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Effect of ferrous iron concentration on anaerobic bio-hydrogen production from soluble starch

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Abstract

Both batch experiments (initial pH value was 7.0 and 8.0, respectively) were conducted to convert soluble starch to hydrogen at 35 °C. Anaerobic mixed bacteria acclimated with soluble starch was used as inoculum. At initial pH = 8.0, the H_2 yield significantly increased from 106.4 to 274.0 ml/g starch with increasing iron concentration from 0 to 200 mg $FeSO_4$/l. When iron concentration continued to increase from 200 to 4000 mg $FeSO_4$/l, iron inhibition did not happen. On the contrary, hydrogen production was efficiently accelerated. Here, lag-phase time and end time all had about 22 h shortened. However, corresponding cumulative H_2 volumes were adjacent to the maximum value (225 ml) required at [$FeSO_4$] = 150 mg/l. As for pH 7.0 systems, though flocculation still appeared, superfluous iron (over 800 mg $FeSO_4$/l) slightly inhibited hydrogen production. The difference in two strains of iron experiments resulted from the variance in initial solubility of iron under various initial pH values. The experiment results show that superfluous soluble iron shows slightly inhibitive influence on H_2 production, and that the optimum soluble iron concentration is 150 mg $FeSO_4$/l from soluble starch (10.0 g/l). Furthermore, the effect of the starch concentration (5.0–40.0 g/l) on hydrogen production also was investigated under 150 mg $FeSO_4$/l. The result shows that high starch concentration has no remarkable effect on H_2 production. The maximum cumulative hydrogen was 260.5 ml at starch concentration of 20 g/l.

Keywords: Soluble starch; Anaerobic bacteria; Bio-hydrogen production; Iron concentration

1. Introduction

Expensive fossil fuel and natural gas prices, in addition to pollution concern, warrant the need for non-polluting, alternative energy sources. Hydrogen may be an ideal, clean and sustainable energy source for the future due to its high conversion efficiency and non-polluting nature [1,2]. Compared to electrochemical and thermo-chemical hydrogen production processes, bio-hydrogen production via converting biomass to hydrogen is more environmentally friendly and attracts attention all over the world [3,4]. Associated with organic wastewater or other wastes treatment, bio-hydrogen generation would further the global environmental conservation and become more attractive [5,6]. In the past years, some complex waste substrates (municipal solid waste [7], food waste [8] and sugar beet [9]) had been used in bio-hydrogen production.

Current production of biological hydrogen is generally divided into two categories: photosynthetic and anaerobic fermentation [10]. The production of

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hydrogen from low-cost organic substrates using anaerobic fermentation methods may be one of the most promising methods [11]. Up to now, many researches have been performed on anaerobic fermentation. This method is known as a system requiring less energy intensive and low energy input. Apart from H_2 and CO_2, the main soluble metabolites generally are acetic acid (HAc), propionic acid (HPr), butyric acid (HBu), and alcohols. Anaerobic fermentative bacteria used include *Clostridia, Escherichia and Citrobacter* [12–14]. It has been proved that the mixed microflora taking anaerobic bacteria as predominant species can become a good microflora source for organic waste fermentative hydrogen production [15].

Bio-hydrogen production requires essential micronutrients for bacterial metabolism during fermentation. Sodium, magnesium, zinc and iron are all important trace metals affecting hydrogen production. Among them iron is an important nutrient element to form hydrogenase or other enzymes which almost all biohydrogen production needs fundamentally [16]. Furthermore, in waste water treatment, iron (III) compound has often been used to organic contamination biodegrading as electron acceptor, for instance, benzene and toluene degradation. And some of the reported effects of nitrate on hydrocarbon biodegradation may be indirect through the reoxidation of iron (II) [17]. However, these studies on iron mainly focused on utilizing ferrous sulfate as flocculants in water treatment. There is thus little information on how the iron concentration influences hydrogen production via fermentative bacteria.

In this study, in order to convert soluble starch to high-value hydrogen efficiently, we investigated the role of ferrous ion concentration on anaerobic mixed bacteria for hydrogen production. In light of low pH inhibiting the activity of iron-containing hydrogenase [18], two series of experiments were employed for studying the effect of iron on starch at various initial pH values. Furthermore, the series three experiments investigated the influence of starch concentration on the hydrogen production at optimum iron concentration. Since there does not exist remarkable effect of SO_4^{2+} on hydrogen production in previous studies, we used $FeSO_4 \cdot 7H_2O$ as addition [19,20].

2. Materials and methods

2.1. Inoculum

The anaerobic mixed cultures used here were enriched in the completely stirred tank reactor (CSTR) in chemostat for about 1 month [20]. The reactor was operated at 35 °C, a 12 h hydraulic retention time (HRT), and stirred by gas circulation. The organisms responsible for hydrogen production were dominated by *Clostridium pasteurianum* [21]. Feed solution for the reaction contained 10.0 g/l of soluble starch and synthetic substrate. Each liter of the synthetic substrate contained NH_4HCO_3, 3770 mg; K_2HPO_4, 125 mg; Na_2CO_3, 2000 mg; $CuSO_4 \cdot 5H_2O$, 5 mg; $MgCl_2 \cdot 6H_2O$, 100 mg; $MnSO_4 \cdot 4H_2O$, 15 mg; $FeSO_4 \cdot 7H_2O$, 25 mg; $CoCl_2 \cdot 6H_2O$, 0.125 mg. The substrate reservoir was kept at 4–5 °C by a water bath.

2.2. Experimental procedures

Batch experiments were conducted in 120 ml vials. After addition of 20 ml inoculum, 40 ml nutrient solution (prepared by adding soluble starch, 20 000 mg; NH_4HCO_3, 7540 mg; $NaHCO_3$, 4000 mg; K_2HPO_4, 250 mg; $CuSO_4 \cdot 5H_2O$, 10 mg; $MgCl_2 \cdot 6H_2O$, 200 mg; $MnSO_4 \cdot 4H_2O$, 30 mg; $CoCl_2 \cdot 6H_2O$, 0.25 mg) was added into 1 l of distilled water. After that, 20 ml of the $FeSO_4$ solution was added into it. The concentration of $FeSO_4$ ranged from 0, 25, 100, 200, 400, 800, 1600, 2400, 3200 to 4000 mg/l in the batch experiment, and it excluded the residual iron concentration in the inoculum. The total work volume in the bottles was 80 ml, which resulted in a solution with a desired concentration of 10.0 g starch/l. The air was removed from the headspace by argon gas sparging for 5 min before the bottles were capped with rubber septum stoppers and placed in a reciprocal shaker (reciprocation: 5 cm × 120 strokes per min). The batch experiments were conducted at 35 °C in the dark. The solution was adjusted to an initial pH = 8.0 or 7.0 with 1 M HCl or 1 M NaOH. Each experimental condition was carried out in triplicate.

2.3. Analysis

The H_2 gas production was calculated from headspace measurements of gas composition and the total volume of biogas production, at each time interval, using the mass equation:

$$V_{H,i} = V_{H,i-1} + C_{H,i}(V_{G,i} - V_{G,i-1}) + V_{H,0}(C_{H,i} - C_{H,i-1}),$$

where $V_{H,i}$ and $V_{H,i-1}$ are cumulative H_2 volumes at the current (i) and previous ($i-1$) time intervals, $V_{G,i}$ and $V_{G,i-1}$, are the total biogas volumes in the current and previous time intervals which was measured by a plunger displacement method using appropriately sized glass syringes, ranged from 10 to 100 ml. $C_{H,i}$ and

$C_{H,i-1}$ are the fraction of H_2 in the head space of the bottle at the current and previous intervals and $V_{H,0}$ is the total volume of headspace in the bottle [22]. All gas production data reported were standardized to standard temperature (0 °C) and pressure (760 mmHg). The fraction of H_2 was determined with a gas chromatograph (SHIMADZU 14B, Japan) equipped with a thermal conductivity detector (TCD). A 2-m stainless iron column packed with Porapak Q (50/80 mesh). Argon was used as the carrier gas at a flow rate of 30 ml/min. The operating temperatures of the injection port, the oven, and the detector were 100, 60 and 100 °C, respectively. Detection of the solvent metabolites (VFAs, C_2–C_4) was also analyzed by a gas chromatograph (SHIMADZU 14B, Japan) using a flame ionization detector (FID). A 2-m glass column packed with Unisole F-200 (30/60 mesh). The temperatures of the injection port, the oven, and the detector were 200, 150, and 220 °C, respectively. The carrier gas was also argon gas at a flow rate of 30 ml/min. The concentrations of soluble starch after the reaction were determined by the phenol–sulfuric acid method using sucrose as a standard [23]. The concentrations of volatile suspended solids (VSS) were determined according to the procedures described in the standard methods [24]. The pH was measured using a pH meter (PHS-3B Shanghai, China).

3. Results and discussions

3.1. Effect of iron concentration on H_2 production at initial pH 8.0

In view of the activity of hydrogenase and the effect of pH on H_2 production, an initial pH 8.0 was used firstly in this study. Batch experiments were examined until the H_2 production end. The effect of different amounts of iron on the H_2 production and the solvent products is shown in Table 1. Gas product analyses represent that the biogas only contains H_2 and CO_2 without any detectable CH_4 and H_2S during the course of H_2 production. The result suggests that no sulfate-reducing bacteria and methanogens in the culture were used, and that high SO_4^{2+} concentration has no influence on H_2 production. The result is not consistent with previously reported result [25], probably due to difference of mixed microflora and reaction conditions. The H_2 content firstly increased from 42% (at 0 mg $FeSO_4$/l) to a peak value of 55.8% (at 100 mg $FeSO_4$/l) and then varied from 52% to 55% with increasing the iron concentrations.

The influence of iron on cumulative hydrogen volume is shown in Fig. 1(a). The H_2 production rapidly increased with increasing the iron concentration within the range of 0–150 mg/l, and the maximum hydrogen yield (279.9 ml/g-starch) was obtained at the iron concentration of 150 mg $FeSO_4$/l. When iron concentration ranged from 150 to 800 mg $FeSO_4$/l, the cumulative hydrogen volume slightly decreased. While the iron concentrations were more than 800 mg $FeSO_4$/l, the cumulative H_2 volumes did not decrease, instead, and formed a high H_2-producing plateau, on which every volume value was close to the maximum value required under the 150 mg $FeSO_4$/l. Namely, with high iron concentrations of 1600, 2400, 3200 and 4000 mg $FeSO_4$/l, the corresponding total cumulative H_2 volumes were 204.1, 217.8, 221.3 and 207.0 ml, respectively. In addition, as shown in Fig. 1(b), when the concentrations of iron were within 1600–4000 mg $FeSO_4$/l, the H_2 production ended earlier (about 70 h) than other bottles (more than 100 h) did. As compared with the bottles with 150 mg $FeSO_4$/l, lag-phase time and end time of them had all about 22 h shortened, though 96 h at iron concentration of 150 mg $FeSO_4$/l was far less than other results (over 100 h, even more than 130 h). Furthermore, as for lower concentration of iron (less than 400 mg $FeSO_4$/l), the slopes were much lower than that of the systems at higher concentration of iron (over 800 mg $FeSO_4$/l). These results seem to represent that the addition of increased iron into the medium cause the H_2 evolution to rapidly happen and end at pH 8.0. This result was unexpected and the detailed investigation followed it.

Through further research, we found that under the anaerobic conditions, green flocculent sediment always occurred in these systems as soon as an amount of iron was added at initial pH 8.0. In fact, via calculating toward ferrous iron chemistry in closed carbonate buffers system, we found the sediment is mainly $FeCO_3$ at initial pH 8.0 and the amount of iron is about 38.0 mg/l. The high hydrogen production plateau may be due to this kind of special sediment. After the addition of overfull iron, the $FeCO_3$ sediment rapidly fall together with the soluble organic substrate, and formed a sort of floccule. Meanwhile, the mixed bacteria cell was also immobilized on it by sorption. And the floccule enhanced the chance of bacteria to meet the substrate, and accelerated the assimilation of them to the substrate. At the same time, it also reduced the concentration of soluble ferrous iron in the closed system, consequently, and lessened the inhibitive influence of iron to H_2 evolution. Furthermore, as shown in Table 1, the final pH values (more than 50 mg $FeSO_4$/l) were all adjacent to 5.3,

Table 1
The effect of the ferrous iron concentration on the H_2 yield, the fermentation products and the H recovery at initial pH 8.0

$FeSO_4$ (mg/l)	Final pH	H_2 content[a] (%)	H_2 yield (ml/g-S)	DBW[b] (mg-VSS/g-S)	VFAs (mg/g-S)			H recovery (%)
					HAc	HPr	HBu	
0	5.08	42.3	106.4	136.6	160.7	36.2	163.8	76.8
25	5.26	47.8	140.3	160.2	164.7	15.3	152.3	80.3
50	5.37	50.2	189.3	161.1	144.6	18.2	146.9	85.0
100	5.46	55.8	248.5	178.5	126.3	9.7	145.1	92.1
150	5.53	55.6	279.9	185.4	122.9	6.9	156.8	98.4
200	5.54	54.3	274.0	179.3	109.3	4.4	158.3	95.3
400	5.29	53.6	246.6	178.5	109.5	1.1	170.4	92.6
800	5.32	54.6	234.0	192.2	108.1	6.1	185.7	95.1
1600	5.36	54.7	260.1	189.1	119.9	4.9	196.4	101.2
2400	5.31	54.9	272.2	192.0	148.9	3.5	225.7	112.0
3200	5.35	55.4	276.6	198.0	156.4	4.1	249.9	116.3
4000	5.32	53.9	262.4	187.4	138.2	4.1	237.9	109.3

[a] H_2 content means percentage of the total cumulative hydrogen produced to total biogas.
[b] The dry biomass weight (DBW) is the biomass production yield of 80 ml liquid reagent in each bottle of the experiments.

which were greater than final that of previous studies [19,21], showing an advance in buffer intensity because of addition of overfull iron. Meantime, it also shows that pH is not the inhibitory factor which stops H_2 production. Besides, the cumulative H_2 volumes with sharper slopes were all adjacent to 225 ml in systems. The result shows that the soluble starch was used up in a shorter time and the consumption of substrate might be mainly responsible for reaction end.

In fact, it was found that the soluble iron concentration has a slightly inhibitive influence on hydrogen production. When the concentration of iron is over 150 mg $FeSO_4$/l, a slight decline occurred from 225 ml ([$FeSO_4$] = 150 mg/l) to 180 ml ([$FeSO_4$] = 800 mg/l). But the declination was counteracted by the flocculation. Considering iron limitation and flocculation, the optimal concentration of iron is 150 mg $FeSO_4$/l in the closed system. Over it, iron concentration would inhibit the starch fermentation. The initial soluble iron concentration (ca. about 40 mg $FeSO_4$/l) in closed system met the first need of bacteria growth. But with the reaction processing and VFAs appearing, iron was released gradually out of the floccule. It made soluble iron concentration maintain a proper level till reaction end. It is certain that the proper level changes with different initial pH in the system.

As an outer improving approach, adsorptive sedimentation may enhance chances of bacteria to meet organic substances by contact adsorption. It may be helpful to improve bio-hydrogen production. But ferrite sediment might be more biological catalysis via various iron-chelated enzymes, unlike simple inorganic flocculation.

3.2. Effect of iron concentration on H_2 production at initial pH 7.0

Fig. 2 depicts that iron concentration influences bio-hydrogen production when initial pH is 7.0. As described in Fig. 2(a), the cumulative H_2 volume first increased from 90 to 236 ml in the range of 0–150 mg $FeSO_4$/l and decreased gradually from 236 to 100 ml when iron exceeded 150 mg $FeSO_4$/l. The maximum value was 236 ml obtained at [$FeSO_4$] 150 mg /l, similar to the result found at initial pH 8.0. In contrast with Fig. 1(a), it was obvious that a high H_2-producing plateau under increased iron concentration (over 800 mg $FeSO_4$/l) was not formed. The reason for the absence of high plateau under pH 7.0, though $FeCO_3$ flocculation also occurred at pH 8.0, may be due to the fact that the distinction of initial pH causes the change of initial solubility of ferrous iron (ca. from about 40 to 200 mg $FeSO_4$/l in closed system). Namely, the initial concentration of iron (more than 200 mg $FeSO_4$/l added) exceeded the optimum concentration of iron (about 150 mg $FeSO_4$/l). After the addition of $FeSO_4$, with the iron concentration increased from 200 to 4000 mg $FeSO_4$/l, the excess soluble iron was harmful to the mixed microbe, and then resulted in final decrease of hydrogen production in these systems. So, whether the initial concentration of soluble iron met the need of bacteria growth or not made the difference of bio-hydrogen production under various pH conditions. The decrease in H_2 production when iron concentration was higher than 150 mg $FeSO_4$/l shows that iron concentration has slightly inhibitive influence on H_2 production,

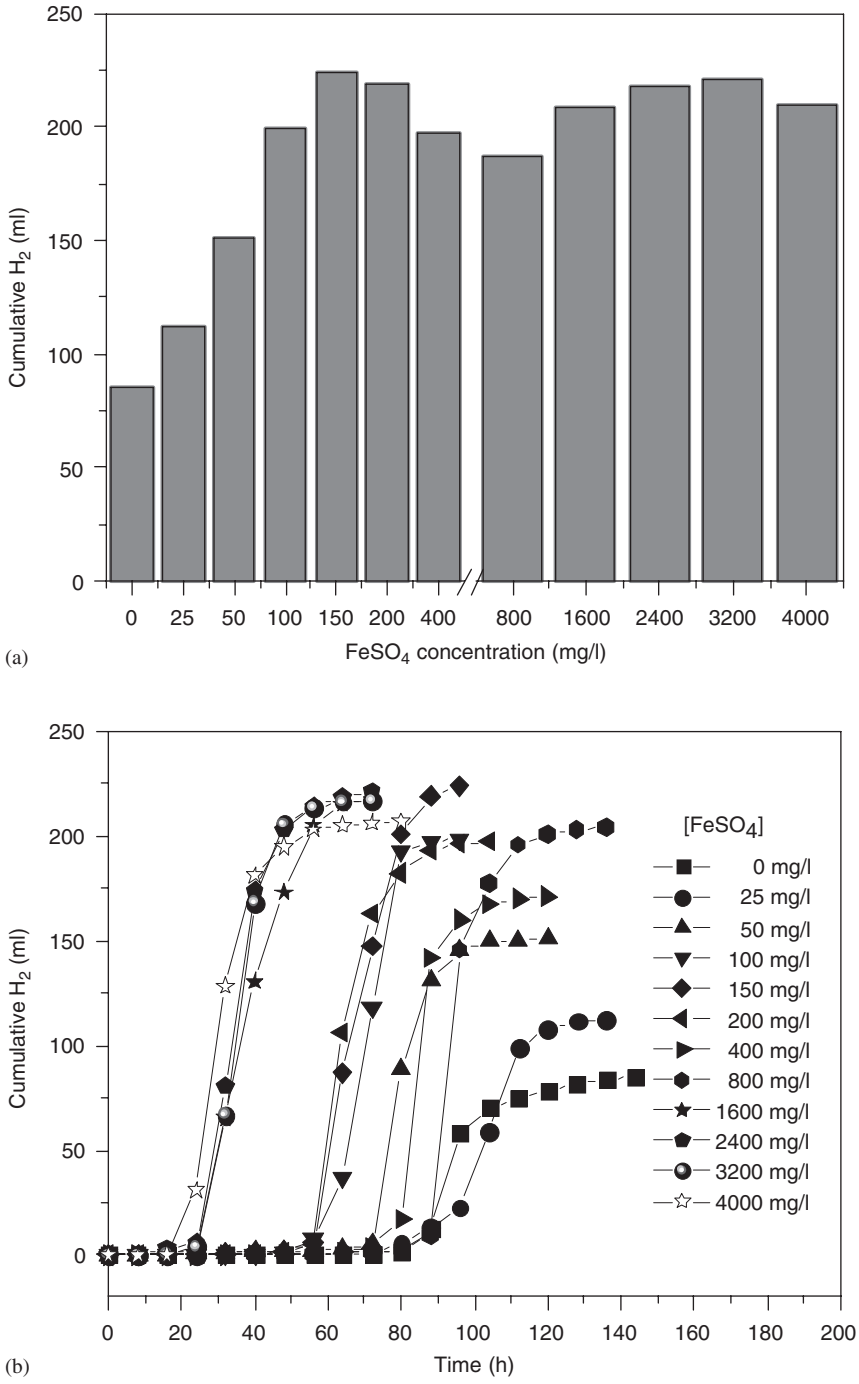

Fig. 1. (a) Cumulative hydrogen volume versus different iron concentrations at initial pH 8.0. The iron concentrations ranged from: 0 to 4000 mg/l. (b) Cumulative hydrogen volume versus corresponding fermentation time. In this study, hydrogen production was measured at 8 h intervals during the incubation. The data of only 20 ml culture added was not shown here for no H_2 production.

and that bio-hydrogen production from soluble starch has the optimum iron concentration (150 mg $FeSO_4$/l). Namely, considering iron inhibition and flocculation, 150 mg $FeSO_4$/l was the optimized value at pH 7.0 in these systems. As initial iron concentration was higher than 150 mg $FeSO_4$/l, superfluous soluble iron would

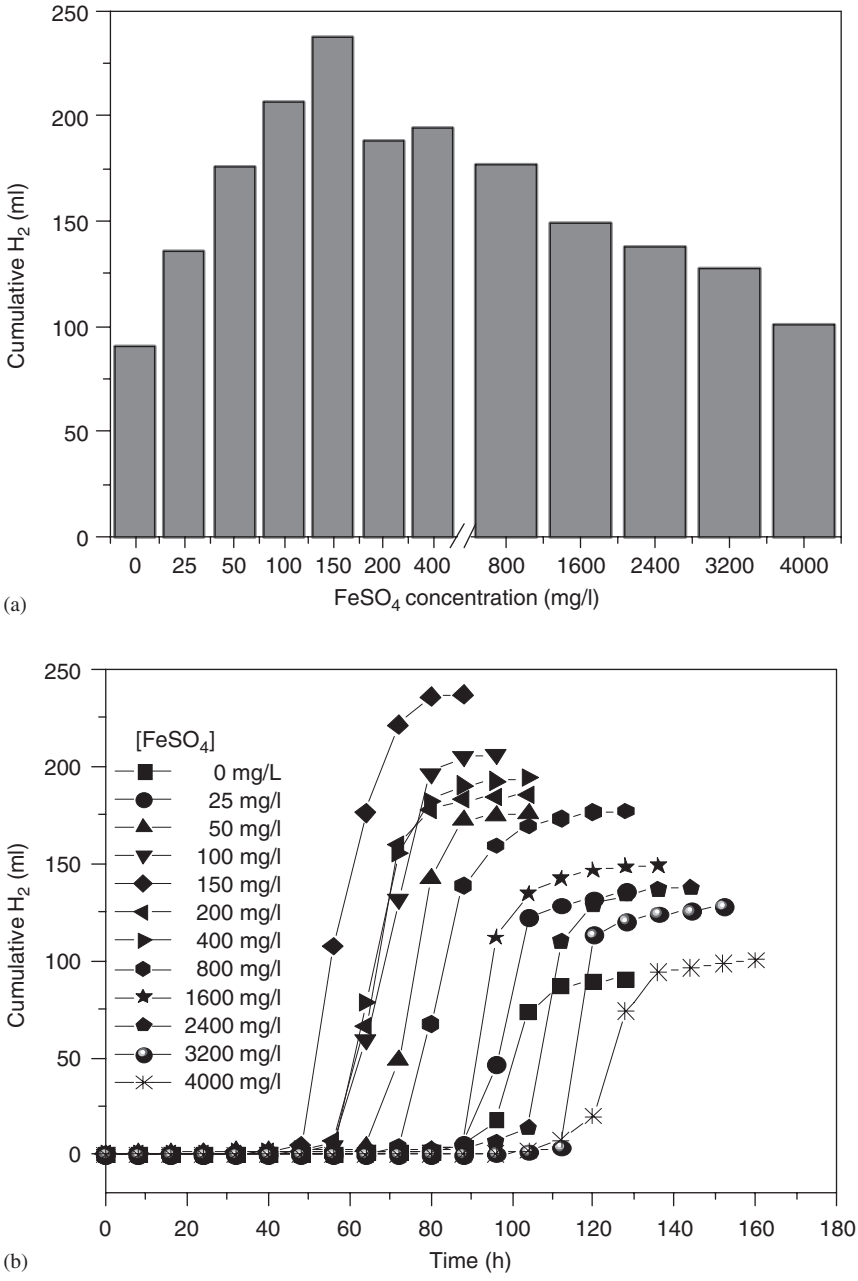

Fig. 2. (a) Cumulative hydrogen volumes versus different iron concentrations at initial pH 7.0. The iron concentration ranged from: 0 to 4000 mg $FeSO_4$/l. (b) Cumulative hydrogen volumes versus corresponding fermentation time. In this study, hydrogen production was measured at 8 h intervals during the incubation.

become an inhibitor to H_2 production. The inhibition will last to the end and become stronger and stronger with pH decrease due to the appearance of VFAs.

As shown in Fig. 2(b), all lag-phase time and end time within the range of 0–400 mg $FeSO_4$/l were less than the corresponding data required at pH 8.0. It indicates that initial pH 7.0 favors more bacteria propagation comparatively. Meanwhile, as indicated in Table 2, the final pH decreased slightly (from 5.5 to 5.1) when iron was over 400 mg $FeSO_4$/l (but not less than 5.0), whereas the consumption of organic substrate was not complete. The results indicate that the inhibition of

Table 2
The effect of the ferrous iron concentration on the H_2 yield, the fermentation products and the H recovery at initial pH 7.0

$FeSO_4$ (mg/l)	Final pH	H_2 content (%)	H_2 yield (ml/g-S)	DBW (mg-VSS/g-S)	VFAs (mg/g-S)			H recovery (%)
					HAc	HPr	HBu	
0	5.62	38.8	113.3	118.8	134.4	30.2	198.1	79.2
25	5.24	41.8	169.3	124.2	136.8	20.4	189.9	85.1
50	5.43	50.4	219.2	137.7	136.1	13.1	227.8	97.9
100	5.36	57.7	257.9	159.3	141.5	7.9	261.3	110.4
150	5.43	56.9	296.2	153.9	148.7	5.2	262.6	115.7
200	5.29	48.9	235.3	124.2	140.2	4.9	259.3	102.1
400	5.34	51.4	242.2	129.6	133.5	4.2	262.2	103.3
800	5.27	47.9	221.0	124.2	130.5	17.4	223.5	96.2
1600	5.08	40.2	186.4	118.8	131.5	16.6	217.8	89.7
2400	5.17	43.5	171.8	116.1	112.1	20.1	189.7	81.8
3200	5.19	41.7	159.6	118.8	110.5	19.1	177.8	78.2
4000	5.09	38.4	125.4	113.4	103.2	22.8	187.8	74.1

reaction would have resulted from iron inhibition and not from the formation of VFAs. Furthermore, the soluble products in all bottles did not change, without other alcohols or acids being produced. And with the reaction processing, this kind of green sediment observed gradually faded and the system became almost transparent finally. The fact suggests that the inhibition did not bring the disappearance of H_2-producting bioactivity of microbe and the switching among metabolic approaches. So the inhibition is less and not fatal.

3.3. Effect of iron on solvent products and H recovery

The variety of solvent products and their distributions are important indicators for analyzing hydrogen production during the anaerobic fermentation. As shown in Table 1, during the anaerobic digestion process, any other solvent products like methonal or ethanol were not found in all bottles. However, with trace amount of propionate detected, significant amount of butyrate and acetate were produced all the time under these conditions. Especially, the four values (1600, 2400, 3200, and 4000 mg $FeSO_4$/l) on the high H_2-producing plateau, for example, occurred with higher solvent metabolites (total amount of VFAs was over 370 mg/g-starch). For these systems at pH 7.0, as indicated in Table 2, a smaller amount of HPr (about 20 mg/g-starch) was detected likewise, the solvent products were mainly HBu and HAc. Besides, the major VFAs yield always became greater with increase in H_2 yield, for instance, at initial pH 7.0, the large amount of HBu (262.6 mg/g starch) and HAc (148.7 mg/g-starch) always accorded with the high H_2 yield (296.2 ml/g-starch) required at an iron concentration of 150 mg $FeSO_4$/l. These results show that converting soluble starch to HBu and HAc is the major metabolism pattern in batch culture. Considering the distributions of VFAs, for earlier end system, the ratio of HBu/HAc was always more than that of other systems with late end time. The ratio frequently has been used as the parameter for evaluating the efficiency of H_2 production [26,27]. The result may suggest that HBu pathway more favors efficient H_2 production.

Furthermore, by comparison of starch fermentation at initial pH 8.0 and 7.0, we can reach some conclusions. First, for pH 8.0 systems, since unchanged VFAs formation was observed, the reason why the flocculation could accelerate the hydrogen production was not due to metabolic pathway shifts. Second, in two series of iron experiments no change of solvent products also indicated that iron limitation did not change the metabolic routes of predominant bacteria. However, the activity of hydrogenase, some iron-containing enzymes, was not affected, which probably contributed to the H_2-producing plateau. Third, soluble starch rapid consumption and the larger amount of HBu was coupled under various iron concentrations, suggesting that the producing butyrate bacteria were the main force of H_2 production. Finally, with reaction end time prolonged, the quantities of HAc gradually increased, showing that partial HBu probably was further converted to HAc at the end of reaction.

The total organic substrate degradation efficiency (TOSDE) was completely ($99.1 \pm 0.2\%$) consumed in all bottles. The main difference between two series of tests of irons was H recovery. The H recovery at pH 7.0 generally exceeded corresponding to that obtained at pH 8.0, e.g. 115.7% under pH 7.0 was more than 98.4% under pH 8.0 at the same iron concentration of

Table 3
Final pH, VFAs and alcohols production at various soluble starch concentrations

Starch (g/l)	Final pH	H_2 yield (ml/g-S)	DBW (mgVSS/g-S)	VFAs and Alcohols (mg/g-S)				
				HAc	HPr	HBu	Methanol	Ethanol
50	6.48	209.0	181.5	139.2	11.8	144.3	0	4.7
100	5.37	258.8	209.4	159.1	6.6	167.7	2.1	3.8
200	4.69	172.6	147.7	167.7	10.1	158.3	4.9	10.0
300	4.46	115.6	101.8	176.0	13.3	161.9	5.2	13.6
400	4.38	85.4	89.2	169.7	15.3	149.9	5.7	19.4

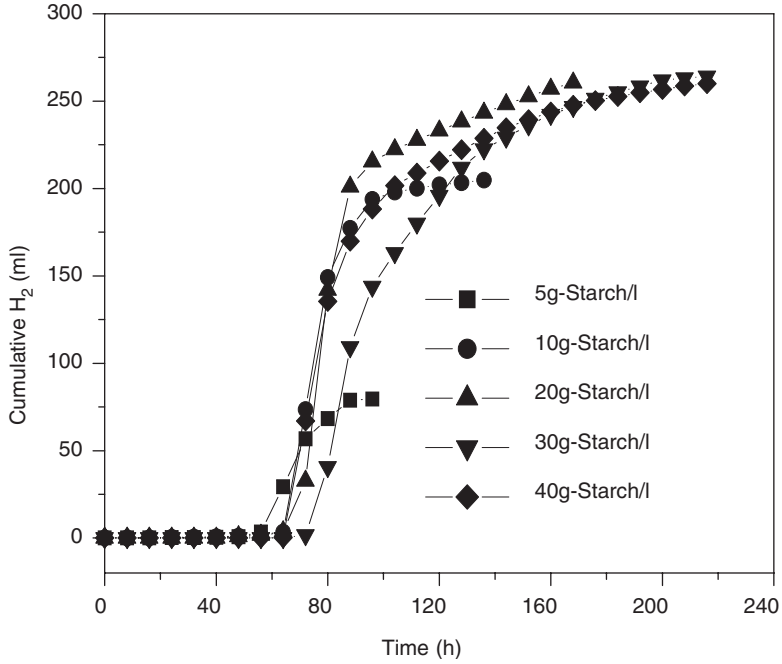

Fig. 3. Hydrogen production at various soluble starch concentrations.

150 mg $FeSO_4$/l. It indicates that the initial pH 7.0 is a better pH condition to the mixed bacteria. Furthermore, at initial pH 8.0, the high H_2-producing plateau always corresponded to high H recovery, for example, at iron concentration of 3200 mg $FeSO_4$/l, the H recovery (116.3%) was the maximum value among all experimental data. It shows that the H element of soluble starch is efficiently converted at higher iron concentrations.

In both series of experiments, the biomass yield (dry biomass weight) all increased rapidly with increasing iron concentrations (below 150 mg $FeSO_4$/l). The maximum values were 185.4 mg/g-starch (pH 8.0) and 159.3 mg/g-starch (pH 7.0), respectively. When iron concentration exceeded 150 mg $FeSO_4$/l, as for initial pH 8.0, the biomass yield first slightly decreased from 185.4 ([$FeSO_4$] 150 mg/l) to 178.5 mg/g-starch ([$FeSO_4$] 400 mg/l), and then increased from about 178.5 to 187.4 mg/g-starch ([$FeSO_4$] 4000 mg/l), accompanied with the high H_2 yield plateau appearing. But, as for initial pH 7.0 systems, the biomass yield gradually decreased from the peak value, 159.3–113.4 mg/g-starch. The result indicates that the starch consumption and bacteria growth are coupled under iron limitation. Meanwhile, redundant solvent ferrous iron affected both the cultivation and the hydrogen-producing activity of the bacteria. From the distribution of the dry biomass weight, we could also conclude the same result that the high H_2 production is always with greater dry biomass weight.

3.4. Substrate concentration effects

Table 3 summarized the results of series three experiments on investigating the effect of starch

concentration (from 5 to 40 g/l) on H_2 production. The soluble ferrous iron concentration, temperature, initial pH was operated at 150 mg $FeSO_4$/l, 35 °C and 7.0, respectively. In batch culture, the soluble starch was degraded as possible (TOSDE over 97.0 ± 0.2%) and was converted to hydrogen gas (H recovery fraction over 23.6%) by the mixed bacteria. As described in Fig. 3, the cumulative volumes of hydrogen increased gradually from 80 at 5 g/l to 260 ml at 20 g/l, and then kept the same level (around 260 ml) with starch concentration increasing from 20 to 40 g/l. The results demonstrate that the starch-utilizing hydrogen fermentation has no starch concentration inhibition. The stopping of final hydrogen production may be due to the complete consumption of other nutrients. The optimum soluble starch concentration is 20 g/l with the maximum value of cumulative H_2 volume (260.5 ml) and the H recovery of 94.4%. But when starch concentration exceeded 20 g/l, end time was longer (from 160 h at 20 g-starch/l to 220 h at 40 g-starch/l) and trace amount of alcohols (15–20 mg/g-starch) appeared in final solvent products. The composition and distribution of VFAs/alcohols were only slightly dependent on the starch concentration. This was similar to the previous observation [28]. The result suggests that the metabolic pathway may shifts with the reaction being prolonged. Furthermore, the starch concentration (10 g/l) used above had the optimized H recovery (100.5%) and the high hydrogen yield (258.8 ml/g-starch). The result suggests that starch concentration of 10.0 g/l favors bacterial metabolism and other nutrient substrates are ample and produce no influence on results attained in iron batch tests.

4. Conclusions

Two series of experimental results demonstrate that soluble iron has inhibitive influence on bio-hydrogen production from soluble starch when soluble iron concentration exceeds 150 mg $FeSO_4$/l. As initial pH was equal to 8.0, because of flocculation of $FeCO_3$, containing ferrous sediment and other substrates, the concentration of soluble iron was lower in these systems. This kind of flocculation accelerated hydrogen production and substrate conversion via sorption, similar to cell-immobilized mechanism. Otherwise, the accelerated hydrogen production may contribute to soluble ferrous iron as supplement improved the bioactivity of hydrogenases. With the increase of VFAs gradually, this kind of flocculation was dissolved and ferrous iron was released gradually. Released iron maintained bacteria, but did not result in inhibitive influence. As for initial pH 7.0 systems, when iron concentration was more than 150 mg $FeSO_4$/l, though flocculation still occurred, the superfluous soluble iron slightly inhibited H_2 production from the beginning and led to disappearance of H_2-producing acceleration finally. The optimal iron concentration was also 150 mg $FeSO_4$/l with H_2 yield (296.2 ml/g-starch) via anaerobic mixed microflora. In addition, the concentration of soluble starch had no remarkable influence on the bio-hydrogen production. The optimum starch concentration and the maximum cumulative hydrogen were 20 g/l and 260.5 ml, respectively.

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