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H_2 Production by a Purple Sulfur Bacterium Blooming in Lake Kaiike

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Abstract : H_2 production by *Chromatium* sp., a large purple sulfur bacterium blooming in lake Kaiike, under various environmental conditions was examined. *Chromatium* sp. produced H_2 only in the presence of light and H_2S. Maximum H_2 production (0.01 μmol/hr/(mg dry cell weight)) was obtained in the solution of 20 mg H_2S-S/*l* under low light intensity (1000 lux) at 30℃. H_2 production was severely inhibited by the presence of N_2 or NH_4^+. The rate observed for *Chromatium* sp. was relatively low compared to that of other phototrophic bacteria. *Chromatium* sp. is probably a most potent H_2 producing species in lake Kaiike, since the bacterium readily produced H_2 photoautotrophically even at low light intensities by the application of suboptimal H_2S concentrations. Based on the photoautotrophic characteristics of bacterial H_2 production, it is suggested that *Chromatium* sp. can be an economic and practical species for biological H_2 production system, particularly in temperate region.(Received May 30, 1996; accepted November 21, 1996)

Introduction

Lake Kaiike is located at the Kamikoshiki island, Kagoshima Prefecture, Japan, and of which surface area and maximum depth are 0.15 km^2 and 11.6 m, respectively.[1]

The lake is conspicuous by its two environmental characteristics. One is thermal stratification which leads to two vertically different environments, oxic and anoxic one, and the other is the presence of H_2S-rich water below the mid-depth of the lake.[1] This physically characteristic conditions may be deeply related to the formation of the interesting biological community found in Lake Kaiike,[2-4] and which gives an attraction for studies of biological processes, e.g., bacterial photosynthesis, N_2 fixation, and H_2 production, resulting from the interrelationship between biological community and physicochemical environment.

Chromatium sp. , a large purple sulfur bacterium, is blooming at an upper boundary of the H_2S layer in the lake throughout the seasons. The dense population of *Chromatium* sp. at the mid-depth of the lake is called the bacterial plate.[5]

Most phototrophic bacteria can produce large quantities of H_2 when grown photoheterotrophically and supplied with certain amino acids, e.g., glutamate, as nitrogen source.[6] This H_2 production by anaerobic phototrophic bacteria in the light is now known to be performed by the action of nitrogenase.[7]

In our previous study, it is observed that *in situ* H_2 production by *Chromatium* sp. is mediated by the nitrogenase.[8] More detailed information on how H_2 is produced by *Chromatium* sp. under experimental conditions are not yet known.

A number of environmental factors may affect the energy-dependent H_2 production by the phototrophic bacteria. Most important factors affecting photoproduction of H_2 would be light and proper inorganic or organic compounds as electron donors.[9] N_2 reduction by *Chromatium* sp. is shown to be dependent on both light and H_2S.[10,11] In addition, NH_4^+ and N_2 are known to be inhibiting factors for the photoproduction of H_2.[12]

H_2 can be biologically produced by solar energy, and has a great significance from the fact that H_2 is utilizable as an alternative fuel source.[13] *Chromatium* sp. isolated from the lake Kaiike is capable of converting solar energy into H_2.[8]

In the present study, the characteristics of H_2 production by *Chromatium* sp. and the bacterial differences in response to environmental factors are established.

Materials and Methods

Chromatium sp. was isolated from the bacterial plate of Lake Kaiike on May 1983.[4] The bacterium was only similar to *Chromatium buderi* of members of Chromatiaceae family in requirement for vitamin B_{12} as well as in its size, shape, motility, plemorphism, lack of for-

Key words : Purple sulfur bacterium, *Chromatium* sp., H_2 production, Environmental conditions, Lake Kaiike.
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mation of aggregates, appearance of both single cells and cell suspension and requirement for NaCl.[4,14] However, in the point of different time-serial change of cell size during growth, different absorption spectrum of living cell suspension, and different use of sulfur and carbon compounds, the bacterium was regarded as different species from *Chromatium buderi.*, and thought to be a new one of Chromatiaceae family.[4]

In this paper, a classification of strain, if necessary, will be specified in the text for making distinction between *Chromatium* sp. of Kaiike strain and the other member of Chromatiaceae shown in the following text, e.g., *Chromatium* sp. of Miami PBS 1071. If there is no specification about a strain of *Chromatium* sp., it will be regarded as one of Kaiike strain.

Chromatium sp. was grown at the conditions of 1000 lux, 25℃, pH 7.9~8.4 and 130 mg H_2S-S/*l* with the medium of Pfennig.[15] NaCl and $MgSO_4 \cdot 7H_2O$ in the medium were increased to 25 g and 3.5 g/*l*, respectively, for marine habitat of the bacterium, and the trace element solution of SL 7 was replaced with a solution of SL 10.[10]

NH_4^+-grown bacterial cells in the exponential growth phase were harvested by centrifugation (670×g, 15 min), and the pellets were resuspended with NH_4^+-free medium (3 times), and were used for the following experiment.

A 50-m*l* bacterial suspension prepared by the above procedure was poured into a 100-m*l* syringe. Then, 50 m*l* of gas mixture of C_2H_2 and Ar (C_2H_2 20%, Ar 80%, V/V) was injected into the syringe, and was placed into a waterbath. Syringes in waterbath were gently agitated and rotated manually at intervals for ameliorating the gas exchange between the gas and the liquid phases.

In the absence of NH_4^+, the formation of H_2 and C_2H_4 was concurrently observed from the gas mixture (C_2H_2 20%, Ar 80%) under the anaerobic condition in the light, and rates of which were shown to be distributed in about an equal molar ratio.[8] This reflects that the H_2 production is catalysed by nitrogenase.[16]

Ilumination was provided by 100-W incandescent lamps, perpendicularly positioned over the waterbath. A black nylon net was used for obtaining different light levels (0~7000 lux) by rolling around syringes.

Water temperatures were adjusted to 10 to 37℃, and H_2S concentration in the bacterial suspension was adjusted by addition of H_2S solution (pH 7.0), reaching the final concentrations of 0 to 100 mg H_2S-S/*l*.

Each NH_4^+ concentrations in suspensions was adjusted by addition of NH_4^+ standard solution (pH 8.0). N_2 content in a gas phase of syringe was prepared by diluting N_2 with Ar gas.

All the detection of H_2 produced was done with a semi-conductor detector-gas chromatograph (Sensortec. Inc. Ltd.). Known volume of sample from the gas phase was collected using a microsyringe, and was analyzed and quantitated. The limit of detection of H_2 was 2×10^{-7} moles/*l*.

H_2 production rates were calculated from the values of H_2 formed as a function of time, using linear regression analysis. This was based on dry weight of the bacterial cells containing intracellular sulfur globules, determined by centrifugation of bacterial suspensions, drying at 105℃ for 2 hrs, and keeping in a desicator to reach room temperature.

Field work was done on May 21, 1996, for obtaining the vertical profiles of Lake Kaiike.

Sampling was done at a central point of the lake. Water temperature was measured with a guaranteed glass thermometer. Salinity was measured with digital salinometer E-202 (Tsurumi Seiki Co. Ltd.). Dissolved oxygen concentration was measured by the Winkler titration method. Hydrogen sulfide concentration was measured by the iodometric titration of CdS precipitate, which was formed by adding $CdCO_3$ suspension into water sample.[17] Bacterial number was microscopically counted using a Thoma hemacytometer.

Results and Discussion

Fig. 1 shows the microphotograph of *Chromatium* sp. containing sulfur globules. The bacterial cell was ovoid to rod-like in shape, and cell dimensions were 3.5 to 8.0 by 5.5 to 11.0 μm during the exponential growth phase.

Figs. 2 and 3 show the environmental conditions of temperature, salinity, O_2 and H_2S concentrations of Lake Kaiike and vertical distribution of two predominant species of the bacterial plate in the lake, respectively. Formation of strong stratification, mainly attributed to vertical changes of temperature and salinity, and the presence of light and H_2S at the depth of just beneath the stratified layer offered an habitat of *Chro-*

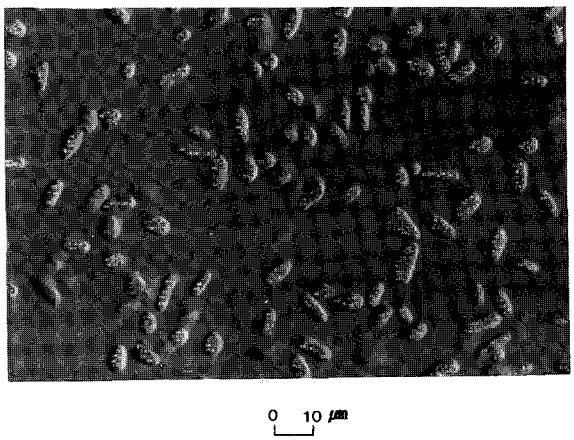

Fig. 1. Photomicrograph of *Chromatium* sp. at the exponential growth phase. Intracellular sulfur globules were observed. Scale bar is 10 μm.

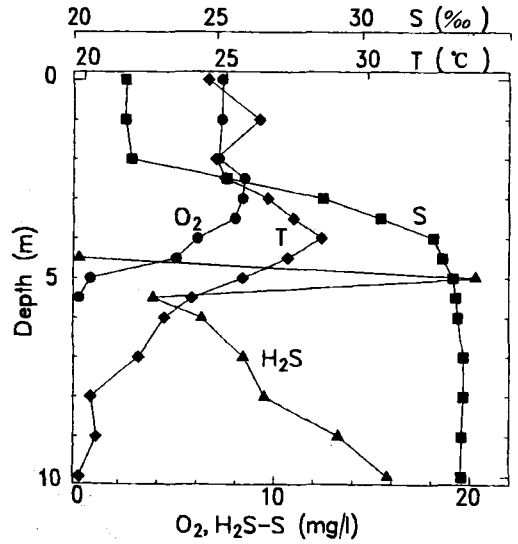

Fig. 2. Vertical profiles of salinity (‰), temperature (℃), O_2 and H_2S-S concentrations (mg/l) in lake Kaiike, on May 21, 1996.

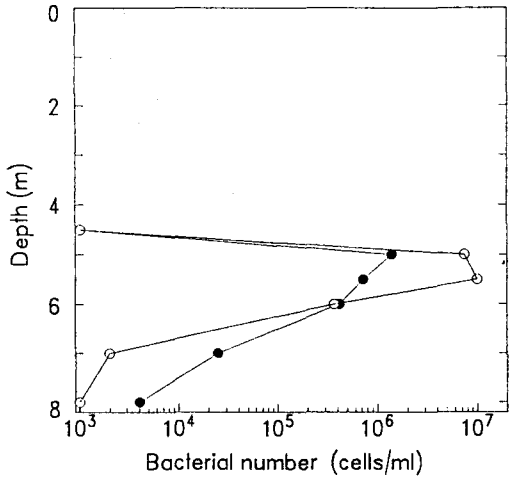

Fig. 3. Vertical distribution of two predominant bacteria at the bacterial plate of lake Kaiike, on 21 May 1996. One was phototrophic bacterium, *Chromatium* sp. (●—●), and the other was chemolithotrophic bacterium, *Macromonas* sp. (○—○).

matium sp..

Fig. 4 shows H_2 production rate of *Chromatium* sp. at light intensities of 0 to 7000 lux. The optimum light condition was 1000 lux. Decrease in rate by light was observed at more than 2000 lux, while no apparent H_2 production was observed in the dark. H_2 production by *Chromatium* sp. was shown to be light-dependent.

Effect of H_2S upon H_2 production rate is shown in Fig. 5. Different addition of neutralized H_2S solution to suspensions of *Chromatium* sp. showed different H_2 production rates (Fig. 5). However, H_2S concentrations of 20 to 100 mg H_2S-S/*l* gave no apparent differences in rate of H_2 production. Without H_2S addition, *Chromatium* sp. could not produce H_2.

Effect of temperature upon H_2 production is shown in Fig. 6. At the broad range of temperatures H_2 was produced, however, most vigorously at 30℃. At more than 35℃, an abrupt decrease in H_2 production was occurred (Fig. 6).

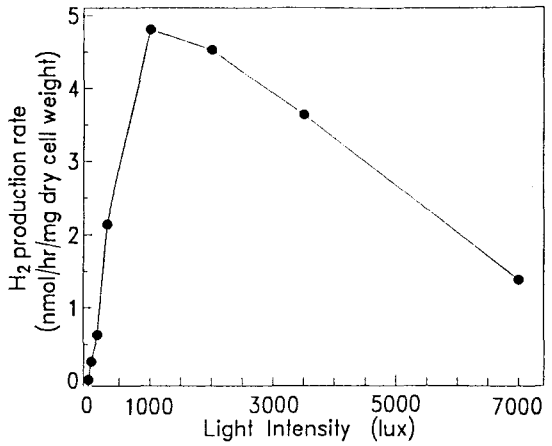

Fig. 4. H_2 production rate of *Chromatium* sp. at varying light intensities (0 to 7000 lux). The highest rate, 5 nmol/hr/(mg dry cell weight) was obtained at 1000 lux. pHs of the bacterial suspensions were 7.9 and 8.4 (0 to 2000 lux, pH 8.4; 3500 to 7000 lux, pH 7.9). The suspensions were incubated at 25℃. The bacterial number in the suspension was 2.7×10^6 cells/ml, corresponding to 0.061 mg dry weight/ml.

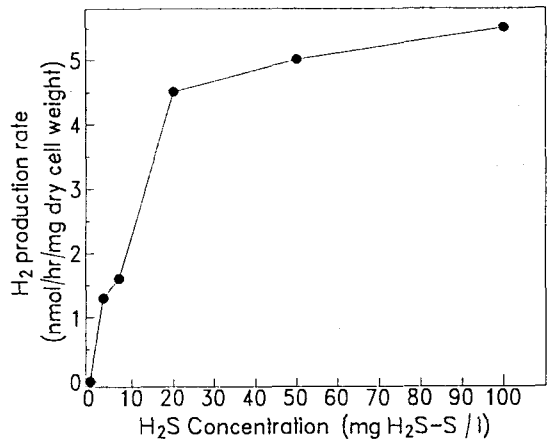

Fig. 5. H_2 production rate of *Chromatium* sp. at different H_2S concentrations. The highest rate was 5.5 nmol/hr/(mg dry cell weight) at 100 mg H_2S-S/*l*. At 20 to 100 mg H_2S-S/*l* little difference between rates was occurred. The bacterial suspensions were incubated at 25℃ and 1000 lux(0 to 20 mg H_2S-S/*l*, pH 8.3; 50 to 100 mg H_2S-S/*l*, pH 8.4). The bacterial number was 2.1×10^6 cells/ml, corresponding to 0.048 mg dry weight/ml.

Figs. 7 and 8 show that H_2 production by *Chromatium* sp. was severely inhibited by NH_4^+ and N_2, respectively. The H_2 production rate was decreased by increasing NH_4^+ addition (Fig. 7). H_2 was not detected when NH_4^+ was added as 700 μM. 45% of maximum H_2 production rate (Control) was obtained at 100 μM of NH_4^+.

Increasing N_2 concentrations decreased the rate of H_2 production by *Chromatium* sp. (Fig. 8). H_2 production was performable even in the presence of 100% N_2, while nearly a half of the maximum production rate

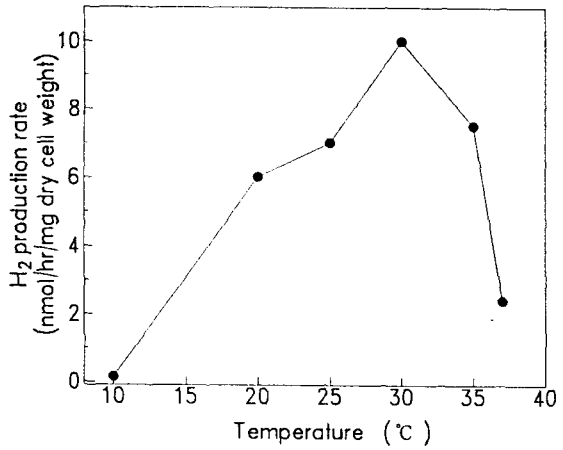

Fig. 6. H_2 production rate of *Chromatium* sp. at different temperatures. Under the conditions of 30℃, 1000 lux, and 100 mg H_2S-S/*l*, the maximum H_2 production rate, 10 nmol/hr/(mg dry cell weight), was obtained. Light was illuminated as 1000 lux. pH of the suspension was 8.3 at 10℃, and at the other temperatures pH was 8.4. The bacterial number was 2.0×10^6 cells/m*l*, corresponding to 0.045 mg dry weight/m*l*.

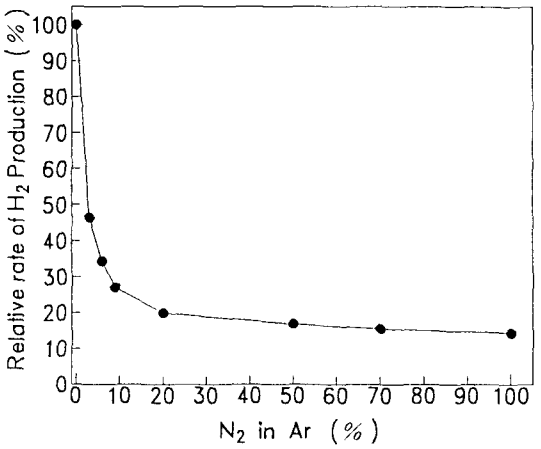

Fig. 7. Effect of NH_4^+ upon H_2 production rate of *Chromatium* sp.. NH_4^+ was added to bacterial suspension at each final concentrations specified in the Fig. Control suspension was not added with NH_4^+. Experimental conditions of light, temperature, pH and H_2S were 1000 lux, 25℃, pH 8.3 and 100 mg H_2S-S/*l*, respectively. The bacterial number was 4.5×10^7 cells/m*l*, corresponding to 1.02 mg dry weight/m*l*.

(Control) was obtained under a gas phase of 3% N_2 (Fig. 8). H_2 production and the N_2 reduction are catalysed by the same enzyme, nitrogenase, and the inhibition of N_2 upon H_2 production is known to be competitive.[7] However, in considering that H_2 was produced even under a gas atmosphere of 100% N_2 (Fig. 8), H_2 seems to be indispensably produced whenever the nitrogenase reaction is performed.[26]

Phototrophic bacteria show a light-dependent H_2 production when grown photosynthetically with proper inorganic or organic compounds as carbon or nitrogen sources in the absence of exogenous NH_4^+.[16,27,28] However, in non phototrophic bacteria, the energy source or electron donor for H_2 production (nitrogenase-mediated) is supported from only organic compounds.[29] Nitrogenase-mediated H_2 production by some diazotrophic bacteria and their various production rates are shown in Table 1. The production rate of *Chromatium* sp. was smaller than that of other bacteria.

Most phototrophic bacteria shown in Table 1 produce large quqntities of H_2 from a variety of organic compounds. Light for their growths is mostly supplied with around 5000 lux. As stated above, *Chromatium* sp. produced H_2 maximally at a light of 1000 lux and H_2S of 20 mg H_2S-S/*l* or more under inorganic growth conditions. *Chromatium* sp. is shown to have not much availability to use carbon compounds, except for limited organic compounds such as fructose and lactate, for the growth.[4]

In marine *Chromatium* sp., Miami PBS-1071, grown on a Pfennig's medium containing organic acids at a light intensity of about 8000 lux, it was shown to have a capability of producing larger quantity of H_2 than the bacterium.[21,30] Miami PBS 1071 also showed its maximum H_2 production at temperatures of 35 to 37℃, while no H_2 was produced at a temperature lower than 20℃.[21] Miami PBS 1071 was considered to have high growth potential and to be the most potent species for the H_2 production in coastal marine envirnment of tropical or subtropical places.[21,30]

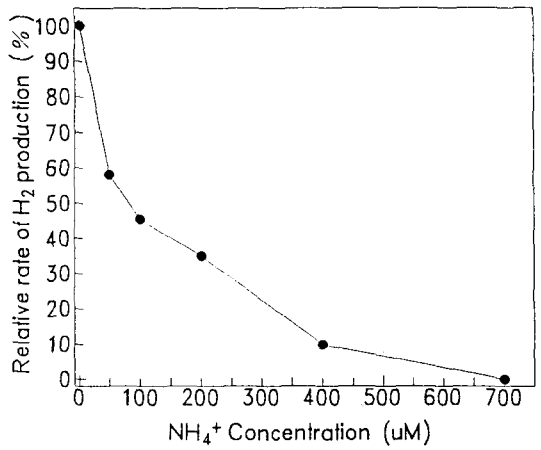

Fig. 8. Effect of N_2 partial pressure upon H_2 production rate of *Chromatium* sp.. N_2 was added to the Ar gas phase of the suspension at each final concentrations specified in the Fig.. Final volume of the gas phase was adjusted to 50 m*l*. Experimental conditions as in Fig 7. The bacterial number was 4.5×10^6 cells/m*l*, corresponding to 0.102 mg dry weight/m*l*.

In aspect of light, *Chromatium* sp. of Kaiike strain can produce H_2, even with a small quantity, at a low light intensity of 50 lux (Fig. 4), and also its optimum light condition for H_2 production is much lower than those of other phototrophic bacteria, shown in Table 1. In case of temperature condition, the bacterium could produce H_2 moderately at temperatures of 20 to 35℃ (Fig. 6).

Table 1. H_2 production rates in some diazotrophic bacteria

Species	Maximum H_2 Production Rate (Nitrogenase-Mediated)	Reference
Rhodopseudomonas capsulata	5.8*	Hillmer & Guest(1977)[6]
Rhodospirillum rubrum	6.6**	Kondrat'eva *et al.*(1979)[18]
Rhodopseudomonas palustris	1.7**	Gogotov *et al.*(1973)[19]
Rhodopseudomonas sphaeroides	1.3**	Jones & Monty(1979)[20]
Thiocapsa roseopersicina	7.6**	Kondrat'eva *et al.*(1979)[18]
Chromatium sp. (Miami PBS-1071)	6.0**	Ohta & Mitsui(1981)[21]
Marine Blue-green Alga (Miami BG 7)	230***	Mitsui & Kumazawa(1977)[13]
Anabaena cylindrica	4.9*	Benemann & Weare(1974)[22]
Klebsiella pneumoniae	12.7**	Andersen & Shanmugan(1977)[23]
Azospirillum brasilense	3.3**	Lespinat & Berlier(1981)[24]
Azotobacter chroococcum	0.3*	Smith *et al.*(1976)[25]
Chromatium sp.	10.0****	This research

*: μmol/hr/ (mg dry cell weight), **: μmol/hr/(mg protein) ***: μmol/hr/(mg chlorophyll), ****: nmol/hr/(mg dry cell weight): The rate was found under the conditions of 1000 lux, 100 mg H_2S-S/*l*, and 30℃, as in Fig. 6.

H_2 production by phototrophic bacteria in natural conditions would not be largely expected, since environmental conditions, e.g., light, organic or inorganic compounds, would become not easy to be optimum for the nitrogenase reaction, when compared to particular experimental conditions.[6,18-20] Thus, in natural environment where N_2-fixing conditions are formed, a bacterium of low environmental requirement for the growth would be a potent species of N_2 reduction or H_2 production. Though a relatively large quantity of H_2 was not produced by *Chromatium* sp. (Table 1), it is proposed that *Chromatium* sp. would be a species predominantly and readily producing H_2 in the lake.

In another aspect, biological H_2 production for development of an alternative fuel source may depend on a species capability of producing H_2. When an efficient biological H_2 production system is envisioned, the most important factors for the system would be a selection of a species which can produce high rate of H_2, and a finding of economic energy and electron source for the H_2 production.[13] In this respect, some species of blue-green algae were nicely met with the biological H_2 production system from the fact that the only major inputs into the system were solar energy and water (salt water).[13,31]

However, in respect of reusing and purifing a waste water containg H_2S, purple sulfur bacteria may become a candidate for the H_2 production system. In case of H_2 production by *Chromatium* sp. of Kaiike strain, only H_2S and CO_2 as an electron and carbon source for the growth are required, and the growth of other microbes as a contaminant source in the culture of *Chromatium* sp. of Kaiike strain are easily controlled because the bacterium could well produce H_2 without an addition of external organic compounds for the H_2 production. In addition, a light condition of 1000 to 2000 lux for the maximum H_2 production, and a temperature condition of 20 to 35℃ for the moderate H_2 production may give an economic and pratical merits for the management of the system.

Miami PBS 1071 was thought to be the best candidate for the H_2 production in relation to the fast growth and high H_2 production rate in tropical or subtropical regions.[21,30]

However, it is suggested that *Chromatium* sp. of Kaiike strain can become also a practical H_2 producer from the points of above mentioned merits for the H_2 production, in temperate region.

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카이이케호에서 농밀하게 분포하는 Purple Sulfur Bacterium의 수소생산

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초록 : 카이이케호에서 농밀하게 분포하는 *Chromatium* sp.의 수소생산을 몇몇 환경조건하에서 측정하였다. 최대 수소생산 (0.01 μmol/hr/(mg dry cell weight))은 낮은 조도 (1000 lux), 20 mg H_2S-S/*l*의 황화수소 농도와 30℃의 조건하에서 얻을 수 있었다. 수소생산은 질소가스 또는 암모늄에 의해 상당히 저해를 받았다. *Chromatium* sp.의 수소생산속도는 타 광합성세균에 비해 낮았다. 본 균은 광독립영양적으로 낮은 조도 또는 낮은 H_2S 농도하에서도 쉽게 수소를 생산하는 점으로부터 카이이케호의 가장 유력한 수소생산자라고 고려되었다. 이러한 *Chromatium* sp.의 광독립영양적 수소생산특성으로부터, 본 광합성세균은 온대역에서의 생물적 수소생산시스템에 대한 경제적이고 실용적인 생물종이 될 수 있으리라 제안되었다.

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