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Evaluation of biohydrogen production from glucose and protein at neutral initial pH

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ABSTRACT

Organic wastes are considered as potential substances for economical biohydrogen production, because the carbohydrate and protein are main components. Previous investigations indicate that an optimum hydrogen production appear in acidic conditions to carbohydrates, or in alkali condition to protein. However, in practice, the treatment of these organic wastes by anaerobic fermentation usually carries out at neutral pH condition, in which biohydrogen production is only a middle process. So, the purpose of this paper is to evaluate the biohydrogen production at neutral pH condition from carbohydrates or protein. Batch tests were conducted to investigate the differences in biohydrogen production by anaerobic fermentation at neutral initial pH using carbohydrate and protein (glucose and peptone) as the sole carbon source. The experimental results showed that the maximal hydrogen yields of two substrates were about 0.14 ml H₂/mg glucose and 0.077 ml H₂/mg protein, respectively, at neutral initial pH. Although the hydrogen yields of glucose is far greater than that of protein at neutral pH, they were lower than previous results of hydrogen production in acidic condition to carbohydrate or in alkali condition to protein. This result shows that the neutral pH is not an optimal condition for biohydrogen production. In this experiment of biohydrogen production, a phenomenon has been observed that the hydrogen production and hydrogen consumption occurred simultaneously in the fermentation of protein, whereas the hydrogen production occurred only in the fermentation of glucose. Furthermore, the different evaluation of the main components of the organic liquid by-products produced by fermentation of each substrate implied that the biohydrogen production pathways of these two substrates were different. Molecular analysis indicated that the dominant microorganisms during the anaerobic fermentation of these two substrates are greatly different.

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1. Introduction

Hydrogen is regarded as an optimal energy carrier and fuel and it has become a topic of intensive research efforts worldwide. Due to utilization of renewable energy resources and operation at ambient temperature and atmospheric pressure, biological hydrogen production is becoming an important method of fuel

production and attracting the attention of many researchers. Due to its environmental friendliness, biohydrogen production from organic wastes has been attracted increased attention [1,2]. Biohydrogen production has been evaluated in many organic wastes, including waste molasses [3], dairy wastewater [4], sewage sludge [5,6] and so on. The hydrogen yields from these wastes differ due to their different organic composition

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and the primary organic components in these wastes are usually carbohydrates and/or proteins (Table 1). For example, Guo et al. [3] obtained a hydrogen yield of 3.47 mol H₂/mol sucrose from molasses (a carbohydrate-rich wastewater), while Cai et al. [6] found that a hydrogen yield of 16.6 ml H₂/g DS could be obtained from alkali pre-treated sludge (a protein-rich waste) under an initial pH of 11.

Although the previous studies found that the pH is an important influence factors of biohydrogen production and the optimal pH of biohydrogen production from carbohydrate is acidic pH (about 4.5–6.0) [1,2] and that from protein is alkaline pH (about 8.5–10) [6,9], the traditional treatment of these organic wastes by anaerobic fermentation usually carries out at neutral pH condition, in which biohydrogen production is only a middle process. Additionally, the initial pH of these wastes and wastewater usually is neutral pH and controlling pH at acidic pH or alkaline pH will increase the cost of anaerobic fermentation and the requirement of reactor on acid-resistant and alkaline-resistant. So, the study about biohydrogen production from organic wastes at neutral initial pH is important for its application.

Since carbohydrate and protein are main components of these organic wastes, it would be very helpful to study the processes and differences in biohydrogen production at neutral initial pH from carbohydrate and protein to understand of the biohydrogen production from these organic wastes. However, few studies are about these processes and differences. Therefore, this study was conducted to evaluate the processes and differences in the production of biohydrogen from carbohydrate and protein used as the sole carbon sources with glucose and peptone as the model compounds, respectively.

2. Materials and methods

2.1. Seed microflora

Activated sludge obtained from the aerobic tank of a municipal sewage treatment plant in Beijing, China, was used as the seed microflora after pretreatment as described below. The collected sludge was settled gravitationally for about 1 h, after which the

sediments were stored at 4 °C until use. The pH value of the raw sludge was 6.9 ± 0.1 and the concentrations of suspended solids (SS) and volatile suspended solids (VSS) for the sludge used in the test were 7300 ± 95 mg/l and 5050 ± 61 mg/l, respectively.

Because most hydrogen-producing microorganisms in excess sludge are spore-formers, the settled sludge was sterilized at 121 °C and 1.2 kfb/cm² for 30 min in an autoclave (VARIOKLAV steam sterilizer, 300/400/500 EP) to inhibit or kill hydrogen-consuming microorganisms and select hydrogen-producers [10]. To remove the soluble organics from the seed microflora, the pre-treated sludge was centrifuged at 5000 rpm for 10 min and then washed twice with sterile deionized water. The centrifuged sludge sediments were then used as the seed microflora for the biohydrogen production tests.

2.2. Experimental procedures to evaluate biohydrogen production

Batch tests of biohydrogen production were conducted in a series of 300 ml reactors that were equipped with two ports for sampling gas and sludge. For each reactor, the seed microflora (about 5 ml), organic substrates (glucose or protein), 10 ml of inorganic minerals solution and 135 ml deionized water were added and mixed. The concentrations of the organic substrates in the mixtures are shown in Table 2. The concentrations of these organic materials were selected based on their contents in the organic wastes. No other organic materials were added to the reactors. As shown in Table 3, the components of the inorganic minerals solution were prepared according to the method described by Chen and Lin [11]. The SS and VSS of the mixtures added into the reactors were 5710 mg/l and 3740 mg/l, respectively. To ensure that the original characteristics of the added organic materials were unchanged, the pH of the mixtures was not adjusted. The reactors were flushed with nitrogen gas for 30 min and then sealed prior to being placed in an air shaker (HKZ-C, China) at 140–150 rpm and 37 ± 1 °C. The tests were conducted in triplicate for each concentration and all results were the mean value of replicate analyses.

2.3. Analytical methods

Biogas production was measured periodically based on the displacement of saturated salt solution making corrections for atmospheric pressure and temperature [12]. The biogas in the headspace of the reactors was sampled with a 1-ml gastight syringe. Hydrogen production was calculated by measuring

Table 1 – Primary organic components of some wastes.

Wastes	Main organic components	Ref.
Waste molasses	Carbohydrates (48%–58% of dry matter)	[3]
Waste sludge	Proteins (25.41 mg/l) and Carbohydrates (18.72 mg/l)	[5]
Alkaline hydrolyzate of sewage sludge	Proteins (1.04 g/l) and Carbohydrates (0.27 g/l)	[6]
Wheat straw	Carbohydrates (848.5 g/kg dry matter)	[7]
Dairy wastewater	Carbohydrates (26.0% of dry matter), Proteins (25.4%, of dry matter), Fats (26.0% of dry matter)	[8]

Table 2 – The concentrations of organic materials in the mixtures of batch tests.

No	Glucose (mg/l)	No	Protein (mg/l)
C1	266.08	P1	321.59
C2	461.71	P2	472.59
C3	946.31	P3	822.14
C4	1603.65	P4	1593.95
C5	2443.17	P5	2272.08
C6	2985.65	P6	2733.48

Table 3 – Components of the inorganic minerals solution.

Substance	Concentration (g/l)	Substance	Concentration (mg/l)
NaHCO ₃	100.8	MnSO ₄ ·6H ₂ O	0.225
K ₂ HPO ₄	1.875	CuSO ₄ ·5H ₂ O	0.075
MgCl ₂ ·6H ₂ O	1.5	CoCl ₂ ·5H ₂ O	0.001875
FeSO ₄ ·7H ₂ O	0.375		

the gas components and the total volumes of biogas produced. The contents of hydrogen in the biogas were analyzed using a gas chromatograph (GC122, China) equipped with a thermal conductivity detector (TCD). The contents of methane in the biogas were analyzed using a gas chromatograph (Agilent 6890, USA) equipped with a flame ionization detector (FID).

The liquid was sampled with a 5-ml gastight syringe. The concentrations of volatile fatty acids (VFA) and ethanol in the liquid phase were determined using another gas chromatograph (Shimadzu GC-9A, Japan) equipped with a flame ionization detector (FID). The concentrations of soluble protein in the liquor samples were measured by Lowry's method using bovine serum albumin as the standard solution [13], while those of glucose were determined by the phenol–sulfuric acid method using glucose as the standard solution [14]. The pH of the liquid samples was measured using a pH meter (PHS-3C, China). The biomass was measured by SS and VSS according to the standard methods [15]. Statistical analyses were conducted using SPSS 11.0 (SPSS Company, USA).

2.4. DNA extraction, PCR amplification and DGGE analysis

The sludge was sampled at the end of the anaerobic fermentation (58th h), which was extracted the total community DNA as described by Zhang and Fang [16]. The DNA was then used as the template for polymerase chain reaction amplification – denaturing gradient gel electrophoresis (PCR-DGGE) analysis.

The PCR primers F357GC (5'-CGCCCGCGCGCGCGCGGCGCGGGGCGGGGGC ACGGGGGCCTACGGGAGGCAGCAG-3') and R518 (5'-TTACCGCGGCTGCTGG-3') [17] were used to amplify a segment of the eubacterial 16S rDNA. A GC-clamp was added to the forward primers to facilitate DGGE. PCR amplification reaction was performed using a PTC-200 thermal cycler (BioRad Laboratories, USA) at a final volume of 50 µl PCR. The reaction mixture contained 20 pmol of both primers, 20 µmol of each dNTPs, 5 µl of 10× buffer, and 1.25 units of Taq DNA polymerase. The temperature cycling conditions were 94 °C for 3 min, followed by 31 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. A final extension at 72 °C for 10 min was used. 5 µl aliquot of the PCR product was separated on a 0.8% (w/v) agarose gel at 100 V for 30 min to verify amplification prior to DGGE.

DGGE of the PCR amplified 16S rDNA was performed using a D-Code system (BioRad Laboratories, USA). The denaturing gradients used to separate the amplified 16S rDNA were 40–60%. The gel was electrophoresed in 1× TAE buffer at 70 V and 60 °C for 10 h. The resulting gel was then visualized using Gel Red (Biotium USA). The analysis of the DGGE gel was conducted using the Bio-Rad software, Quantity One.

3. Results and discussion

3.1. Hydrogen fermentations of glucose and protein at neutral initial pH

Batch anaerobic fermentation tests of glucose and protein were conducted at neutral initial pH and without pH controlling to compare the biohydrogen production when each was used as the sole carbon source. As shown in Fig. 1, the accumulated hydrogen production of protein formed a parabola, whereas that of glucose produced a trapeziform curve. Many studies have reported similar results about biohydrogen production of glucose, other carbohydrates and carbohydrate – contained wastes [2]. Although the result of protein was similar with the study conducted by Bai [18] and Akutsu et al. [19] in which peptone was also used as the hydrogen-producing substrate, it was not inconsistent with the studies of our previous studies [6,9] and Guo et al. [5], in which sludge (a protein-contained waste) was used. The cumulative hydrogen production showed similar increases as the substrate increased, and the cumulative hydrogen production was much higher when glucose was used as the substrate than when protein was used. Indeed, the maximum cumulative hydrogen production was about 61 ml when glucose was used as the substrate, while it was only 5 ml when protein was used. Fig. 2 shows the hydrogen yields of unit organic substrates during the anaerobic fermentation. The changes of hydrogen yields were similar to that of the accumulated hydrogen production since the hydrogen yields of these organic substrates were calculated by dividing the cumulative hydrogen production by their initial quantity. Additionally, the hydrogen yields were nearly the same when there were high initial concentrations (namely, >1603.65 mg/l), and the maximum yield was about 0.14 ml/mg glucose (1.1 mol/mol glucose). Table 4 summarized some hydrogen yields in some studies. It could be found from Table 4 that the hydrogen yields of this study were similar with some previous studies and lower than some studies. The diversity may be caused by the diversity of the hydrogen-producing microorganisms, substance concentration, pH, and so on, since these factors are important for biohydrogen production. The similar hydrogen yields observed when high initial glucose concentrations (>1603.65 mg/l) were used showed that the initial concentration had little impact on the biohydrogen yield when high concentrations were used in this test. The hydrogen yields produced when protein was used as the substrate were negatively correlated with its concentration, and the maximum (0.077 ml/mg protein) was observed at 321.59 mg/l (P1). Comparison the hydrogen yields from protein and protein-contained wastes (Table 4), it could be found that the hydrogen yields of protein observed in the present study were similar to the results of Bai [18,19] and lowered some previous studies [5,6]. The diversity may also be caused by the hydrogen-producing microorganisms, substance concentration, pH, and so on. Both the accumulated hydrogen production and hydrogen yields from protein were much lower than those observed when glucose was used as the carbon source, which suggested that protein was not good for biohydrogen produced by the anaerobic fermentation method used in the test. Additionally, since the optimal pH of biohydrogen production from

Fig. 1 – Accumulated hydrogen production during the anaerobic fermentation of glucose or protein.

carbohydrate (including glucose) and protein was acidic pH range and alkaline pH range, respectively, in order to produce maximally hydrogen from carbohydrate and protein, respectively, the fermentative pH should be in the optimal pH range.

Notably, there was a large difference in hydrogen consumption during biohydrogen production by the substrates evaluated here. Specifically, during the anaerobic fermentation of glucose, almost no hydrogen consumption was observed, especially at high initial concentrations. However, obvious hydrogen consumption occurred during the anaerobic fermentation of protein, and the hydrogen consumption almost began immediately after the cumulative hydrogen production reached its maximum (Figs. 1 and 2). In the anaerobic fermentation, glucose tend to be donated electrons (Eq. (1) and Eq. (2)), and the biodegradation of glucose can produce hydrogen [2]. Conversely, during the anaerobic fermentation of protein, some intermediates produced by the anaerobic degradation of protein, such as amino acids, could be reductive and act as an electron donor (e.g., glutamate, Eq. (3)), while some could be oxidative and act as an electron acceptor (e.g., proline, Eq. (4)) and others can act as both electron donors and acceptors (e.g., threonine, Eq. (5) and Eq. (6)) [23]. The electrons produced by oxidation of amino acids would be consumed by the reduction of others amino acids, thereby leading to few available electrons for the production of hydrogen via ferredoxin and hydrogenase [24]. In other words, hydrogen may be produced and/or consumed during the anaerobic degradation of protein [18,25]. Additionally, initial pH is an important factor that influences hydrogen

consumption in the anaerobic fermentation of protein. For example, Cai et al. [6] found hydrogen consumption existed at low initial pH (4–10.5) and not at high initial pH (>11.0) in the biohydrogen fermentation of alkaline pre-treated sludge. While in our previous study [9] few hydrogen consumption was observed in the anaerobic fermentation of the alkaline pre-treated sludge supernatant (which contained 2.14 g protein/l) at high initial pH value (>9.0).

3.2. Consumption of the two organic substrates and production of liquid organic by-products

During the anaerobic fermentation, the glucose and protein were consumed by the microflora (Fig. 3A and B) and the liquid organic by-products (VFA and ethanol) were produced (Fig. 4).

Fig. 2 – Biohydrogen yields from glucose or protein during the anaerobic fermentation.

Table 4 – Comparison of hydrogen yield between this study and some previous studies.

System	Inoculum	Substrate (g/l)	pH (initial pH)	T (°C)	Hydrogen yield	Ref.
Batch	Heat-treated anaerobic sludge	Glucose (2.82)	(6.2)	25	0.968 mol/mol	[20]
Batch	<i>Ethanoligenens harbinense</i> B49	Glucose (10)	(6.0)	35	2.21 mol/mol	[21]
Batch	Heat-treated anaerobic sludge	Sucrose (24.8)	5.5	34.8	3.4 mol/mol	[22]
EGSB	Mixed culture	Molasses ^a	4.2–4.4	35	3.47 mol/mol sucrose	[3]
Batch	Heat-treated anaerobic sludge	Peptone (5) ^b	(7.0)	35	0.034 ml H ₂ /mg protein	[18]
Batch	Heat-pretreated natural microflora	Peptone (5) ^b	(6.5)	35	8.37 ml/l	[19]
Batch	Alkaline pre-treated sludge	Alkaline pre-treated sludge	(11)	36	16.6 ml/g DS	[6]
Batch	<i>Pseudomonas</i> sp. GZ1	Sterilized sludge	(7.38)	35	30.4 ml/g DS	[5]
Batch	Sterilized sludge	Glucose (3.0)	(7.7)	37	1.1 mol/mol glucose	This study
Batch	Sterilized sludge	Peptone (0.32)	(7.5)	37	0.077 ml H ₂ /mg protein	This study

a The concentration of substrate was not given.

b The unit was gCOD/l.

For glucose, the substrate was rapidly consumed in the initial stage of fermentation, but little was consumed in the latter stage. Additionally, the rapid consumption stages were affected by the glucose concentration. Conversely, during the anaerobic fermentation of protein, the substrate was consumed throughout the fermentation period and the consumption in the initial stage of fermentation occurred more rapidly than in the latter stage. The consumption rates of these two organic substrates during the anaerobic fermentation were calculated in each sampling stage and the results are summarized in Fig. 3

(C and D). For the anaerobic fermentation of glucose, the maximal consumption rates occurred at the 10th h when low concentrations were used (C1–C3); therefore, 0–10 h was the maximal consumption stage. Conversely, the maximal consumption rate was observed at 23.5th h when higher concentrations were used (C4–C6); therefore, stage of 10–23.5 h was the maximal consumption stage. The trends in the consumption of glucose were similar to those of previous studies [1,2,26]. However, for the anaerobic fermentation of protein, the maximal consumption rates occurred at the 23.5th h

Fig. 3 – Consumption and consumed rate of glucose and protein during the anaerobic fermentation.

Fig. 4 – Changes of VFA and ethanol during the anaerobic fermentation.

for all tested concentrations. The above results suggested that the consumption of glucose was affected by its concentration and that of protein was not.

With the consumption of two substrates, the liquid organic by-products (VFA and ethanol) were produced and the yields were increased with the fermentation time and the concentrations of substrates (Fig. 4). The compositions of the liquid organic by-products for the two organic substrates differed. Specifically, the main compositions of the liquid organic by-products for glucose were acetate, butyrate and ethanol and the content of propionate was lower, whereas those for protein were acetate, butyrate and propionate and the content of ethanol was lower. The different compositions of the liquid organic by-products illuminated that the hydrogen-producing pathways of the two substrates were different: the pathway of glucose was ethanol-type fermentation and that of protein was butyrate-type fermentation.

Comparing the three processes, consumption of two substrates, productions of hydrogen and liquid organic by-products, it could be found that the production of liquid

organic by-products was synchronous with the consumption of two substrates and the production of hydrogen was lag behind the other two processes. It is due to hydrogen-producing microorganisms in the seed microflora had to acclimatize the new environment. Analyzing the hydrogen yields of these two organic substrates during the anaerobic fermentation based on their initial concentration and consumption (Table 5), it could be revealed that the hydrogen yield was significantly correlated with their initial concentration and the consumption during the anaerobic fermentation. These results showed that the hydrogen produced during the anaerobic fermentation of the glucose and protein was actually produced by the degradation of these substrates.

3.3. Changes of pH

The degradation of both glucose and protein produced several low weight molecular materials, such as H_2 , CO_2 , H_2O , VFA or amino acids, NH_4^+-N , and so on [1,2]. The production of VFA, amino acids and NH_4^+-N could result in a change of pH

Table 5 – Pearson correlations of some parameters during hydrogen fermentation of two substrates.

Item	Glu _{in} ^a	Glu _{con1} ^b	Glu _{con2} ^c	Item	Pro _{in} ^a	Pro _{con1} ^b	Pro _{con2} ^c
H ₂ -yield	.998 ^e	.875 ^d	.998 ^e	H ₂ -yield	.874 ^d	.847 ^d	.873 ^d
Glu _{in}		.893 ^d	1.000 ^e	Pro _{in} ^a		.995 ^e	.999 ^e
Glu _{con1}			.893 ^d	Pro _{con1} ^b			.992 ^e

a Glu_{in}, Pro_{in}: The initial concentrations of glucose and protein, respectively.

b Glu_{con1}, Pro_{con1}: The consumption of glucose and protein from 0 to 23.5 h and 21.0 h, respectively.

c Glu_{con2}, Pro_{con2}: The consumption of glucose and protein from 0 to 58.5 h and 64.0 h, respectively.

d Correlation is significant at the 0.05 level (2-tailed).

e Correlation is significant at the 0.01 level (2-tailed).

during the anaerobic fermentation (Fig. 5). During the anaerobic fermentation of glucose, the pH decreased rapidly within 0–10 h when low concentrations were used due to the production of VFA, after which it became stable, and even increased slightly (≤ 1603.65 mg/l, namely C1–C4). Additionally, the pH dropped to nearly 5.0 when high concentrations were used (>1603.65 mg/l, namely C5 and C6). When the low substrate concentrations were used (≤ 1603.65 mg/l), the hydrolysis and acidification of seed sludge produced NH_4^+ -N, which could neutralize the VFA produced during fermentation [23]. Conversely, when high substrate concentrations were used (>1603.65 mg/l), the production of VFA was much higher than that of NH_4^+ -N. During the anaerobic fermentation of protein, the VFA produced was neutralized by some of the degradation products, such as NH_4^+ -N and amino acids, which resulted in buffering capacity and the maintenance of a stable pH [18]. Specifically, the pH of the sludge during the anaerobic fermentation of protein was maintained around 7.0 (Fig. 5).

3.4. Microbial diversity

The diversity of microbial communities in the sludge in these two organic substrates was analyzed and compared by PCR-DGGE using the profiles of 16S rDNA gene fragments (Fig. 6). Only the sludge samples obtained from the tests in which the maximal hydrogen production was obtained (C6 and P3) were analyzed due to similarities in the microbial communities when the hydrogen-producing substrate was the same.

Each band on the DGGE profile corresponded to a gene fragment of one of the 16S rDNA sequences and accordingly represented a specific species in the microbial community. The number of bands revealed the relative diversity of the bacterial community, while the intensity of the band was correlated with the abundance of each bacterial group. The DGGE profiles clearly demonstrated that the bands of two sludge samples were very similar, which indicated that their microbial communities were similar. However, the band pattern and intensity of the DGGE fingerprint showed that the dominant microorganisms in the two sludge samples were different. Many factors can affect the growth of microorganisms, like seeds, substrate, nutrient, incubation time, pH, and so on. In the test, although the seeds were same, the substrate and pH for fermentative hydrogen production of two substrates were different. The differences in the substrate and pH would lead to the growth of different microorganisms. While it seemed the short fermentation time (only 58 h) in the test could not completely change the microbial species of sludge but change the dominant microorganisms of sludge. Comparing the results of this study with the studies at alkaline pH for protein and ones at acid pH for glucose [9,27,28], it could be found that the DGGE band numbers of this study were far more than the previous studies, which suggested that these microorganisms in the anaerobic fermentation of this study were more abundant than these previous studies. The abundant microorganisms may be due to the anaerobic fermentation at initial pH was favorable the growth of microorganisms. Additionally, the

Fig. 5 – Changes of pH during the anaerobic fermentation.

Fig. 6 – DGGE profiles of the microbial community produced by the anaerobic fermentation of glucose (C6) and protein (P3).

abundant microorganism may be a reason of lower hydrogen yield of this study.

4. Conclusions

The results of this study demonstrated that the maximal hydrogen yield of glucose at neutral initial pH was about 0.14 ml H₂/mg glucose which was far higher than that of protein (about 0.077 ml H₂/mg protein) in their anaerobic fermentation at neutral initial pH. Unlike glucose, the hydrogen consumption could also be observed in the anaerobic fermentation of protein. Although, the hydrogen production was lag behind the consumption of two substrates and production of liquid organic by-products, statistical analysis revealed that the biohydrogen produced during the anaerobic fermentation of two substrates was significantly correlated with their degradation. In the anaerobic fermentation at neutral initial pH, the hydrogen-producing pathways of two substrates were different: ethanol-type fermentation for glucose and butyrate-type fermentation for protein. In the anaerobic fermentation of protein, the pH maintained a stable pH near due the production of NH₄⁺-N and the amino acids. Finally, the molecular analysis indicated that the dominant microorganisms during the anaerobic fermentation of two substrates differed greatly.

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