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Biohydrogen Production under Aerial Conditions by a Nitrogen-Fixing Bacterium Isolated from a Steel Signboard

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Abstract: Hydrogen gas is attractive as a clean fuel source if it can be produced efficiently without relying on fossil fuels. Biohydrogen production using photosynthetic bacteria may enable environmentally friendly hydrogen production but is currently limited by factors such as low oxygen tolerance. In this study, we isolate a new strain of bacteria that can produce hydrogen under aerial-phase conditions compared with those under liquid-phase conditions in a nitrogen gas or an argon gas atmosphere. Bacterial strains were cultured from scrapings taken from a steel signboard. Investigation of the hydrogen production of the strains under aerial- and liquid-phase conditions and subsequent DNA sequencing led to identification of the bacterium *Cereibacter* sp. KGU-NF001. Aerial-phase conditions were achieved by filter membranes with the bacterial strains and placing the membranes on medium-soaked cotton wool. The gas atmosphere affected the behavior of the isolated bacterial strains under both aerial- and liquid-phase conditions. *Cereibacter* sp. KGU-NF001 showed promising oxygen tolerance and was able to maintain hydrogen production of 1.33 mL/mg/d even when the atmosphere contained 12% oxygen. Our findings illustrate that biohydrogen production may be achieved by photosynthetic bacteria under oxygen-containing aerial-phase conditions, indicating a possible pathway to help lower our reliance on fossil fuels.

Keywords: biohydrogen; screening; aerial conditions; *Cereibacter* sp.; nitrogen gas; argon gas; oxygen gas; nitrogenase; hydrogenase; *Rhodobacter sphaeroides*

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1. Introduction

Currently, the use of fossil fuels is causing serious climate change because it is increasing airborne concentrations of greenhouse gases such as carbon dioxide (CO_2). There are also concerns about resource depletion because of the mass consumption of fossil fuels. To solve these problems, there is a need for clean energy that can replace fossil fuels and does not burden the global environment [1]. Hydrogen (H_2) is attracting attention as a clean energy source. H_2 has a very high combustion efficiency—2.75 times that of fossil fuels—and because only water remains after combustion, it does not burden the environment. However, H_2 is almost nonexistent in Earth's atmosphere [2,3]. Currently, the primary method of producing H_2 is steam reformation, which uses methane gas as its main reagent; this method relies on fossil fuels and thus does not solve the fundamental problem of fossil fuel use [4,5]. In a bioprocess using photosynthetic bacteria, H_2 can be produced by an enzymatic reaction that uses light as an energy source and an organic compound as an electron donor [6–8]. As such, this reaction has a low environmental impact. Bioprocess-based H_2 production using organic waste has been reported [9,10].

H_2 production through bioprocesses can generally be divided into two broad categories. The first involves H_2 production from water decomposition by green algae and cyanobacteria under anaerobic conditions, which is accompanied by oxygen (O_2) generation [11–13]. The second is H_2 production by photosynthetic bacteria. Photosynthetic bacteria are bacteria that grow by receiving light energy from the sun; they are classified into

green sulfur bacteria, red sulfur bacteria, and red non-sulfur bacteria [6,14]. Among them, red non-sulfur bacteria use light energy to obtain reducing power from carbon sources to produce H_2. H_2 production by algae uses water, which is an abundant resource on Earth, but the energy required to decompose water and form H_2 is large ($\Delta G^{0'} = -242$ kJ/mol), and it is difficult to maintain anaerobic conditions because O_2 is produced by photosynthesis. In contrast, H_2 production by non-oxygen-evolving photosynthesis in red non-sulfur bacteria is energetically achievable; for example, $\Delta G^{0'} = -25$ kJ/mol for glucose [8]. Many bacterial species are also able to use various carbon sources as substrates for H_2 production, including starch and aromatic compounds [15].

The enzymes used by red non-sulfur bacteria to generate H_2 as a byproduct are nitrogenases and hydrogenases [16]. Nitrogenases contain molybdenum and catalyze adenosine triphosphate (ATP)-dependent and irreversible nitrogen fixation reactions, usually in the presence of molecular nitrogen (N_2) [17–19]. Nitrogenase receives electrons from nicotinamide adenine dinucleotide and ferredoxin by utilizing ATP produced by ATP synthase and acquired from the photosynthetic apparatus. The protons are subsequently transformed into H_2 [6,16]. When nitrogen is plentiful, one mole of H_2 is produced, along with ammonia, whereas in the absence of nitrogen, four molecules of H_2 are generated. The reactions are represented by Equations (1) and (2).

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi, \quad (1)$$

$$2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP + 4Pi, \quad (2)$$

The reaction involves the reduction of atmospheric N_2 to ammonia (NH_3), but in the absence of N_2, the reaction also involves proton reduction with wide substrate specificity. Although nitrogen fixation and proton reduction reactions compete, H_2 production occurs at a ratio of 20%–30% even in the presence of pure N_2 [20].

Hydrogenases are enzymes that catalyze the reversible redox reaction of H_2 (here, X is an electron carrier):

$$\text{reduced X} + H^+ \rightleftharpoons \text{oxidized X}^+ + 1/2H_2, \quad (3)$$

$$2H^+ + 2e^- \rightleftharpoons H_2, \quad (4)$$

Nitrogenases and hydrogenases are normally inactivated by O_2. Although some hydrogenases have been found to be relatively tolerant of O_2, H_2 production by hydrogenases occurs in the absence of O_2, making culture under anaerobic conditions essential for H_2 production [21]. Conversely, N_2 gas displacement is essential for H_2 production using nitrogen-fixing bacteria.

Biofilms in which terrestrial microalgae are attached to a base such as a membrane filter and cultured under aerial conditions have been reported [22–24]. In batch culture, aerial conditions allow for more direct light exposure to the bacteria in the culture vessel than is the case under normal liquid-phase conditions, improving light utilization and lowering external energy costs by eliminating the need for agitation of the culture medium, leading to a decrease in total operating costs. Cost is an issue in bioprocess-based H_2 production, so H_2 formation by biofilm under aerial conditions is an attractive prospect [25,26].

Photosynthetic bacteria are found primarily in the hydrosphere and soil under anaerobic conditions. In a previous study, aerial microalgae were isolated from the surface of a nutrient-poor steel billboard and photosynthetic nitrogen-fixing bacteria were found near the algae bodies [27]. In this study, photosynthetic bacteria are isolated from a wall surface, and suitable strains for H_2 production under aerial-phase conditions are selected. H_2 production by the isolated bacteria under various O_2 concentrations is then examined.

2. Materials and Methods

2.1. Subsection

Bacteria were collected from the surfaces of a steel signboard at the Hachioji campus of Kogakuin University in Japan (35°68′54″ N, 139°31′88″ E) in 2013 by removal and direct

deposition into plastic tubes. Then, bacterial samples were placed on SA medium (Medium No. 360) supplemented with 1% *w*/*v* agar in a Petri dish. The SA medium consisted of 1 g sodium acetate, 1 g sodium succinate, 0.5 g KH_2PO_4, 0.6 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g NaCl, 0.05 g $CaCl_2 \cdot 2H_2O$, 0.1 g $Na_2S_2O_3 \cdot 5H_2O$, 0.1 g yeast extract, 0.5 mg thiamin-HCl, 0.5 mg niacine, 0.3 mg *p*-aminobenzoic acid, 0.1 mg pyridoxal-HCl, 0.05 mg biotin, 0.05 mg vitamin B_{12}, 1 mg 2Na-EDTA, 2 mg $FeCl_3 \cdot 6H_2O$, 0.1 mg $ZnCl_2$, 0.1 mg $MnCl_2 \cdot 6H_2O$, 0.1 mg H_3BO_3, 0.1 mg $CoCl_2 \cdot 6H_2O$, 20 µg $Na_2MoO_4 \cdot 2H_2O$, 10 µg $CuCl_2 \cdot 2H_2O$, 10 µg $NiCl_2 \cdot 6H_2O$, and 5 µg Na_2SeO_3 in 1 L of distilled water. The pH was adjusted to 6.7 with NaOH prior to autoclaving. After more than 1 week of growth, single colonies that formed on the agar-solidified medium were manually isolated under a microscope (BX51; Olympus, Tokyo, Japan). Isolated strains, which were named BRCNF-001 to BRCNF-008, and *Rhodobacter sphaeroides* (NBRC 12203) were precultured in SA medium and maintained for 7 days at 25 ± 2 °C and 40 µmol photons/m^2/s using a cool-white fluorescent lamp under anaerobic conditions in a 200 mL Erlenmeyer flask that was flushed with N_2 gas for 1 min and then capped with a lid.

2.2. Liquid and Aerial Culture Conditions

After preculture for 7 days, the cells were incubated for 7 days at 25 °C and 25 µmol photons/m^2/s using a cool-white fluorescent lamp under two sets of conditions (see Figures 1 and S1): (1) liquid-phase conditions—where 0.5 mg cells were cultured in 21 mL glass tubes containing 3 mL N-free SA medium with 0.3% glucose as the carbon source, and (2) aerial-phase conditions, which were achieved as follows. Precultured cells (0.5 mg) were layered on 0.20 µm polytetrafluoroethylene membrane filters, which are water permeable under reduced pressure, and then washed with distilled water. The biofilm on the membrane filter was placed on cotton wool wetted with 3 mL N-free SA medium with 0.3% glucose as the carbon source in a 21 mL glass tube. The headspace of each tube was flushed with N_2 gas (with 1.5, 2.0, 2.5, 4.0, 6.0, 10.0, 12.0, 13.0, or 15.0% O_2 gas), Ar gas, or air (21% O_2) for 1 min, and then the tubes were sealed with a rubber cap.

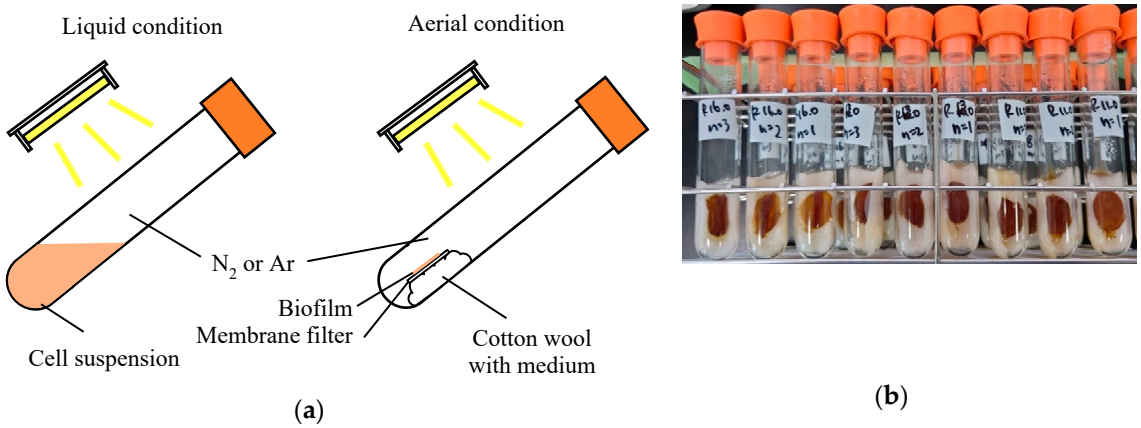


Figure 1. (**a**) Images of cultivation under liquid and aerial culture conditions and (**b**) photograph of biofilms cultivation under aerial culture conditions.

2.3. Hydrogen Analysis

Gas components in the headspace of each glass tube on day 7 of culture were analyzed as described elsewhere [28]. H_2 produced during the one-week fermentation was collected with a microsyringe and analyzed using a gas chromatography (GC) system (GC-2014, Shimadzu, Kyoto, Japan) equipped with a thermal conductivity detector (GC-TCD, Shimadzu, Kyoto, Japan) under the following conditions. Column: Shincarbon ST column 50–80 (4.0 m × 3.0 mm internal diameter, Shinwa Chemical Industries, Kyoto, Japan), injection volume: 0.5 mL, carrier gas: Ar (43.5 mL/min), and column temperature: 80 °C. The detection limit of the GC-TCD for H_2 quantification was 0.02 µL.

2.4. 16S rRNA Analysis and Phylogenetic Study

Crude DNA was extracted from bacterial cells using the bead-beating method [29]. The partial 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal primers [29]. The PCR products were checked using 1.5% (*w*/*v*) agarose-gel electrophoresis and purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Singapore). DNA sequencing was performed using the PCR primers and then determined by Fasmac Co., Ltd. (Atsugi-shi, Japan). BLAST searches were used to compare the sequences of the PCR products with those of known microbial species in the GenBank. A phylogenetic tree was constructed with *Cereibacter* spp. and closely related bacteria based on 16S rRNA gene sequences. Positions at which the alignment resulted in gaps or ambiguities were omitted from the analysis. The DNA sequence of the 16S rRNA gene for the isolated strain have been deposited in GenBank under accession number LC814985. Gram staining was carried out as described in [30].

2.5. Statistical Analysis

The mean and standard deviation for each treatment were calculated. Data were subjected to analysis of variance. Differences between groups were considered statistically significant at $p < 0.05$. Statistical analyses were conducted using Microsoft Excel 2019. Statistical analyses of the experimental data were performed using Student's *t*-test.

3. Results and Discussion

3.1. Biohydrogen Production under Aerial- and Liquid-Phase Conditions

Eight strains denoted as BRCNF001–008 were isolated from a steel signboard. These eight strains and *R. sphaeroides* were cultured for 7 days under liquid- or aerial-phase conditions with N_2 as the displacement gas. BRCNF001, BRCNF004, BRCNF007, BRCNF008, and *R. sphaeroides* had significantly lower cell weights under aerial-phase conditions than under liquid-phase conditions (Figure 2a). Only BRCNF003 displayed a larger cell weight under aerial-phase conditions than under liquid-phase ones. Among these strains, the weights of BRCNF004 and BRCNF007 increased about 16-fold from 0.5 mg of bacteria at the beginning of culture under liquid-phase conditions. The weight of BRCNF003 increased about 12-fold under aerial-phase conditions. These results indicate that all strains except BRCNF003 grew with nitrogen fixation under liquid-phase conditions. Compared to the other strains, BRCNF003 grew more easily under aerial-phase conditions.

H_2 production rates were greater under aerial-phase conditions than under liquid-phase ones for BRCNF001, BRCNF005, BRCNF007, BRCNF008, and *R. sphaeroides* (Figure 2b). *R. sphaeroides* resulted in higher bacterial abundance and lower H_2 production rates under liquid-phase conditions than under aerial-phase conditions. The solubility of N_2 gas in the liquid medium was only about 0.016 mL mL^{-1} under standard conditions (0 °C, 1013 hPa), suggesting that the bacteria grew by increasing nitrogenase activity, actively fixing nitrogen, and obtaining a nitrogen source. Conversely, under aerial-phase conditions, the bacteria had easier access to the N_2 gas, so the N_2 gas utilization efficiency was higher than that in the liquid phase. Due to the fact that the nitrogen source in the cells under liquid-phase conditions was insufficient for growth as a result of low nitrogenase activity, the carbon source in the medium was used for hydrogenase activity, which generated H_2 [31]. In general, H_2 generation and cell proliferation are reported to be in a competitive relationship, with NH_3 generated by nitrogenases and carbon sources in the medium being used for growth [32]. H_2 generated by nitrogenases is trapped by hydrogenases in the cell and becomes a source of electrons and protons through oxidative reactions [33]. Strains with more reductively reactive hydrogenases are more promising as biohydrogen-producing bacteria than strains with high nitrogenase activity.

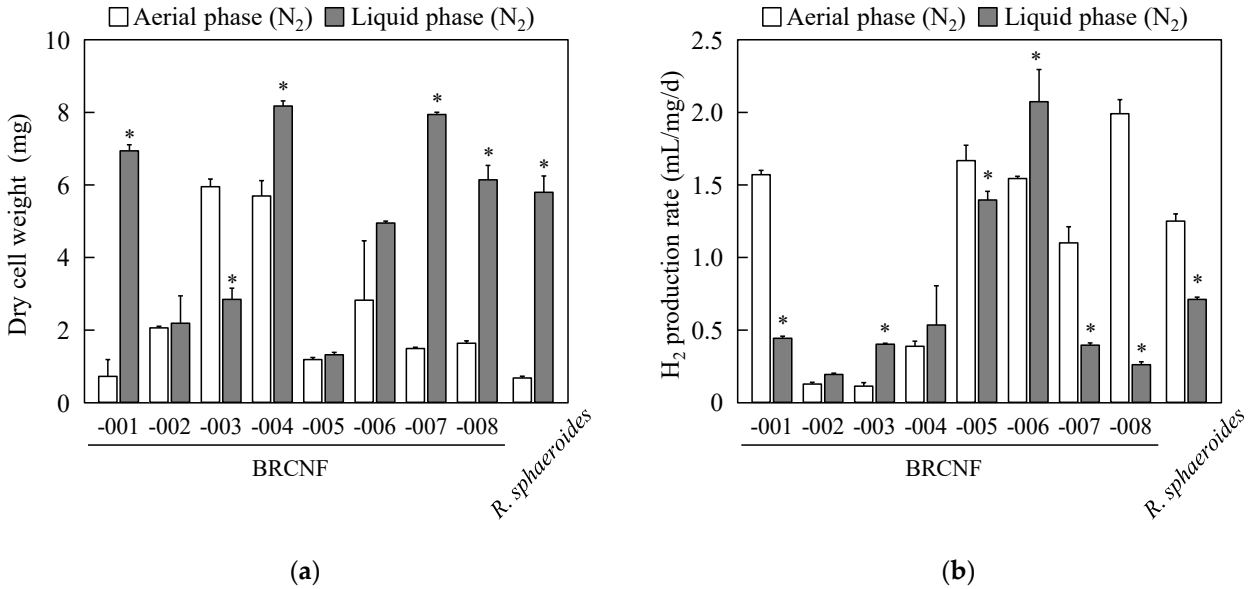
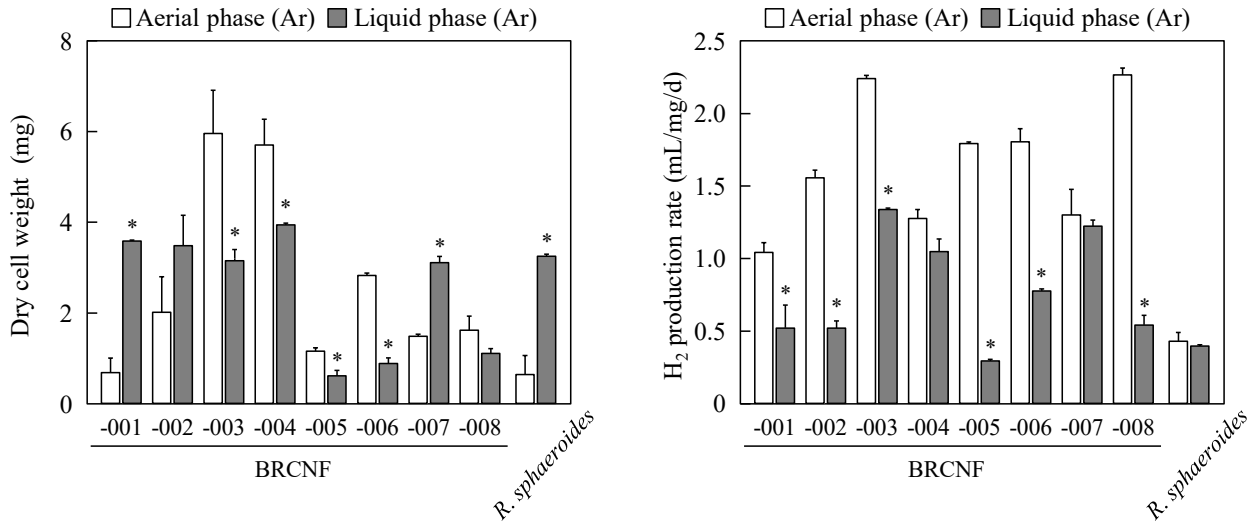


Figure 2. (**a**) Dry cell weight and (**b**) H_2 production of the isolated bacteria (BRCNF001–008) and *Rhodobacter sphaeroides* cultured under aerial- or liquid-phase conditions. Dry cell weights are per membrane filter (aerial-phase conditions) or per 3 mL of medium (liquid-phase conditions). The carbon source in the N-free SA medium was glucose. The headspace of each tube was flushed with N_2 gas. Data are mean ± standard deviation of three replicate incubations. Asterisks indicate statistically significant differences at $p < 0.05$.

Next, BRCNF001–008 and *R. sphaeroides* were cultured under liquid- or aerial-phase conditions in an argon (Ar) gas atmosphere for 7 days. Ar is commonly used as a replacement gas in biohydrogen production studies because it is inert and does not react with the bacteria. In an Ar atmosphere, nitrogenases remain inactive because of the lack of a substrate, and biohydrogen is produced by hydrogenases [34]. In an Ar atmosphere, BRCNF001, BRCNF007, and *R. sphaeroides* had significantly lower cell weights under aerial-phase conditions than under liquid-phase ones, the cell weights of BRCNF002 and BRCNF008 were not significantly different, and the cell weights of BRCNF003, BRCNF004, and BRCNF006 were larger under aerial-phase conditions than under liquid-phase ones (Figure 3a). Cell weights of BRCNF001–004, BRCNF008, and *R. sphaeroides* increased about 7–8-fold from 0.5 mg of bacteria at the beginning of incubation under liquid-phase conditions in an Ar atmosphere. In contrast, cell weights of BRCNF003 and BRCNF004 increased by about 12 times from the initial bacterial weight under aerial-phase conditions in an Ar atmosphere. The inactivity of nitrogenases under an Ar atmosphere and the lack of a nitrogen source indicate that these strains took up carbon to grow rather than growing by cell division. BRCNF003 grew better under aerial-phase conditions than liquid-phase ones in both N_2 and Ar atmospheres.

The H_2 production rate in an Ar atmosphere was greater or equal for all strains cultured under aerial-phase conditions than was the case under liquid-phase conditions (Figure 3b). Although nitrogenases did not function in the Ar atmosphere and H_2 was produced by hydrogenase activity, the aerial-phase conditions were more efficient than the liquid-phase conditions because of the more uniform light exposure to bacterial cells in the former compared with that in the latter [8]. Comparing the results for N_2 and Ar gas, BRCNF002–004 displayed significantly ($p < 0.05$) greater H_2 production rates under both aerial- and liquid-phase conditions compared with those of the other strains. In other words, these strains exhibited low nitrogenase activity and high hydrogenase activity (Figures 2b and 3b). It is believed that N_2 gas-fixing nitrogenases produce NH_3 simultaneously with H_2 and NH_3 acts as an inhibitor of nitrogenase activity, so nitrogenase activity gradually weakens as NH_3 and H_2 are produced [35]. N_2 is important as a replacement

for air because it makes up 78% of Earth's atmosphere and is inexpensive. Although Ar is an inert gas that does not affect H_2 production, it is much more expensive than N_2 and therefore not desirable as an atmosphere for cost-effective biohydrogen production. BRCNF008 showed the highest H_2 production rate among the nine strains in both N_2 and Ar gas atmospheres under aerial-phase conditions, indicating that it is attractive for H_2 production under aerial-phase conditions.



(**a**) (**b**)

Figure 3. (**a**) Dry cell weight and (**b**) H_2 production of the isolated bacteria (BRCNF001–008) and *Rhodobacter sphaeroides* cultured under aerial- or liquid-phase conditions. Dry cell weights are shown per membrane filter (aerial-phase conditions) or per 3 mL of medium (liquid-phase conditions). The carbon source in the N-free SA medium was glucose. The headspace of each tube was flushed with Ar gas. Data are mean ± standard deviation of three replicate incubations. Asterisks indicate statistically significant differences at $p < 0.05$.

3.2. Phylogenetic Analysis of the Bacterial Strain of BRCNF008

Using PCR amplification and subsequent DNA sequencing, we determined almost the full length of the 16S rRNA gene of the strain BRCNF008, which showed promise for H_2 production under aerial-phase conditions. The strain was observed as a Gram-negative bacillus (Figure 4a). The sequence information obtained for BRCNF008 was compared with those of known related species (Figures 4b and S2). The strain was most closely related to *Cereibacter* species of the class Alphaproteobacteria and named *Cereibacter* sp. KGU-NF001 (Accession Number: LC814985). The most closely related species, *Cereibacter johrii* JA192, is a strain discovered in 2010 and recently reanalyzed to be best placed in the genus *Cereibacter* [36,37]. The nitrogenase and hydrogenase activities (H_2 production rate) of *Cereibacter* are not well understood. *Cereibacter* sphaeroides (strains IFO12203 and KC2139) has recently undergone a phylogenetic tree reanalysis and been renamed from *Rhodobacter sphaeroides* [37]. According to the National Institute of Technology and Evaluation's NBRC database, strain NBRC12203 is derived from IFO12203, so *Rhodobacter sphaeroides* strain NBRC12203 and BRCNF008 are relatively close species.

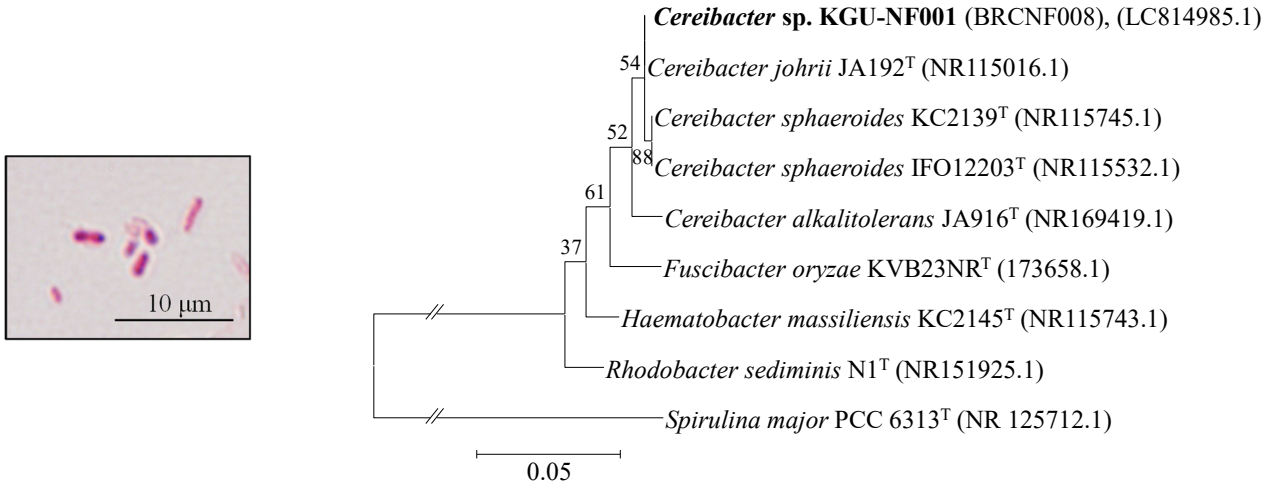


Figure 4. Micrographs of Gram staining cells of the strain BRCNF008 (**a**) and phylogenetic tree of the isolated bacteria with its known neighbors based on 16S rRNA gene sequences constructed using the maximum-likelihood method (**b**). The scale bar in phylogenetic tree represents an evolutionary distance (Knuc) of 0.05. Numbers at branch nodes are bootstrap values (1000 replicates) obtained using the maximum-likelihood method. Accession numbers for DNA sequences are indicated in parentheses. ^{T} is type strain.

3.3. Influence of O_2 on H_2 Production

The influence of O_2 concentration on dry cell weight (Figure 5a) and H_2 production rate (Figure 5b) was investigated under aerial-phase conditions in a N_2 gas atmosphere. First, when the tubes were filled with air (i.e., 21% O_2) instead of N_2 gas and incubated for 7 days under otherwise identical aerial-phase conditions, dry cell weight did not increase and H_2 was not produced. When the cells were incubated under aerial-phase conditions in a N_2 gas atmosphere with an O_2 concentration lower than that of air, BRCNF008 and *R. sphaeroides* showed increases of dry cell weight in the 10–15% O_2 concentration range that were similar to those at 0% O_2, approximately 10 times higher than at the start of incubation. BRCNF008 and *R.* sphaeroides produced 1.33 and 1.00 mL/mg/d of H_2 under 12% O_2, respectively, and did not produce H_2 under O_2 concentrations above 13.0% (Figure 5b). The H_2 production rate of BRCNF008 was similar to that of *R. sphaeroides* under anaerobic conditions in the O_2 concentration range of 6–12%, whereas that of *R. sphaeroides* was approximately half that under anaerobic conditions in the O_2 concentration range of 6–12%. *R. capsulatus* JP91 was reported to produce H_2 under microaerobic dark conditions (2–10% O_2) [38]. BRCNF008 showed higher O_2 resistance than *R. sphaeroides* and *R. capsulatus* JP91. O_2 showed 50% inhibitory activity against a nitrogenase in a system with an acetylene concentration of 0.37 μM (e.g., below 0.001% O_2) [39]. In comparison, the O_2 concentration of 12.0% used here is far greater, indicating that H_2 can be generated sufficiently by BRCNF008 in the presence of O_2 in the concentration range of 0–12%. Although it is difficult to accurately measure the concentration of CO_2 generated by BRCNF008, the rate of CO_2 generation was able to be measured by GC (Figure 5c). In an O_2 concentration range of 0–21%, CO_2 was produced in a concentration range of about 0.15–0.25 mL/mg/d. Because *R. sphaeroides* is a facultative anaerobe, BRCNF008, which is closely related to *Rhodobacter*, also respires when O_2 is present. Even in the absence of O_2, anaerobic respiration and fermentation produce CO_2 [40]. Even at an O_2 concentration of 13.0%, CO_2 was produced and the H_2 production rate decreased despite the respiration (Figure 5b,c), suggesting that the O_2 concentration remaining after some is consumed by respiration during the 7-day incubation period may affect H_2 production. After 7 days of incubation, 9.0% (74.7 μmol) O_2 remained in the BRCNF008 culture when the initial O_2 concentration was 12.0%, and 10.7% (102.7 μmol) O_2 remained when the initial O_2 concentration was 13.0%. For *R. sphaeroides*, 8.0% (84.8 μmol)

O_2 remained when the initial O_2 concentration was 12.0% and 11.0% (100.7 μmol) O_2 remained when the initial O_2 concentration was 13.0%. The nitrogenase activity of *R. capsulatus* MNL was sufficiently inhibited by 5%–7.5% O_2 [41], indicating that BRCNF008 shows high O_2 tolerance. It was shown that H_2 production is possible because O_2 is consumed to some extent by respiration.

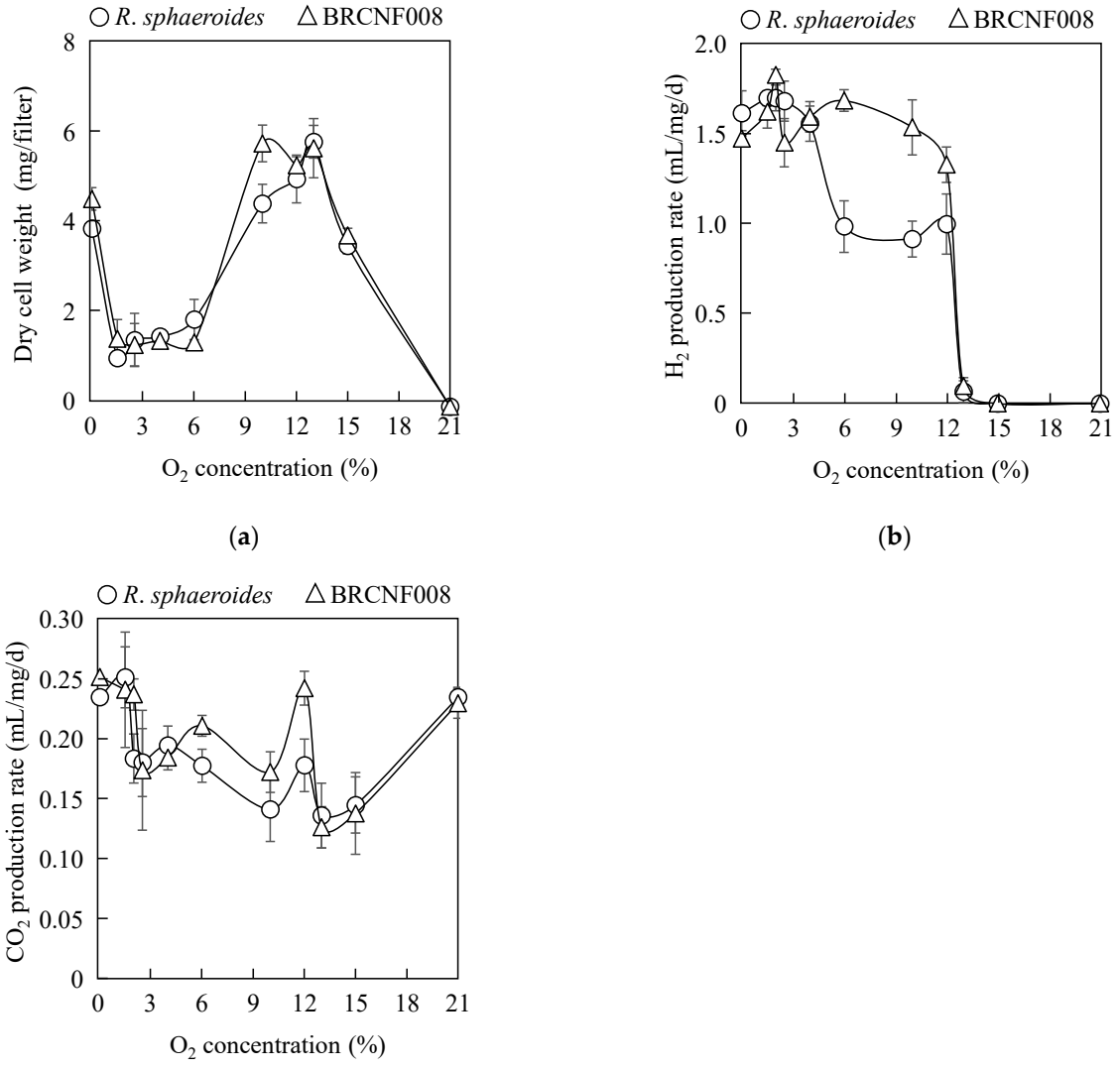


Figure 5. Influence of oxygen (O_2) concentration on the (**a**) dry cell weight, (**b**) H_2 production, and (**c**) CO_2 production of *Rhodobacter sphaeroides* and the strain BRCNF008 cultured under aerial-phase conditions. The carbon source in the N-free SA medium was glucose. The headspace of each tube was flushed with N_2 and O_2 gas (or air for the 21% O_2 conditions). Data are mean ± standard deviation of three replicate incubations.

The same culture conditions in air were extended to 14 days, and the rate of H_2 production was measured. H_2 evolution was observed, albeit weakly, in air, with H_2 production rates of 0.239 and 0.157 mL/mg/d for BRCNF008 and *R. sphaeroides*, respectively. The CO_2 production rates for BRCNF008 and *R. sphaeroides* were 0.493 and 0.359 mL/mg/d, respectively. At the end of incubation (day 14), the residual O_2 gas in the systems with BRCNF008 and *R. sphaeroides* was 15.0% (140.9 μmol) and 12.8% (119.9 μmol), respectively. When incubated in air without replacement gas such as N_2 or Ar, H_2 was not produced after 7 days, but was produced after 14 days of incubation. It is thought that not only the

high tolerance to O_2, but also the accumulation of excess ATP by respiration, which was used for hydrogenase activity, resulted in the generation of H_2 after 14 days [42].

4. Conclusions

To construct a H_2 production system using photosynthetic bacteria isolated from a wall, strains that produced H_2 under aerial-phase conditions were identified. The effect of the replacement gas composition in the reaction-vessel headspace on H_2 production by the systems was investigated. In both N_2 and Ar gas, BRCNF001, BRCNF005, and BRCNF008 showed higher H_2 production rates under aerial-phase conditions than under liquid-phase conditions, with BRCNF008 showing the highest H_2 production rate of the strains. BRCNF008 was also shown to have relatively high O_2 tolerance. Examination of the O_2 concentration in the aerial phase showed that BRCNF008 and *R. sphaeroides* were able to produce H_2 at up to 12.0% O_2 concentration over 7 days of culture. CO_2 was also produced, suggesting that the bacteria were respiring and consuming O_2 to produce anaerobic conditions in the aerial phase. When the bacteria were incubated in air for 14 days, H_2 production was observed, even though H_2 was not produced after 7 days under these conditions. This study demonstrates the possibility of H_2 production under aerial-phase conditions using photosynthetic bacteria and the potential for low-cost biohydrogen production in air. In the future, more efficient H_2 production will be achieved by optimizing various basic culture conditions such as light, pH, temperature, and scale.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10050248/s1, Figure S1: Photograph of biofilms seen from an angle; Figure S2: Phylogenetic tree of the isolated bacteria with its known neighbors based on 16S rRNA gene sequences constructed using the bootstrapped neighbor-joining method.

Author Contributions: Conceptualization, N.A.; methodology, N.A., H.T., H.K. and T.S.; validation, N.A.; investigation, N.A., H.T., H.K. and T.S.; writing—original draft preparation, H.T., H.K. and T.S.; writing—review and editing, N.A.; supervision, N.A.; project administration, N.A.; funding acquisition, N.A. All authors have read and agreed to the published version of the manuscript.

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