Title

Biohydrogen production by isolated halotolerant photosynthetic bacteria using long-wavelength light-emitting diode (LW-LED)

Authors

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Abstract

Biohydrogen is expected as one of the alternative energy to fossil fuel. In this study, halotolerant photosynthetic hydrogen producing bacteria (ht-PHB) were isolated from a sediment of tideland, and hydrogen gas (H_2) production by isolated ht-PHB from mixed short-chain fatty acids (SFAs) using a long-wavelength light emitting diode (LW-LED) was investigated. The isolated ht-PHB grow on a culture containing three kinds of SFAs (lactic acid, acetic acid, butyric acid) and produced H_2 with their complete consumption at NaCl concentration in the 0 % to 3 % range in the light of tungsten lamp. The isolated ht-PHB was phylogenetically identified as *Rhodobacter* sp. KUPB1. The KUPB1 showed well growth and H_2 production even under LW-LED light irradiation, indicating that LW-LED is quite useful as an energy-saving light source for photosynthetic H_2 production.

Keywords

Photosynthetic hydrogen production, Halotolerant bacteria, Purple non-sulfur bacteria, Long-wavelength LED

1. Introduction

Hydrogen gas (H_2) is expected as one of the promising future energy [1] because that it is carbon-neutral and a candidate of material for power fuel cell. Recently, biohydrogen producing technologies from organic wastes have been attracting attention in the perspective of ecological advantage. There are two principal types of methods available in biohydrogen production [2-4], i.e., anaerobic H_2 fermentation (dark fermentation) [5,6] and photosynthetic H_2 production [7-9]. In commonly dark fermentation technology, carbohydrates contained in biomass-materials are utilized to produce H_2 by strictly anaerobic bacteria, however, they are only converted to metabolic intermediates such as short-chain fatty acids (SFAs) and remained as byproducts in culture [10]. Therefore, dark fermentation is currently regarded as an alternative to an acid fermentation step (solubilization step) in methane fermentation process [11]. On the other hand, photosynthetic H_2 production is performed by photosynthetic bacteria such as cyanobacteria [12] or purple bacteria [13]. In particular, purple non-sulfur bacteria has the advantage of being able to utilize various carbon sources [13], even the byproducts of dark H_2 fermentation such as SFAs are completely treated with H_2 production [14-16]. However, there are still problems to be solved, e.g., low conversion efficiency of light energy [17] and adverse effect of ammonium nitrogen on H_2 productivity [18,19].

The high energy cost for light irradiation is one of those problems because that well bacterial growth and H_2 production require an intensive light energy containing specific light wavelength depending on bacterio-chlorophylls. For this reason, light source such as tungsten lamp which possesses a variety of light wavelength has generally been used in the laboratory. We have currently focused on the use of light source other than tungsten lamp and demonstrated a photosynthetic H_2 production by using solar lighting system in previous study [20], but sunlight irradiation was unstable because of depending on weather condition and useless at night. We therefore focused attention on long-wavelength light-emitting diode (LW-LED), which possesses a suitable light wavelength range (770 ~ 920 nm) for bacterio-chlorophyll α. In addition, we conceived halotolerant photosynthetic H_2 producing bacteria (ht-PHB) for stable H_2 production even in high salinity condition from the practical perspective because that we sometimes find an organic waste or wastewater high in salt (a small percent level).

In this study, we isolated ht-PHB from tideland sediment and investigated photosynthetic H_2 production from mixed SFAs by ht-PHB using LW-LED light.

2. Materials and Methods

2.1. Enrichment culture and isolation of ht-PHB from tideland sediment sample

Sediment samples were collected from the tideland of Ariake Sea in Japan, and used as inoculum for enrichment culture of ht-PHB. The sediment sample was dispersed by a ultrasonicator (Model JS-150T, NISSEI, Tokyo) for 2.5 min and filtrated with 1mm-mesh metal screen. The filtrate was collected by centrifugation at 10,000 g x 5 min, and then added into the 200 ml Erlenmeyer flask containing 200 ml of salt-PHB medium, which prepared with reference to previous paper [21,22] (20 g/l NaCl, 1.5 g/l KH_2PO_4, 1.0 g/l K_2HPO_4, 170 mg/l glutamic acid, 200 mg/l $MgSO_4 \cdot 7H_2O$, 70 mg/l $CaCl_2 \cdot 2H_2O$, 5.0 mg/l thiamine-HCl, 5.0 mg/l nicotinic acid, 0.05 mg/l biotin, 2.0 mg/l $MnSO_4 \cdot 5H_2O$, 20 mg/l $FeSO_4 \cdot 7H_2O$, 0.95 mg/l $CoCl_2 \cdot 6H_2O$, 1.0 mg/l $Na_2MoO_4 \cdot 2H_2O$, 3.0 mg/l H_3BO_3, 0.24 mg/l $ZnSO_4 \cdot 7H_2O$, 0.04 mg/l $Cu(NO_3)_2 \cdot 3H_2O$, 0.02 mg/l $NiCl_2 \cdot 6H_2O$, 20 mg/l yeast extract, pH 7.0) with 20 mmol/l lactic acid (LA), 25 mmol/l butyric acid (BA), and 20 mmol/l (35 mmol/l for the culture of isolated bacteria) acetic acid (AA). The culture flask was sparged with argon gas for 5 minutes to be deoxidized and maintained in an incubator at 30 ºC. The flask was exposed to a light at 6,000 lx on the surface of flask by a tungsten lamp as shown in Fig. 1. After cultivation for 7 days, the culture liquid was added into fresh medium in another flask at 10 % inoculum-ratio (v/v) and next culture was conducted under the same condition. Subculture was repeated for enrichment of ht-PHB until stable H_2 production was observed.

2.2. Isolation of ht-PHB from enrichment culture

All operations for isolation of ht-PHB below were carried out under aseptic conditions. The 0.1 ml of enriched culture liquid of ht-PHB was spread onto a salt-PHB agarplate (salt-PHB medium with 1.7 g/l agar) and cultured at 30 ºC for several days. The colonies on the plate were arbitrarily picked up to test for photosynthetic H_2 productivity under same culture condition as above. The colonies showing H_2 productivity were repeatedly streak-cultured, and finally the purified single colony was picked up as a candidate of ht-PHB isolate. The each candidate was tested again for the phototrophic H_2 productivity in the light of tungsten lamp and LW-LED.

2.3. Culture of isolated ht-PHB in the light of tungsten lamp or LW-LED

The isolated ht-PHB was cultured using the salt-PHB medium to investigate cell growth, H_2 production, and consumption of SFAs under tungsten lamp light irradiation. The effect of NaCl concentration on all growth and H_2 productivity of the isolated ht-PHB were investigated by varying NaCl concentration at 0 %, 1.0 %, 2.0 % and 3.0 %. The LW-LED, which has light wavelength

ranging from 77~92 % and maximum intensity at 850 nm, was used (ISL-150x150-II85, CCS Inc., Kyoto, Japan) in this study. When LW-LED was used as a light source, culture flask was exposed to LW-LED light at 2000 lx on the surface of the flask, and other culture conditions were same as the above.

2.4. Analytical methods

The culture liquid was taken at intervals to measure the cell concentration and SFAs concentration. The produced gas was collected in a syringe by water-replacement method, and then gas amount and H_2 concentration were determined. The optical density at 660 nm (OD_{660}) was measured as cell concentration. The H_2 concentration and SFAs concentration were determined by GC-TCD and HPLC, respectively according to our previously reported method [21]. The physiological test such as gram-staining, oxidase activity, catalase activity, and scanning electron microscope (SEM) observation were conducted according to conventional methods. Phylogenic identification was carried out by 16S rDNA sequence analysis. The colony of KUPB1 was provided for DNA amplification by PCR using the bacterial universal primers 27f and 1492r [23]. The PCR amplification and the following analysis for phylogenic study were performed according to our previously reported method [10]. Sequence alignment and phylogenetic analysis were conducted by Clustal W program [24] and MEGA software version 4 [25] using FASTA homology search database [26].

3. Results and Discussion

3.1. Enrichment and Isolation of ht-PHB from tideland sediment sample

Establishment of the enrichment culture of ht-PHB was conducted by successive subculture method using tideland sediment sample as inoculum. Bacterial growth was observed in the first to third batch cultures, but the color of culture liquid showed off-white and H_2 production was not observed. However, in the forth culture, the color of culture liquid showed dark red and H_2 production was confirmed, thus subcultures were further repeated. In the sixth and seventh batch cultures, H_2 production was stable in a concentration of 35 %, suggesting that enrichment culture of ht-PHB was established. Therefore, the seventh batch culture liquid was spread onto salt-PHB medium plates and isolation of ht-PHB was performed. Ten colonies on the plate were arbitrarily picked up and purified by streak-culture method, and then their ability of H_2 production from the SFAs in salt-PHB medium was tested. A purified colony showing relatively high H_2 productivity was provided for subsequent

study as candidate of ht-PHB. Figure 2 shows the cell growth of isolated bacteria (OD_{660}), cumulative H_2 amount, and SFAs concentration in batch culture. The cell concentration increased with SFAs consumption and H_2 production was observed 24 hours later. The concentrations of LA and AA decreased soon after beginning of cultivation and they were rapidly consumed within 200 hours. On the other hand, BA concentration decreased slowly compared to other SFAs. H_2 production was observed until SFAs were consumed, which indicates that the isolated bacteria has the ability of H_2 production from the mixed SFAs. The isolated ht-PHB shows ovoid as shown in Fig. 3, gram negative, catalase (+), and oxidase (+). As a result of comparing partial 16S rDNA sequence with those in FASTA database, the sequence of this bacteria showed 99.6 % similarity in 1,450 bps to *Rhodobacter sphaeroides* ATCC17029 (Access. no.: CP000577). From these results, the isolated ht-PHB was affiliated with purple non-sulfur bacteria genus *Rhodobacter* and named *Rhodobacter* sp. strain KUPB1. There have been only a few issues concerned with H_2 production utilizing phototrophic bacteria in marine environment [27] to the best of our knowledge.

3.2. Effect of NaCl concentration on H2 production by KUPB1

Halotolerant ability of KUPB1 was determined from a practical perspective. Figure 4 shows the cell growth (OD_{660}), cumulative H_2 amount, and SFAs concentration with time in the culture of KUPB1 using salt-PHB mediums containing NaCl of 0 % (a), 1.0 % (b), 2.0 % (c), and 3.0 % (d). In all cultures, well growth of KUPB1 was observed with complete SFAs consumption. However, the H_2 productivity in the culture using 3.0 % NaCl was remarkably small of about only 10 % to that in other cultures, suggesting the relationship between the H_2 production and NaCl concentration. Although the exact reason of this phenomenon is currently unclear, but the effect of salinity on the metabolism related with H_2 production was presumed. In the cultures at the range from 0 % to 2 % NaCl concentration, the variation in SFAs concentration showed almost same trend, i.e., LA and AA were initially consumed within 200 h cultivation, and later BA was consumed for 250 h.

3.3. Photosynthetic H2 production from mixed SFAs under LW-LED light irradiation

Since KUPB1 was determined to be closely related to *Rhodobacter* species which commonly possess bacterio-chlorophyll α, LW-LED has maximum wavelength at 850 nm and considered as a suitable light source for culture of KUPB1 and hydrogen production. Figure 5 shows the cell growth (OD_{660}), cumulative H_2 production and SFAs concentration with time. As shown in Fig. 5, the cell concentration rapidly increased within 24 h, indicating that LW-LED was suitable light source for the growth of KUPB1. The culture liquid showed dark purple-red color, which was a little different

from the color (dense red) of the culture liquid in the light of tungsten lamp. The variation trend in SFAs concentration that LA and AA decreased in advance of BA was observed as well as the result of the culture in the light of tungsten lamp. The H_2 production was occurred along with the decrease of LA and AA, and continued even after the cell growth reached steady state. The H_2 production continued until BA was consumed, and the H_2 yield to BA was about 1.0 mol-H_2 / mol-BA which is the same value as that in the tungsten lamp culture although the intensity of LW-LED was only about 20 % (2000 lx) of tungsten lamp (8000 lx). On the other hand, since the value of H_2 yield obtained in this study was lower than those of previous reports [21,28], further study on the optimization of cultivation and lighting condition for H_2 yield improvement will be necessary.

From these results, photosynthetic H_2 production from SFAs by ht-PHB using LW-LED light irradiation was demonstrated. Commonly, life time of LED (20,000~30,000 hours) is ten times as long as that of tungsten lamp (1,000~2000 hours), and consumption electricity of LW-LED and tungsten lamp used in this study is 28 W and 150 W, respectively, suggesting that it is possible to reduce the energy cost by 98 % when LED is used in place of tungsten lamp. Maeda et al. reported availability of sunlight as non-electric light source for photosynthetic H_2 production [29], and the authors also demonstrated the availability of a solar lighting system [20]. Furthermore, the combination of these light sources is expected to establish the energy-saving and low-cost photosynthetic H_2 production technology in the future.

4. Conclusions

1) Halotolerant photosynthetic H_2 producing bacteria (ht-PHB) was isolated from tideland sediment.
2) The isolated ht-PHB can grow and produce H_2 with complete consumption of mixed three kinds of SFAs (lactic acid, acetic acid, butyric acid) in phototrophic culture containing 2 % NaCl .
3) The isolated ht-PHB was phylogenetically identified to be the bacteria closely related to *Rhodobacter shphaeroides* and named *Rhodobacter* sp. strain KUPB1.
4) The KUPB1 can produce H_2 in the culture containing NaCl in the 0 % to 3.0 % range, however, H_2 yield decreased at 3 % NaCl.
5) The KUPB1 can grow and produce H_2 under LW-LED light irradiation indicating that LW-LED is quite useful as an energy-saving light source for photosynthetic H_2 production.

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Figure captions

Fig. 1. Schematic diagram of anaerobic phototrophic cultivation for enrichment and culture of the isolated ht-PHB (tungsten lamp or LW-LED was used as a light source).

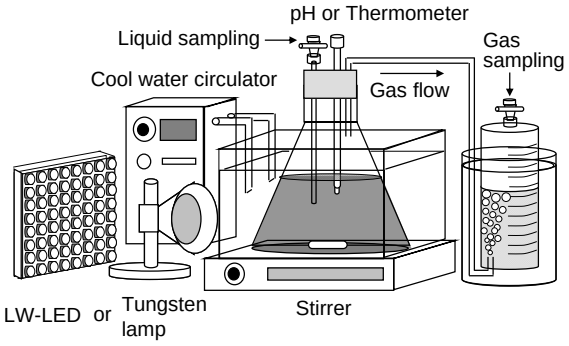
Fig. 2. Growth curve of ht-PHB strain KUPB1 and cumulative H_2 production with SFAs consumption in the salt-PHB medium under the irradiation of tungsten lamp light.

Fig. 3. SEM photograph of ht-PHB strain KUPB1 (15,000 fold).

Fig. 4. Growth curve of KUPB1 and H_2 production with SFAs consumption in the salt-PHB medium containing NaCl of 0 % (a), 1.0 % (b), 2.0 % (c) and 3.0 % (d) under the irradiation of tungsten lamp light.

Fig. 5. Growth curve of KUPB1 and H_2 production with SFAs consumption in the salt-PHB medium under the irradiation of LW-LED light.

Fig. 1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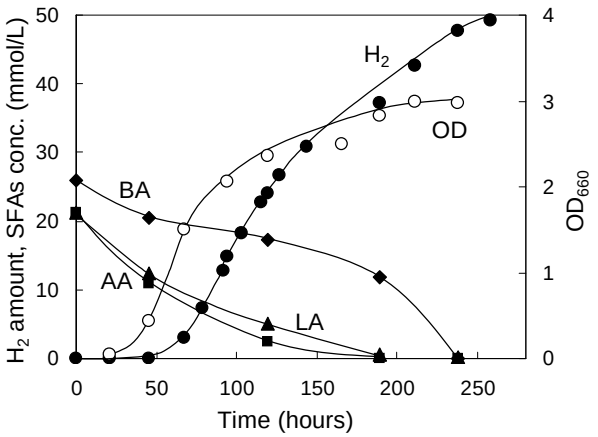
Liquid sampling
Gas sampling
Cool water circulator
Gas flow
LW-LED or
Tungsten lamp
Stirrer

Fig. 2


H2 amount, SFAs conc. (mmol/L)
OD660
Time (hours)
H2
OD
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AA
LA
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Fig. 3

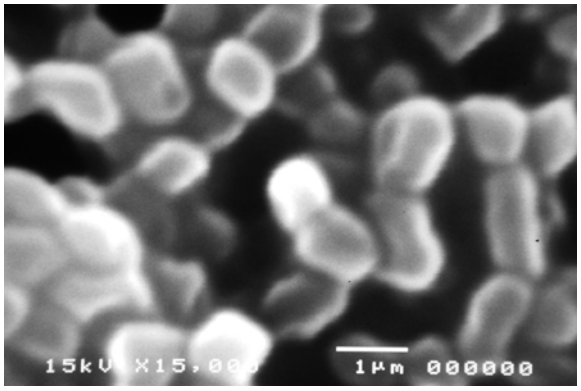


Fig. 4

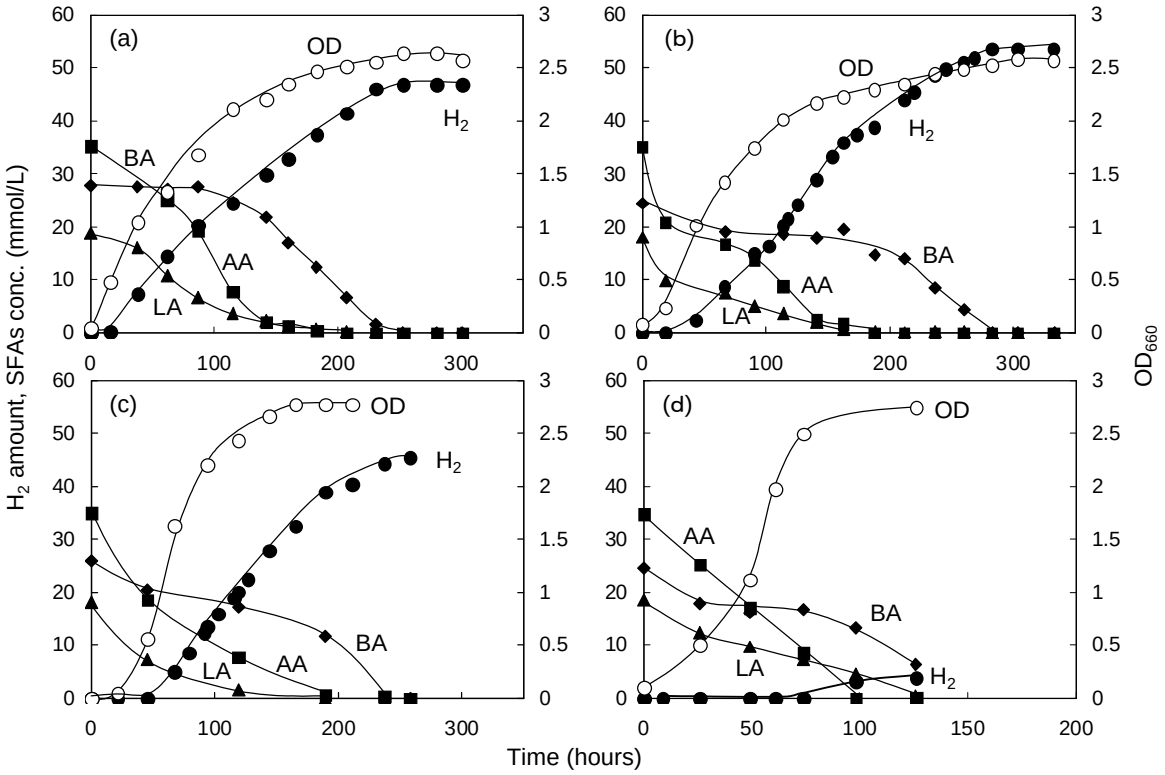
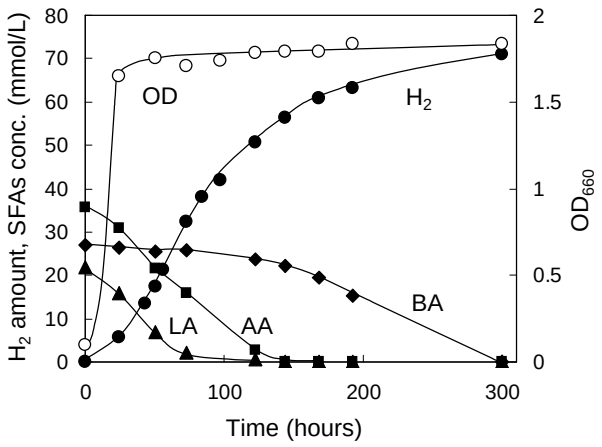


Fig. 5


H2 amount, SFAs conc. (mmol/L)
OD660
Time (hours)
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