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Effect of ferrous ion on photo heterotrophic hydrogen production by *Rhodobacter sphaeroides*

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Abstract

The effect of ferrous ion (0–3.2 mg/l) on photo heterotrophic hydrogen production was studied in batch culture using sodium lactate as substrate. The results showed that hydrogen production by *Rhodobacter sphaeroides* was significantly suppressed when Fe^{2+} was limited. Hydrogen production increased linearly with an increase in Fe^{2+} concentration in the range of 0–1.6 mg/l; reaching a maximum at 2.4 mg/l. When hydrogen production was suppressed in the above medium, a pH increase to 8.9 was observed, and the ratio of lactate utilized to total organic carbon removal was found to be increased, indicating that more soluble organic products were produced. Under the Fe^{2+} limited conditions, ferrous iron was shown to have a greater effect on hydrogen production by *Rb. sphaeroides* than that by the anaerobic heterotrophic bacterium *Clostridium butyricum.*

Keywords: Ferrous effect; Biological hydrogen production; Photo heterotrophy; *Rhodobacter sphaeroides*; pH; Total organic carbon

1. Introduction

Photo heterotrophic hydrogen production is continuously attracting research interests [1–7]. It is partly because the waste organic matters and solar energy can be utilized for this process. However, studies on the optimal conditions of this process, especially the nutritional ingredients of the feedstock, are still rare. Lack of this information could directly affect the application of this process.

Iron is essential for the growth of almost all microorganisms. The basic physiological function of Fe is as a cofactor for some proteins, most of which are related to energy metabolism. Anoxygenic phototrophic bacteria produce hydrogen as a mechanism of disposing excess reducing equivalences which accumulate during growth in nutritionally rich anaerobic conditions [8]. This process of hydrogen photo fermentation is strongly coupled with the photosynthetic electron transport system, through which the bacteria obtain energy [8]. Many electron carriers of the system, including cytochromes, are protein complexes containing Fe. Moreover, nitrogenase, the functional enzyme of hydrogen production in these bacteria, has been reported to contain 24 Fe in each molecule [9]. Ferredoxin, which is an electron carrier for nitrogenase also contains Fe. Because of the importance of Fe to the light energy utilization and hydrogen production system of anoxygenic phototrophic bacteria, it is believed that the availability of Fe in the culture medium may greatly influence hydrogen production in these microorganisms.

Studies incorporating Fe in the medium for hydrogen production have used 2.4 mg/l Fe^{2+} (ca. 11.8 mg/l $FeSO_4 \cdot 7H_2O$). This concentration of Fe^{2+} was first adopted by Ormerod et al. for the cultivation and hydrogen production of *Rhodospirillum rubrum* [10]. Other studies using this concentration of Fe^{2+} have been for the growth and hydrogen production of *Rhodopseudomonas capsulata* (current name *Rhodobacter capsulatus*) [11] and *Rhodobacter sphaeroides* [12]. Nevertheless, there has not been report on how and in what extent the Fe^{2+} influences the photo heterotrophic hydrogen production by those anoxygenic phototrophic bacteria. Knowledge on the effect of

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Fe^{2+} on the photo heterotrophic hydrogen production is important from an engineering perspective, especially when wastewater or waste biomass is used as a feedstock. Since these kinds of feedstock are diverse in their Fe^{2+} concentration, exogenous Fe^{2+} may be of critical importance for effective hydrogen production.

In this research, the effect of various Fe^{2+} concentrations on hydrogen production by anoxygenic phototrophic bacteria was systematically studied. Bacterial growth, substrate utilization, pH variation and total organic carbon (TOC) removal during the hydrogen production were examined. Iron in the bulk medium and biomass was determined, as well as the optical absorption spectrum of the culture medium. The effect of Fe^{2+} on the hydrogen production by *Clostridium* was also examined for comparative purposes.

2. Materials and methods

2.1. Bacterial strains and growth conditions

An anoxygenic phototrophic bacterium *Rb. sphaeroides* DSM 158 was used in this study. An aSy medium [12] containing 1.25 g/l ammonium sulfate, 9.8 g/l sodium succinate, 1 g/l yeast extract and a basal solution was employed for the growth and maintenance of this bacterium. The aSy medium was used because ammonium, the intermediates of tricarboxylic acid cycle and some vitamins are essential for the heterotrophic growth of this bacterium [13]. In the basal solution mineral salts, including 12 mg/l $FeSO_4 \cdot 7H_2O$, and trace amount of vitamins were contained. All water used was ultra pure (EASYpure UV/UF, Barnstead, Iowa, USA). The medium was flushed with nitrogen gas for 10 min and autoclaved at 121 °C for 30 min before use. The bacterium was grown in a test tube (13 ml) or flat tissue flask (200 ml), which was completely filled with the medium, capped and incubated at 30 °C under a radiation of tungsten lamp at a flux of 200 W/m^2. The bacterial culture used for hydrogen production was incubated for about 12 h.

A heterotrophic anaerobic bacterium, *Clostridium butyricum* DSM 10702, was used for comparative purpose. The growth medium for this bacterium was Medium 104 (DSMZ) with a minor modification by replacing the salt solution with aSy basal solution. The bacterium was cultivated in a serum bottle placed in an anaerobic chamber (Forma Scientific, Ohio, USA) at 35 °C for overnight. The medium was flushed with nitrogen gas and autoclaved at 121 °C for 30 min before inoculation.

2.2. Hydrogen production experiment

Before being used to examine the effect of Fe^{2+} on hydrogen production, the *Rb. sphaeroides* from the growth cultivation step was harvested by centrifuge at 4000 rpm for 20 min and cultivated in an intermediate step in a sterilized gL medium to initialize hydrogen production activity [12]. The gL medium contained sodium glutamate (10 mmol), sodium lactate (83 mmol) and sodium hydrogen carbonate (1.5 g/l) dissolved in the aSy basal solution. The intermediate cultivation was performed in 200 ml flat tissue flasks as described previously [14,15] with some modifications. The flasks were inoculated with the harvested bacteria from growth cultivation step, filled up with gL medium (a small headspace was left for the gas collection purpose), sealed with silicon stoppers and immersed in a 30 °C water bath made from polyacrylic resin. A tungsten light, with a flux of 135 W/m^2, was radiated to the culture liquid from the side of the water bath. The radiation area of the flask was 75 cm^2. The gas produced from each flask was collected in a 200 ml cylinder which was immersed in water and connected to the flask through apipetting needle (18 G, 5.1 cm) fixed in the stopper and silicon tubing, and measured by water displacement method. The cylinder was connected to a septum at the end of the experiment for gas sample collection purpose. A second pipetting needle (10.2 cm) was fitted in the stopper for liquid sampling purposes. A valve and a syringe filter (0.22 μm) were connected to this needle to prevent contamination during sampling and sampling intervals.

Once a steady rate of hydrogen production was detected from the gL medium in the intermediate cultivation step, the bacterium was harvested again (centrifugation at 4000 rpm, 20 min), washed twice using a modified gL medium with $FeSO_4 \cdot 7H_2O$ removed, and re-suspended in the same modified medium to a final cell density of 22 mg dry weight/ml for further Fe^{2+} effect experiments. The experiment to determine the Fe^{2+} effect on hydrogen production was the same as the method used in the intermediate cultivation step, except various concentrations of $FeSO_4 \cdot 7H_2O$ were added to the gL medium. Each flask was inoculated with 1.5 ml of washed culture suspension containing 33 mg dry weight of cells. In the experiments to determine the effect of Fe^{2+} on hydrogen production, each experiment was repeated 2–3 times. However, the amount of the inoculum in the repetition experiments was not controlled, varying from 17.4 to 27.6 mg dry weight per flask.

The experiments to compare Fe^{2+} effects on hydrogen production by *C. butyricum* and *Rb. sphaeroides* were carried out with the same methods described above, except that the medium used for hydrogen production was changed to a glucose medium [14] containing 54 mmol glucose, 10 mmol sodium glutamate, 0.1 g/l meat extract, 0.2 g/l yeast extract, and 0.1 mol sodium phosphate buffer (pH 7.9) in 1 l aSy basal solution. Intermediate cultivation of *Rb. sphaeroides* was performed to activate its hydrogen production activity from glucose. There was no intermediate cultivation step for *C. butyricum* because this bacterium produced hydrogen during the growth cultivation. The medium for the Fe^{2+} effect experiment was the glucose medium with $FeSO_4 \cdot 7H_2O$ removed. The volume of the culture inoculated in each reaction flask (200 ml) was 40 ml, with cell concentrations of 0.95 and 1.35 mg dry weight/ml for *C. butyricum* and *Rb. sphaeroides*, respectively.

2.3. Chemical analysis

Gas analysis was performed using a gas chromatograph (Hewlett Packard 5890 II, Florida, USA) equipped with a thermal conductivity detector and a 2 m × 2 mm column packed with Porapak N (80–100 mesh) [16]. Lactate in the culture medium was examined by high performance liquid chromatography

(LC-10 ADVP, Shimadzu, Kyoto, Japan) equipped with a Aminex column (300 mm × 7.8 mm, HPX-87H, Bio-Rad Laboratories, California, USA) and run with a mobile phase containing 8 mmol sulfuric acid, flow rate of 0.60 ml/min, column temperature of 30 °C and UV detector of 210 nm at 30 °C. TOC in liquid phase was analyzed in a total organic carbon analyzer (TOC-5000A, Shimazu, Kyoto, Japan) and the pH examined with a compact pH meter (TWINpH B-213, Horiba, California, USA). Volatile fatty acids and alcohols were analyzed by a second GC of the same model equipped with a flame ionization detector and a 10 m × 0.53 mm HP-FFAP fused-silica capillary column [16].The optical density OD660 and the absorption spectrum (400–1100 nm) of the culture medium were measured with a spectrophotometer (UV-160, Shimadzu, Kyoto, Japan).

The concentration of total Fe in the culture medium (filtered by a 0.22 μm filter) was determined using an atomic adsorption analyzer (AAnalyst 300, Perkin Elmer, USA). To examine the Fe assimilated in the bacterial cells, the cells were harvested by centrifugation at 4000 rpm for 20 min and washed twice with 1.42% (v/v) nitric acid solution. The pellets were re-suspended in 70% nitric acid solution for digestion, and then diluted to different concentrations in a volumetric flask for analysis on the atomic adsorption analyzer.

3. Results

3.1. Effect of Fe^{2+} on hydrogen production

The concentration of Fe^{2+} added in the culture medium ranged from 0 to 3.2 mg/l in this study. It is important to note that trace Fe^{2+} may enter the experiments without Fe^{2+} being added through the inoculum. Therefore, the experiments without Fe^{2+} added are referred to as iron limiting. Hydrogen production by *Rb. sphaeroides* was greatly influenced by Fe^{2+} in the experimental range of the Fe^{2+} concentration (Fig. 1, Table 1). Hydrogen production was suppressed under Fe^{2+} limiting conditions with only 53 ml hydrogen evolved per flask. It increased linearly with an increase of Fe^{2+} concentration in the range of 0–1.6 mg/l. It reached the maximum when the Fe^{2+} concentration was 2.4 mg/l. A further increase of Fe^{2+} concentration to 3.2 mg/l did not result in further increase of the hydrogen production, in fact, the hydrogen production decreased slightlycompared with the Fe^{2+} concentration of 2.4 mg/l. Hydrogen production in flasks with a Fe^{2+} concentration range of 1.6–3.2 mg/l lasted for over 100 h. When Fe^{2+} concentration was 2.4 mg/l, the total hydrogen evolved and production rate were 459 ml/flask and 0.15 ml/h/mg dry weight, respectively, resulting in a substrate conversion efficiency of 28.2% and a light conversion efficiency of 1.9%. The substrate conversion efficiency and the light conversion efficiency were at an intermediate level compared with that reported in literature [17].

3.2. Effect of Fe^{2+} on bacterial growth and substrate utilization

As shown in Fig. 2A, ferrous ion did not influence bacterial growth as significantly as hydrogen production. The effect on bacterial growth was only evident after 25 h in Fe^{2+} limited medium. In all the cases, the optical density of the bacterial cultures reached a maximum value in 35–40 h, after which, the O.D. decreased. Similar to the bacterial growth, lactate utilization (Fig. 2B, Table 1) was only influenced by Fe^{2+} to a minor extent. In comparison to the flasks with Fe^{2+} added, the utilization of lactate in the flask without addition of Fe^{2+} slowed down after 25 h. No obvious difference was observed among flasks with Fe^{2+} concentrations greater than 0.8 mg/l.

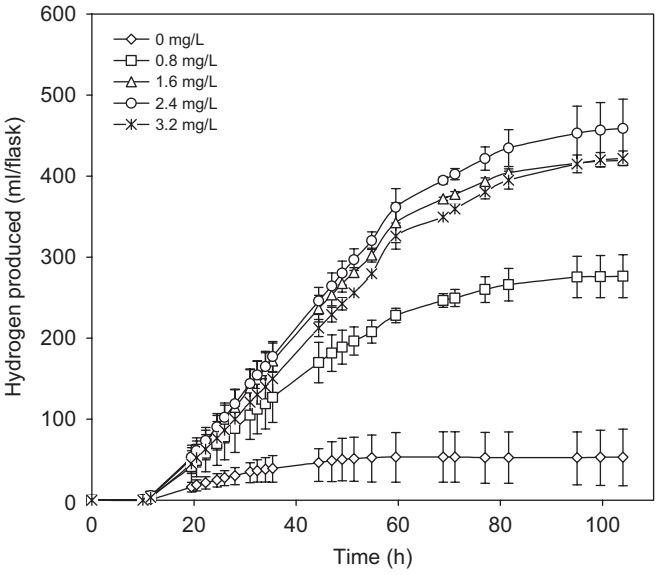

Fig. 1. The effect of Fe^{2+} concentration on phototrophic hydrogen production. The average deviation depicted was calculated from data obtained from the three experiments.

These results demonstrated that the Fe^{2+} affected the hydrogen production to a larger extent than the bacterial growth.

3.3. Effect of Fe^{2+} on TOC removal

TOC removal is a function of both substrate utilization and production of soluble metabolites. TOC removal, with respect to Fe^{2+} concentrations, is showed in Fig. 2C and Table 1. Ferrous ion concentration had a greater affect on TOC removal than on bacterial growth and lactate utilization. TOC removal rate from Fe^{2+} limited medium was only 24%, while TOC removal rate was approximately 60% when Fe^{2+} was above 2.4 mg/l. The observation that TOC removal was lower than the lactate utilization indicates that other organic metabolites were being produced. The ratio of lactate utilization to TOC removal indicates the portion of carbon source converted to soluble metabolic organic compounds. In the case of the Fe^{2+} limited medium, the ratio was 1.75, much greater than all other Fe^{2+} concentrations (Table 1).

3.4. Effect of Fe^{2+} on pH variation

Fig. 2D shows the effect of Fe^{2+} on pH variation during hydrogen production. After a 10 h delay, the pH increased

Table 1
Summary of Fe^{2+} effect on hydrogen production, substrate utilization and TOC removal

Conc. of Fe^{2+} (mg/l)	H_2 produced (ml/flask)	H_2 production rate (ml/h/mg dry weight)	Lactate utilized (%)	TOC removal (%)	Lactate utilized/ TOC removal
0	53	0.03	42	24	1.75
0.8	277	0.08	61	46	1.32
1.6	420	0.14	75	59	1.27
2.4	459	0.15	76	60	1.26
3.2	422	0.14	72	61	1.18

drastically from 7.2 to 8.9 in Fe^{2+} limited medium in 50 h. The pH value increased from 7.2 to 7.7 when the Fe^{2+} concentration was 0.8 mg/l, and from 7.2 to around 7.5 at all other Fe^{2+} concentrations. The pH value reached the maximum at 50 h and then dropped slightly until the end of the cultivation. VFAs and alcohols analysis indicated that no significant quantities of fatty acids and alcohols were produced during the experiments at all Fe^{2+} concentrations (data not shown).

It was assumed that the increase of pH was a result of the suppression in hydrogen production and the metabolism shifting to soluble products other than VFAs and alcohols. With the suppression in hydrogen production, the excessive reducing equivalents accumulated in the cells during the cultivation are possibly diverted to other metabolic processes such as the production of intra or extra cellular organic matter. The observation on the difference between the lactate utilization and the TOC removal supported this hypothesis. *Rb. sphaeroides* has been reported to accumulate poly-β-hydroxybutyrate (PHB) during hydrogen production [18]; the accumulation of PHB is competitive for excess reducing equivalents and can result in a drop in hydrogen production. As a result, more carbon sources accumulate inside or outside of the cells as a form of organic metabolites without being decomposed to hydrogen and CO_2. The reduction of lactate, the only carbon source used in the medium, may change the acid–base balance of the cultivation liquid and result in an increase in pH. When hydrogen is produced, the co-evolved CO_2 may neutralize the alkalinity and moderate the pH variation.

3.5. Fe concentration in solution and in the bacterial cells

Total Fe concentrations in solution at the end of the hydrogen production experiments (i.e., 100 h) were about 50% of their initial values in all flasks except the flask with no Fe^{2+} added and the flask with 3.2 mg/l Fe^{2+}, which was only 30% of the initial value (Fig. 3A). Total Fe concentration in the cells also increased with the increase of initial Fe^{2+} concentration in the medium, with the exception of 3.2 mg/l Fe^{2+} (Fig. 3B). As noted in Section 3.1, a trace amount of Fe was detected in samples without Fe^{2+} added; 0.017 mg/l in bulk medium and 0.061 mg/g dry weight in bacterial cells. When 3.2 mg/l Fe^{2+} was present, both measured concentrations in solution and bacterial cells decreased compared with that observed in the sample with 2.4 mg/l Fe^{2+}. $FeSO_4 \cdot 7H_2O$ is a typical inorganic coagulant. It was possible that, when the $FeSO_4 \cdot 7H_2O$ concentration increased and surpassed a certain value, for example 23.5 mg/l (equivalent to 3.2 mg/l Fe^{2+}) in this study, its coagulation effect became significant, thereby negatively influencing the hydrogen production. With the coagulation effect, more Fe^{2+} was entrapped in the floccule or precipitated on the surface of the bacterial cells, and lost during the acidic wash when the sample was being processed for Fe analysis.

3.6. Optical absorption spectrum

The optical absorption spectra of the culture liquid at the beginning and end of the experiments for Fe^{2+} limited and 2.4 mg/l are shown in Fig. 4. Several peaks in the infrared region (802–968 nm) were observed in all three adsorption spectra, which are characteristic absorption peaks for anoxygenic phototrophic bacteria and are indicators for their light reaction center [19]. Another peak at 590 nm was possibly a characteristic peak related to bacteriochlorophy II [20], which was also observed in all three spectra, although it was more obviously seen at the end of the experiments in flasks with Fe^{2+} added. The difference of the peak size was probably due to the difference of cell concentrations in different samples. According to the observation of the optical absorption spectrum, there was no evidence that Fe^{2+} affected the synthesis of structural components such as the light harvesting system in the bacteria.

3.7. Comparison of Fe^{2+} effect on hydrogen production by C. Butyricum and Rb. sphaeroides

Both bacteria produced significant hydrogen in their intermediate step or growth cultivations from the glucose medium with sufficient Fe^{2+} (data not shown). After transferring to the medium without addition of Fe^{2+}, they showed very different hydrogen production capability (Fig. 5). Hydrogen production by *C. butyricum* was about 1 mol/mol glucose, not significantly affected by the removal of Fe^{2+}, while hydrogen production by *Rb. sphaeroides* was almost completely suppressed. This observation demonstrated that the Fe^{2+} effect on hydrogen production by *Rb. sphaeroides* is much greater than that by *C. butyricum*, in other words, the threshold Fe^{2+} concentration which suppresses hydrogen production by *Rb. sphaeroides* is much higher than that which suppresses the hydrogen production by *C. butyricum*.

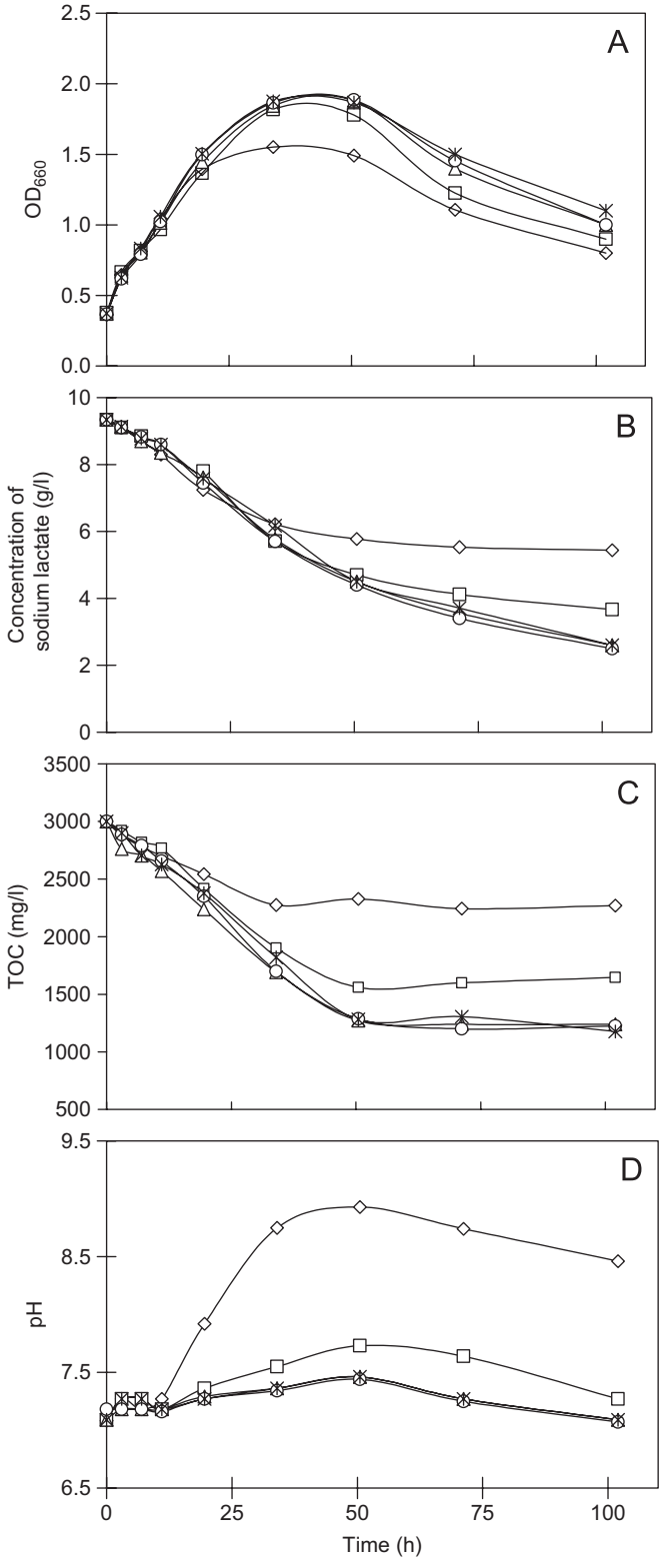

Fig. 2. Bacterial growth, substrate utilization, TOC removal and pH variation during hydrogen production under the effect of Fe^{2+} concentration. ◇, 0 mg/l; □, 0.8 mg/l; △, 1.6 mg/l; ○, 2.4 mg/l; ×, 3.2 mg/l. (A) optical adsorption; (B) lactate concentration; (C) TOC; (D) pH.

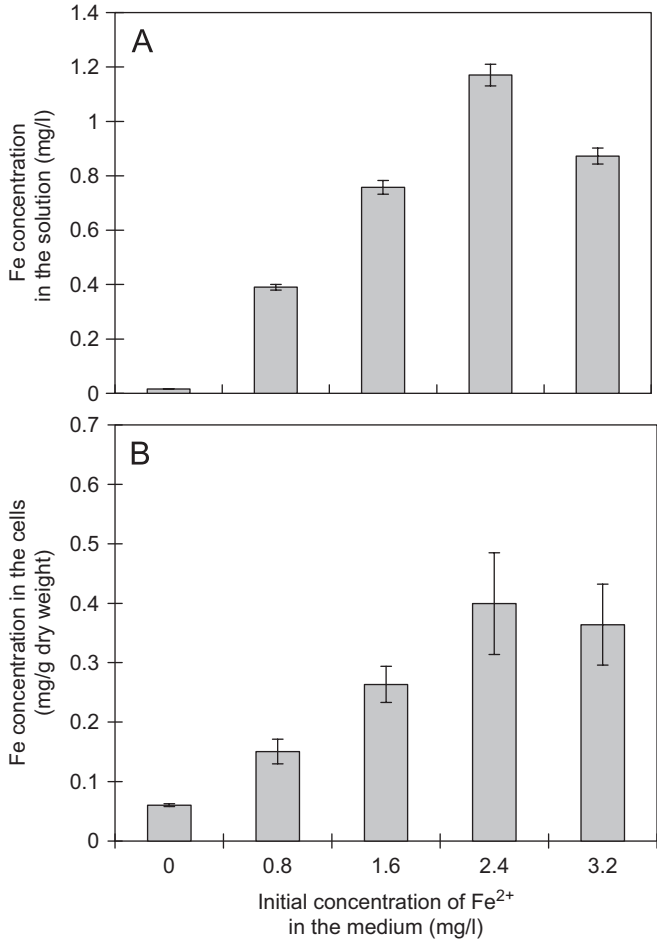

Fig. 3. The concentration of soluble Fe in culture medium (A) and assimilated Fe in the bacterial cells (B) at the end of cultivation (100 h).

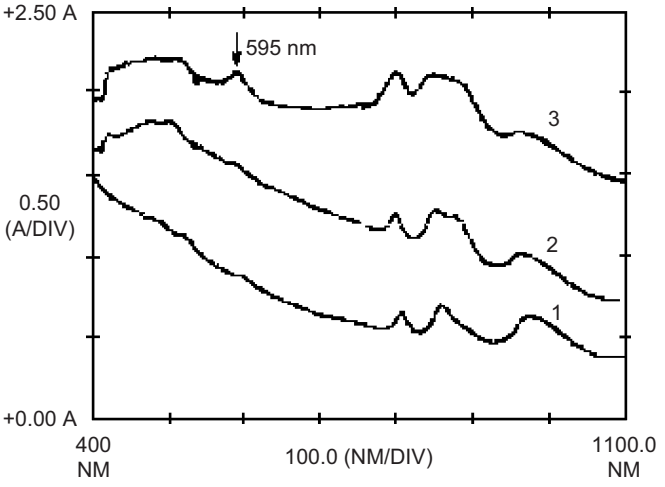

Fig. 4. The optical adsorption spectrums of the intact cells at the beginning of cultivation (1), at the end of cultivation with Fe^{2+} limitation (2), and at the end of cultivation with Fe^{2+} of 2.4 mg/l (3).

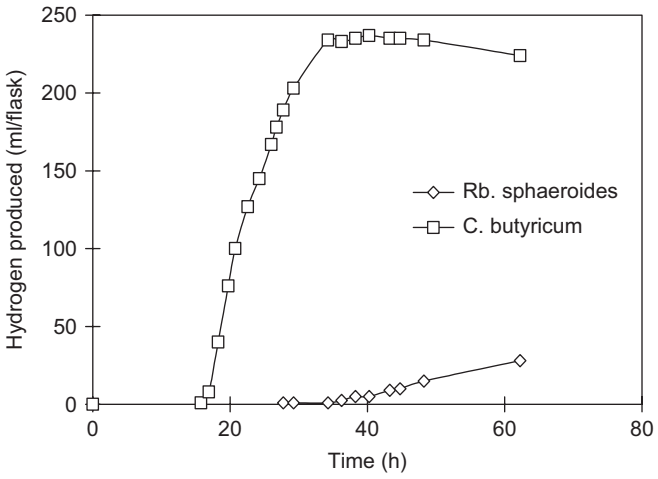

Fig. 5. Hydrogen production from a glucose medium by *C. butyricum* DSM 10702 and *Rb. sphaeroides* DSM 158 under Fe^{2+} limited conditions.

4. Discussion

The minimum concentration of ferrous ion for active hydrogen production by anoxygenic phototrophic bacteria was found to be around 2.4 mg/l. This concentration was identical with the Fe^{2+} concentration contained in the Omerod medium [10]. Under Fe^{2+} limitation condition, the hydrogen production activity was significantly reduced to about 10%. Probably due to the coagulation effect of the $FeSO_4 \cdot H_2O$, when the Fe^{2+} concentration was higher than 2.4 mg/l (equivalent to 17.6 mg/l $FeSO_4 \cdot 7H_2O$), no further increase in hydrogen production was observed. The coagulation effect could significantly change the charge distribution on the surface of bacterial cells and make them aggregated together, thereby negatively affecting the bacterial activity.

Ferrous ion effects on the anoxygenic phototrophic bacteria appear to be somehow specific to hydrogen production. Its effect on bacterial growth and substrate utilization (lactate) was less obvious in the Fe^{2+}range examined in this research. The less obviousness of Fe^{2+} effect on the absorption peaks of light harvesting components in the optical absorption spectra (Fig. 4) also support these observation. It implied that Fe^{2+} influences hydrogen production through some factors directly involved in the hydrogen production, such as nitrogenase and ferredoxin, when its concentration is below 2.4 mg/l.

Hydrogen production by anoxygenic phototrophic bacteria can be effected by the Fe^{2+} to a larger extent than that by heterotrophic anaerobic bacteria. Lee et al. [21] have reported that dark fermentation of hydrogen by anaerobic sludge was not affected by $Fe^{2+}$when $FeCl_2$ concentrations were in the range of 3–80 mg/l, but by Fe^{2+} larger than 80 mg/l. The Fe^{2+} effect observed by Lee et al. was probably not in a metabolic level, because the Fe^{2+} concentration larger than 80 mg/l is much higher than the normal physiological requirement of bacteria. There is no other report on the Fe^{2+} effect on the dark fermentative hydrogen production in a lower concentration range ($\leqslant$3 mg/l). Our study demonstrated that the dark fermentative hydrogen production is not significantly effected by Fe^{2+} at a much lower concentration level. Since the hydrogen production by anaerobic heterotrophic bacteria is mediated by hydrogenase, this observation provided evidence that the effectiveness of the Fe^{2+} on hydrogen production mediated by nitrogenase is much higher than that mediated by hydrogenase.

As discussed previously, the suppression of hydrogen production by the deprivation of Fe^{2+} may cause the reducing equivalents to be diverted to other organic metabolites like PHB, thereby causing a faster pH rising. The pH also dropped down after it reached a maximum value. The phenomenon was also observed by Hillmer and Gest [22]; they attributed this to the production of some acidic products, however, they did not indicate the name of these acidic products. Further study is warranted in this connection.

Ferrous ion could be a critical factor to affect photo hydrogen production when Fe^{2+} was less than 2.4 mg/l. So, it will be important to make certain that sufficient Fe^{2+} is contained in substrates when phototrophic anoxygenic bacteria are used as the hydrogen production carrier. However when $FeSO_4 \cdot 7H_2O$ is considered as the source of Fe^{2+}, it should not be higher than 23.5 mg/l (equivalent to 3.2 mg/l of Fe^{2+}) to avoid possible non-physiological effect such as coagulation effect.

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