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Two-stage photo-biological production of hydrogen by marine green alga *Platymonas subcordiformis*

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Abstract

A marine green alga, *Platymonas subcordiformis*, was demonstrated to photobiologically evolve hydrogen (H_2) after the first stage of photosynthesis, when subjected to a two-phase incubation protocol in a second stage of H_2 production: anaerobic incubation in the dark followed by the exposure to light illumination. The anaerobic incubation induced hydrogenase activity to catalyse H_2 evolution in the following phase of light illumination. H_2 evolution strongly depended upon the duration of anaerobic incubation, deprivation of sulphur (S) from the medium and the medium pH. An optimal anaerobic incubation period of 32 h gave the maximum H_2 evolution in the second phase in the absence of sulphur. Evolution of H_2 was greatly enhanced by 13 times when S was deprived from the medium. This result suggests that S plays a critical role in the mediation of H_2 evolution from *P. subcordiformis*. A 14-fold increase in H_2 production was obtained when the medium pH increased from 5 to 8; with a sharp decline at pH above eight. H_2 evolution was enhanced by 30–50% when supplementing the optimal concentrations of 25 mM acetate and 37.5 mM glucose.

Keywords: Photo-biological; Green algae; *Platymonas subcordiformis*; Sulphur-deprivation; Hydrogen; Hydrogenase

1. Introduction

Some bacteria and green algae are capable of biologically evolving H_2 under certain conditions [1]. The first report on H_2 production by green algae was dated back to 1942 [2]. Gaffron and Rubin [2] found that green alga *Scenedesmus obliquus* under anaerobic conditions could evolve H_2 in both dark and light. However, it is only until recently that this discovery comes into the spotlight as it could become an ideal system for renewable generation of large quantities of H_2 gas [3]. H_2 evolution in green algae requires a certain period of anaerobic incubation in the dark to induce the reversible hydrogenase. The hydrogenase then functions to combine protons and electrons to form H_2 [4–7]. The high quantum yield of photosynthesis makes it feasible to produce H_2 by green algae using the two most abundant resources of light and water in our planet. Moreover, it is even more preferable to photobiologically generate H_2 using seawater.

Photosynthetic O_2 formation and H_2 evolution occur simultaneously in green algae as electrons and protons released from photosynthetic H_2O oxidation are used in the hydrogenase catalysed H_2 evolution [7,8]. In this one-stage process, H_2 evolution is transient and cannot be sustained due to strong deactivation of hydrogenase activity by O_2 (at as low as 2% partial pressure) evolved from photosynthesis [9]. This mutually exclusive nature of the O_2 and H_2 photoproduction reactions has halted the development of H_2 production process by green algae under ambient conditions [9].

To overcome this problem, a two-stage protocol has been developed to evolve H_2 from green algae, in which photosynthetic O_2 evolution and carbon accumulation (stage 1) are temporally separated from the consumption of cellular metabolites and concomitant H_2 production (stage 2) [10,11]. A transition from stage 1 to 2 has been achieved by deprivation of sulphur (S) from the medium [11,12]. S is an important component of cystein and methionine [7], and plays a role in protein biosynthesis and the photosystem II (PSII) repair cycle [13]. Oxygenic photosynthesis is greatly inhibited by S-deprivation [13], while the respiration rate is relatively insensitive [11]. Over a period of time, an anaerobic environment in sealed cultures can thus be established when the absolute activity of photosynthesis is reduced to a level lower than that of respiration [11,12]. As a result, a

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sustained H_2 evolution by a freshwater green algae *Chlamydomonas reinhardtii* was demonstrated using this two-stage protocol [11,12].

The aims of the current study are to investigate whether S-deprivation could stimulate H_2 evolution in a marine green alga *P. subcordiformis*, and to optimise the conditions for H_2 evolution. In this context, we investigated H_2 production by *P. subcordiformis*, using a modified two-stage incubating protocol. A period of dark anaerobic incubation was introduced to induce hydrogenase activity before light illumination for H_2 evolution. Effect of medium pH, acetate and glucose on H_2 evolution was also examined.

2. Materials and methods

2.1. Culture of P. subcordiformis

The marine green alga, *P. subcordiformis*, an ideal aquaculture feed with known genetic organisation and energy metabolism [14] was used. The organism was grown in seawater medium supplemented with micronutrients consisting of (mg l^{-1}): 1.3 $FeCl_3$, 0.36 $MnCl_2$, 33.6 H_3BO_3, 45.0 EDTA, 20.0 NaH_2PO_4, 100 $NaNO_3$, 0.21 $ZnCl_2$, 0.20 $CoCl_2$, 0.09 $(NH_4)_4Mo_7O_{24}$, 0.20 $CuSO_4$, 0.1 μg VB_{12} and 1.0 μg VB_1. The pH was adjusted to 8.2 prior to autoclaving. The cultures were illuminated at 100 μmol photos m^{-2} s^{-1} by cool-white-fluorescent light (14 h light: 10 h dark) at 25 °C, and shaken by hand for 2–5 min every 3 h from 8 a.m. to 5 p.m. for four times in the daytime.

Culture density was measured by an ultra-plane hemocytometer under light microscope. Cells at the late logarithmic phase ((1.0–1.3) × 10^6 cells ml^{-1}) were collected by centrifugation at 500 × *g* for 5 min, washed three times and finally resuspended to about 1.8 × 10^6 cells ml^{-1} in fresh medium. The S-deprived medium was made by replacing all sulphates with chloride salts at the same concentrations, consisting of (g l^{-1}): 27.23 NaCl, 5.079 $MgCl_2$, 1.123 $CaCl_2$, 0.667 KCl, 0.196 $NaHCO_3$, 0.098 H_3BO_3, 0.098 KBr, 0.024 $SrCl_2$ and 0.003 NaF, and the micronutrients with $CuSO_4$ replaced by $CuCl_2$.

2.2. Photo-biological H_2 production

For H_2 production experiments, 295 ml algal suspension cells in seawater medium with or without sulphur were placed in a serum bottle of 300 ml volume, sealed with retroflexed serum stoppers and tightly fitted with a syringe. To induce H_2 production in the second stage, the algal cells were subjected to a two-phase incubation. In the first phase, the algal cells were maintained under dark anaerobic conditions for induction of hydrogenase activity. Anaerobosis was achieved by two methods: (1) depletion of O_2 by algal cells through respiration incubated at 25 °C in dark; (2) combining method (1) and N_2 flushing for 5 min at the beginning. In the second phase, the cultures were illuminated continuously at 160 μmol photo m^{-2} s^{-1} and shaken at 150 rpm on an orbital shaker at 25 °C. A schematic of the experimental setup for H_2 production is shown in Fig. 1. To identify the key parameters affecting H_2 production, the kinetics of H_2 evolution was studied in the presence and absence of S at varying pH and levels of acetate and glucose.

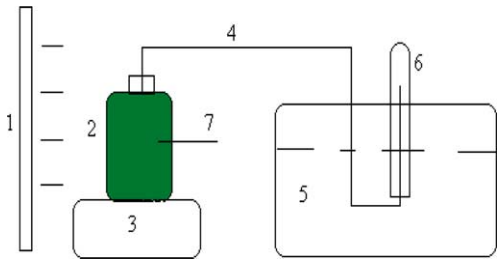

Fig. 1. Schematic of the experimental setup for photoproduction of hydrogen using *P. subcordiformis* algal cultures. 1: the bank of fluorescent lamps; 2: the photo-bioreactor; 3: the orbital shaker; 4: the PVC tubule conducting gas; 5: beaker filled with water; 6: measuring burette; and 7: the algal cells.

2.3. Gas collection and volume measurements

Culture bottles were sealed with retroflexed serum stoppers which were tightly fitted with a syringe for efflux of gases from the culture. PVC tubule was attached tightly to the syringe for gas collection. The PVC tubule conducted gas to an upside-down burette filled with water. The burette was immersed in a beaker filled with water. Gas produced by the culture eventually accumulated in the inverted burette by displacing an equal volume of water, and was measured from the graduated divisions of the burette until no gas was released as described by Cao et al. [15].

2.4. Determination of H_2 concentration and volume

A gas chromatography (Model SRI 8610C, SRI Instruments, USA) with data analysis software was used to determine the concentration of H_2 evolved. A 3 mm × 2 m 13X Molecular Sieve column with Ar as the carrier gas was used to separate O_2, N_2 and H_2. Signals were detected by thermal conductivity detector and calibrated by injection of known amounts of H_2 and O_2. The volumes of H_2 and O_2 gas were calculated by their concentration multiplying the total volumes of gas collected, respectively.

3. Results

3.1. Enhanced H_2 production by S-deprivation

Fig. 2 shows typical results for the time courses of H_2 evolution rate upon illumination from *P. subcordiformis* that

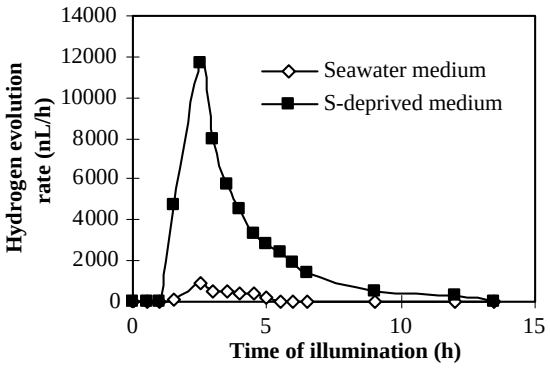

Fig. 2. Time courses of the hydrogen evolution rate upon illumination in seawater medium (open symbols) and S-deprived seawater medium (closed symbols). Dark anaerobic incubation is 32 h.

have been anaerobically incubated in dark for 30 h in both seawater medium and S-deprived medium. H_2 evolution rate was defined as the instantaneous rate of H_2 produced at the specific time of the culture. In seawater medium, H_2 evolution began after 0.5 h illumination and H_2 evolution rate reached a peak value of 900 nl h^{-1} after ca. 2.5 h of illumination. Thereafter, H_2 evolution rate exhibited a time-dependent decline and became undetectable after 5 h illumination. When sulphur was removed from the seawater medium, a remarkable increase in H_2 evolution rate was observed, suggesting that sulphur plays a key role in modulating H_2 production from *P. subcordiformis*. The maximum H_2 evolution rate from S-deprived seawater medium was 11,720 nl h^{-1}, a 13-fold increase when compared with that from the seawater medium. The accumulated H_2 volume in seawater medium and S-deprived seawater was 3000 and 39,000 nl, respectively.

3.2. Effect of the duration of dark anaerobic incubation on H_2 production

Anaerobic incubation is a prerequisite for induction of reversible hydrogenase activity in algal cells before photoproduction of H_2. Effect of the duration of dark anaerobic incubation upon H_2 production under S-deprived condition was presented in Fig. 3. The accumulated H_2 volume increased with increasing duration of incubation and reached a maximum after 40 h incubation (Fig. 3A). When the incubating medium was flushed with N_2 for 5 min at the beginning of the incubation, higher increase in H_2 production was obtained (Fig. 3A). In addition, N_2 flushing reduced the time period of incubation required for maximum H_2 production from 40 to 32 h (Fig. 3A). A similar dependence of H_2 concentration on the duration of incubation was found for the cultures with and without N_2 flushing (Fig. 3B). After 40 h incubation, the H_2 concentration of 1.38 and 1.03% (v/v) was obtained with and without the N_2 flushing, respectively. Higher H_2 evolution and shorter incubation period required for maximum H_2 production when flushed with N_2 (Fig. 3A)

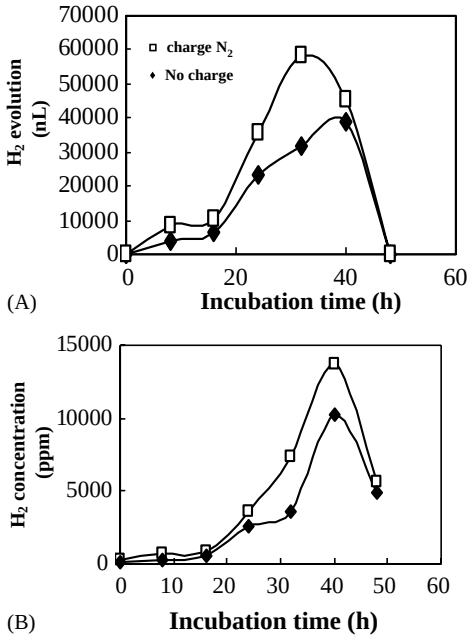

Fig. 3. Effect of the duration of dark anaerobic incubation on accumulated H_2 volume and hydrogen concentration in the second phase of light illumination with (open squares) and without (closed squares) 5 min N_2 flushing. (A) Accumulated H_2 volume and (B) accumulated H_2 concentration.

can be accounted for by a more rapid anaerobiosis induced by N_2 flushing.

In contrast to H_2 evolution, O_2 evolution that reflects the photosynthetic activity in the second phase of light illumination, decreased dramatically as incubation time increased. O_2 evolution eased completely after 48 h dark incubation (data not shown).

3.3. Effects of pH, acetate and glucose on H_2 production

To examine the dependence of H_2 evolution on external acetate, glucose and incubating pH, *P. subcordiformis* cells were incubated in S-deprived medium in dark for 32 h (flushed with N_2 for 5 min at the start) and then illuminated under light. H_2 evolution was very sensitive to the medium pH (Fig. 4). Low H_2 production was observed at pH below 6 and above 10. Maximum H_2 evolution was achieved at pH 8, which is about 7.8- and 5.4-fold higher than that at pH 6 and pH 10, respectively (Fig. 4).

Fig. 5 shows the dependence of H_2 evolution on the addition of external sodium acetate and glucose. Acetate enhanced H_2 yield at concentrations less than 40 mM, and inhibited H_2 production at higher concentrations (e.g. 50 mM NaAc). The maximum increase in H_2 evolution by 48% was obtained at 25 mM NaAc, in comparison with the control (Fig. 5). Like the effect of acetate, the supplement with glucose led to an increase in H_2 evolution with a maximum H_2 yield achieved at 37.5 mM glucose, which is 36% higher than that of the control (Fig. 5).

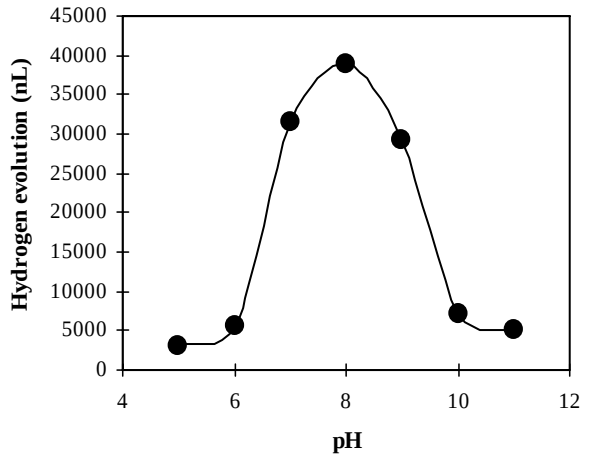

Fig. 4. Effect of initial extracellular pH in the sulphur-deprived medium on H_2 production. After 32 h dark anaerobic incubation and H_2 was collected for 5 h under continuous light illumination.

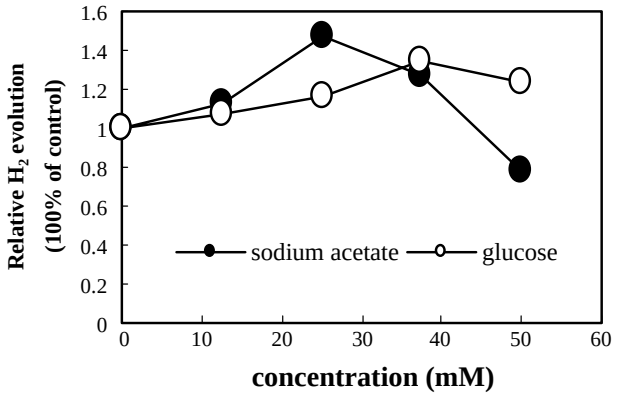

Fig. 5. Effect of external sodium acetate (closed circle) and glucose (open circle) in the sulphur-deprived medium on H_2 production. After 32 h dark anaerobic incubation and H_2 was collected for 5 h under continuous light illumination. Control is 100%.

4. Discussion

When microalgae grow under nutrient deficiency, an increase in activity of chloro-respiration and a decrease in activity of photosynthesis occur to help the survival of microalgae [11,13,15,16]. Of those nutrient deficiencies, inorganic sulphate, the commonly available form of S has a marked effect on photosynthetic O_2 evolution in some algae. It demonstrated that anaerobiosis in the sealed cultures would be developed when O_2 evolution by photosynthesis decreases to a level lower than O_2 uptake by respiration in S-deprived medium [11,15]. Anaerobiosis will activate hydrogenase, leading to a sustained release of H_2 [11,15]. In the present study, the absence of sulphur in the medium alone did not suffice for the establishment of anaerobiosis for *P. subcordiformis* cultures. It indicated that the photosynthetic O_2 evolution sustained at a higher level than respiration. A dark anaerobic incubation phase was thus introduced before proceeding to the phase of light illumination for H_2 production. This modified protocol successfully led to an anaerobiosis in the culture. It was found that the duration of incubation was critical to achieve optimal H_2 production (Fig. 3). At the beginning of dark anaerobic incubation, 5 min N_2 flushing helps a quicker establishment of anaerobiosis at about 30 h (40 h without N_2 flushing). As a result, increases in both H_2 yield (50%) and concentration (34%) were achieved (Fig. 3). In contrast, dark anaerobic incubation phase was not required for *C. reinhardtii* culture, to establish anaerobiosis when S was deprived from the medium [11].

Initial extracellular pHs exhibited a dramatic effect on H_2 production by *P. subcordiformis* with a maximum H_2 production achieved at pH 8 (Fig. 4). Kosourov et al. [17] have recently reported that the maximum rate and yield of H_2 production by *C. reinhardtii* occur when the pH at the start of the sulphur deprivation is 7.7 and decrease when the initial pH is lower to 6.5 or increased to 8.2. Although the optimal pH is close for both strains, H_2 production by *C. reinhardtii* is actually more sensitive to pH changes. H_2 production at optimal pH 8 was reduced by only 19.3% (pH 7) and 25% (pH 9) with one pH shift for *P. subcordiformis* (Fig. 4). In contrast, H_2 production at optimal pH 7.7 decreased by 56% at pH 6.9 and by 93% at pH 8.2 for *C. reinhardtii* [17]. This feature may be viewed as additional advantages for using *P. subcordiformis*.

Intracellular acetate and starch have been partially consumed during H_2 production from *C. reinhardtii* [11]. Acetate and glucose are intermediary metabolites and may be photo-assimilated by respiration as sources for electrons for H_2 production [18,19]. *P. subcordiformis* could grow heterotrophically using glucose [20], and *C. reinhardtii* using acetate [14,20]. It is expected that acetate and glucose would supply some of the energy for cells to survive under the stressed conditions of H_2 production. Our findings that H_2 evolution is stimulated by the addition of acetate and glucose are in line with this proposition. However more energy may be directed to the oxidation of these external carbon sources at higher concentrations, which could results in a decrease in H_2 production as observed in this study.

In summary, freshwater green alga *C. reinhardtii* has been the focus for photo-biological production of H_2 gas using green algae. The present work demonstrates, for the first time that S-deprivation can also significantly enhance H_2 evolution using a marine green alga *P. subcordiformis*, but to a lesser extent. Furthermore, anaerobiosis could not be established for *P. subcordiformis* culture under light illumination by applying S-deprivation alone. It is possible that *P. subcordiformis* may possess a complex mechanism in response to S-deprivation. It may be necessary to study other green algal strains for a better understanding of the H_2 metabolism under S-deprived conditions. The current study serves as a pertinent example that further refined research and developments are required.

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