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STUDIES ON THE OPTIMIZATION OF CULTURAL CONDITIONS FOR MAXIMUM HYDROGEN PRODUCTION BY SELECTED CYANOBACTERIA

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ABSTRACT

Maximum production of hydrogen by the cyanobacterial isolates was obtained with 0.3 per cent CO_2, 2 per cent, N_2, pH 7.5, incubated under 3500 lux light intensity with 16:8 h dark: light cycle at 27°C. Supplementation of photo system II inhibitor DCMU enhanced the hydrogen production when incubated with longer light duration. Supplementation of iron in the growth medium enhanced the hydrogen production in *Anabaena* - TE1 and *Nostoc* - TE1, whereas supplementation of both nickel and iron increased the hydrogen efficiency of *Fischerella* - TE1. Sulphur stress had a pronounced effect on the production of hydrogen that it sustained higher production rate under 8:16 h dark: light cycle and highest production was recorded in *Anabaena* - TE1 (26.28 mL g^{-1} dry wt. L^{-1}).

Keywords: bio-hydrogen, cyanobacteria, culture conditions standardization.

INTRODUCTION

Molecular hydrogen is widely regarded as the energy carrier of the future. It can be used in fuel cells for generation of electricity. The electricity generated can be employed to drive engines and fuel cell engines, which are thought to be a future replacement for combustion engines. The main benefit of this type of engine is that the only exhaust fume is water. Nearly 90 per cent of hydrogen at present is obtained by the reaction of natural gas or light oil with steam at high temperature. Coal gasification and electrolysis of water are other industrial methods for hydrogen production.

These industrial methods mainly consume fossil energy sources and sometime hydroelectricity. In order to make use of the hydrogen powered fuel cells as a real environmental advantage, the hydrogen used should come from a renewable source and no pollution should be generated in the production. Photo production of hydrogen gas by eukaryotic micro algae is of keen interest as they posses the enzyme hydrogenase (Ghirardi *et al*., 2000). Since, the pioneering discovery by Gaffron and Co-workers over 60 years ago (Gaffron, 1939), the ability of unicellular green alga *Scenedesmus* to produce hydrogen gas upon illumination has been mostly a biological curiosity. Cyanobacteria have been implicated in the photo biological hydrogen production due to the fact that they produce molecular hydrogen using water as their ultimate electron substrate and solar energy as an energy source (Markov *et al*., 1995). Cyanobacteria could produce hydrogen gas as a by-product during nitrogen fixation with nitrogenase (Bothe *et al*., 1978) and also with hydrogenase (Kondratieva, 1983). Immobilization of cyanobacteria in polyurethane foam stabilized PS I and PSII photo activities in *N. muscorum* with enhanced hydrogen evolution (Hall *et al*., 1985). It has been suggested by several investigators that, the most suitable candidate for the development of an environment acceptable technology for hydrogen production was cyanobacteria (Bothe and Kentemich, 1990) as it holds the promise of generating a renewable fuel from nature's most plentiful resource, light and water. There are conditions under which the efficiency for hydrogen production can be increased. Further optimization of the process, standardization of optimal conditions for hydrogen production and strain improvement are considered as feasible options to make the technology viable. It is in this background, the present investigation was undertaken to select suitable cyanobacterial cultures for maximum hydrogen production and to optimize all the cultural conditions under *in vitro* conditions by the cyanobacteria by standardizing one by one and pooling all the conditions to have maximum production.

MATERIALS AND METHODS

Screening of algal isolates for hydrogen production

Hydrogen production was estimated both in cell pellet and in suspension culture. A total of 50 different cyanobacterial cultures and one green alga *Chlorella* sp maintained at Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore were screened for hydrogen production. The hydrogen evolution from cell pellet and suspension culture was estimated and found to be same in both the preparations. Hence; further experiments were carried with exponential phase suspension cultures.

Estimation of hydrogen production

Hydrogen evolution was measured using a Gas Chromatograph (Schimadzu - 14B) fitted with thermal conductivity detector (TCD) and Poropak - Q column. Nitrogen was used as the carrier gas with a flow rate of 20 mL min^{-1}. The temperature settings were

Column temperature - 60°C
Injector temperature - 90°C and

Detector temperature - 100°C (Fibler, 1995)

The hydrogen gas evolved was estimated using the formula

$$\text{Amount of hydrogen evolved (mL g}^{-1}\text{ dry weight h}^{-1}\text{)} = \frac{\text{Peak area for the sample x amount of gas injected into GC x attenuation}}{\text{Peak area of the standard x hours of incubation x dry weight of sample (g)}}$$

Estimation of hydrogen production under different growth conditions

`Different factors influencing hydrogen production under *in vitro* conditions were studied with the selected cyanobacterial isolates *Anabaena* - TE1, *Nostoc* - TE1, *Fischerella* - TE1 and the green alga *Chlorella*-TE1 for optimizing the conditions for enhanced hydrogen production. Gas samples were withdrawn at regular intervals for 48 h from the cyanobacterial isolates and for 32 h for the green algal isolate and were analyzed in GC for hydrogen production.

Effect of varied levels of carbon-di-oxide on hydrogen production

The cultures were grown separately in 150 mL serum vials containing respective medium (pH 7.5) to exponential phase. Argon gas (50%) was flushed into vials and sparged with CO_2 gas at 0.1, 0.3, 0.5 and 1 per cent level on 25th day. The vials were incubated in cabinets with fluorescent light (3000 lux, 23^{o}C and 16:8 h light: dark). Gas samples were withdrawn and analysed.

Effect of varied levels of nitrogen on hydrogen production

Two per cent homogenized cyanobacterial isolates were inoculated separately in N-free BG-11 medium (pH 7.5). N_2 gas at selected levels *viz*., 1, 2, 5 and 10 per cent and argon (50%) were sparged into the vials on 25th day. CO_2 at selected level from the previous experiment (0.3%) was sparged. Gas samples were withdrawn and analysed.

Effect of light intensity on hydrogen production

The exponential phase algal cultures were sparged with selected levels of argon /CO_2/N_2 and incubated under three different light intensities *viz*., 3000 lux, 3500 lux and 4000 lux continuously for 16:8 h light: dark cycle at 23°C (Prabaharan and Subramanian, 1996). Gas samples were withdrawn and analysed.

Effect of pH on hydrogen production

Samples were prepared with various pH levels *viz*., 5.5, 6.5, 7.5, 8.5 and 9.5 buffered with 2x10^{-4} M phosphate buffer. Cultures were sparged with selected levels of argon/CO_2/N_2 and incubated under optimum light intensity of 3500 lux (Jeffries *et al*., 1978). The gas samples were analyzed in GC for hydrogen production.

Effect of temperature on hydrogen production

With optimum levels of gases, pH and light intensity from previous experiments, three different temperature regimes *viz*., 23, 27 and 30°C were tested for hydrogen production **(**Miyamoto *et al*. 1979**)**. The gas samples were analyzed in GC for hydrogen production.

Effect of varying photoperiod on hydrogen production

Different dark: light durations tested were 0:24h, 3:21h, 8:16h, 12:12h, 16:8h, 24:0h (Prabaharan and Subramanian, 1996) for a week. The co-production of oxygen at varied photoperiod was estimated at the end of the experimental period by injecting a known quantity of gas sample in GC.

Effect of DCMU on hydrogen production

To study the effect of photo system II inhibitor DCMU [3- (3,4 dichlorophenyl)-1,1 dimethyl urea] on hydrogen production, 2x10^{-2}M DCMU was prepared using ethanol as solvent and added to N-free BG-11 medium to bring the final concentration to 2x10^{-5} M. The optimum conditions obtained from the above mentioned factors were set and hydrogen gas production at different light intensities was measured in GC (Miyamoto *et al*., 1979).

Effect of metal ions on hydrogen production

Nickel and iron each 2 mg L^{-1} and 3 mg L^{-1} were supplied to N-free BG-11 medium as nickel chloride and ferric chloride with optimum growth conditions for maximum hydrogen production obtained with the above experiments and hydrogen production was analyzed in GC at regular intervals (Markov *et al*., 1995).

Effect of fixed nitrogen on hydrogen production

To examine the effect of addition of fixed nitrogen on H_2 production, ammonium chloride and sodium nitrate were added separately at concentrations of 0.2 mM NH^{+}_{4} and 0.2 mM NO^{-}_{3} with all the optimum conditions obtained from previous experiments and incubated. Gas samples were withdrawn at regular intervals and analyzed in GC for hydrogen production (Markov *et al*., 1995). (The experiment was carried for cyanobacterial isolates only).

Effect of oxygen concentration on hydrogen production

To assess the tolerance level of oxygen for hydrogen production by the algal isolates, under selected gaseous phase, oxygen was sparged at 1, 2, 3, 5 and 10 per cent levels to exponential phase cultures. The cultures

were incubated in growth chamber and H_2 production was measured in GC at regular intervals (Markov *et al*., 1995).

Effect of sulphur deprivation on hydrogen production

To study the effect of sulphur deprivation on hydrogen production, which is important for the completion of photosynthesis, the isolates were grown initially under 3000 lux light intensity, at 23°C with 16:8 h light:dark cycle. Then, the isolates were centrifuged at 5000 rpm for 5 minutes and resuspended in BG-11 medium free of sulphur nutrients (i.e.) without magnesium sulphate, zinc sulphate and copper sulphate. The isolates were incubated with selected optimum conditions obtained from previous experiments under two different light intensities. The gas sample was analyzed for hydrogen production and oxygen production in GC. Once the hydrogen production dropped the isolates were resuspended in medium containing sulphur for 2 days, again sulphur deprivation was given and gas sample was analysed in GC for hydrogen production (Ghirardi *et al*., 2000).

RESULTS AND DISCUSSIONS

Hydrogen is starting to move from a fuel of the future to an energy carrier of the present, promising greatly reduced pollution and increased fuel efficiencies. A major goal is the production of renewable hydrogen at affordable costs. The growth conditions of algae can be optimized to find the best balance between growth rate and hydrogen production at lower cost. The hydrogen production ability can vary greatly with species. More research has been under taken with heterocystous cyanobacteria and a few with non-heterocystous cyanobacteria (Markov *et al*., 1995). Among green algae, the widely studied one for hydrogen production is *Chlamydomonas reinhardtii* (Ghirardi *et a*l., 2000).

In the present study, without altering the growth conditions, the hydrogen production was estimated initially and it varied widely with cultures. The heterocystous *Anabaena*-TE1 and *Nostoc*-TE1 produced higher hydrogen compared to the non-heterocystous *Fischerella*-TE1 with a production of 4.60 and 4.30 mL g^{-1} dry wt. h^{-1}, respectively. The hydrogen production in non-heterocystous *Fischerella*-TE1 was low and was 3.00 mL g^{-1} dry wt. h^{-1}.

Gaseous atmosphere influencing hydrogen production

Cyanobacteria and algae use carbon di oxide for photosynthesis. Jones and Bishop (1976) discussed that, cyanobacterial cultures grown under limiting CO_2 conditions have hydrogen production rates proportional to their growth rates. Lambert and Smith (1981) observed maximum hydrogen production rate in *Anabaena cylindrica* under 3 per cent CO_2 in a gas phase balanced by argon. Semin *et al*. (2003) observed higher hydrogenase activity of *Chlamydomonas reinhardtii* with 3 per cent CO_2.

Maximum hydrogen production was recorded with 0.3 per cent CO_2 in a gas phase with 50 per cent argon. With increasing levels of CO_2 beyond 0.3 per cent, the hydrogen production started declining. The maximum average production recorded was 9.68 mL g^{-1} dry wt. h^{-1}in *Anabaena* - TE1, 5.02 mL g^{-1} dry wt. h^{-1}in *Fischerella* - TE1 and 10.35 mL g^{-1} dry wt. h^{-1} was recorded in *Nostoc* - T E1 (Table-1)**.** In non-heterocystous cyanobacteria, the reduction in hydrogen production with higher levels of CO_2 may be due to the inhibition of nitrogenase probably by competing for ATP and reductant (Houchins, 1984) thus reducing nitrogenase mediated hydrogen evolution.

Table-1. Effect of varying levels of CO_2 on the H_2 production (mL g^{-1} dry wt. h^{-1}) by the cyanobacterial isolates under *in vitro* conditions

Incubation time (h)	***Anabaena* -TE 1**					***Fischerella* -TE 1**					***Nostoc* -TE 1**				
	Carbon -di -oxide levels (%)														
	0	0.1	0.3	0.5	1	0	0.1	0.3	0.5	1	0	0.1	0.3	0.5	1
4	1.10	1.61	2.12	1.60	1.40	0.61	1.90	1.12	1.16	1.21	0.93	1.36	1.94	0.5	1.49
8	3.64	3.70	9.00	7.73	4.42	2.10	4.72	6.31	4.46	3.60	2.47	7.30	8.61	1.70	6.53
12	3.91	7.11	11.23	10.96	9.40	2.43	5.21	6.80	4.60	3.93	3.60	11.21	12.42	9.16	8.90
16	4.36	9.63	14.70	11.71	10.63	2.92	6.91	7.32	5.11	4.90	4.91	12.23	13.90	12.10	9.30
20	6.62	9.90	15.27	14.60	11.91	4.20	7.13	8.41	6.90	6.30	5.60	13.30	16.61	12.31	10.40
24	7.90	13.32	19.60	17.92	13.30	4.44	3.27	8.23	4.31	2.17	6.81	14.91	18.93	15.20	11.77
28	8.10	14.13	22.91	20.10	13.12	ND	0.62	9.20	3.62	1.40	10.30	22.40	23.40	17.32	15.30
32	2.41	16.14	14.30	13.20	7.21	ND	ND	7.51	2.10	ND	7.10	11.70	13.60	21.94	7.42
36	ND	7.90	7.10	6.10	ND	ND	ND	5.40	1.10	ND	ND	8.30	10.10	12.60	3.20
40	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	4.70	ND	ND
44	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
48	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Mean	3.17	6.90	9.68	8.66	5.94	1.39	2.48	5.02	2.78	1.96	3.47	8.56	10.35	9.31	6.19

(3000 lux, 23°C, pH 7.5, 16:8 h light: dark, 50% argon)

	SEd	**CD** (0.05%)
C (Cultures)	0.04	0.08
L (CO_2 levels)	0.09	0.17
H (Incubation time)	0.05	0.11
C x L	0.09	0.19
L x H	0.19	0.37
C x H	0.15	0.29
C x L x H	0.33	0.65

Next is the molecular nitrogen, which is the substrate for nitrogenase, inhibits nitrogenase mediated hydrogen production in some cyanobacteria (Lambert and Smith, 1977). The presence of nitrogen in the growth medium is essential for long-term hydrogen production since, it is necessary for nitrogen fixation and thus ultimately for cell metabolism. (Bothe and Kentemich, 1990). In the present investigation, all the three cyanobacterial isolates recorded maximum production with 2 per cent N_2 (Table-2)**.** Kondratieva (1983) observed increased hydrogen production in *Anabaena cylindrica* upto 1 per cent N_2 and complete inhibition with 15 per cent N_2. In *Mastigocladus laminosus* maximum hydrogen production was observed with 0.7 per cent N_2 (Miyamoto *et al*., 1979). Again, gas phase nitrogen level for maximum hydrogen production varies with cultures.

Table-2. Effect of varying levels of N_2 on the H_2 production (mL g^{-1} dry wt h^{-1}) by the cyanobacterial isolates under *in vitro* conditions.

Incubation time (h)	***Anabaena* -TE 1**					***Fischerella* -TE 1**					***Nostoc* -TE 1**				
	Nitrogen levels (%)														
	0	1	2	5	10	0	1	2	5	10	0	1	2	5	10
4	2.06	2.41	2.63	2.70	2.26	0.82	1.36	1.66	1.32	1.0	2.01	2.00	2.13	1.60	1.82
8	8.60	7.33	9.80	9.72	8.41	2.40	5.70	7.11	4.21	3.63	8.73	9.31	9.40	9.12	8.64
12	11.51	11.70	12.14	12.60	11.30	3.11	6.92	8.30	5.20	7.91	12.60	12.70	14.64	10.30	11.31
16	14.42	14.81	15.96	14.73	12.68	3.61	7.30	9.11	6.91	4.80	13.27	14.32	14.90	12.67	13.20
20	15.50	16.02	16.30	15.90	14.47	4.50	7.97	9.90	7.30	2.11	16.65	16.73	17.37	17.20	15.13
24	19.34	19.00	20.41	21.11	19.71	5.13	9.01	10.62	9.40	ND	18.40	15.96	18.60	17.69	14.60
28	23.13	23.40	24.60	23.02	16.30	3.72	9.14	11.40	9.33	ND	24.01	24.10	24.96	20.40	20.32
32	14.70	14.74	17.15	16.30	14.62	ND	10.40	13.63	8.10	ND	14.20	16.71	18.10	23.61	21.30
36	6.80	12.30	15.30	12.11	11.30	ND	8.71	9.11	4.70	ND	9.30	14.60	14.31	20.40	15.31
40	ND	ND	12.40	9.30	6.70	ND	5.20	3.40	2.40	ND	6.70	12.14	12.02	14.12	7.30
44	ND	ND	8.30	6.20	ND	ND	3.20	2.10	ND	ND	ND	6.30	9.60	7.30	2.40
48	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	4.20	2.60	ND
Mean	9.67	10.14	12.92	11.97	9.81	1.94	6.24	7.19	4.91	1.62	10.49	12.07	13.36	13.08	10.94

(3000 lux, 23°C, pH 7.5, 16:8 h light: dark, 50% argon, 0.3% CO_2)

	SEd	**CD** (0.05%)
C (Cultures)	0.05	0.09
L (N_2 levels)	0.06	0.12
H (Incubation time)	0.09	0.18
C x L	0.11	0.21
L x H	0.21	0.41
C x H	0.16	0.32
C x L x H	0.36	0.71

Enzymes involved in hydrogen production are so sensitive to oxygen that even 1 per cent of oxygen has been reported to arrest hydrogen production. (Philips and Mitsui, 1986). In this study, oxygen did not totally arrest the hydrogen evolution process even at 10 per cent in *Anabaena* - T E1, *Nostoc* - T E1 and *Chlorella* - T E1, indicating a certain degree of protection. In *Fischerella* - T E1 with 10 per cent oxygen, hydrogen evolution completely ceased. Prabaharan and Subramanian (1996) reported that in *Phormidium valderianum* 20 per cent oxygen did not totally arrest the process of hydrogen evolution. In *Anabaena* - TE1 and *Nostoc* - TE1 oxygen at one and two per cent did not significantly inhibit hydrogen evolution and in contrast, oxygen at and above concentrations of one per cent drastically reduced hydrogen evolution in the non-heterocystous *Fischerella* - T E1. Low oxygen tensions of one and two per cent did not inhibit hydrogen evolution in heterocystous *Anabaena cylindrica* and oxygen tensions of five and ten per cent inhibited hydrogen formation. In non-heterocystous *Oscillatoria* sp. oxygen concentrations above one per cent inhibited hydrogen production (Philips and Mitsui, 1983). Again, the oxygen tolerance for hydrogen production varied with organisms.

Light, pH and temperature

Though, biological hydrogen production by microorganisms has created much interest, commercial scaling-up requires the identification of suitable conditions for efficient and continuous production. Among the different factors influencing hydrogen production, light is primary in photosynthetic organisms. Light dependence of nitrogenase - mediated hydrogen production and a light intensity dependent increase in production have been documented in cyanobacteria (Asada *et al*., 1979; Spiller and Shanmugam, 1987). In the present investigation, all the three cyanobacterial isolates produced maximum hydrogen at 3500 lux. At higher light intensities, the hydrogen production decreased in all the algal isolates. Prabaharan and Subramanian (1996) showed higher hydrogen production in *Phormidium valderianum* at lower light intensity than at higher intensity. In contrast, Jeffries *et al.* (1978) reported increase in hydrogen production with increase in light intensity. The reduction in hydrogen production at intensity of 4000 lux in the present study might be due to the co-production of oxygen at higher light intensities as reported by Agar *et al*. (1991).

In the present study, with decreased light duration higher hydrogen evolution was recorded in *Anabaena* TE 1 and *Nostoc* - TE1, but, the hydrogen production decreased under 0:24h dark: light condition. In *Fischerella*

- TE 1 increase in hydrogen evolution with decrease in light duration was noticed (Table-3). Even though, 24:0 h dark : light condition produced higher hydrogen, 16:8 h dark: light cycle was chosen for further optimization in *Fischerella* - TE1 as the organism would not thrive under 24:0 h dark: light cycle. Sixteen hours of darkness followed by eight hours light gave the best hydrogen production, while 24 h light without a dark period resulted in minimum hydrogen in all the isolates. Oxygen co-production occurred in 0:24, 3:21, 8:16, 12:12 h dark: light cycle and other 16:8, 24:0 h dark: light durations failed to show oxygen co-production.

The absence of net release of oxygen is believed to be due to the total consumption of oxygen production under these intensities (Mitsui *et al*., 1979) and the absence of oxygen might be the reason for higher production at 16:8 h dark: light cycle. Prabaharan and Subramanian (1996) recorded 18:6 dark: light cycle as optimum condition for higher hydrogen evolution in *Phormidium valderianum* and noticed oxygen co-production only in 0:24 h, 3:21h dark: light cycle.

Table-3. Effect of varying photo periods on the hydrogen production (mL g^{-1} dry wt. h^{-1}) by the cyanobacterial isolates under *in vitro* conditions.

Incubation time (days)	*Anabaena* -TE 1						*Fischerella* -TE 1						*Nostoc* -TE 1					
	Dark : light durations (h)																	
	0:24	3:21	8:16	12:12	16:8	24:0	0:24	3:21	8:16	12:12	16:8	24:0	0:24	3:21	8:16	12:12	16:8	24:0
1	18.42	17.36	22.31	23.91	26.8	27.91	ND	4.63	10.41	11.93	14.09	14.12	12.12	16.34	21.30	26.02	25.53	28.11
2	21.41	21.67	27.91	29.60	33.12	34.24	ND	9.12	17.14	18.32	21.3	22.76	14.30	17.70	29.64	29.13	28.44	30.6
3	7.93	9.32	25.13	25.13	36.21	38.92	ND	3.78	7.73	9.60	11.31	16.14	7.63	8.32	27.10	30.41	30.72	34.70
4	ND	ND	19.74	18.64	33.20	21.60	ND	ND	ND	7.97	7.44	8.60	3.21	4.11	25.26	29.66	30.11	32.70
5	ND	ND	15.32	17.30	25.98	13.31	ND	ND	ND	ND	ND	ND	ND	ND	20.61	25.63	24.63	20.14
6	ND	ND	12.10	13.41	17.30	4.21	ND	ND	ND	ND	ND	ND	ND	ND	9.32	17.03	19.14	7.27
7	ND	ND	6.82	7.90	11.43	ND	ND	ND	ND	ND	ND	ND	ND	ND	7.31	12.40	13.40	ND
Mean	6.82	6.91	18.58	19.41	26.28	20.03	0	2.50	5.04	6.82	7.73	8.80	5.32	6.64	20.08	24.32	24.57	21.93

(3500 lux, 27°C, pH 7.5, 0.3% CO_2, 2% N_2)

	SEd	**CD** (0.05%)
C (Cultures)	0.06	0.12
L (dark: light durations)	0.09	0.17
D (Incubation time)	0.09	0.18
C x L	0.15	0.29
L x D	0.23	0.45
C x D	0.16	0.32
C x L x D	0.39	0.77

Reports on the optimum temperature and pH for hydrogen production suggest variation with the organism studied (Ernst *et al*., 1979; Miura *et al*., 1980). In the present study, the rate of hydrogen production varied widely at different pH levels of 5.5,6.5,7.5,8.5 and 9.5 and all the three cyanobacterial isolates *viz.*, *Anabaena*-TE 1, *Fischerella*-TE 1 and *Nostoc*-TE 1 showed higher hydrogen evolution of 20.86, 9.80. and 21.69 mL H_2 g^{-1} dry wt h^{-1} respectively at near neutral pH of 7.5. The lower pH of 5.5, 6.5 and higher pH of 9.5 significantly reduced hydrogen evolution in all the algal isolates and the decrease in hydrogen photo production was much more pronounced at acidic than at alkaline pH. Similar finding was reported by Markov *et al.* 1995. The reduction in hydrogen evolution of higher pH might be due to the activation of uptake hydrogenase that functions optimally at pH 9 (Markov *et al*., 1995). Kosourov *et al*. (2003) showed reduction in hydrogen production in the green alga *Chlamydomonas reinhardtii* below 6.5 and above 8.2.Even though, optimum temperature for higher hydrogen production varied considerably with the organism, in this study, 27°C was found to be ideal for hydrogen production in all the cyanobacterial isolates. The alga, *Phormidium valderianum* produced higher hydrogen at 27°C as reported by Prabaharan and Subramanian, 1996.

Fixed nitrogen and DCMU

The presence of combined nitrogen in the growth medium inhibits nitrogenase synthesis and differentiation of heterocysts from vegetative cells, thereby affecting nitrogenase mediated hydrogen evolution in cyanobacteria. However, the effect of ammonium is reversible, and when this compound is assimilated by the cells, nitrogenase becomes active again.

In this study, addition of ammonical and nitrate nitrogen destabilized the system and suppressed hydrogen production, as compared with the control. Weissman and

Benemann (1977) reported maximum hydrogen production by *Anabaena cylindrica* after 1 to 2 days of nitrogen starvation and it can de concluded that high stable efficiencies are attained in the absence of nitrogenous addition as reported by Jeffries *et al*. (1978).

Miyamoto *et al.* (1979) reported 100 per cent stimulation of hydrogen activity in *Anabaena cylindrica* with addition of DCMU, which is a photosystem II inhibitor. Under anaerobic conditions in the presence of DCMU, accumulated reducing equivalents from the fermentative catabolism of the algae cannot be oxidized *via* respiration because of the absence of terminal electron acceptor (O_2) (Sazanov *et al.*, 1998). Inhibitor experiments have yielded evidence in support of a thylakoid membrane localized NAD (P) H reductase in *Chlamydomonas reinhardtii* (Godde and Trebst, 1980) suggesting that electrons derived upon the oxidation of endogenous substrate may feed into the plastoquinone pool. Thereafter, electrons are driven upon light absorption by PSII to ferredoxin, which is an efficient electron donor to the Fe hydrogenase that efficiently combines these electrons with protons to generate molecular hydrogen (Florin *et al.*, 2001).

In this study, in the experiments carried out with addition of DCMU under varied photoperiods, there was an increase in hydrogen production in all the algal isolates as the duration of photoperiod was increased. DCMU supplementation did not show significant increase or decrease in hydrogen production under 16:8 h and 24:0 h dark: light duration. Higher hydrogen production under longer light duration might be due to the availability of endogenous substrate for oxidation that generates electrons for feeding into plastoquinone pool (Florin *et al.*, 2001) through photosynthesis. Miyamoto *et al.* (1979) reported reduction in hydrogen production in *Mastigocladus laminosus* supplemented with DCMU, after depletion of endogenous reductant pools. Weissman and Benemann (1977) observed drastic reduction in oxygen evolution at all stages of hydrogen production by the addition of DCMU that could stimulate nitrogenase activity by eliminating the competition between nitrogenase and oxygen scavenging processes for the reductant. Florin *et al.* (2001) obtained high rates of H_2 production upon illumination of *Chlamydomonas reinhardtii* supplemented with DCMU.

Metal ions

Metal ions like zinc, nickel, manganese, magnesium, cobalt, iron, molybdenum, vanadium are activity involved in the stimulation of hydrogenase and nitrogenase activity (Asada *et al*., 1987). In the present study, nickel at 3 mg L^{-1} reduced hydrogen evolution in *Anabaena* - TE1 and *Chlorella* - TE1. Tredici *et al*. (1990) found reduction in net hydrogen production due to the enhancement of hydrogen uptake activity at higher concentrations of nickel. But, nickel at 3 mg L^{-1} enhanced hydrogen evolution in *Fischerella* - TE1. In *Nostoc* - TE1 nickel at both the concentrations did not show significant increase or decrease in hydrogen production suggesting, the optimum concentration of nickel for enhancing hydrogenase or uptake hydrogenase activity varied with organisms. Nickel is involved in several biological processes and low concentrations are required for the synthesis of active hydrogenase (Smith *et al*., 1992).

Hydrogen evolution by cyanobacteria depends on the supply of growing cultures with iron (Jeffries and Leach, 1978) and suggested an iron concentration of 5 mg L^{-1} in the medium is sufficient for active hydrogen production. In the present study, enhanced hydrogen production with 2 and 3 mg L^{-1} of iron was noticed in all the three algal isolates *viz*., *Anabaena* - TE1, *Fischerella* - TE1 and *Nostoc*- TE1

Sulphur

Sulphur is critical for the completion of normal photosynthesis and in the absence of the element; the algae ceased emitting oxygen and stopped storing energy as carbohydrates, protein and fats. Instead, the algal cells use an alternative metabolic pathway to exploit stored energy reserved anaerobically in the absence of oxygen. The hydrogenase was activated, splitting large amounts of hydrogen gas from water and release it as a by product (Melis and Happe, 2001). Imbalance in the photosynthesis - respiration relationship by sulphur deprivation resulted in net consumption of oxygen by the cells causing anaerobiosis in the growth medium, a condition that automatically elicited hydrogen production by the algae (Melis *et al*., 2000) and the expression of the Fe hydrogenase could be induced in the light, so long as anerobiosis is maintained within the culture (Ghirardi *et al*., 2000).

In the present study, temporal separation of photosynthesis and hydrogen production was carried out by growing the algal isolates in medium supplemented with sulphur. Then, sulphur deprivation was imposed carefully by transferring the cells to sulphur deprived medium. Compared to sulphur grown samples, marked increase in hydrogen production was noticed in all the algal isolates under sulphur deprived condition. Also, higher hydrogen production by sulphur deprived culture was noticed in isolates incubated under longer light duration whereas, sulphur grown isolates showed higher production with longer dark period (Table-4).

Table-4. Effect of sulphur deprivation at two different photoperiods on the hydrogen production (mL g^{-1} dry wt. h^{-1}) by the cyanobacterial isolates under *in vitro* conditions.

Incubation time (days)	**Dark: light: durations** (h)					
	***Anabaena* -TE 1**		***Fischerella* -TE 1**		***Nostoc* -TE 1**	
	8:16	16:8	8:16	16:8	8:16	16:8
1	40.12	7.21	20.36	4.21	41.36	5.26
2	51.36	3.26	23.46	1.16	52.24	2.19
3	55.21	ND	25.36	ND	56.78	ND
4	58.36	ND	27.36	ND	60.12	ND
5	52.17	ND	20.42	ND	58.36	ND
6	50.10	ND	15.36	ND	54.21	ND
7	45.36	ND	12.36	ND	42.36	ND
8	35.28	ND	7.20	ND	38.12	ND
9	-	-	-	-	-	-
10	-	-	-	-	-	-
11	38.41	ND	18.36	ND	45.20	ND
12	45.63	ND	14.95	ND	52.84	ND
13	54.91	ND	12.42	ND	55.26	ND
14	57.63	ND	9.32	ND	50.21	ND
15	51.24	ND	4.50	ND	46.72	ND
16	43.36	ND	-	ND	42.36	ND
Mean	48.65	0.65	15.10	0.33	49.73	0.47
Control mean	18.58	26.28	5.04	7.73	20.08	24.57

(3500 lux, 27°C, pH 7.5, 0.3% CO_2, 2% N_2)

	SEd	**CD** (0.05%)
C (Cultures)	0.21	0.41
L (Dark :light durations)	0.17	0.37
D (Incubation time)	0.39	0.79
C x L	0.29	0.58
L x D	0.56	1.12
C x D	0.69	1.37
C x L x D	0.97	1.93

Also, intermittent addition of sulphur in the growth medium sustained higher hydrogen production for longer period. Melis *et al.* (2000) observed sustained higher amount of hydrogen production in *Chlamydomonas reinhardtii* under sulphur deprived condition. The algae would die if the nutrient stress was maintained for more than a few days. They can be fattened with intermittent exposure to sulphur and sunlight, allowing for repetitions of the process and continued harvesting of hydrogen.

In this study, the hydrogen production by sulphur deprived algal isolates under 16:8 h dark: light cycle was negligible but gave promising results under 16:8 h light: dark cycle. Melis and Happe (2001) could observe hydrogen production in sulphur deprived *Chlamydomonas reinhardtii* in the light and not in the dark.

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REFERENCES

Agar J., S. Suda, H. Takeyama, W. Lee and A. Mitsui. 1991. Hydrogen and oxygen photoproduction by marine unicellular cyanobacterium under high light intensities equivalent to mid-day intensities. Paper presented in the 2nd International Marine Biotechnology Conference, Baltimore, USA. p. 75 (Abstr.).

Asada Y., K. Tonomura and O. Nakavama. 1979. Hydrogen evolution by an isolated strain of *Anabaena*. J. Ferment. Technol. 57: 280-286.

Asada Y., S. Kawamura and K.K. Ho. 1987. Hydrogen production by *Anabaena cylindrica*: Effect of metal ions. Soviet Plant Physiology. 26: 637.

Bothe H and T. Kentemich. 1990. Potentialities of H_2 production by cyanobacteria for solar energy conversion programs. In: Hydrogen Energy Progress VIII. Proc. 8th World Hydrogen Energy Conference, (eds.) T.N. Veziroglu and P.K. Takahash, USA. p. 729.

Bothe H., E. Distler and G. Eisbrenner. 1978. Hydrogen metabolism in blue-green algae. Biochimie. 60: 277-289.

Ernst A., W. Kerfin, H. Spiller and P. Boger. 1979. External factors influencing light - induced H_2 evolution by the blue-green alga *Nostoc muscorum*. Z. Natur. Achung. 34: 820-825.

Gaffron H. 1939. Reduction of CO_2 with H_2 in green plants. Nature. 143: 204-205.

Godde D and A. Trebst. 1980. NADH as electron donor for the photosynthetic membrane of *Chlamydomonas reinhardtii*. Arch. Microbiol. 127: 245-252.

Fibler M. 1995. Conditions for estimation of hydrogen production. Appl. Microbiol. Biotechnol. 96: 24-26.

Florin L., A. Tsokoglou and T. Happe. 2001. A novel type of Fe-hydrogenase in the green alga *Scenedesmus obliquus* is linked to the photosynthetical electron transport chain. J. Biol. Chem. 276: 6125-6132.

Ghirardi M.L., L. Zhang, J.W. Lee, T. Flynn, M. Slibert, E. Greenboum and A. Melis. 2000. Microalgae: a green source of renewable hydrogen. TIBTECH. 18: 506-511.

Hall D.O., D.A. Affolter, M. Brouers, D.J. Shi, L.W. Wang and K.K. Rao. 1985. Photobiological production of fuels and chemicals by immobilized algae. In: Ann. Proc. Phytochem. Soc. Eur. Vol. 26. Oxford University Press, Oxford. pp. 161-85.

Houchins J.P. 1984. The physiology and biochemistry of hydrogen metabolism in cyanobacteria. Biochem. Biophys. Acta. 768: 227-255.

Jeffries T.W and K.T. Leach. 1978. Effect of iron on hydrogenase activity. Appl. Envrion. Microbiol. 35: 1228.

Jeffries T.W., H. Timourian and R.L. Ward. 1978. Hydrogen production by *Anabaena cylindrica*. Effects of varying ammonium and ferric ions, pH and light. Appl. Environ. Microbiol. 35(4): 704-710.

Jones B.B and N.I. Bishop. 1976. Growth rate and hydrogen production by cyanobacteria. Plant Physiol. 57: 659.

Kondratieva E.N. 1983. Production of molecular hydrogen in microorganisms. In: Advances in Biochemical Engineering /Biotechnology (ed.) A. Fiechter, Springer-Verlag, New York. pp. 139-180.

Kosourov S., M. Seibert and M.L. Ghirardi. 2003. Effect of extracellular pH on the metabolic pathways in sulphur deprived, H_2 - producing *Chlamydomonas reinhardtii* culture. Plant Cell Physiol. 44(2): 146-155.

Lambert G.R and G.D. Smith. 1981. The hydrogen metabolism by cyanobacteria. Biol. Rev. 56: 589-660.

Lambert G.R and G.D. Smith. 1977. Nitrogenase catalysed hydrogen evolution in cyanobacteria. FEBS Lett. 83: 159.

Markov S.A., M.J. Bazin and D.O. Hall. 1995. The potential of using cyanobacteria in photobioreactors for hydrogen production. In: Advances in Biochemical Engineering/Biotechnology (ed.) A. Fiechter, Springer Verlag, New York.

Melis A. and Happe T. 2001. Hydrogen production. Green algae as a source of energy. Plant Physiol. 127: 740-748.

Melis A., L. Zhang, M. Forestier, M.L. Ghirardi and M. Seibert. 2000. Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. Plant Physiol. 122: 127-136.

Mitsui A., S. Duerr, E. Kumazawa, H. Philips and H. Skjodal. 1979. Hydrogen production and nitrogen fixation by marine blue - green algae. In: Biological Solar Energy Conversion (ed.) K.W. Boer and B.H. Glenn, Pergamon Press, New York. pp. 31-35.

Miura Y., M. Yokoyama, K. Kanaoka, S. Saita, K. Iwasa, M. Okazaki and S. Komemushi. 1980. Hydrogen evolution by a thermophilic blue-green alga *Mastigocladus laminosus*. Plant Cell Physiol. 21: 149-156.

Miyamoto K., P.C. Hallenbeck and J.R. Benemann. 1979. Hydrogen production by the thermophilic alga *Mastigocladus laminosus*: Effects of nitrogen, temperature and inhibition of photosynthesis. Appl. Environ. Microbiol. 38(3): 440-446.

Philips E.J and A. Mitsui. 1986. Immobilization of cyanobacteria for enhanced nitrogen fixation. Int. J. Hydrogen Energy. 11: 83.

Philips E.J and A. Mitsui. 1983. Photoproduction of hydrogen by *Oscillatoria* sp. strain Miami BG 7. Appl. Environ. Microbiol. 45: 1212.

Prabaharan D and G. Subramanian. 1996. Oxygen-free hydrogen production by the marine cyanobacterium

Phormidium valderianum BDU 20041. Biores. Technol. 57: 111-116.

Sazanov L.A., P.B. Burrows and P.J. Nixon. 1998. The plastid *ndh* genes code for an NADH - specific dehydrogenase: isolation of a complex I analogue from pea thylakoid membranes. Proc. Natl. Acad. Sci., USA. 95: 1319-1324.

Semin B.K., L.N. Darletshina, A.A. Novakova, T.Y. Kiseleva, V.Y. Lanchinskaya, A.Y. Alekasandrov, N. Seifulina, I.I. Ivanov, M. Seibert and A.B. Robin. 2003. Accumulation of ferrous iron in *Chlamydomonas reinhardtii*. Influence of CO_2 and anaerobic induction of the reversible hydrogenase. Plant Physiol. 131: 1756-1764.

Smith G.D., G.D. Ewart and W. Tucker. 1992. Metals ions and active hydrogenase and uptake hydrogenase. Int. J. Hydrogen Energy. 17: 695.

Spiller H and T. Shanmugam. 1987. Physiological conditions for nitrogen fixation in a unicellular marine cyanobacterium *Synechococcus* sp. strain SF1. J. Bacteriol. 132: 5379-5384.

Weissman J.C and J.R. Benemann. 1977. Hydrogen production by nitrogen-starved cultures of *Anabaena cylindrica*. Appl. Environ. Microbiol. 33(1): 123-131.