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REGULAR PAPER

Hydrogen photoproduction in green algae *Chlamydomonas reinhardtii* sustainable over 2 weeks with the original cell culture without supply of fresh cells nor exchange of the whole culture medium

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Abstract Unicellular green algae *Chlamydomonas reinhardtii* are known to make hydrogen photoproduction under the anaerobic condition with water molecules as the hydrogen source. Since the hydrogen photoproduction occurs for a cell to circumvent crisis of its survival, it is only temporary. It is a challenge to realize persistent hydrogen production because the cells must withstand stressful conditions to survive with alternation of generations in the cell culture. In this paper, we have found a simple and cost-effective method to sustain the hydrogen production over 14 days in the original culture, without supply of fresh cells nor exchange of the culture medium. This is achieved for the cells under hydrogen production in a sulfur-deprived culture solution on the {anaerobic, intense light} condition in a desiccator, by periodically providing a short period of the recovery time (2 h) with a small amount of TAP(+S) supplied outside of the desiccator. As this operation is repeated, the response time of transition into hydrogen production (preparation time) is shortened and the rate of hydrogen production (build up time) is increased. The optimum states of these properties favorable to the hydrogen production are attained in a few days and stably sustained for more than 10 days. Since generations are alternated during this consecutive hydrogen production experiment, it is suggested that the improved hydrogen production properties are inherited to next generations without genetic mutation. The properties are reset only when the cells are placed on the {sulfur-sufficient, aerobic, moderate light} conditions for a long time (more than 1 day at least).

Keywords *Chlamydomonas reinhardtii* · Green algae · Hydrogen photoproduction · Anaerobic condition · Sulfur deprivation · Non-genetic inheritance

Introduction

Technical terms, abbreviated words, and definition of terminology in the present paper are described in the final part of this section and in Fig. 1.

In recent years, the resolution of energy and environmental issues is urgent: there is a growing interest in hydrogen, which yields water even if it is combusted, as a clean energy resource (Allakhverdiev 2012; Atsumi et al. 2008; Babu et al. 2012; Masukawa et al. 2014; Nath et al. 2015; Omata and Ogawa 1985; Sakurai et al. 2013). We pay attention to the fact that the green alga *Chlamydomonas reinhardtii* generates hydrogen gas, originating from the photolysis of water, in the oxygen-deficient state (anaerobic condition) (Melis and Happe 2001; The *Chlamydomonas* Sourcebook 2 2008).

A conventional hydrogen production method consists of two stages as a single cycle, i.e., an oxygen consumption stage and a subsequent hydrogen production stage. In the oxygen consumption stage, oxygenic photosynthesis is suppressed against aerobic respiration as explained below (Melis and Happe 2001).

In oxygenic photosynthesis, the electrons and hydrogen ions (protons) are obtained by decomposing water with light energy to evolve oxygen by photosystem II (PSII) in the light reaction (Allakhverdiev et al. 2010; Najafpour

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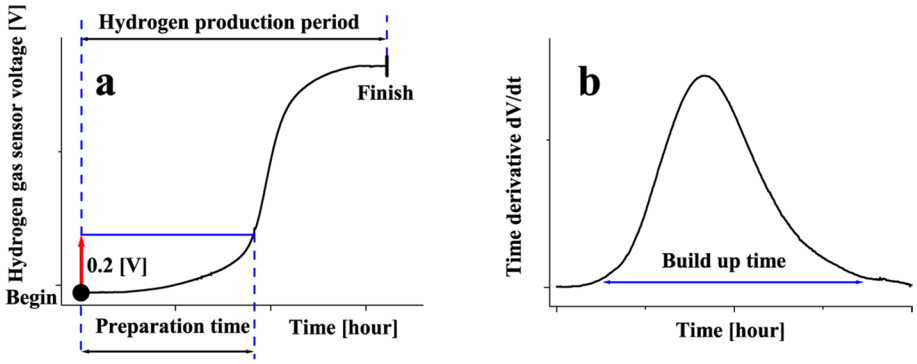

Fig. 1 Definition of terminology. **a** Time evolution of hydrogen concentration during the hydrogen production period. **b** Time derivative of the hydrogen production curve in **a**

and Allakhverdiev 2012). Thereby a concentration gradient of hydrogen ions is established across the thylakoid membrane and the electron transport chain including PS I is driven with light, producing ATP and NADPH. Using these products, carbon dioxide is converted (reduced) into glucose in the dark reaction. Thus performing the oxygenic photosynthesis under the normal aerobic environment, *Chlamydomonas* cells are furnished with energy through ATP by decomposing stored glucose by aerobic respiration.

Hydrogen is produced by the reaction with ferredoxin (Fd), $2H^+ + 2Fd^- \rightarrow H_2 + 2Fd$ through the [Fe–Fe]-hydrogenase (Clowez et al. 2015; Fritsch et al. 2011; Sakurai et al. 2013). With respect to metal composition in the active site, hydrogenases are classified into three types: [FeFe]-hydrogenase, [Fe]-hydrogenase, and [NiFe]-hydrogenase (Das et al. 2006). "Hydrogenase" referred to in this study always indicates the [FeFe]-hydrogenase of *C. reinhardtii*. Since the hydrogenase is expressed under anaerobic conditions and is inactivated in the presence of oxygen, in order to make cells to produce hydrogen continuously, the cells must be maintained under anaerobic conditions by suppressing the oxygenic photosynthesis (Allakhverdiev et al. 2009; Hankamera et al. 2007; Melis and Happe 2001). As the standard way to obtain the anaerobic condition, sulfur is deprived from the cell culture in such a way that the cell suspension is centrifuged to extract cells, then they are re-suspended in a culture solution deprived of sulfur [TAP(−S), tris–acetate–phosphate]. Then, the PS II, which is responsible for decomposition of water in photosynthesis, is inactivated because the photo-damaged PS II is prevented from being repaired PSII (Grewe et al. 2014; Saito et al. 2013). As a result, the oxygen generation rate by photosynthesis is suppressed below the oxygen consumption rate by aerobic respiration in the oxygen consumption stage. It takes time for the hydrogen production stage to be initiated until the oxygen is consumed below a threshold concentration. Origin of the reducing power which contributes to the hydrogen evolution reaction is photolysis of water and photosynthetic anabolic products that have been synthesized by oxygenic photosynthesis (Antal et al. 2003, 2009, 2011, 2014; Chochois et al. 2009; Volgusheva et al. 2013; Zhang and Vassiliadis 2015). Accordingly, by the hydrogen production method on this technique, it is difficult to realize sustainable hydrogen production since cells are subjected to damage and a lot of steps and time are required in the process from cultivation to hydrogen production.

In order to increase the hydrogen production efficiency, major efforts have been devoted to rapid depletion of sulfur from green algae, efficient illumination of light, and mutations of cells towards higher hydrogen production properties (Kruse et al. 2005). To characterize the conditions suitable for hydrogen production in algal cells, various conditions have been examined such as the change due to rotation of the bright and dark environment, the chlorophyll content, the simulation of hydrogen production in the presence of a small amount of sulfur (Williams and Bees 2014), the cell concentration dependence (Kim et al. 2005), the light intensity dependence (Kim et al. 2006), and the cell-age dependence (Kim et al. 2005). Many other experimental efforts have been made to enhance the hydrogen production properties (Cox et al. 2013; Li et al. 2013; Saleem et al. 2012; Stojkovic et al. 2015; Tsygankov et al. 2006; Volgusheva et al. 2015).

It was found that under sulfur deficient conditions in the culture, more hydrogen is produced at intense illumination than at weak illumination (Kim et al. 2006). When the light is too strong, however, excessive damage is given to the cells, reducing the amount of the cell number, chlorophyll content, and produced hydrogen quantity. If the concentration of the cell number in the culture is too low, illuminated light is not efficiently absorbed by the cells. If the concentration is too high, illuminated light is not penetrated throughout the culture volume to decrease the production efficiency. Thus the light intensity should be adjusted to an appropriate value (Kim et al. 2005). For cell age, it was found that the amount of hydrogen production is maximized in the late logarithmic growth period when chlorophyll content is largest (Kim et al. 2005). It is reported that when the culture solution is alkaline, the amount of

produced hydrogen is larger and the onset time of hydrogen production is earlier for a higher pH without irradiated light and for a lower pH with light irradiation (Saleem et al. 2012). It is also reported that hydrogen production takes place on a magnesium deficient state (Volgusheva et al. 2015).

The main stream of current research on hydrogen production by algae is to enhance the efficiency of hydrogen production in the single cycle as described above. One-cycle hydrogen production, however, is not persistent but terminated within a few days. Therefore, in order to sustain hydrogen production, the cycle should be repeated by recovering the cells, which have finished hydrogen production, in TAP(+S) under the aerobic condition, and again by depriving the cell culture of sulfur [re-suspending centrifuged cells in TAP(−S)], and by waiting for initiation of hydrogen production [24–48 h from sulfur deprivation (Amos 2004)].

The purpose of the present study is to find protocols to realize highly efficient, long-term hydrogen production by repeating the cycle. The protocols should be as simple and cost-effective as possible. In this paper, we have succeeded in sustaining hydrogen production as long as 14 days, by periodically providing a short recovery period to the cells under hydrogen production. In this protocol, the preparation time is minimized and the usual sulfur deprivation operation and subsequent disposal of the culture solution, which are cost and time consuming, are avoided.

It is known that the preparation time is significantly reduced by placing the cell culture in the anaerobic atmosphere (Amos 2004). In addition, even when sulfur is contained in the culture, hydrogen can be produced by intense light illumination, which immediately damages PSII to prevent water photolysis (Markov et al. 2006). Thus, the conditions for the hydrogen production period are set to {anaerobic, intense light}.

It is known that acetic acid in TAP(+S) plays an essential role in establishing the anaerobic conditions and in producing hydrogen (Fouchard et al. 2005). It is also reported that the sulfur uptake is accelerated by 10 times in the sulfur-deficient state compared to the normal state (Morsy 2011; Yildiz et al. 1994). Thus, it is expected that sulfur is exhausted in a short time when a small amount of TAP(+S) is added. Motivated by these studies, the protocol as follows is carried out: The cell culture under hydrogen production is taken out of a desiccator to be placed on the usual culture conditions {aerobic, moderate light} with a small amount of TAP(+S) (5 % of the culture medium weight) added for the short recovery period, and the culture of recovered cells is placed again on the hydrogen production conditions in the desiccator. This protocol avoids the complicated operation of sulfur deprivation and consumption of the TAP medium.

Definition of terminology

TAP(+S)	Tris–acetate–phosphate medium
TAP(−S)	Sulfur-free tris–acetate–phosphate medium
Hydrogen production period	Period when produced hydrogen is measured as shown in Figs. 1a and 3
Recovery period	Period between two hydrogen production periods as shown in Fig. 3
Preparation time	Time needed for hydrogen production to start since the cell culture is placed in an anaerobic atmosphere. The start (onset) time of hydrogen production is defined at the time when the hydrogen gas sensor voltage is increased by 0.2 V from the initial one as shown in Fig. 1a
Build up time	Rise time of hydrogen evolution curve in Fig. 1a, or the width of the time derivative curve in Fig. 1b.

Growth of algae

A small amount of *Chlamydomonas* (C-541) is transferred from an agar culture to a screw tube containing 20 mL of tris–acetate–phosphate (TAP) medium. The cells are precultivated for 3 days with a doubly folded aluminum foil on the top, illuminated by light intensity of 25–40 $\mu E/m^2s$ (photon flux density, defined as *moderate light*) (Markov et al. 2006). Then, they are transferred to a 100 mL beaker with a doubly folded aluminum foil on the top, to which TAP (+S) is added to make a 100 mL TAP(+S) culture. Then, they are mass cultured for 4 days, in order to increase produced hydrogen concentration from the culture to meet the sensitivity of the hydrogen sensor. The cell cycle at moderate light illumination is estimated to be about 0.5–1 days from the light intensity (Matsumura et al. 2003; Saenz et al. 2015). In order to put the cells on the hydrogen production conditions, sulfur as a nutrient is deprived of the cell culture to inactivate the PSII, thereby suppressing ordinary oxygen-generating photosynthesis. Then, the cells consume the oxygen by aerobic respiration.

Sulfur deprivation

By placing the culture solution under a low oxygen concentration atmosphere from the beginning, anaerobic conditions can be attained more quickly. On the anaerobic conditions, hydrogenases are activated for hydrogen production to be realized. Specifically, first, the cell suspension is centrifuged to switch the culture from TAP (+S) to TAP (−S): For switching of the TAP, the TAP (+S) culture is centrifuged for 5 min at 4,500*g* with a centrifuge (Beckman AvantiJ-25).

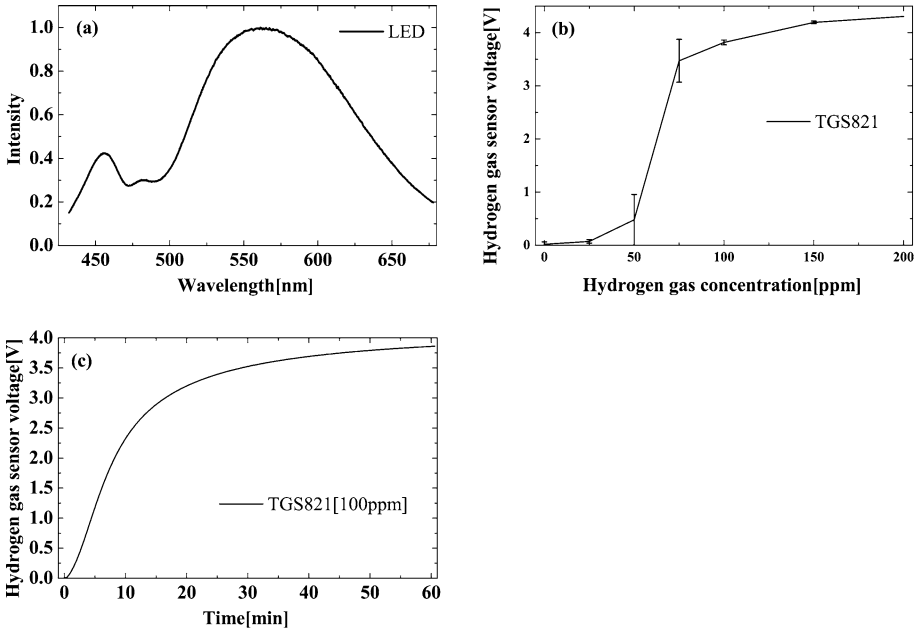

Fig. 2 **a** Emission spectrum of the white LED, the light source used in the experiment. **b**The correction curve of the hydrogen gas sensor for conversion from the output voltage to the hydrogen gas concentration. **c**Typical time response curve of the hydrogen sensor exposed to H_2 gas at 100 ppm

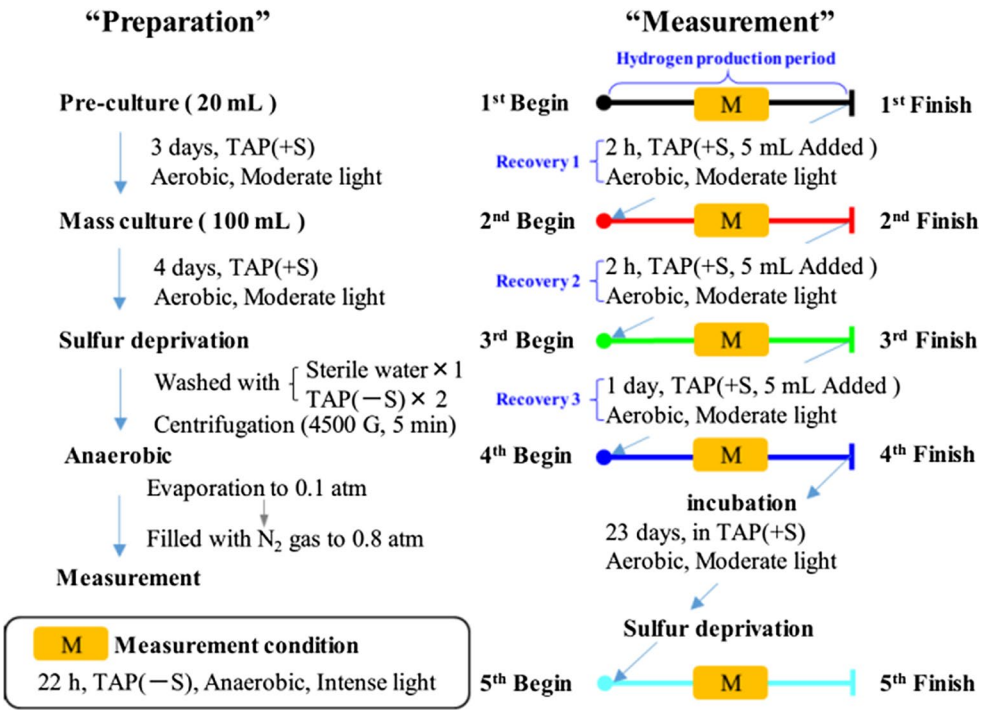

Fig. 3 Protocol of repeated hydrogen production measurement

The supernatant is discarded and the cells are washed by stirring with sterile water. After that, they are washed with TAP (−S) two times. Then cells are suspended in TAP(−S) of 100 mL. This completes the switching to TAP (−S).

Setting for anaerobic state

After the switching the culture from TAP (+S) to TAP (−S), the cell suspension is placed within a 30 L desiccator for the hydrogen production measurement, and it is illuminated by intense light of 1 500 μE/m^{2}s (defined as *intense light*). The light source is a white LED whose spectrum is shown in Fig. 2a. Putting a stirrer in, the cell suspension is always agitated for all the cells to be illuminated uniformly during the hydrogen production condition. A hydrogen sensor is placed in the desiccator, wherein the air is stirred by a fan. The desiccator containing the cell suspension is evacuated to 0.1 atm. with a vacuum pump, then nitrogen

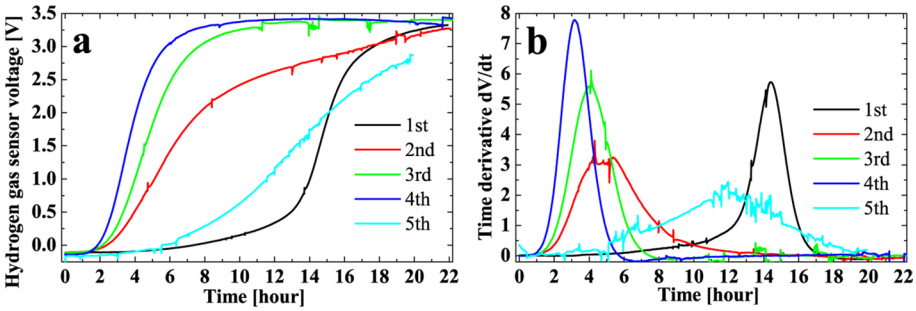

Fig. 4 **a** Time evolution of the output voltage from the hydrogen sensor during the hydrogen production period from the 1st to 4th, and 5th (after 23 days since the 4th). Due to the saturated output shown in Fig. 2b, it is difficult to convert the sensor voltage for >3.5 V to the precise value for the hydrogen concentration, which is considered to rise steadily with the elapsed time. **b** The time derivatives of the hydrogen production curves in **a**. The *onset* time is advanced for the 2nd period against the 1st. For the 2nd to 4th, as the hydrogen production period is repeated, the build up time is shortened and the production rate is increased as demonstrated by the peak values (highest rate) of the time derivative curves. For the 5th period after the {aerobic, moderate light, with sulfur} period for 23 days, the enhanced hydrogen production properties are degraded to show the production curve close to the 1st

is introduced until 0.8 atm. This makes the cell suspension to be placed in the space at the oxygen concentration of about 1.5 %, leaving the cells ready to receive hydrogen photoproduction conditions. After a certain time has passed which is needed for dissolved oxygen to be exhausted to a certain threshold level by respiration, the cells start hydrogen production. In this paper, this state, where the cell culture in a 100 mL beaker covered by a folded aluminum foil is placed in a nitrogen substituted desiccator for produced hydrogen to be measured, is defined as *the anaerobic state*. The cell culture exposed to the atmosphere is defined as *the aerobic state*.

Gas measurement

The hydrogen gas sensor TGS821 (FIGARO) was used at the circuit voltage Vc = 5 V, the heater voltage Vh = 5 V, and the shunt resistance R_l = 200 Ω. The results for the output voltage versus the hydrogen gas concentration was measured for calibration as shown in Fig. 2b.

In this paper, the {anaerobic, intense light illumination} conditions are always referred to as the hydrogen photoproduction conditions (states).

Repeated hydrogen production measurement

Hydrogen production was measured repeatedly for the same group of cells. The cell suspension which was mass cultured on {aerobic, moderate light, with sulfur} conditions was put in the cycle of

1. the hydrogen production period for 22 h on {anaerobic, intense light, without sulfur} conditions,
2. addition of TAP(+S) at the mass ratio of 5 %,
3. the recovery period on {aerobic, moderate light} conditions, which was repeated by three times. After that, it was cultured for 23 days on {aerobic, moderate light, with sulfur} conditions while repeatedly sub-cultured to fresh TAP (+S), and then it was subjected to hydrogen production for 22 h on {anaerobic, intense light, without sulfur} conditions. Detailed experimental conditions are depicted in Fig. 3 and the result is shown in Fig. 4.

Next, in order to confirm the reproducibility, the repeated hydrogen production experiment with TAP(+S) added in the recovery period was performed for a longer period (14 days). The result is shown in Fig. 5.

Finally, as the control experiment, the repeated hydrogen production experiment was performed without addition of TAP(+S). The result is shown in Fig. 6.

Results and discussion

The result for the repeated hydrogen production experiment with TAP(+S) added in the recovery period is shown in Fig. 4. The preparation time for the 1st hydrogen production period is 9 h. This is remarkable improvement, compared with 24–48 h, which is usually required as the preparation time for conventional sulfur-deprivation hydrogen production methods. This is accomplished because the anaerobic condition required for hydrogen production is attained earlier by placing the cells under the anaerobic atmosphere to make the onset time earlier and by irradiating intense light to damage the PS II immediately (Mameli 2004). Compared with the 1st, the preparation time for the 2nd to 4th is significantly shortened. This indicates that the

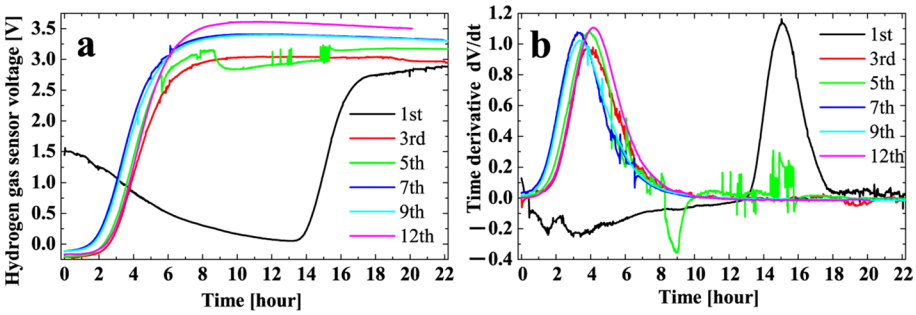

Fig. 5 Repeated hydrogen production measurement reexamined for a longer period. **a** Time evolution of the output voltage from the hydrogen sensor during the hydrogen production period from the 1st to 12th. The initial voltage of the 1st is high because the measurement started when the hydrogen sensor was not well prepared (its heater was not sufficiently warmed up). Actually, hydrogen production started in 13 or 14 h after the cell culture was placed in the anaerobic atmosphere. Then, the same tendency as in Fig. 4 was observed from the 1st to the 3rd, exhibiting improvement of the hydrogen production properties. Further, until the 12th period (14 days after the start of measurement) the improved hydrogen production properties were maintained at similar levels. **b** The time derivatives of the hydrogen production curves in **a**. All the curves from the 3rd are nearly the same, demonstrating that the hydrogen production properties were maintained at a high level until the 12th

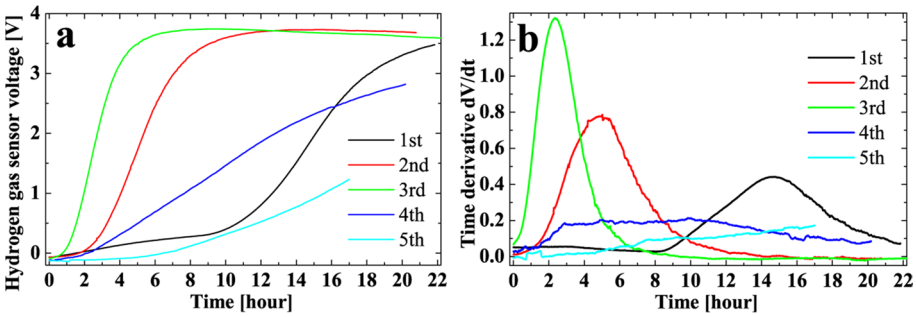

Fig. 6 Control experiment: the repeated hydrogen production experiment without addition of TAP(+S) in the recovery period. For the recovery period, the cell culture was just taken out of the desiccator to be placed under {aerobic, moderate light, without sulfur} condition. **a** Time evolution of the output voltage from the hydrogen sensor during the hydrogen production period from the 1st to 5th. **b** The time derivatives of the hydrogen production curves in **a**

enhanced hydrogen production properties expressed in the 1st hydrogen production period are not lost but maintained even after the exposure to oxygen and the addition of a small amount of TAP(+S) in the recovery period (Fig. 3, Recovery 1), although hydrogenases should be inactivated by oxygen. The reason why the preparation time is not further reduced is because it takes time for hydrogenases to be newly synthesized and for the hydrogen concentration in the desiccator to reach a threshold value of the hydrogen gas sensor, and there is the time lag in response time of the sensor as shown in Fig. 2c. In addition, as shown in Fig. 4b, from the 2nd to the 4th, the build up time (width) is shortened and the production rate (peak value) is increased as the hydrogen production period is repeated. This is understood as collective behavior of the cell ensemble as follows: Individual cells exhibit a different preparation time. As the hydrogen production period is repeated, the number of the cells is increased which have a shorter preparation time. It is suggested that once the transition to a shorter preparation time is made, the enhanced hydrogen production properties of the cell are not lost by oxygen exposure and by addition of a small amount of sulfur [TAP(+S) at the mass ratio of 5 %] during the short recovery period.

For the residual sulfur content in the hydrogen production period, it is believed that a sulfur-deficient state is realized even after the addition of sulfur in the recovery period (Yildiz et al. 1994). *Chlamydomonas* accustomed to sulfur deficiency is known to absorb a large amount of sulfur. Initial rate of sulfate ion absorption time at 25 μM is about 200 fmol/s for 10^5 cell, as estimated from Fig. 2c in Yildiz et al. (1994). For the culture solution of 100 mL with the density of about 30 000 cells/mm^3, the initial speed of sulfate ion absorption is estimated to be about 22 μmol/h. Since the amount of sulfate ions added to the culture solution was 2.5 μmol, most of the sulfur was presumably consumed in the recovery period.

In order to confirm whether a longer recovery period resets the enhanced hydrogen production properties or not, the recovery period between the 3rd and the 4th is set to be 1 day, longer than others (Fig. 3 Recovery 3). As the result, the properties are further enhanced as seen in the 4th curve, demonstrating that the recovery period as long as 1 day does not reset the properties. Rather, the absorbed sulfur is utilized not only for sustaining cell life but also for repairing the PS II to provide reduction power required for hydrogen production. In addition, acetic acid contained in the TAP(+S) medium should be utilized for a respiratory substrate or reduction power in favor of hydrogen production. Since the total period from the 1st to the 4th is long enough for cell division to occur (Matsumura et al. 2003), the experimental result suggests that properties adapted to hydrogen production are inherited non-genetically to daughter and granddaughter cells (Jablonka and Lamb 1998; Jablonka et al. 1992; Levin 2003; Pal and Miklos 1999; Solomon 1981; Spudich and Koshland 1976; Wakamoto and Yasuda 2006). In order to confirm how long the enhanced properties are inherited, the cells after the 4th period are normally cultured in TAP(+S) for 23 days and then they are placed on the hydrogen production conditions. The result (Fig. 4, 5th) demonstrates that the enhanced properties are nearly reset when sufficient time is elapsed after released from stress of severe hydrogen production conditions. The sustainable enhanced hydrogen production properties, therefore, are presumably not due to genetic but non-genetic inheritance, triggered by stress under hydrogen production environment.

Next, in order to examine the reproducibility of the experiment as well as how long the enhanced hydrogen production properties are sustained in the cell culture, we carried out a long-term (14 days) repeated hydrogen production experiment. The result is shown in Fig. 5. The reproducibility of the result in Fig. 4 is confirmed, and once the state with the short preparation time (2–3 h) and the high production rate is realized, the state is stably sustained for a long time (Fig. 5, 3–12th). The continuous hydrogen production without sulfur deprivation up to several weeks is reported (Amos 2004), by placing cells with suppressed PSII on the anaerobic condition. In this method, however, the continuous production was achieved by periodically removing older cells nearly at the end of hydrogen production cycle while adding fresh cells with a high production rate. In the present method, by contrast, only with a small amount of TAP(+S) supplied, we have realized the hydrogen production in the same cell culture sustainable for 14 days, which is the longest sustainable hydrogen production method without supply of fresh cells, to the best of our knowledge. We finished this experiment at the 12th production period (in 14 days) because there had been enough time elapsed without substantial change after the hydrogen production properties were enhanced.

In order to evaluate the effect of addition of TAP(+S), we carried out the control experiment with the recovery period without addition of TAP(+S). The result is shown in Fig. 6. The hydrogen production properties were improved from the 1st to the 3rd, but rapidly deteriorated from the 3rd to the 4th. Hydrogen production was nearly terminated for 5th. This elucidates the crucial role of addition of a tiny amount of TAP(+S) for the long-term sustainable hydrogen production under severe hydrogen production conditions. The overall hydrogen production period (3–4 days) without adding TAP(+S) is coincident with the period of the conventional one-cycle hydrogen production experiment. This might not be an accidental coincidence but be correlated.

Conclusion

For the conventional hydrogen production method with a sealed, sulfur deprived culture, 24–48 h are needed for the preparation time, and hydrogen production is terminated in 4 days (Amos 2004). For the production method in the present study, the preparation time is reduced to 2–3 h and the enhanced hydrogen production properties are sustained over 14 days.

Sustained expression of enhanced hydrogen production properties requires that *C. reinhardtii* is placed on the hydrogen production conditions for a long time. A long-term anaerobic hydrogen production is found to be effectively supported by periodic addition of a tiny amount of TAP(+S) into the culture in the recovery period. This method does not involve sulfur deprivation operation for the culture solution to be exchanged by extracting the cells using a centrifuge at each cycle, which is a usual process in the conventional hydrogen production method, and *C. reinhardtii* in this study is a strain which is not genetically engineered. Hence, this is a low-cost, widely applicable method to be brushed up to the next stage as described below.

There are many tasks to be left for commercialization. Firstly, the optimum conditions should be identified, such as the cell concentration and illumination intensity for the hydrogen production period, and the minimum period and the minimum amount of TAS(+S) needed for the recovery period. Secondly, detailed mechanisms should be identified to show how the enhanced hydrogen production properties are expressed and sustained. To this end, genes expressed for the hydrogenase and a quantity of the hydrogenase synthesized in the hydrogen production period should be analyzed, respectively, by real-time PCR and by Western blotting. Third, the transition time into the hydrogen production mode should be clarified for individual cells, with

single-cell measurement techniques (Isono et al. 2015; White et al. 2014).

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