Bull. Mater. Sci., Vol. 10, No. 4, July 1988, pp. 393–400. © Printed in India.

Hydrogen photoproduction by algae and higher plants: Optimal conditions for polarographic determination

G KULANDAIVELU, V RAVI and A THANGARAJ
School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021, India

Abstract. Photosynthetic H_2 production capacity has been studied using a H_2 sensitive electrode in green and blue-green algae, higher plant cells and chloroplasts and photosynthetic bacteria. Among the cell types tried, *Anacystis* showed the maximum rate of H_2 production. All the cell types require either an electron acceptor or a reductant (sodium dithionite) for H_2 production. In the presence of an electron acceptor or reductant H_2 production was observed even in unadapted cells. Cultures of *Anacystis* grown for a maximum period of 70 days showed a high rate of H_2 production up to 40 days. The light saturation curves and pH optima for H_2 production were similar to that of O_2 evolution in the cell types studied.

Keywords. Hydrogen production; algae; mesophyll cells; polarography; optimal conditions.

1. Introduction

During the past few years, research on alternate energy sources has been gaining serious attention. Biological conversion of solar energy to H_2 is one such emerging field of interest. All 'biological' energy conversion systems depend on the process of photosynthesis. The enormous potential of biological H_2 producing system has been well documented in several reviews (Calvin 1976; Hall 1978; Mitsui 1979).

Intact cells of the heterocystous, filamentous cyanobacteria have been reported to evolve O_2 and nitrogenase-mediated H_2 under continuous illumination. In eukaryotic green algae, H_2 production via hydrogenase has been suggested to occur either by photosystem II (PS II) or photosystem I (PS I) and these organisms have also been the subject of a great deal of research towards solar energy conversion. Over the past two decades many workers have demonstrated that photosynthetic electron flow of isolated higher plant chloroplasts could be coupled to hydrogenase to allow H_2 production. However, there is no experimental evidence to suggest the existence of hydrogenase in higher plants.

In this paper, we present our results in establishing ideal measuring conditions for high rate of H_2 production in a variety of algae, bacteria and higher plant species. For most of the experiments, polarographic method was used for its simplicity and also for its capacity to detect the immediate release of H_2 by the photosynthetic organisms.

2. Materials and methods

2.1 *Algal and bacterial cultures*

The eucaryotic, unicellular green alga *Scenedesmus obliquus* D3 strain was grown photoautotrophically in culture tubes using the medium devised by Bishop and Senger (1971). The green alga *Chlorella protothecoides* was grown photoautotrophi-

cally in Sorokin and Krauss (1958) liquid culture medium. Aeration was given throughout the culture period of the cells and care was taken in every step to avoid bacterial and fungal contamination.

The unicellular blue green alga *Anacystis nidulans* (IU 625), the filamentous heterocystous blue green algae, *Anabaena* and *Nostoc corneum* were grown in modified Allen's (1968) medium.

The unicellular photosynthetic bacterium *Rhodopseudomonas palustris* (DSM No. 123) and *Rhodopseudomonas capsulata* (DSM No. 1710) were grown photoautotrophically in the medium devised by Bose (1963).

2.2 *Plant materials*

Seedlings of *Pisum sativum*, *Dolichos lab lab* and *Ipomoea* were grown under field condition in botanic garden.

2.3 *Isolation of chloroplasts and mesophyll cells*

Type C broken chloroplasts were isolated from *Pisum* leaves as described by Takaoki *et al* (1974). Mesophyll cells from fresh fully expanded leaves of *Dolichos* and *Ipomoea* were isolated according to the method of Gnanam and Kulandaivelu (1969).

2.4 *Measurements*

Photosynthetic O_2 exchange reaction was monitored using a Clark type O_2 electrode. For H_2 measurements, the same kind of electrode polarized at +0·8 V as suggested by Wang *et al* (1971) was used. The reaction was carried out in a 3 ml water jacketed glass vessel at 25°C. Saturating white light was provided by a slide projector. Irradiation was measured using a Li-Cor Ll-188B quantum/radiometer. Total chlorophyll was determined by the method of Arnon (1949). The bacteriochlorophyll content was estimated using the mM extinction coefficient of 75 cm^{-1} (Clayton 1963). Packed cell volume (PCV) was determined according to Senger (1970) using hematocrit tubes.

3. Results and discussion

In an attempt to optimise the measurement conditions for H_2 evolution in a variety of organisms we have selected unicellular and filamentous cyanobacteria, green algae and isolated mesophyll cells and chloroplasts from a few higher plant species. Typical kinetics of H_2 evolution in *Anacystis* are shown in figure 1. The cells require addition of either an electron acceptor (BQ) or a reductant (dithionite) for H_2 evolution. In the absence of any of these compounds, no H_2 evolution could be detected. This reaction is coupled to photosynthesis as addition of either BQ or dithionite alone in the reaction mixture yielded no H_2 evolution. The kinetics of H_2 evolution observed in these organisms resemble those reported for *Scenedesmus* (Senger and Bishop 1979), *Anacystis* (Peschek 1979a, b) and *Anabaena* (Houchins and Burris 1981a, b).

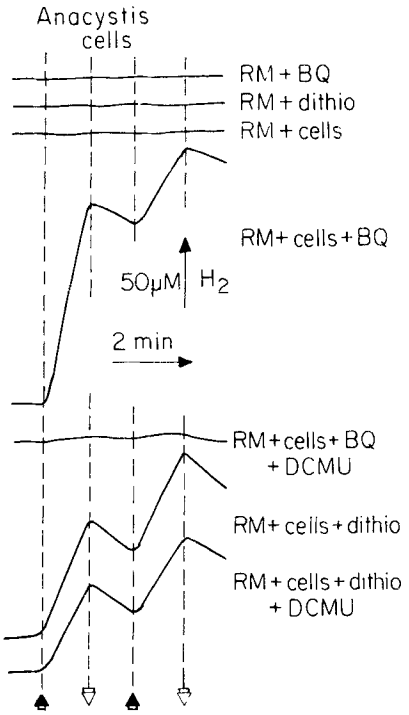

Figure 1. Original polarographic tracings of H_2 evolution in *Anacystis* cells. Open and closed arrows indicate light on and off respectively.

Comparative rates of H_2 evolution mediated by BQ and dithionite and photosynthetic O_2 evolution in a variety of organisms are presented in table 1. Among the organisms tried, the cyanobacteria showed higher rate of H_2 evolution than photosynthetic bacteria, green algae and isolated mesophyll cells of higher plants. The green algae and mesophyll cells showed approximately equal rate of H_2 evolution while *Anacystis* among cyanobacteria showed the highest rate of H_2 evolution in the presence of either BQ or dithionite. In all the oxygenic photosynthetic systems, the BQ-mediated H_2 evolution exceeded 50% of the rate of O_2 evolution.

3.1 *H_2 evolution in ageing algal and bacterial cultures*

As the culture ages, the nutrients in the medium get depleted, the average intensity of light within the culture decreases, the average spectral distribution of light within the culture changes and the metabolic rate of cells also changes from day to day. Hence the influence of culture age on the rate of H_2 evolution was followed in the selected cell types.

Changes in the rate of H_2 evolution and uptake as a function of culture age in *Anacystis* are shown in figure 2. In this culture the log phase lasts for almost 25 days and the stationary phase up to 40 days. The culture starts decaying after 40 days. The maximum rate of BQ mediated H_2 and O_2 evolution was observed in the log and early stationary phase (5–30 days). Contrary to the changes observed in BQ

Table 1. Rates of H_2 and O_2 evolution in different cell systems. For details on measurement conditions see materials and method.

Cells	H_2 μmol. mg. Chl^{-1} h^{-1}		O_2 μmol. mg. Chl^{-1} h^{-1}
	BQ	+Dithionite DCMU (10 μM)	
Rhodopseudomonas capsulata	87·8	52·1	0
Rhodopseudomonas palustris	96·3	47·4	0
Anacystis nidulans	178·2	79·3	247·7
Anabaena sp.	162·0	68·7	223·0
Calothrix sp.	98·4	53·0	196·0
Nostoc corneum	146·4	44·6	266·0
Oscillatoria sp.	157·7	43·1	245·0
Tolypothrix sp.	129·0	56·2	237·0
Chlorella protothecoides	103·6	53·4	258·5
Scenedesmus obliquus	112·6	42·1	256·3
Arachis hypogea	105·0	59·2	237·6
Dolichos lab lab	108·2	71·3	247·7
Erythrina indica	101·9	56·9	228·8
Ipomoea pentaphylla	94·1	67·3	231·4
Musa paradisiaca	96·2	61·8	203·5
Thunbergia grandiflora	102·9	41·5	226·8

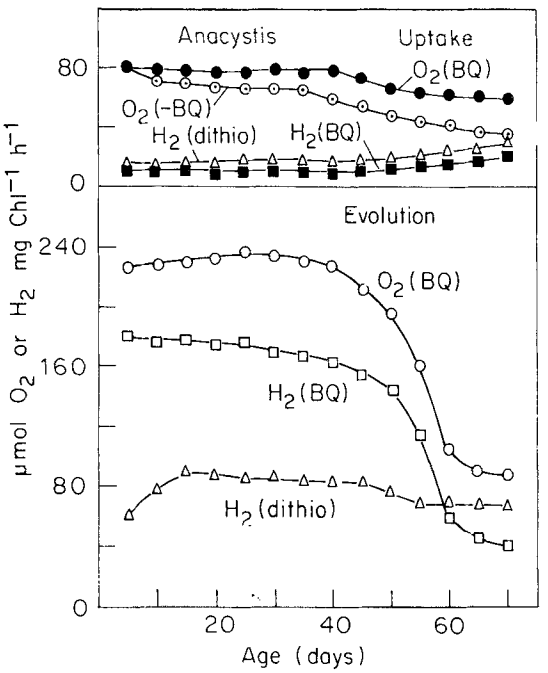

Figure 2. Changes in the rate of H_2 and O_2 evolution (open symbols) and uptake (closed and partially closed symbols) as a function of age in autotrophically grown cultures of *Anacystis*.

mediated reaction, dithionite-mediated rates remained highly stable even in the decay phase of the culture. This could be due to the fact that the dithionite mediated reaction which requires only PS I remains active even in highly aged cultures. Similar pattern of changes at different age of the culture, was also observed in *Scenedesmus* and *Nostoc* (data not shown here). Stable PS I activity in late stationary and decay phases of culture has been reported in *Scenedesmus* (Kulandaivelu and Senger 1976).

3.2 *Effect of anaerobic incubation on H_2 evolution*

Anaerobic incubation of algal and bacterial cells has been suggested to result in reductive activation of H_2 ase and H_2 evolution. In order to determine whether the anaerobic incubation is a prerequisite for H_2 evolution and further enhancement of the initial rate, log phase cells of *Anacystis* were incubated under anaerobic condition by continuously flushing with argon in dark or light for 20 h at 30°C. Changes in the photosynthetic O_2 and H_2 evolution as a function of time of anaerobic incubation are presented in figure 3. In the presence of BQ or dithionite, H_2 evolution could be observed even before anaerobic incubation.

Cells incubated in dark showed greater loss of BQ mediated reactions than those incubated in light, whereas the dithionite-mediated reaction showed no difference under both light and dark conditions. When the cells were kept under dark, the activity of water-oxidizing enzyme decreases (Gaffron 1940a, b) and the photo-

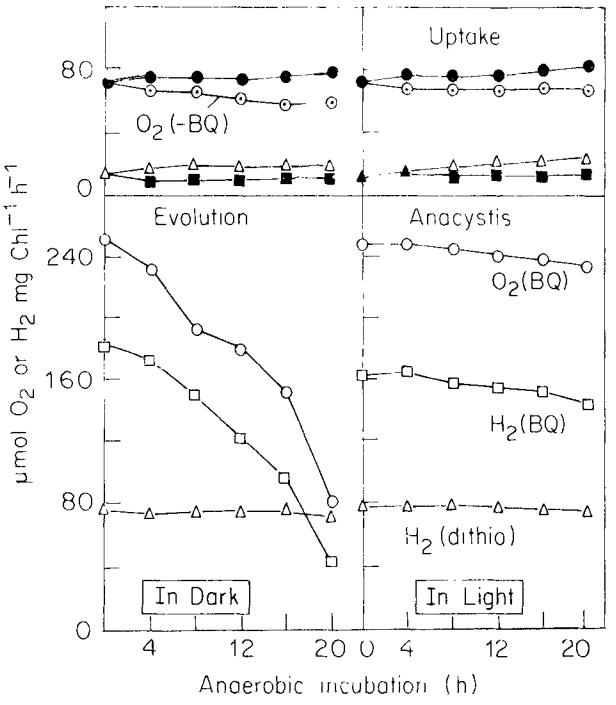

Figure 3. Effect of dark and light (75 W.m^2) anaerobic incubation on the rate of H_2 and O_2 evolution (open symbols) and uptake (closed and partially closed symbols) in *Anacystis* cells.

synthetic electron flow becomes inhibited (Shiau and Franck 1947; Kessler 1960). However, under light the decrease is only marginal. This indicates greater stability of cells incubated in light than in dark.

3.3 *Effect of different light intensities of H_2 evolution*

The rate of photosynthetic electron transport depends upon the intensity of incident light, besides several other factors. Since the BQ and dithionite-mediated H_2 evolution depends upon the electrons from the photosynthetic electron transport, the rate of H_2 evolution is greatly influenced by the light intensity. In autotrophically grown log phase cells of *Anacystis* and *Scenedesmus* and isolated mesophyll cells of *Dolichos* the BQ-mediated O_2 as well as H_2 evolution was measured under different light intensities. In *Anacystis* the maximum rate of BQ mediated O_2 and H_2 evolution was observed at 150 $W.m^{-2}$, whereas in *Scenedesmus* and in isolated mesophyll cells of *Dolichos* the maximum rate was found only at 450 $W.m^{-2}$ (figure 4). In all the cases, no reduction in the rate of H_2 evolution was observed even at very high light intensities. H_2 evolution has been usually measured at intensities ranging from 100 to 300 $W.m^{-2}$ (Schick 1971; Hillmer and Gest 1977a, b; Zurrer and Bachofen

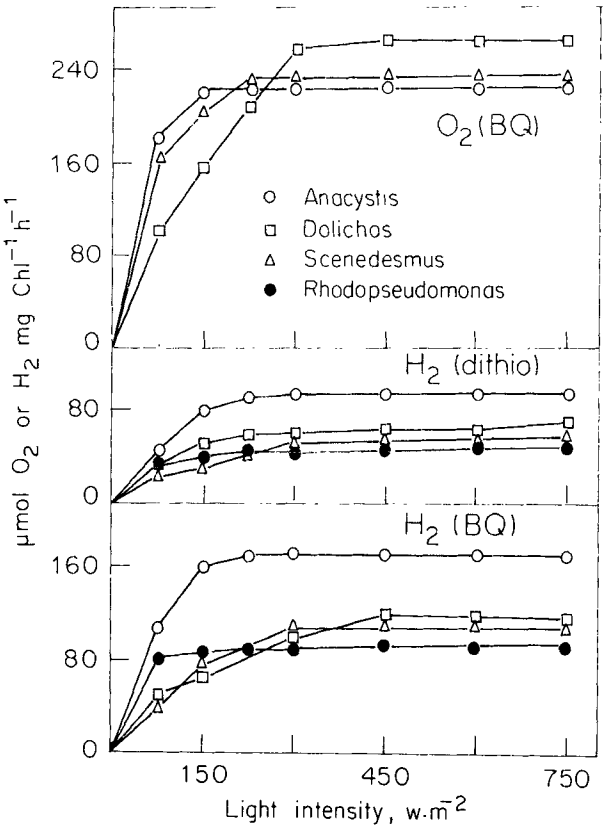

Figure 4. Effect of light intensity on the rate of H_2 and O_2 evolution in different cell systems. Intensity of white light was varied using calibrated neutral density filters.

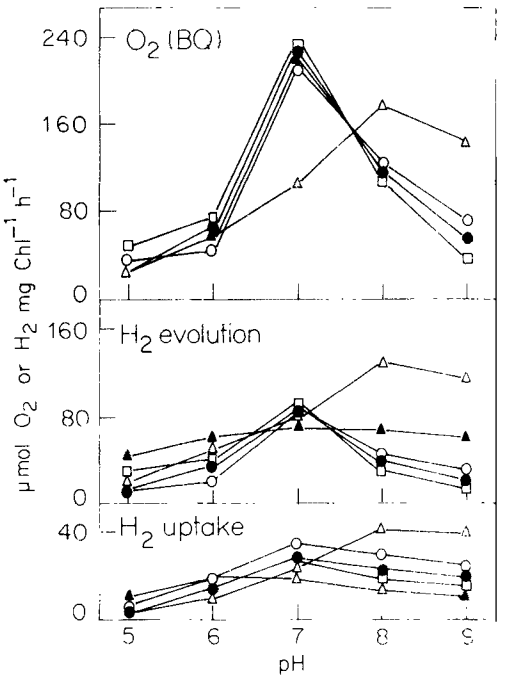

Figure 5. Effect of pH on the rate of H_2 and O_2 evolution and uptake with BQ in different cell and chloroplast systems. Measurement conditions as in text

1979). Under our measuring conditions, the changes in the rate of H_2 evolution at different light intensities followed the same pattern as that of O_2 evolution.

3.4 *Effect of pH on H_2 evolution*

H_2 ase-mediated H_2 evolution and uptake reactions are highly sensitive to the pH of the media. Since protons are the substrates in the H_2 evolution and products in the uptake reactions the proton concentration in the media may influence the extent of these reactions. To assess the effect of pH, cells were incubated for 15 min in different pH media before assay. In all the cell types, the BQ-mediated O_2 and H_2 evolution showed maximum rate at neutral or weak alkaline pH range (figure 5). The low rate of aerobic H_2 evolution observed at both acidic and alkaline pH might be due to the fact that both extreme pH ranges inhibit the water oxidation and subsequent electron flow (Yocum *et al* 1984). High pH requirement for H_2 ase activity has been reported in *Anacystis* (Peschek 1979b, c), *Anabaena* and *Nostoc* (Tel-Or *et al* 1978).

All the above experiments clearly indicate that the rate of H_2 evolution mediated by BQ and dithionite responds in a way similar to the photosynthetic electron transport reactions with respect to various measuring conditions.

Acknowledgements

We thank the Department of Non-Conventional Energy Sources for financial support.

References

Allen M M 1968 *J. Phycol.* **4** 1
Arnon D I 1949 *Plant Physiol.* **24** 1
Bishop N I and Senger H 1971 In: *Methods Enzymol.* **23** 53
Bose S K 1963 In: *Bacterial photosynthesis* (eds) H Gest, A San Pietro and L P Vernon (Yellow Springs: Antioch Press) p. 501
Calvin M 1976 *Photochem. Photobiol.* **23** 425
Clayton R K 1963 *Biochim. Biophys. Acta* **75** 312
Gaffron H 1940a *Am. J. Bot.* **27** 273
Gaffron H 1940b *Science* **91** 529
Gnanam A and Kulandaivelu G 1969 *Plant Physiol.* **44** 1451
Hall D O 1978 *Fuel* **57** 322
Hillmer P and Gest H 1977a *J. Bacteriol.* **129** 724
Hillmer P and Gest H 1977b *J. Bacteriol.* **129** 732
Houchins J P and Burris R H 1981a *J. Bacteriol.* **146** 215
Houchins J P and Burris R H 1981b *Plant Physiol.* **68** 71
Kessler E 1960 In *Handbuch der pflanzenphysiol.* Vol. V (ed.) W Ruhland (Berlin: Springer) p. 951
Kulandaivelu G and Senger H 1976 *Physiol. Plant.* **36** 157
Mitsui A 1979 In *Solar hydrogen energy systems* (ed.) T Ohta (Oxford and New York: Pergamon Press) p. 171
Peschek G A 1979a *Biochim. Biophys. Acta* **548** 187
Peschek G A 1979b *Biochim. Biophys. Acta* **548** 203
Peschek G A 1979c *Arch. Microbiol.* **123** 81
Schick H J 1971 *Arch. Microbiol.* **75** 102
Senger H 1970 *Planta* **90** 243
Senger H and Bishop N I 1979 *Arch. Biochem.* **145** 53
Shiau Y G and Franck J 1947 *Arch. Biochem.* **14** 253
Sorokin C and Krauss R W 1958 *Plant Physiol.* **33** 109
Takaoki T, Torres-Pereira J and Packer L 1974 *Biochem. Biophys. Acta.* **352** 260
Tel-Or E, Lujic L W and Packer L 1978 *Arch. Biochem. Biophys.* **185** 185
Wang R, Healey F P and Myers J 1971 *Plant Physiol.* **48** 108
Yocum C F, Sandusky P O, Ghanotakis G and Babcock G T 1984 *Advances in photosynthetic research* (ed.) C Sybesma, (Den Haag: Martinus Nijhoff/Dr. Junk, W.N.V. Publishers) Vol. 2, p. 341
Zurrer H and Bachofen R 1979 *Appl. Env. Microbiol.* **37** 789