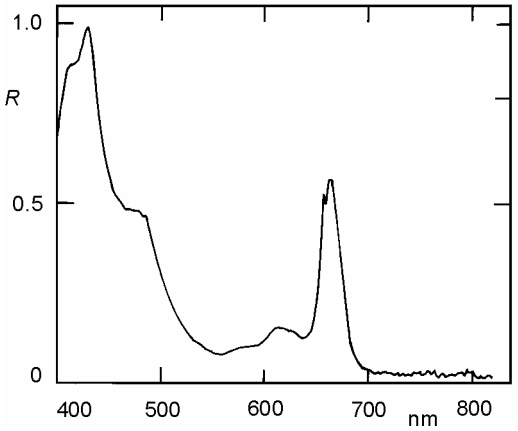

Fig. 3. Absorption spectra (wavelength, nm) of *N. muscorum* grown in the presence of nitrogen source; *R* – detector response, relative units.

Table I. Effect of dark/light periods and temperature on hydrogen production by *N. muscorum*

Light/dark[a] h	Hydrogen production[b]	Temperature[c] °C	Hydrogen production[b]
24/0	9.5	25	1.9
20/4	9.4	30	3.7
16/8	9.2	35	7.6
12/12	8.5	40	9.3
8/16	7.8	45	6.9
4/20	7.3	50	0
0/24	6.3		

[a]Incubated at 40 °C for 1 d.
[b]nmol H_2 per h per mg dry mass.
[c]Incubated for 1 d under constant light.

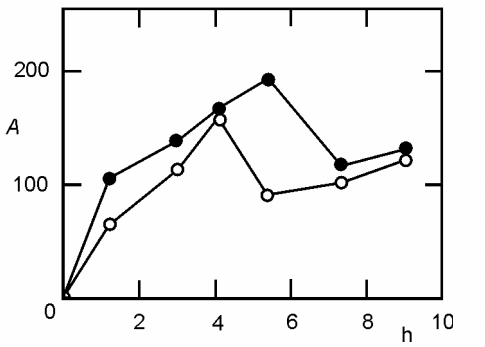

Fig. 4. Nitrogenase activity (*A*, nmol ethylene produced per mg dry mass; Turner and Gibson 1980) of *N. muscorum* in the presence (*closed symbols*) and absence (*open symbols*) of light.

N. muscorum produces hydrogen and can utilize organic compounds as electron donors. This ability can be used for wastewater treatment and simultaneous H_2 production, at higher temperatures it may be of use in various applications especially in warm regions.

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Table II. Effect of carbon and nitrogen sources on hydrogen production by *N. muscorum*[a]

Carbon source	Hydrogen production[b]	Nitrogen source	Hydrogen production[b]
None[c]	9.12	None[c]	9.12
Glucose	46.0	Isoleucine	4.19
Maltose	25.9	Glycine	3.36
Sucrose	21.5	Alanine	2.52
Mannose	16.7	Methionine	2.37
Fructose	12.0	Leucine	2.28
Sodium pyruvate	5.48	Threonine	2.07
Sodium acetate	4.18	Tryptophan	1.71
Trisodium citrate	3.36	Glutamic acid	1.10
$NaHCO_3$	1.67	Cysteine	0
Na_2CO_3	0.80	NO_2^-	4.54
		NO_3^-	0.78
		NH_4^+	1.99

[a]Incubated at 40 °C for 1 d under constant light.
[b]nmol H_2 per h per mg dry mass.
[c]Control.

Table III. Effect of NaCl (0–200 mmol/L) and metal ions (10 mmol/L) on hydrogen production by *N. muscorum*[a]

NaCl	Hydrogen production[b]	Metal ion	Hydrogen production[b]
0[b]	9.12	None[b]	9.12
25	8.85	Mn^{2+}	4.53
50	8.72	Co^{2+}	4.02
100	8.11	Ni^{2+}	2.51
200	7.00	Zn^{2+}	2.49
		Cu^{2+}	2.42
		Fe^{3+}	0

[a,b,c]*See* respective footnotes in Table II.

REFERENCES

ARNON D.L., MCSWAIN B.D., TSUJIMOTO H.T., WADA K.: Photochemical activity and components of membrane preparation from blue green algae. *Biochim.Biophys.Acta* **357**, 231–245 (1974).

CARR N.G.: The occurrence of poly-β-hydroxybutyrate in the blue green alga, *Chlorogloea fritschii*. *Biochim.Biophys.Acta* **120**, 308–310 (1966).

DAWAR S., MOHANTY P., BEHERA B.K.: Sustainable hydrogen production in the cyanobacterium *Nostoc* sp. ARM 411 grown in fructose- and magnesium sulfate-enriched culture. *World J.Microbiol.Biotechnol.* **15**, 289–292 (1999).

DREWS G.: Fine structure and chemical composition of the cell envelopes, pp. 99–117 in *The Biology of Blue Green Algae* (N.G. Carr, B.A. Whitton, Eds). Blackwell, Oxford –London 1973.

HANSEL A., LINDBLAD P.: Towards optimization of cyanobacteria as biotechnologically relevant producers of molecular hydrogen, a clean and renewable energy source. *Appl.Microbiol. Biotechnol*. **50**, 153–160 (1998).

KOMÁREK J., CEPÁK V.: Cytomorphological characters supporting the taxonomic validity of *Cyanothece* (*Cyanoprokaryota*). *Plant Syst.Evol*. **210**, 25–39 (1998).

LEFORT-TRAN M., POUPHILE M., SPATH S., PACKER L.: Cytoplasmic membrane changes during adaptation of the fresh water cyanobacterium *Synechococcus* 6311 to salinity. *Plant Physiol*. **87**, 767–775 (1988).

MADAMWAR D., GARG N., SHAH V.: Cyanobacterial hydrogen production. *World J.Microbiol.Biotechnol*. **16**, 757–767 (2000).

MADIHAH M.S., ARIFF A.B., KHALIL M.S., SURAINI A.A., KARIM M.I.A.: Anaerobic fermentation of gelatinized sago starch-derived sugars to acetone–1-butanol–ethanol solvent by *Clostridium acetobutylicum*. *Folia Microbiol*. **46**, 197–204 (2001).

MOORE D.J., REED R.H., STEWART W.D.P.: Responses of cyanobacteria to low-level osmotic stress: implication for the use of buffers. *J.Gen.Microbiol.* **131**, 1267–1272 (1985).

PRASANNA R., PRASANNA B.M., MOHAMMADI S.A., SINGH P.K.: Evaluation of *Tolypothrix* germplasm for phycobiliprotein content. *Folia Microbiol*. **48**, 59–64 (2003).

RAI A.K., ABRAHAM G.: Relationship of combined nitrogen sources to salt tolerance in freshwater cyanobacterium *Anabaena doliolum*. *J.Appl.Bacteriol*. **78**, 501–506 (1995).

RAO K.K., HALL D.O.: Hydrogen production by cyanobacteria: potential problems and prospects. *J.Mar.Biotechnol*. **4**, 10–15 (1996).

SHAH V., GARG N., MADAMWAR D.: Record of the cyanobacterial flora present in the Hamisar pond of Bhuj, India. *Acta Bot.Malacitana* **25**, 175–180 (2000).

SHAH V., GARG N., MADAMWAR D.: Ultrastructure of fresh water cyanobacterium *Anabaena variabilis* SPU 003 and its application for oxygen-free hydrogen production. *FEMS Microbiol.Lett*. **194**, 71–75 (2001).

SINHA R.P., LEBERT M., KUMAR A., KUMAR H.D., HADER D.P.: Spectroscopic and biochemical analysis of UV effects on phycobiliproteins of *Anabaena* sp. and *Nostoc carmium*. *Bot.Acta* **108**, 87–92 (1995).

TEL-OR E., MELHAMED-HAREL H.: Adaptation to salt of photosynthetic apparatus in cyanobacteria, pp. 455–462 in *Photosynthesis* (G. Akoyunoglou, Ed.). Bablan International Science Services, Philadelphia (USA) 1981.

THOMAS J., APTE S.K.: Sodium requirement and metabolism in nitrogen-fixing cyanobacteria. *J.Biosci*. **6**, 771–794 (1984).

TURNER G.L., GIBSON A.H.: Measurement of nitrogen fixation by indirect means, pp. 111–138 in *Methods for Evaluating Biological Nitrogen Fixation* (F.J. Bergersen, Ed.). John Wiley and Sons, New York 1980.

VERMA P., MADAMWAR D.: Decolorization of synthetic textile dyes by lignin peroxidase of *Phanerochaete chrysosporium*. *Folia Microbiol*. **47**, 283–286 (2002).