Development of Fixed-bed Bioreactor for Higher Bio-hydrogen Production

July 2019

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A Dissertation Submitted to

the Graduate School of Life and Environmental Sciences,

the University of Tsukuba

in Partial Fulfilment of the Requirements

for the Degree of Doctor of Philosophy in Biotechnology

(Doctoral Program of Bioindustrial Sciences)

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Abstract

Nowadays, the excessive usage of fossil fuels has led to a lot of environmental problems. Hydrogen is a promising alternative energy carrier due to its high energy content of 122 KJ/g, and biological hydrogen production from anaerobic digestion process has drawn a lot of attention because of its environment friendly and cost effective. Nonetheless, there are some limitations such as low production and low microbial activity. On the other hand, bedding material has been widely used in methane fermentation, which could improve the efficiency of anaerobic digestion effectively. However, the research about the usage of fixed-bed in the hydrogen fermentation is limited. Therefore, the main purpose of this study is development of fixed-bed bioreactor for biohydrogen production effectively.

Firstly, the effects of the fixed-bed bioreactor in hydrogen fermentation was investigated. A series of experiments with three typical bedding materials including loofah sponge (LS), chlorinated polyethylene (CPE) and porous nylon (PN) were carried out, the reactor without bedding material as the control. As the result, the highest cumulative hydrogen production of 676.4 mL/L was achieved in CPE fixed-bed bioreactor, which is 4 times higher than the LS (161.6 mL/L), 9 times higher than the PN (71.8 mL/L) and 65 times higher than the control (10.4 mL/L). Moreover, SEM observation revealed that the stable and non-toxic CPE fixed-bed could provide favorable environment for microorganisms immobilization, contributing to extraordinary bio-hydrogen productivity. After that, series of batch operations under various concentration of substrate (5 g/L to 35 g/L) was carried out. CPE fixed-bed

bioreactor obtained higher hydrogen production and carbohydrate degradation rate even at high concentration of 35 g/L, whereas no fixed-bed of control reactor showed negligible hydrogen production, indicating that CPE fixed-bed could tolerate extremely high organic loading. Moreover, in the semi-continuous experiment, the developed CPE fixed-bed bioreactor has proved to be high-efficient and stable for hydrogen production after 32 days operation. The developed CPE fixed-bed bioreactor could be a promising strategy for improving bio-hydrogen production effectively.

On the other hand, micronutrient is important for the growth of the microorganisms. Zeolite which can release micronutrients during anaerobic digestion, could contribute to improve the activity of hydrogen producing bacteria. Although CPE fixed-bed bioreactor could enhance bio-hydrogen production efficiently, the lack of micronutrients would restrain the growth of microorganisms. In order to achieve high and stable biohydrogen productivity in the long-term operation, the zeolite was added into the developed CPE fixed-bed bioreactor. Thus, four kinds of bioreactors including the Hybrid (CPE and zeolite), CPE, Zeolite, and Control (no fixed-bed or zeolite) were conducted. The results presented that the Hybrid bioreactor achieved the highest hydrogen production, and the nutrients change in the bioreactors showed that zeolite could release essential micronutrients for the growth of microorganisms. In addition, the Hybrid bioreactor could maintain the enhancement of bio-hydrogen production high and sustainable in 50 days semi-continuous process, while the CPE fixed-bed bioreactor could not produce hydrogen after 32 days.

Additionally, owing to the limited content of essential nutrients such as trace metal ions in the conventional zeolite, especially the iron, zeolite was modified by iron and the iron-modified zeolite was combined with CPE fixed-bed bioreactor in the hydrogen fermentation. The results indicated that Hybrid-Fe reactor (CPE with iron-modified zeolite) showed sustained ability of bio-hydrogen production during 72-days operation, whereas, there is no hydrogen produced in the no iron-modified Hybrid bioreactor (CPE with zeolite) after 40 days. Moreover, SEM observation and immobilized biomass results demonstrated that the iron-modified zeolite could provide favorable condition for enhancing the diversity and quantity of microbes. Therefore, the Hybrid-Fe bioreactor could enhance bio-hydrogen production under long-term operation effectively, which could provide a unique viewpoint for enhancing hydrogen production for practical application in the future.

Contents

Chapter 1 Introduction

1.1 Background

Nowadays, the energy crisis and environment degradation have been receiving an increasing attention in contemporary society. Growing population and economic development lead to more energy demand [1]. Fossil fuels, which is the most important requirements of the world's energy demand today, are rapidly depleting and produce a vast amount of greenhouse gases upon combustion [2]. To solve these problems, it is urgent to search for renewable, sustainable and clean energy for development of our society [3]. Hydrogen production is attracting more and more attention since it is high energy content of 122 kJ/g, which is 2.75 times higher than fossil fuel such as gasoline, also generates no greenhouse gases during combustion, and only water vapor as the byproduct [4]. Hydrogen can be produced by both physicochemical and biological process [5]. However, biological method plays an important role due to it is mild, environmental friendly, energy efficiently and cost effective as compared to the physicochemical method [6]. Hydrogen production through biological process can be classified into two types: photo fermentation and dark fermentation processes. Dark fermentation hydrogen production (DFHP) has a lot of advantages such as: simple bioreactor, versatility of substrate and the operating costs are relatively lower than photo fermentation [7]. However, the main drawbacks in bio-hydrogen production are low hydrogen formation yields [8]. Fixed-bed reactor is regarded as a promising strategy to solve these problems, recent research indicated that fixed-bed reactor shown a better performance in methane fermentation [9]. However, there are few studies

focused on enhancement of hydrogen production by fixed-bed reactor in dark fermentation. Therefore, developing a fixed-bed bioreactor for higher hydrogen production is necessary.

1.2 Dark hydrogen fermentation process

Generally, bio-hydrogen can be produced by photo fermentation and dark fermentation. Fig. 1.1 shows a brief comparison of these processes [8]. Dark fermentation is the degradation of organic substrates by anaerobic bacteria in a condition without light and oxygen in order to produce bio-hydrogen [10,11]. It is convenient as compare to the photo fermentation which requires particular organisms, light and strict operation condition. In addition, there are a lot of studies indicated that light dependent processes has less inhibition than the light independent, although both of them have their own disadvantages with regard to commercial application [12].

In dark hydrogen fermentation process, different kinds of heterotrophic bacteria can ferment carbohydrates to produce hydrogen, volatile fatty acids (VFAs) and CO_2 under anaerobic condition, without requirement of additional light energy [13,14]. A variety of substrates can be used in the fermentation process, such as lignocellulosic substrate and food waste [15]. Many researches indicated that lots of different factors may results in inhibition in dark fermentation, which can be divided into two parts: pre-process and in-process inhibitors. Pre-process inhibitors are the factor already present before dark fermentation process, such as the substrate, while in-process inhibitors are factors that occur during the dark fermentation process, like the concentration of VFAs [16].

1.2.1 Hydrogen production pathway

Bio-hydrogen through the dark fermentation process has been considered as a sustainable method, hydrogen yields depends on the metabolic pathway followed by the microorganisms [17]. Different kinds of microorganisms have the ability to produce hydrogen from carbohydrates but molecular techniques have identified clostridial species as the H_2 producing strain [18–20]. A range of carbohydrate substrate can be used by clostridia including starch and cellulose [21]. Acetate, butyrate and ethanol are the most common byproducts in the dark fermentation process as showed in equation (1), (2) and (3) [14,22].

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2 \quad (2)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2OH + CH_3COOH + 2H_2 + 2CO_2 \quad (3)$$

According to the equations, when glucose is used as substrate, the theoretical maximum yields are 4 mol of hydrogen with 2 mol acetic acid, or 2 mol of hydrogen with1 mol of butyric acid or ethanol. However, generally the theoretical maximum yield value could not achieve due to the consumption of carbon resource by the growth of microbes and production of byproducts [23,24].

In the end, products such as acetate and lactate, in huge quantity would cause several inhibitions leading to low hydrogen yield [14]. In addition, there are other reasons will influence the hydrogen yields, like glucose may convert to the other byproducts which inhibit hydrogen production, some hydrogen might be consumed in to produce other byproducts in the process, such as propionate [25].

1.2.2 Effect of inoculum on dark fermentation

Inoculum contains different microflora, which can produce hydrogen through dark fermentation [26–29]. And effective hydrolyzing may result in hydrogen production from substrate. Hydrogen producing microbial community depends on the inoculum source and pretreatment methods [30,31]. Some research showed that hydrogen yields influenced by the inoculum sources, and the response of the microorganisms in the dark fermentation process results in inhibiting process [30,32]. Several studies indicate that in order to enhance hydrogen yields, pretreatment on the inoculum is necessary, which result in increasing the hydrogen producing bacteria (HPB). Pretreatment method should not only protect the HPB, but also inhibit hydrogen consuming bacteria (HCB). It is confirmed that hydrogenase activity in the pretreated inoculum shows three times higher than in the non-pretreated inoculum [33], which indicate that pretreatment can increase HPB and enhance hydrogen yields. Pretreatment methods have two ways: physical and chemical methods. Physical methods include heat pretreatment, ultrasonic pretreatment, freeze drying and others, while chemical methods can be divided into pH pretreatment and chemical inhibition. As introduced before, pretreatment methods should be suitable to the HPB, for example, culture containing Propionibacterium granulosum pretreated by the acid methods, only showed 10.4 times less hydrogen as compared to the heat pretreatment, which suggested that pretreatment methods play an important role in dark fermentation [34]. And some studies demonstrated that inoculum pretreatment at 90℃ for 60 mins is helpful for inhibiting the HCB [35]. Therefore, this method was used to enhance HPB for higher hydrogen production.

1.2.3 Effect of initial substrate concentration on Dark fermentation

A wide range of substrates can be used in the dark fermentation [12,36], especially the source which include the organic carbonaceous, such as the sucrose, glucose, xylose, wheat starch and others [37]. Several studies have shown that when using sucrose as the substrate, the performance of the biogas production could arrive at the maximum value, and then decrease to lower or zero value [38], but when using glucose as the substrate, it is steadier in the dark fermentation [39]. From the reports, it can be conducted that different substrate can result in different metabolic pathway, which can lead to different performance. In this research, the steadiest substrate, glucose was chosen as the substrate.

On the other hand, substrate concentration is recognized as another important factor for hydrogen production. Substrate concentration affects the metabolic pathway and microbial community, which would influence the performance of the hydrogen production. Too much substrate will inhibit the process of the dark fermentation, but the lower substrate result in the lower reaction efficiency [40,41]. The optimal substrate concentration for different substrate in the different bioreactor would not be the same. Thus, identification of the optimal initial substrate concentration in this research is necessary.

1.2.4 Effect of micronutrients in hydrogen fermentation

The significant micronutrients including carbon, nitrogen, phosphorous and metal ions involved in hydrogen fermentation [29,42–44], they are co-effects on the fermentation process. With the optimal carbon concentration range, the high carbon

content consistent with super hydrogen yield normally. Nitrogen existing in various portion as ammonium and proteins. Besides, ammonium plays an critical role in the anaerobic digestion because it not only be utilized as nutrient for the hydrogen producing bacterial but also provide a slight pH buffering ability avoid the production of volatile fatty acids (VFAs) [45,46]. However, the inappropriate nitrogen composition might enlarge the lag phase in the fermentation process, the short lag phase is an indicator to obtain higher biogas production, thus with increase lag period would decrease biogas production [47]. The appropriate carbon to nitrogen ratio severs as a factor avoiding this inhibition due to the anaerobic microorganisms consume carbon 25-30 time than the nitrogen [48]. Moreover, the phosphate is one of the important inorganic macronutrients, which also influence final hydrogen production attributed to pH buffer ability for VFAs and other byproducts during hydrogen fermentation. Hawkes et al. [49] has reported that optimal C/P ratio of 130 in the bioreactor correspond with optimum operation condition. Among the different kinds of micronutrients in the hydrogen fermentation, metal ions were exhibited as remarkable effect on hydrogenases which is the key enzyme consist with formation of hydrogen and influencing hydrogen production compared to others. Because they are associate with cell growth, metabolism, enzyme activation and functioning [50–52]. Thus, supplement of necessary and proper metal ions in the bioreactor could enhance biohydrogen production effectively during anaerobic digestion.

1.3 Fixed bed bioreactor as a potential method

Recently, several researches have been reported to produce hydrogen through dark fermentation. At present, the main drawbacks are the low biohydrogen production and low reactor efficiency. There are different methods to enhance the biohydrogen production through the dark fermentation, such as using different pretreatment methods, different substrate, changing parameters like carbon to nitrogen ratio, pH value and temperature. However, all these methods would lead to new problems, like the high energy cost, not economical and the most important is they are not efficient enough. For the above reasons, fixed-bed bioreactor is considered as a promising way to solve these problems. Several studies demonstrated that fixed-bed bioreactor would get a better performance in the methane fermentation by improving the activity and quantity of the microorganisms [53,54]. Fixed-bed bioreactor can immobilize microorganisms and operate at high dilution rate without encountering washout of microorganisms [55]. However, there are very few researches about the fixed-bed bioreactor used in the hydrogen fermentation. Therefore, developing a fixed-bed bioreactor in the hydrogen fermentation for higher hydrogen production is necessary.

1.3.1 Effect of fixed-bed bioreactor on hydrogen fermentation

Fixed-bed bioreactor is regarded as a crucial factor in the dark fermentation, which affect the biomass retention [56]. Zheng et al. [9] supported that the fixed-bed bioreactor showed the shortest start-up period, and reached the highest methane production in the anaerobic digestion. In addition, it also indicated the long-term practical effectiveness of the bioreactor by higher and stable methane concentration.

Fixed-bed bioreactor is a way to provide environment for microorganisms to immobilize, because the remarkable immobilization of the microorganisms could contribute to the enhancement of the anaerobic digestion efficiency. The fixed-bed bioreactor added with optimum bedding material enhanced pH buffer ability of the bioreactor [57]. Due to the immobilized cells offer additional advantages such as being more tolerant to environmental perturbation, being more stable in operation, promoting a higher biological activity due to the higher cell density [58]. Although fixed-bed bioreactor demonstrated high efficiency to promote methane production in anaerobic digestion, there are few researches focused on its application in the dark hydrogen fermentation, especially the optimization of bedding materials for high hydrogen production. Hence, it is urgent to develop a high-efficient fixed-bed bioreactor to enhance biohydrogen production.

1.3.2 Different bedding materials in the fixed-bed bioreactor

The characteristics of bedding material have a significant influence on the immobilization of the microorganisms [54]. The physiochemical characteristics could influence the metabolites and immobilization of the microbes in the fixed-bed bioreactor, such as the porosity, pore size, specific surface area and the roughness of the surface [59]. Zheng et al. [9] indicated that the bedding material plays an important role in the dark fermentation. Therefore, identifying the optimal bedding material in the fixed-bed bioreactor is necessary. As different bedding material had different performance, it is essential to explore a suitable material. When choosing bedding material, the quantity of immobilizing microorganisms and the tolerance to the

unfavorable environmental condition should be regarded as the main factors that would be helpful for the bioreactor system's effectiveness and success.

Besides, the cost should also be regarded as a significant factor. Considering all the factors, three different materials chlorinated polyethylene (CPE), porous nylon (PN) and loofah sponge (LS) were selected as the bedding materials. All of them could be bought from the 100-yen shop in Japan, i.e, 1.5, 1.2 and 1.0 dollars/m^2 for CPE, PN and LS, respectively. The porous structure and porosity characteristics were also higher as compared to other bedding materials. Most importantly, according to the previous study in our laboratory, they were useful in the methane fermentation, and demonstrated high efficiency to enhance methane productivity in the anaerobic digestion.

1.4 Hybrid fixed-bed bioreactor on hydrogen production

The biohydrogen through dark fermentation draw a lot of attention as environment friendly and cost effective, and fixed-bed bioreactor was obtained as a novel strategy to enhance biogas yield by immobilizing microorganisms contributed to a stable biofilm as well as increased bioactivity. Although the fixed-bed bioreactor demonstrated a large quantity of microbes result in effective biogas productivity, there are other factors might limit the hydrogen fermentation process continuously and stably. For instance, pH, temperature, concentration of metabolic products, pressure in the bioreactor and micronutrients [29,36,60]. Among the several factors, the micronutrients such as metal ion was obtained as key factor effect the growth of microbes [61]. The high density of microorganisms in the fixed-bed bioreactor demonstrating superior requirement of nutrients, and lack of significant metal ions could restrict the continuous

operation. However, one of main problems in the fermentation process is the exceed micronutrients concentration cause destructive strength for cells. To solve this problem, the bioreactor has been designed with the requirement of immobilization of cells and supplement of critical metal ions. Thus, it is urgent to develop an effective hybrid bioreactor which could continuously and sustainably stimulate the growth of microbes for increasing biohydrogen production efficiently.

1.4.1 The effect of trace metal in hydrogen fermentation

Generally, various trace metals are critical in the anaerobic digestion due to its association with cell growth, metabolism, enzyme activation and functioning [50–52]. Usually, the metal ions were divided into heavy metal ions and light metal ions. The heavy metal ions including iron, nickel and others. Both of iron and nickel play important role in hydrogen fermentation due to they are the mainly composition of hydrogenases ([Fe-Fe] hydrogenase and [Ni-Fe] hydrogenase) in hydrogen fermentation [33]. It has been reported that addition of Fe would facilitate hydrogen productivity，and the addition of nickel lead to a change in metabolic pathway which affect the biohydrogen yield [27,65]. In terms of light metal ions, Na^+ is associated with the growth of microorganisms and Ca^{2+} has been reported to increase the mechanical strength as well as encourage cell retention [63,64]. The concentration of trace metal at low level could promoting hydrogen fermentation process, while high content of these metal ions would decrease the biohydrogen production by inhibiting transportation of necessary ions, accumulation of metals in the cell and destroying the composition of membrane [65,66]. Yuan et al. [67] reported that the hydrogen yield decreased when

Ca^{2+} added at concentration of 100 mg/L. Owing to the different characteristics of distinctive bioreactor, the addition of necessary metal ion would be important to avoid inhibition in hydrogen producing bacterial.

1.4.2 Combination of zeolite in fixed-bed bioreactor

There is no doubt that the fixed-bed bioreactor could enrich the quantity of microorganisms by provide a suitable condition for the immobilization of bacterial and result in higher biogas yield in anaerobic digestion [9,68]. On the other hand, the micronutrients also play an essential role in growth of hydrogen producing microbes as they are associate with cell growth, metabolism, enzyme activation and functioning [50–52]. Although the optimal fixed-bed bioreactor could immobilize a large quantity of microbes, it could not supply necessary micronutrients. Moreover, zeolite has been reported as an cost-effective material, which with porous structure, since the immobilization of microbes and ion exchange for ammonia removal [69–71]. Montalvo et al. [72] stated that the COD removal and methane production were promoted by adding natural zeolite in the continuous anaerobic digestion. Additionally, zeolite also could supply some micronutrients as well as facilitate electron transfer during fermentation process due to its physical and chemical structure. Both of fixed-bed bioreactor and zeolite showed advantage to enhance biogas yield during anaerobic digestion, while they still have limitation since neither exhibited ability for high efficiency of microbes' immobilization nor supply necessary micronutrients simultaneously. Therefore, combination of fixed-bed bioreactor with zeolite is a promising technique to avoid the inhibition of individual advantages.

1.5 The effect of iron in hydrogen fermentation and development of Fe-modified zeolite

The metal ions draw a lot of attention in hydrogen fermentation process since they could affect cell growth, metabolism, enzyme activation and functioning [50–52]. Furthermore, among the metal ions, Fe is considered as primary micronutrient as it is the essential formation of hydrogenases ([Ni-Fe] hydrogenase, [Fe-Fe] hydrogenase and [Fe] hydrogenase), affecting hydrogen production attributed by proton reduction and biosynthesis of enzymes [52,61,73]. In terms of form of ferredoxins (or iron-sulphur protein), it assists in the transfer of electrons for the oxidation of pyruvate to acetyl-CoA facilitating the biohydrogen production [52,74–76]. Wang and Wan [77] reported that the maximum hydrogen yield was obtained which is increased 55.7% compared to control by addition of Fe^{2+}. And the study from Trchounian et al. [78] also indicated that the Fe^{3+} could be applied for enhancement of biomass in the fermentation process. Moreover, the addition of iron led to a shift of pathway during fermentation, also contributing to stimulate biohydrogen production [79]. However, the normal zeolite is lack of some essential metal ions as iron particularly. Accordingly, development of iron-modified zeolite is a beneficial method to facilitate biogas productivity in the fermentation process. Owing to CPE fixed-bed bioreactor is a novel technic to enhance biohydrogen production, until now, there is no research focused on combining zeolite with CPE fixed-bed bioreactor. Therefore, the main purpose of this study is synthesizing iron-modified zeolite to achieve high-efficient fermentation system.

1.6 Objectives and structure of thesis

From pervious introduction, we know that rising of global energy consumption result in the increasing energy demand. However, the reduction of fossil fuel and environmental problems have led to more attention towards renewable energy sources. Hydrogen is regarded as a sustainable and clean energy, because its high energy content and clean combustion. There are many methods to produce hydrogen, but biological hydrogen production through dark fermentation is considered as a promising way. Nevertheless, there is still some limitations, such as low biohydrogen yield and low bioreactor efficiency. Fixed-bed bioreactor is believed to be a useful method to solve these problems. At present, it is already being used in the methane fermentation and enhancing methane yield effectively, but very few researches about it being used in the hydrogen fermentation is carried out. On the other hand, micronutrient is important for the growth of the microorganisms. Although CPE fixed-bed bioreactor could enhance bio-hydrogen production efficiently, the lack of micronutrients would restrain the growth of microorganisms during long-term operation. Until now, there is no research focus on the addition of micronutrients carrier like zeolite to promote microbial growth in hydrogen fermentation. In order to achieve high and stable biohydrogen productivity in the long-term operation, the zeolite was added into the developed CPE fixed-bed bioreactor. Thus, four kinds of bioreactors including the Hybrid (CPE and zeolite), CPE, Zeolite, and Control (no fixed-bed or zeolite) were conducted. Additionally, owing to the limited content of essential metal ions in the conventional zeolite, especially the iron, zeolite was modified by iron and the synthesized iron-modified zeolite was

combined with CPE fixed-bed bioreactor in the hydrogen fermentation. Therefore, in the research we would like to try our best to overcome the limitation of anaerobic digestion and enhance biohydrogen production sustainably and effectively. The specific objectives of this research are enlisted below:

(1) Optimization of the fixed-bed bioreactor by identifying the optimal bedding material and the capacity of optimal fixed-bed bioreactor.

(2) Investigate the effect of CPE fixed-bed bioreactor combining with zeolite under batch and semi-continuous operation.

(3) Development of Fe-modified zeolite in CPE fixed-bed bioreactor for long-term operation.

The objectives stated above are achieved in the following chapters of this dissertation. The overall structure of the thesis is as outlined below:

(1) Introduction – Giving the reader a clear background and briefly introduction in this field of research. Also, explaining some basic insights that are progressed until now.

(2) The effect of the fixed-bed bioreactor in hydrogen fermentation was investigated, as well as the optimal bedding material. The CPE fixed-bed bioreactor could provide favorable environment for microorganisms, contributing to extraordinary bio-hydrogen productivity. Additionally, series of batch operation under variable concentration of substrate (5 to 35 g/L) was carried out demonstrated that CPE fixed-bed could tolerate extremely high organic loading. Furthermore, the developed CPE fixed-bed bioreactor proved to be highly efficient and stable for hydrogen production after 32 days operation.

(3) Combination of CPE fixed-bed bioreactor and zeolite was conducted to enhance biohydrogen production continuously both under batch and semi-continuous experiments. The results presented that the Hybrid fixed-bed reactor (CPE with zeolite) achieved the highest hydrogen production, and the analysis results of nutrients in different reactor showed that zeolite could release essential micronutrients for the growth of microorganisms. In addition, the Hybrid fixed-bed reactor could also maintain enhanced bio-hydrogen production in 50 days semi-continuous process compared to CPE fixed-bed bioreactor (32 days).

(4) The iron-modified zeolite was synthesized to identify the effect on improving hydrogen production in the CPE fixed-bed bioreactor. The performance indicated that Hybrid-Fe bioreactor (CPE with iron-modified zeolite) showed sustained ability to increase bio-hydrogen production under 72-days operation. Moreover, the proposed improvement mechanism of iron-modified zeolite in hydrogen fermentation was investigated.

(5) Conclusions – Summarizing the results and conclusions in the present research and giving some prospective ideas for future research.

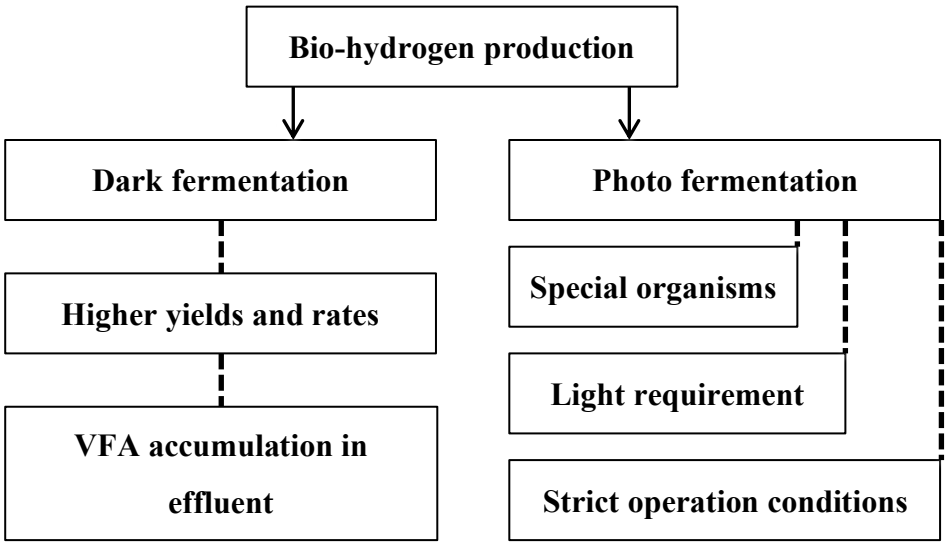

Fig. 1.1 Comparison of the dark and photo fermentation

Argun & Kargi (2011) [8]

Chapter 2 Study on the optimal fixed-bed bioreactor and the capacity of CPE fixed-bed bioreactor

2.1 Introduction

As mentioned in the introduction, the adhesion of the microorganisms plays an important role in the dark fermentation, which can contribute to the process efficiency. Hence, fixed-bed bioreactor is considered as a promising way to enhance hydrogen production in the anaerobic digestion. Wang et al. [70] showed that fixed-bed bioreactor could decrease startup time and immobilize bacteria in the anaerobic digestion. Meanwhile, Zheng et al. [9] reported that fixed-bed bioreactor could be regarded as a support for the biomass attachment. Nevertheless, there are few studies about effect of the fixed-bed bioreactor in the hydrogen fermentation, no research focused on identifying the optimal bedding material, which is necessary for the efficiency of the process. It should be noted that the selection of appropriate materials for bacteria to immobilize draws a lot of attention, because that is crucial for implementation and successful performance in the process. Several species of materials have been used in different types of wastewater treatment, such as low-density polyethylene (LDP), charcoal, expand clay, porous ceramic, synthetic pumice and polyurethane foam (PU) [9]. in addition, the bedding material used in the bioreactor should also be low cost, high efficiency, and easy to get. Hence, according to the different properties of the materials, three typical materials were investigated in the experiments, including the loofah sponge (LS), chlorinated polyethylene (CPE) and porous nylon (PN) were selected as bedding materials. On the other hand, characteristics and concentration of

substrate are important factors for bio-hydrogen production. In addition, the high substrate concentration could lead to excess accumulation of VFA, resulting in the inhibition of hydrogen production [80]. The high accumulated concentration of substrate would inhibit the metabolic pathway of anaerobes as well as the microbial community composition and lead to decreasing hydrogen production [10, 11]. Argun and Onaran [82] found that the inhibition of hydrogen fermentation occurred when concentration of substrate is over 30 g/L. Han et al. [83] also reported that the high concentration of glucose could result in hydrogen producing bacterial restricted. Hence, to develop a bioreactor with high stability and efficiency under high concentration of substrate is necessary for improvement of hydrogen production. However, there are few researches focused on hydrogen anaerobic digestion with high-efficient fixed-bed bioreactor, especially the improvement of digestion capability under high concentration of glucose for long-term hydrogen fermentation.

Therefore, in Chapter 2, the efficiency of the fixed-bed bioreactor in the hydrogen fermentation have been investigated with different bedding materials, and the capacity of optimal fixed-bed bioreactor was also examined under various initial glucose concentrations from 5 to 35 g-glucose/L as well as semi-continuous experiments.

2.2 Materials and methods

2.2.1 Seed sludge preparation and substrate

The original seed sludge was taken from an anaerobic digester at a wastewater treatment plant in Ibaraki prefecture, Japan. Then, the seed sludge was collected in the PET bottles and kept at 4˚C in refrigerator. Before using as inoculum, seed sludge was

acclimatized in 500 mL serum bottles for two weeks, which the total working volume of 400 mL, including the trace mineral solution (200 mL/L) as shown below, $FeSO_4 \cdot 7H_2O$ (1000 mg/L), $CaCl_2 \cdot 2H_2O$ (125 mg/L), $MgCl_2 \cdot 6H_2O$ (125 mg/L), $CoCl_2 \cdot 6H_2O$ (25 mg/L), $MnSO_4$ (25 mg/L), $ZnSO_4 \cdot 7H_2O$ (25 mg/L), $NiCl_2 \cdot 6H_2O$ (25 mg/L), $CuSO_4 \cdot 5H_2O$ (25 mg/L), $Na_2MoO_4 \cdot 2H_2O$ (25 mg/L) and H_3BO_3 (25 mg/L). In addition, after 1 day, 0.5 g glucose was added into serum bottle and continuous every alternate. The cultivation experiment was carried out at 35˚C in incubator.

As mentioned before, in order to enhance hydrogen production, pretreatment for the seed sludge is necessary to enrich HPB, and inhibit other microorganisms, such as the methanogens. Although there are various ways to pretreat seed sludge, considering the energy cost, thermal pretreatment method was chosen among other method. According to the pervious study in our laboratory, it indicated that seed sludge pretreated at 90˚C for 60 min showed best performance. Therefore, this result was used as the pretreatment method in the batch fermentation experiments. The characteristic of the seed sludge is shown in Table 2.1. In order to determine the performance of different bedding material, the steadiest substrate, glucose was used as the substrate in the experiments.

2.2.2 Different bedding material

In the present study, due to the porous structure, low cost and non-toxic, three kinds of materials including porous nylon (PN, T&T, Kainan, Japan), chlorinated polyethylene (CPE, Moritiku, Osaka, Japan) and loofah sponge (LS, Hiroshima, Hiroshima, Japan) were selected as bedding material. They were suspended in the middle of the bioreactor to construct the fixed-bed system, which is the same as

previous study [9]. Prior to use, the bedding materials were washed with distilled water to remove non-adhesive impurities, and then dried in an oven at 105 ºC for 1 h. Fig. 2.1 showed the structure of four different bioreactors: the bioreactor without fixed-bed as control; PN, where the bedding material was PN expand; CPE where the bedding material was CPE expand; LS where used LS expand as the bedding material. The structure of different bedding materials is shown in Fig. 2.2, respectively.

2.2.3 Scanning electron microscope

The morphologies of the microbes present on the bedding materials were observed by a scanning electron microscope (SEM, DS-720, Tokyo, Japan). At the end of the experiment, the bedding material were removed from the bioreactor, and fixed using 2.5% (v/v) glutaraldehyde solution for around 2 h, then, used ultrapure water was used to desalt materials about 6 times and transfered to freezer for about 2 h at -80˚C. After that, the materials were dried in freeze dryer (FD-5, RIKAKIKAI< Tokyo, Japan) for about 24 h. Finally, before observing, the Pt powder was coated or the materials. This method was referred to Yang et al. [84]. Fig. 2.2 showed the original SEM of three different materials.

2.2.4 Batch anaerobic digestion experiment

Fermentation bottles (300 mL, SIBATA) with 200 mL working volume were used as bioreactor. Each bioreactor contained 15% (v/v) thermal pretreated inoculum, the glucose was used as substrate in this experiment. The initial pH in the bioreactors was adjusted to 5.0 ± 0.1 by using HCl (1 M) and NaOH (1 M) and the incubation temperature was set to 35ºC. The bioreactors were then purged with N_2 gas by

deoxygenized gas pressure and replace injector (GR-8, SANSHIN, Japan) to create anaerobic condition and the reactors were tightly sealed with rubber caps. A syringe attached to the reactor was used to collect the daily biogas production. All the tests were carried out in duplicate and the bioreactors were conducted under 35ºC.

To identify the effect of the fixed-bed bioreactor and optimal bedding material, the fixed-bed bioreactors were carried out with different bedding materials PN, CPE and LS. The bedding material was 4.2% of total working volume, and the reactor without bedding materials was performed as control. The initial substrate concentration was 5 g/L.

Then CPE fixed-bed bioreactor was used to identify the capacity of organic loading. Different initial substrate (glucose) concentration of 5, 15, 20, 25 and 35 g/L were used in the experiments.

2.2.5 CPE fixed-bed bioreactor under semi-continuous experiment

The CPE fixed-bed bioreactor with 200 mL working volume was used as anaerobic fermenters. The operation condition and sampling method were the same as the previous experiment. The bioreactors were fed with glucose under different organic loading rates (OLRs) of 0.8 g-glucose/L/d (day 1 to 3), 9.6 g-glucose/L/d (day 4 to 11), 14.4 g-glucose/L/d (day 12 to 19), 19.2 g-glucose/L/d (day 20 to 23), 24.0 g-glucose/L/d (day 24 to 29) and 35.0 g-glucose/L/d (day 30 to 32), respectively. Control was the reactor without bedding material at the same condition. All the tests were performed in duplicate.

2.2.6 Analytical methods

The analysis of total solid content (TS), volatile solid content (VS) were determined according to standard methods (APHA) [85]. In this study, the immobilized biomass was measured at the end of experiment. The bedding material was removed from the bioreactor, washed by the distilled water to remove the biomass. After the biomass sample was dried at 105^{o}C for 24 h, it was taken out and calcined at 600^{o}C for 1 h (Muffle furnace, F-1404-T, Tokyo Garasu Kikai, Japan). Then cooled down to room temperature in a desiccator and weighed. The measurement of broth was the same as mentioned above. The pH, biogas production and composition were detected and recorded every day. The composition of the biogas was analyzed by gas chromatography (GC-8A, Shimazu, Japan), using a machine equipped with a thermal conductivity detector (80^{o}C) and a Porapak Q column (60^{o}C). Nitrogen was used as the carrier gas. The dissolved organic carbon (DOC) was measured with a TOC analyzer (TOC-5000A, Shimadzu, Kyoto, Japan). In this study, ATP value was measured at the end of hydrogen fermentation. Firstly, the biomass attached on the bedding material was washed using the digestate as liquid sample. Then 1 mL of liquid sample added with 1 mL 5% Trichloroacetic Acid (TCA) solution. Next, the sample was adequately mixed and centrifugated. Finally, the 100 μL of supernatant phase added with 100 μL fluorescent agent was measured by a Bac Titer-GLoTM Microbial Cell Viability Assay (Promega, USA), indicating the adenosine triphosphate (ATP) concentration. The degradation of carbohydrate was measured in accordance with standard methods [82]. The concentration of volatile fatty acids (VFAs) was determined using high

performance liquid chromatography (HPLC) (JASCO, PU-4180, Japan) with a UV/Vis detector. The oven temperature was set at 45°C, and 20 mM H_3PO_4 with a flow rate of 1 mL/min was used as the mobile phase.

2.2.7 Kinetic modeling

The amount of hydrogen gas produced was determined according to the measurement of gas composition and the total biogas volume using following equation:

$$V_{H,i} = V_{H,i-1} + C_{H,i}\,(V_{G,i} - V_{G,i-1}) + V_H\,(C_{H,i} - C_{H,i-1}) \quad (4)$$

where $V_{H,i}$ and $V_{H,i-1}$ are the cumulative hydrogen gas volumes at the current (i) and previous time intervals (i-1), respectively. $V_{G,i}$ and $V_{G,i-1}$ are total biogas volumes at the current and previous time intervals. $C_{H,i}$ and $C_{H,i-1}$ are the fractions of hydrogen gas in the headspace at the current and previous time intervals. V_H is the volume of headspace of the serum bottle [86].

The cumulative hydrogen production in the experiments was estimated according to the modified Gompertz equation:

$$H = P\,exp\left\{-\exp\left[\frac{R_m e}{P}(\lambda - t) + 1\right]\right\} \quad (5)$$

where H is the cumulative bio-hydrogen production (mL/L); P is the maximum hydrogen production (mL/L); R_m is the maximum hydrogen production rate (mL/L/h); t is the incubation time (h), e is Euler's number, and is equal to 2.71828, and λ is lag time (h) [87]. The R_m (maximum hydrogen production rate, mL/L/h), λ (lag time, h) and R^2 (R-Squared value) were calculated from the modified Gompertz model by the Origin 8.1 software.

2.3 Results and discussion

2.3.1 Identification of optimal fixed-bed bioreactor

Figure 2.3A showed daily hydrogen concentration from bioreactors with different bedding materials and control. The highest hydrogen concentration (51.8%) was obtained in CPE bioreactor followed by LS and PN bioreactor which achieved hydrogen concentration of 47.5% and 36.8%, respectively. While the control bioreactor showed the lowest hydrogen concentration of only 30.1%. CPE bioreactor achieved a shorter startup period than LS and PN bioreactor. The concentration of biogas is one of the crucial parameters for anaerobic digestion process monitoring, which presented the steady biogas content [88]. Higher hydrogen concentration in the fixed-bed bioreactor indicated higher activity of hydrogen producing bacterial. The superior hydrogen of the CPE fixed-bed bioreactor represented that the CPE is a favorable bedding material to enhance the bio-hydrogen production.

The cumulative biogas production was presented in Fig. 2.3B. As shown in the figure, the CPE fixed-bed bioreactor achieved the highest cumulative biogas production (13010 mL/L) at the first day, which is 2.9 times higher than the LS (455 mL/L), 4.3 times than the PN (355 mL/L) and 32.7 times higher than the control (40 mL/L). In terms of PN and LS bioreactors, the lag phase were 2 and 3 days, respectively, indicating that the shortest lag phase was obtained in CPE fixed-bed bioreactor. The short lag phase consisted with superior biogas production, thus the increment of lag period would decrease biogas production. Moreover, the higher biogas production demonstrated high amount of hydrogen production usually.

Figure 2.3C showed the highest hydrogen production was achieved in the CPE fixed-bed bioreactor (676.4 mL/L), which is 4 times higher than the LS (161.6 mL/L), 9 times higher than the PN (71.8 mL/L) and more than 60 times higher than the control (10.4 mL/L). This result indicated that the addition of the fixed-bed could be an effective method to promote the bio-hydrogen fermentation and CPE is regarded as the optimal bedding material for fixed-bed bioreactor in hydrogen fermentation. Wang et al. [89] obtained a high hydrogen yield (0.80 mol H_2/mol-glucose) from reactor without fixed-bed, and Zheng et al. [90] reported that in a continuous stirred tank reactor (CSTR) system with plastic carriers, the hydrogen yield was only 0.69 mol H_2/mol-glucose. The CPE fixed-bed bioreactor showed higher productivity (0.96 mol H_2/mol-glucose) compared to other researches, revealing that CPE fixed-bed reactor is effective in improving bio-hydrogen yield. According to the result calculated by the modified Gompertz equation, the highest production rate at 140.3 mL/L/h was achieved in CPE bioreactor, which was much higher than that of the control (0.5 mL/L/h) reactors. That was consistent with the practical hydrogen productivity shown in Fig. 2.3C. Moreover, the shortest lag phase (λ) was 11.4 h in the CPE reactor, whereas that of the control bioreactor was 19.1 h. It has been reported that short lag phase could facilitate hydrolysis process, contributing to hydrogen production [60]. Consequently, CPE fixed-bed bioreactor plays a critical role in enhancement of hydrogen production.

Figure 2.4A presented the degradation rate of the carbohydrate after batch experiments. The highest degradation rate of carbohydrate was achieved in CPE (95.5%) followed by LS (92.6%), PN (87.9%) and control (75.2%), respectively. The higher

degradation of the substrate indicated that more substrate has been consumed by the microorganisms in the bioreactor, leading to the higher efficiency of the fermentation process [11, 23]. The higher glucose degradation achieved represented more glucose could be converted to hydrogen in the fixed-bed bioreactor. Consequently, the CPE fixed-bed reactor showed highest bio-hydrogen production (Fig. 2.3C). Zheng et al. [9] reported similar result that the fixed-bed bioreactor could enhance methane production and CPE was obtained as the best bedding material. The results indicated that CPE is the most suitable material in both methane and hydrogen fermentation.

Furthermore, the pH in different bioreactors is shown in Fig. 2.4B. It showed that only CPE reactor could maintain pH around 5.0 at the end of fermentation, while the others were lower than 3.5. pH is regarded as one of the most important operational parameters in H_2 fermentation, since it could directly affect the hydrogen producing bacterial activity, metabolic pathway, and dominant population [82]. Generally, the optimal pH range controlled at 5.0 - 6.5 in hydrogen fermentation [91]. The final pH value in CPE fixed-bed reactor, indicating that CPE fixed-bed bioreactor showed better buffer capacity than other bioreactors. The CPE with rough surface as well as porous structure was favorable for the growth and immobilization of microorganisms (Fig. 2.2B), accelerating the conversion of acids to hydrogen through suitable pathway to maintain the optimal pH range. Consequently, the bioreactor fixed with CPE could provide optimal pH environment for the microorganisms to generate the hydrogen efficiently.

The SEM images of the anaerobic microbes immobilized on the surface of bedding materials were shown in Fig. 2.5. The SEM images revealed that the microbes were successfully immobilized on the bedding materials, and different species of bedding materials gives specific condition for the adherence of distinct microbes (Fig. 2.2). The highest quantity of microbes among the three bedding materials was found on the surface of CPE, indicating that CPE was more suitable for microorganisms to immobilize. This is probably due to the rough surface of CPE with irregular defined channels and apertures (Fig. 2.2B). The roughness of the surface and affinity of CPE offered sufficient contact surface area for the microbes to adhere. In addition, the porous and rough surface structure of CPE provided the microorganisms with favorable condition to immobilize [9,54]. In terms of PN and LS, although both exhibited porous structure, the surface was too smooth to provide an appropriate immobilization condition like CPE (Fig. 2.2B). Therefore, CPE was proved to be the most suitable and promising material in the fixed-bed bioreactor due to its porous structure, roughness of the surface and affinity, which could provide a favorable environment for the microorganism to immobilize.

In order to further support the results and identify optimal bedding materials, the adenosine triphosphate (ATP) concentration in the bioreactors was demonstrated in Fig. 2.6. The higher ATP value of fixed-bed bioreactor indicated higher microorganism activity than that of the control. The CPE bioreactor achieved the highest ATP concentration of 1.56 μmol/L, which was around 70 % higher than LS (0.91 μmol/L) and PN (0.90 μmol/L). The activity of the microorganism in the bioreactor is an

important parameter that influences the performance of anaerobic digestion process [22, 24]. ATP could reflect the activity of microorganism efficiency of anaerobic digestion process, which is considered as an indicator of metabolically active cells and a sign of microbial density [93]. The results are in accordance with the highest hydrogen production in CPE fixed-bed bioreactor (Fig. 2.3C) and could further confirm the effectiveness of CPE fixed-bed bioreactor.

The hydrogen production is accompanied with production of volatile fatty acids (VFAs), such as acetate, butyrate and lactate [94,95]. The VFA value from bioreactor is presented in Table 2.2. In all of the fixed-bed reactors, n-butyrate was found to be the dominant organic acid. The highest n-butyrate concentration was obtained in the CPE fixed-bed bioreactor (11670 mg/L), in which the highest H_2 production was also achieved. While, the n-butyrate concentration was 970, 690 and 580 mg/L of LS, PN and control reactors, respectively.

The *n*-butyrate acid is regarded as one of the most important VFAs in the hydrogen fermentation [96]. Acetate and butyrate routes are the principal hydrogen producing pathways during the hydrogen fermentation. It has been reported that the protein-rich substrate favors the acetate pathway, while the carbohydrate-rich substrate favors the butyrate pathway [97]. In this study, glucose was selected as the substrate and the high concentration of the butyrate acid in the fixed-bed bioreactors is also representing a butyrate pathway dominated hydrogen fermentation (Fig. 2.7). Via the butyrate pathway during the hydrogen fermentation, the glucose is metabolized to pyruvate. The electrons generated from the oxidative decarboxylation of pyruvate are transferred to

protons and then hydrogenase reduces the protons to H_2 [98]. The generated *n*-butyrate acid would contribute to the further energy conversion for hydrogen production. According to the Eq. (6) [99], the high concentration of butyrate always parallels with the higher H_2 production.

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COO^- + 2HCO_3^- + 2H_2 + 3H^+ \quad (6)$$

Consequently, the CPE fixed-bed bioreactor showed the highest *n*-butyrate concentration, resulting in the highest hydrogen production via the butyrate hydrogen generated pathway.

Moreover, the CPE fixed-bed bioreactor showed highest butyrate to acetate ratio (291.75), whereas only 3.12, 2.46 and 2.41 in LS, PN and Control reactors, respectively (Table 2.2). The ratio of the butyrate to acetate ratio could reflect the hydrogen yield to some extent. The high butyrate fraction in liquid metabolite and high ratio of butyrate to acetate might favor fermentative hydrogen production [100]. In Table 2.2, the results of the butyrate to acetate ratio in the different bioreactors, matched their hydrogen producing ability. The CPE bioreactor could provide a favorable environment with sufficient butyrate acid supply and optimal butyrate to acetate ratio.

On the other hand, relative high lactate concentration was observed in the PN, control and LS reactors, which were 1600, 1430 and 550 mg/L, while only 430 mg/L in the CPE fixed-bed reactor. The lactate acid is considered as a main inhibitor decrease the efficiency of bio-hydrogen production [101]. The low lactate concentration in the CPE fixed-bed bioreactor indicated an effective and well-performed hydrogen fermentation.

Therefore, the CPE fixed-bed bioreactor dominated a butyrate pathway fermentation with efficient hydrogen productivity.

2.3.2 The tolerance and potential capacity of CPE fixed-bed bioreactor under semi-continuous operation

The CPE fixed-bed bioreactor showed high potential ability for hydrogen production at initial glucose concentration of 5 g/L. In order to investigate the tolerance of CPE fixed-bed bioreactor for higher organic loading, more experiments were conducted under different initial concentrations of substrate.

The variation of cumulative hydrogen production with different initial concentration of substrate in the bioreactors is presented in Table 2.3. As shown in the table, with the different initial concentration of substrate, the highest value of hydrogen production (950.6 mg/L) was achieved at 20 g/L in the CPE fixed-bed reactor. When the initial substrate concentration increased from 5 to 20 g/L, the production increased from 670.4 to 950.6 mL/L. After that, the production decreased to 77.4 mL/L with further increased substrate concentration to 35 g/L. On the other hand, in the control reactor, when concentration increased from 5 to 15 g/L, the hydrogen production increased from 10.4 to 268.0 mL/L. Further increase the initial concentration from 20 to 35 g/L, the hydrogen production started to decrease dramatically from 142.7 to only 2.4 mL/L, the hydrogen production in the control was around 32 times lower than the CPE fixed-bed bioreactor at the initial concentration of 35 g/L.

In the no fixed-bed (Control) reactor, it was not favorable for H_2-producing microorganism and led to a decline in hydrogen production. This is in accordance with

the study by Argun and Onaran [82]. They reported that if the concentration of glucose is higher than 30 g/L, negligible hydrogen was determined, indicating that the fermentation process could not proceed further. The developed CPE fixed-bed bioreactor demonstrates better capacity to operate under high initial substrate concentration (35 g/L) than normal hydrogen fermentation reactor.

Table 2.3 also showed the degradation rate of carbohydrate under different initial concentration of substrate. In the fixed-bed bioreactor, at the initial concentration of 15 g/L, the highest level (98.5%) of carbohydrate degradation rate was obtained. Most glucose was degraded (90.6 - 98.5%) when the initial glucose concentration increased from 5 to 20 g/L. However, the glucose utilization decreased to 25.6% when the initial concentration was further increased to 35 g/L. Sagnak et al. [7] reported that excess concentration of substrate would lead to inhibition on the microorganism. Comparing with the CPE fixed-bed reactor, the carbohydrate degradation rate in control reactor decreased from 75.2 to 4.0% when the initial concentration of substrate increased from 5 to 35 g/L. Lower degradation of substrate at high concentration (35 g/L) is in accordance with negligible hydrogen production obtained in control reactor. Under different initial substrate concentration, it is obvious that CPE fixed-bed bioreactor always showed higher degradation than control. Consequently, CPE fixed-bed reactor suggested ability to achieve better performance under high concentration of substrate.

The pH in the fixed-bed reactors was always higher than that in control, even with increase in concentration of substrate as seen in Table 2.3. The pH value in the control bioreactor was less than 3.0, while CPE fixed-bed bioreactor could achieve a pH value

more than 3.4 throughout the long-term operation, and even maintain the optimal pH range (5.2) at substrate concentration of 5 g/L. This result indicated that CPE fixed-bed bioreactor showed better buffer capacity than the no fixed-bed bioreactor. The bedding material with buffer capacity in the bioreactor would avoid the VFAs accumulation and contribute to stable pH for anaerobic operation [102]. The increase in concentration of substrate contributed to the VFAs accumulation in the reactor, resulting in low pH value. Abdallah et al. [99] observed the same tendency that the pH value would decline when concentration of substrate increased. While in my study, due to the better buffer capacity of CPE, the CPE fixed-bed reactor could mitigate the decrease of the pH in the bioreactor. Thus, achieving higher pH level (Table 2.3) compared to no bed bioreactor at different substrate concentration.

Comparison of the performance on hydrogen production from previous works are listed in Table 2.4. As shown in the table, the hydrogen yields were from 0.52 to 0.73 mol H_2/mol-substrate when saccharide was used as substrate [89, 103, 108-109]. Lo et al. [103] obtained obviously higher hydrogen yield (0.73 mol H_2/mol-substrate) than the previous literatures, while the CPE bioreactor showed even higher biohydrogen yield at 0.96 mol H_2/mol-glucose. Therefore, the performance proved that bioreactor fixed with CPE is effective in improving bio-hydrogen yield.

Based on these results, it could be concluded that CPE fixed-bed bioreactor has ability to operate under high substrate concentration. The high capacity of CPE fixed-bed bioreactor contributed to high bio-hydrogen production. Furthermore, in order to

investigate the effectiveness of CPE fixed-bed bioreactor under long-term system, semi-continuous hydrogen fermentation should be conducted.

In practical operation, the reactor was operated under high concentration of substrate continuously. Therefore, the semi-continuous operation was carried out to explore the long-term practical effectiveness of the CPE fixed-bed bioreactor during the hydrogen fermentation process. The variation of hydrogen concentration and production in CPE fixed-bed and control bioreactors under 32 days of operation is shown in Fig. 2.8. After the first day's stagnation, the CPE fixed-bed bioreactor started to produce hydrogen and achieved the highest hydrogen concentration of 48.2%. From the 2^{nd} day, the concentration of the hydrogen started to decrease due to malnutrition. Then, the hydrogen concentration was continuously increase to the peak of hydrogen concentration (32.3%) occurred at the 9^{th} day with OLR increased to 9.6 g-glucose/L/d. In this period, the CPE fixed-bed bioreactor showed higher hydrogen concentration and shorter lag phase than the no bed reactor. After that, the hydrogen concentration in the CPE fixed-bed bioreactor dropped sharply, the growing in consumption of glucose resulted in shortage of substrate that decreased the hydrogen concentration. Furthermore, the next peak of hydrogen concentration (31.8%) was achieved on day 22 and 25 with further increasing of OLR to 19.2 and 24.0 g-glucose/L/d, respectively. Along with the OLR increased to 35.0 g-glucose/L/d from day 28 to 32, the hydrogen concentration in the CPE fixed-bed bioreactor decreased. This might attribute to the accumulation of substrate that inhibits the activity of the hydrogen producing bacterial [98]. On the other hand, the concentration in the control dropped to 0 on day 12, and

did not recover until the end of the experiment. These results indicated that the CPE fixed-bed bioreactor is a suitable and promising option for the long-term high OLR hydrogen anaerobic digestion process.

Figure 2.8B shows the cumulative hydrogen production in CPE fixed-bed and control reactors. The CPE fixed-bed reactor started to produce hydrogen on the second day, then increased continuously until the OLR increased to 35.0 g-glucose/L/d. The hydrogen production increased dramatically from day 7, which corresponds with the hydrogen concentration result (Fig. 2.8A). However, in the control reactor, hydrogen was produced at the beginning of the experiment only for three days. It was also agreed well with the result of previous batch experiment that traditional reactor without fixed-bed is not suitable for long-term fermentation operation. In contrast, during the whole semi-continuous operation, the CPE fixed-bed bioreactor presented higher hydrogen production constantly, and the final cumulative hydrogen production (160.0 mL/L) is 7.5 times higher than that achieved in the control (21.3 mL/L). This result demonstrated that CPE fixed-bed reactor could provide a more stable and sustainable process for enhancing hydrogen production effectively during long-term operation compared with control.

A series of SEM photographs from different parts (A: upper part; B: center part; C: bottom part) of CPE suspended in the broth after fermentation were depicted in Fig. 2.9. The observation of the SEM could further understand the performance of hydrogen production in CPE fixed-bed bioreactor. As shown in the Fig. 2.9, after 32-days long-term operation, a large number of microorganisms were immobilized on the fixed-bed

from various aspects, including upper (Fig. 2.9A), center (Fig. 2.9B) and bottom (Fig. 2.9C) parts of CPE suspended in the broth. In addition, the numerous microbial formed a stable biofilm until 32 days, that was attributed by the suitable porosity and the rough surface of CPE, thus provided a favorable place to improve the quantity of microorganisms facilitating the hydrogen fermentation process. The result is in accordance with the previous study that immobilization strategy with optimal bedding material could improve microbial population dynamic during anaerobic digestion [5, 6, 22]. Furthermore, the biomass quantity in fixed-bed and control reactor was determined (Fig. 2.10). According to Fig. 2.10, it is obvious that distinct high value of biomass quantity (1.250 g/L) was achieved on the CPE in the fixed-bed reactor. That is consistent with the SEM phenomenon (Fig. 2.9), proving that CPE is suitable for the immobilization of microbial as well as improve microbial density. In addition, the amount of biomass in the broth of CPE fixed-bed reactor (0.007 g/L) also presented higher biomass quantity than the control (0.004 g/L). It has been reported that biomass density was a critical factor to accelerate the hydrogen fermentation process [9]. As a result, CPE fixed-bed reactor immobilized microorganisms corresponding to better performance on hydrogen production.

On the other hand, ATP is a crucial factor that can influence the performance of anaerobic digestion process and the higher ATP value indicated a higher microorganism activity [5, 22]. The result of final ATP value in the bioreactors at the end of operation is shown in Fig. 2.11. The ATP value in the CPE fixed-bed bioreactor was 0.0120 μmol/L that is 20 times higher than that of control (0.0006 μmol/L). After

32-days of long-term fermentation, even under high OLR of 35.0 g-glucose/L/d, the higher ATP value was found in the CPE fixed-bed bioreactor, which revealed that the CPE fixed-bed bioreactor facilitated the activity of microbial leading to higher hydrogen production (Fig. 2.8B). Therefore, the CPE fixed-bed reactor not only improved the quantity and activity of microorganisms, but also can keep hydrogen fermentation process successfully for 32 days. Usually, the bio-hydrogen could generated for only 2 days in the batch operation [33, 39]. Chang et al. [55] and Wu et al. [107] stated that in the semi-continuous bioreactor without fixed-bed, the bio-hydrogen production could lasted no more than 20 days. In conclusion, the novel CPE fixed-bed bioreactor developed in this study is an appropriate and promising option for the long-term hydrogen fermentation.

2.4 Summary

The CPE fixed-bed bioreactor has been identified to enhance hydrogen production for the first time. It would provide an appropriate condition for immobilization of microorganisms and could also improve the activity of the microbes, contributing to higher hydrogen production. Moreover, the stable and non-toxic CPE fixed-bed bioreactor has ability to operate under high substrate concentration for bio-hydrogen production. The long-term semi-continuous operation verified the practical application of the CPE fixed-bed bioreactor. This study could provide a new viewpoint for enhancing hydrogen production by developing an efficient CPE bioreactor.

Table 2.1 Characteristics of seed sludge used in the experiment.

Parameters	Digested sludge
Chemical oxygen demand (COD, mg/L)	6500
Total nitrogen (TN, mg/L)	5489
Total solid (TS, mg/L)	13,292
Volatile solid (VS, mg/L)	9500
Ammonium nitrogen (NH_4^+-N, mg/L)	1547
pH	7.1

Table 2.2 Concentration of VFAs of each bioreactor during the batch experiments.

	Control	PN	CPE	LS
Lactate (mg/L)	1430	1600	430	550
Acetate (mg/L)	240	280	40	310
n-Butyrate (mg/L)	580	690	11670	970
Total VFAs (mg/L)	2250	2570	12140	1830
HBu/HAc	2.41	2.46	291.75	3.12

Table 2.3 Cumulative hydrogen production, degradation rate of carbohydrate and final pH in CPE fixed-bed and control reactor with different initial concentration of substrate.

Initial substrate concentration (g/L)	Cumulative hydrogen production (mL/L)		Degradation rate of carbohydrate (%)		Final pH	
	Fixed-bed	Control	Fixed-bed	Control	Fixed-bed	Control
5	670.4	10.4	95.5	75.2	5.2	2.8
15	796.4	268.0	98.5	37.5	3.9	2.7
20	950.6	142.7	90.6	35.0	3.5	2.7
25	143.4	61.7	42.4	14.6	3.4	2.8
35	77.4	2.4	25.6	4.0	3.5	3.0

Table 2.4 Comparison of the hydrogen yield in this research with other studies.

Inoculum	Substrate	Fermentation condition (Running time)	Fixed-bed	Hydrogen yield (mol H_2/mol substrate)	Reference
Digested sludge	Glucose (10 g/L)	Batch (36 h) 35°C	-	0.69	[89]
Seed sludge	Glucose (20 g-COD/L)	Batch (72 h) 35°C	-	0.58	[108]
Enterobacter aerogenes	Molasses (0.85 L/L)	Batch (20 h) 38°C	-	0.52	[109]
Clostridium butyricum	Xylose (20 g-COD/L)	Batch (35 h) 37°C	-	0.73	[103]
Digested sludge	Glucose (5 g/L)	CPE Bioreactor Batch (96 h) 35°C	CPE	0.96	This study

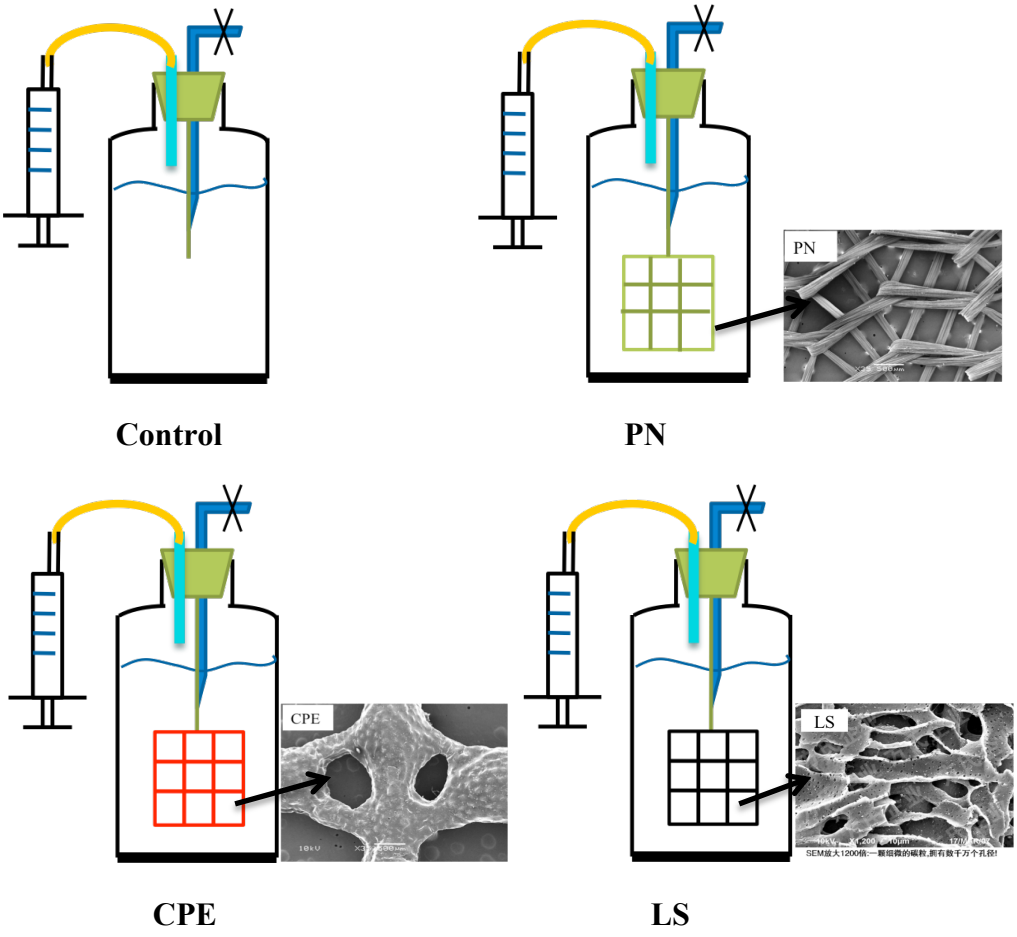

Fig. 2.1 Schematic diagrams of the different bioreactors and SEM images of different materials (PN: porous nylon, CPE: chlorinated polyethylene, LS: loofah sponge).

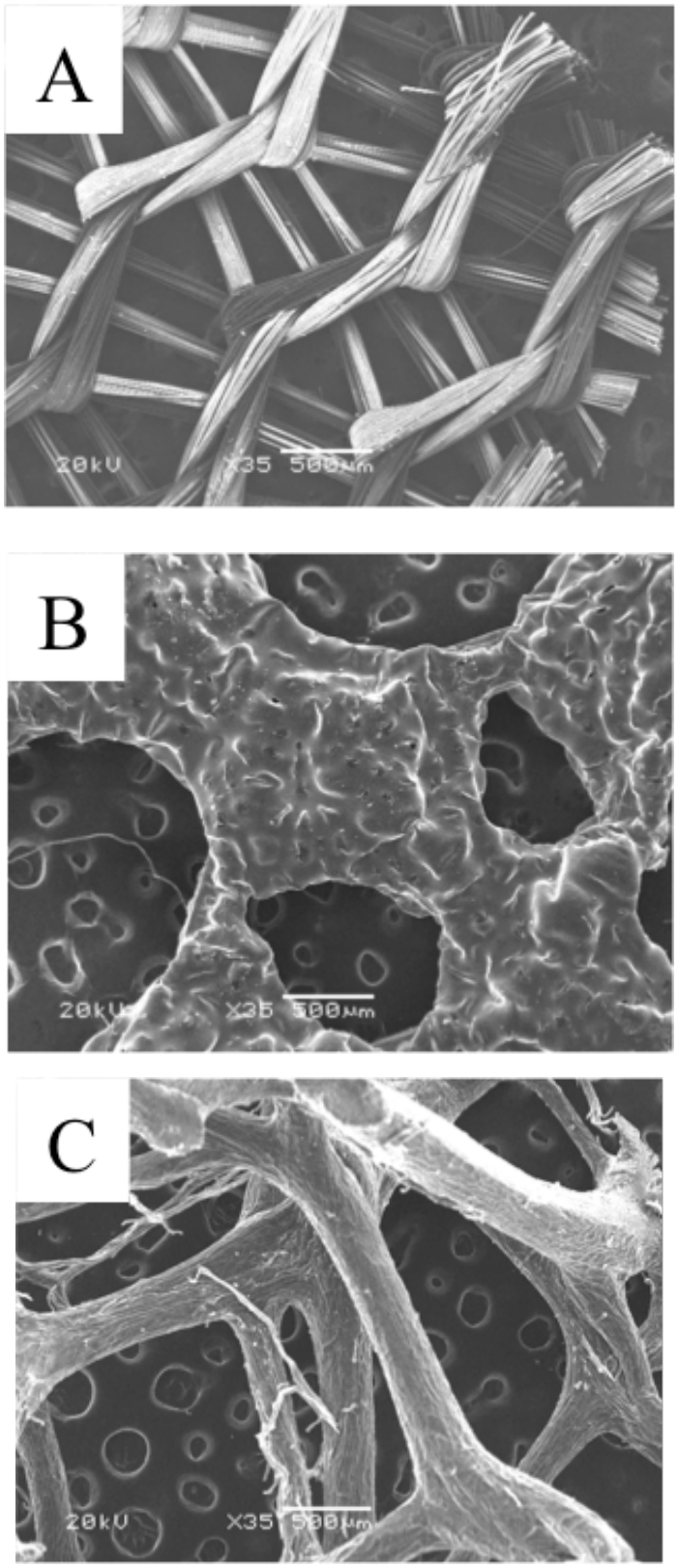

Fig. 2.2 SEM images of different bedding materials: (A) PN, (B) CPE, (C) LS, magnification 35×.

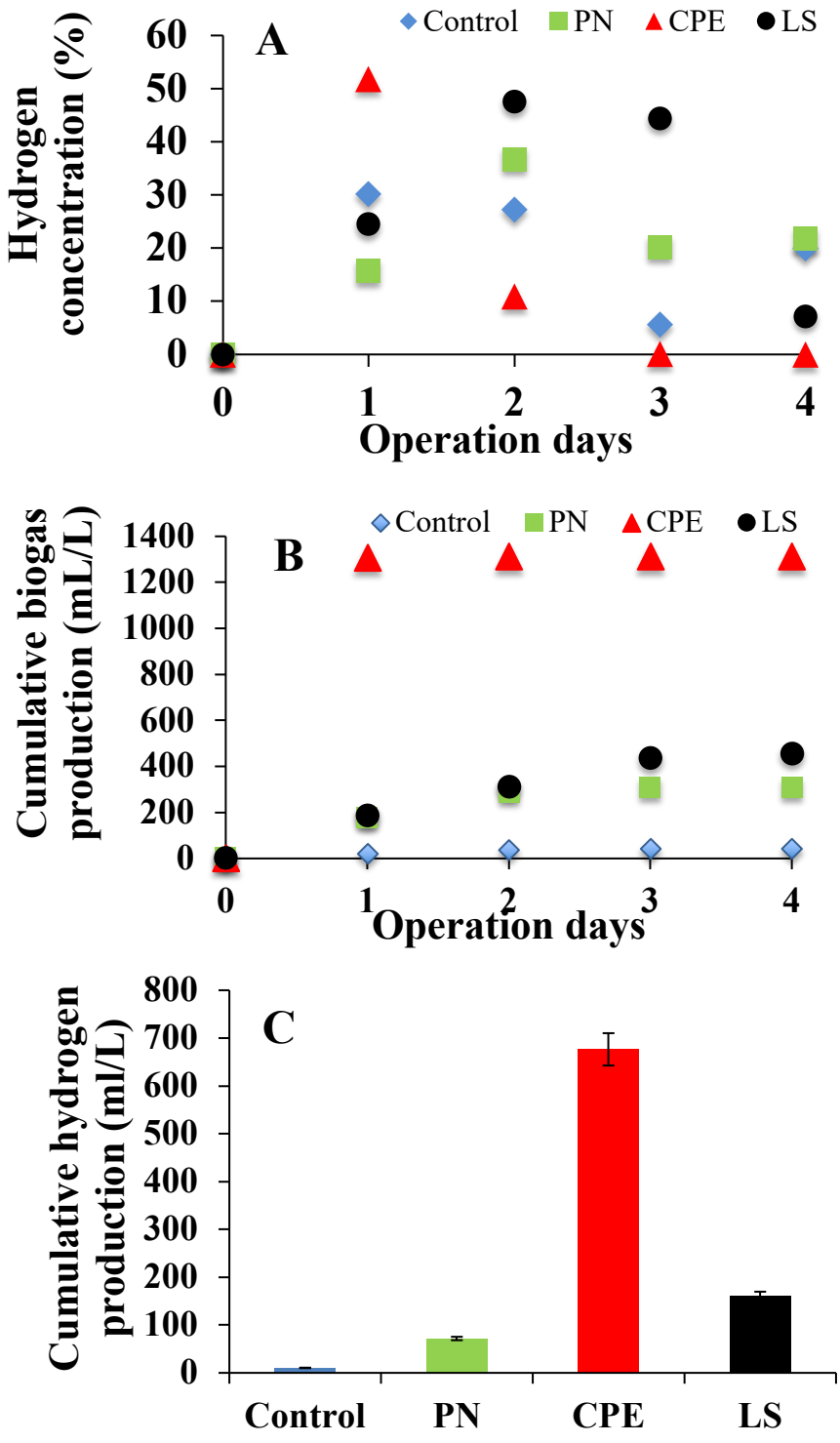

Fig. 2.3 Changes of (A) Daily hydrogen concentration, (B) Cumulative biogas production, (C) Cumulative hydrogen production in bioreactors no bed bioreactor (Control), porous nylon fixed-bed bioreactor (PN), chlorinated polyethylene fixed-bed bioreactor (CPE), and loofah sponge fixed-bed bioreactor (LS).

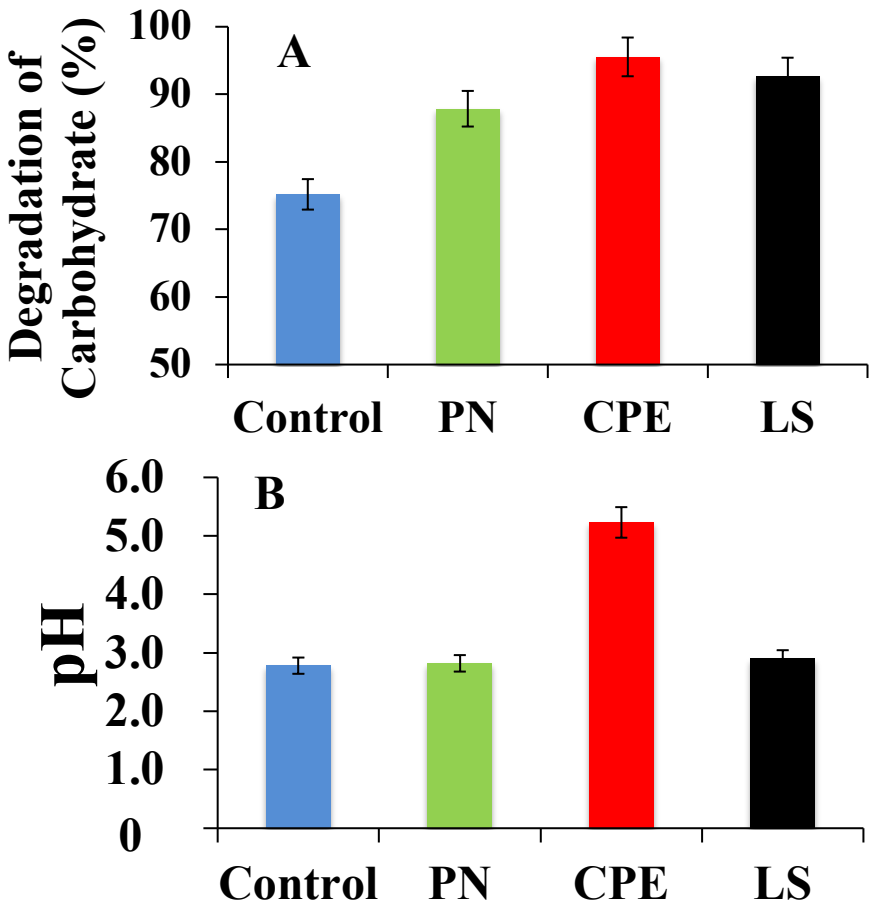

Fig. 2.4 The (A) degradation of carbohydrate, and (B) pH value in bioreactor at the end of the experiment: no bed bioreactor (Control), porous nylon fixed-bed bioreactor (PN), chlorinated polyethylene fixed-bed bioreactor (CPE), and loofah sponge fixed-bed bioreactor (LS).

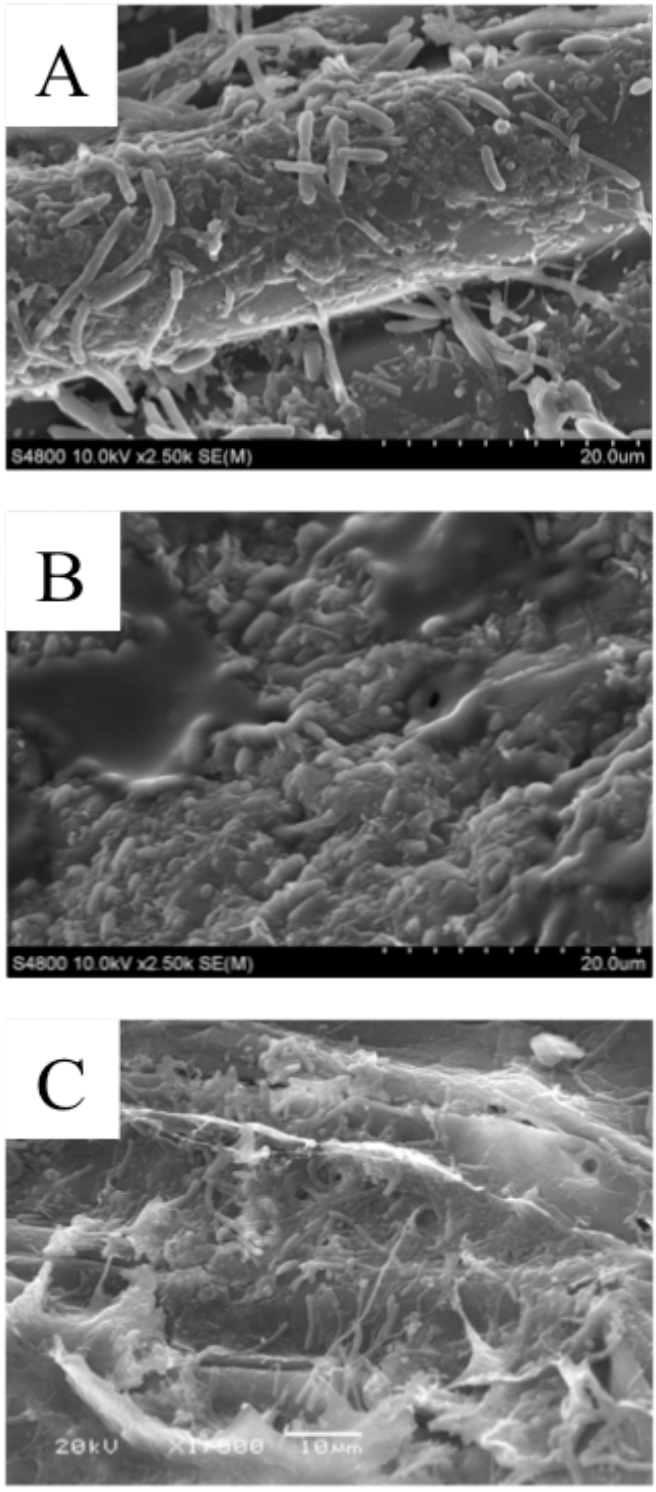

Fig. 2.5 SEM images of anaerobic microbes immobilized on the surface of different bedding materials: (A) PN, magnification 2500×, (B) CPE, magnification 2500×, (C) LS, magnification 1500×.

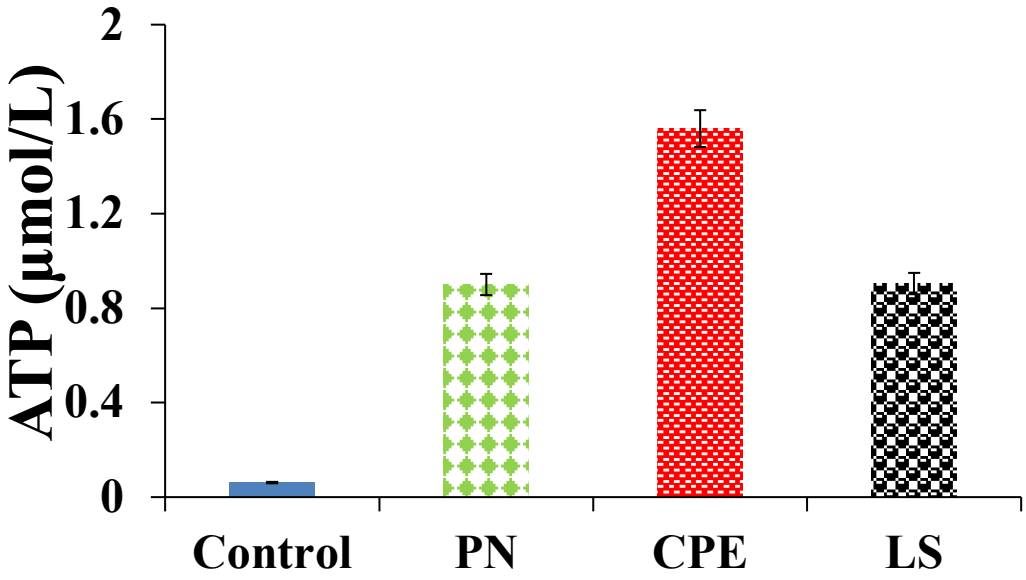

Fig. 2.6 ATP values of the microbes from bioreactors containing different bedding materials and control. The bars designate standard deviations.

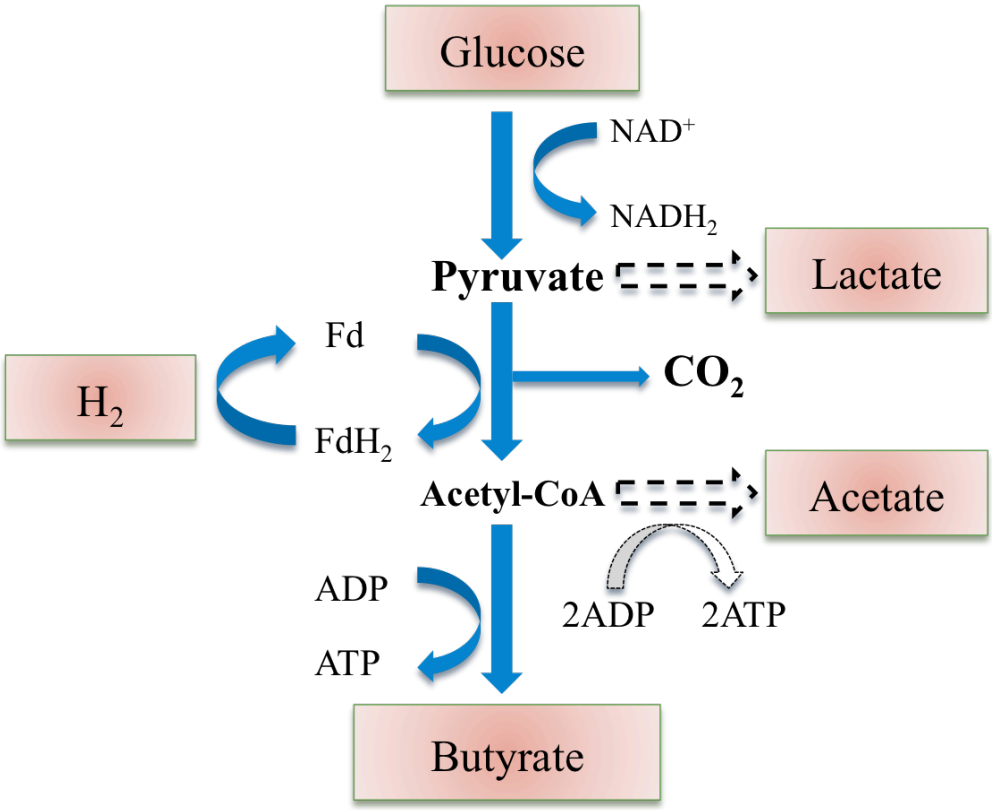

Fig. 2.7 The metabolic pathway for glucose decomposition during hydrogen fermentation.

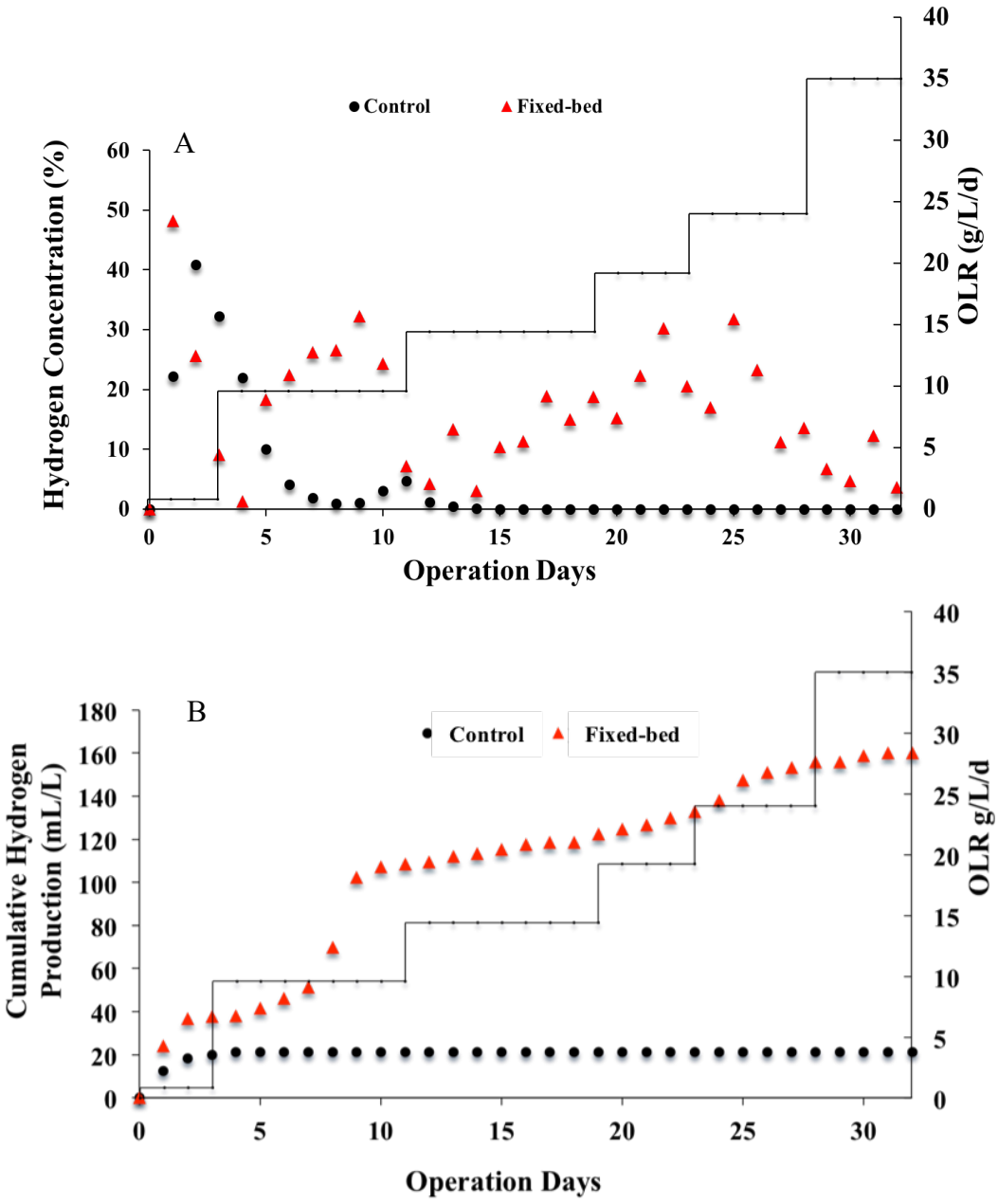

Fig. 2.8 Daily hydrogen concentration (A) and cumulative hydrogen production (B) during semi-continuous anaerobic digestion under different OLRs.

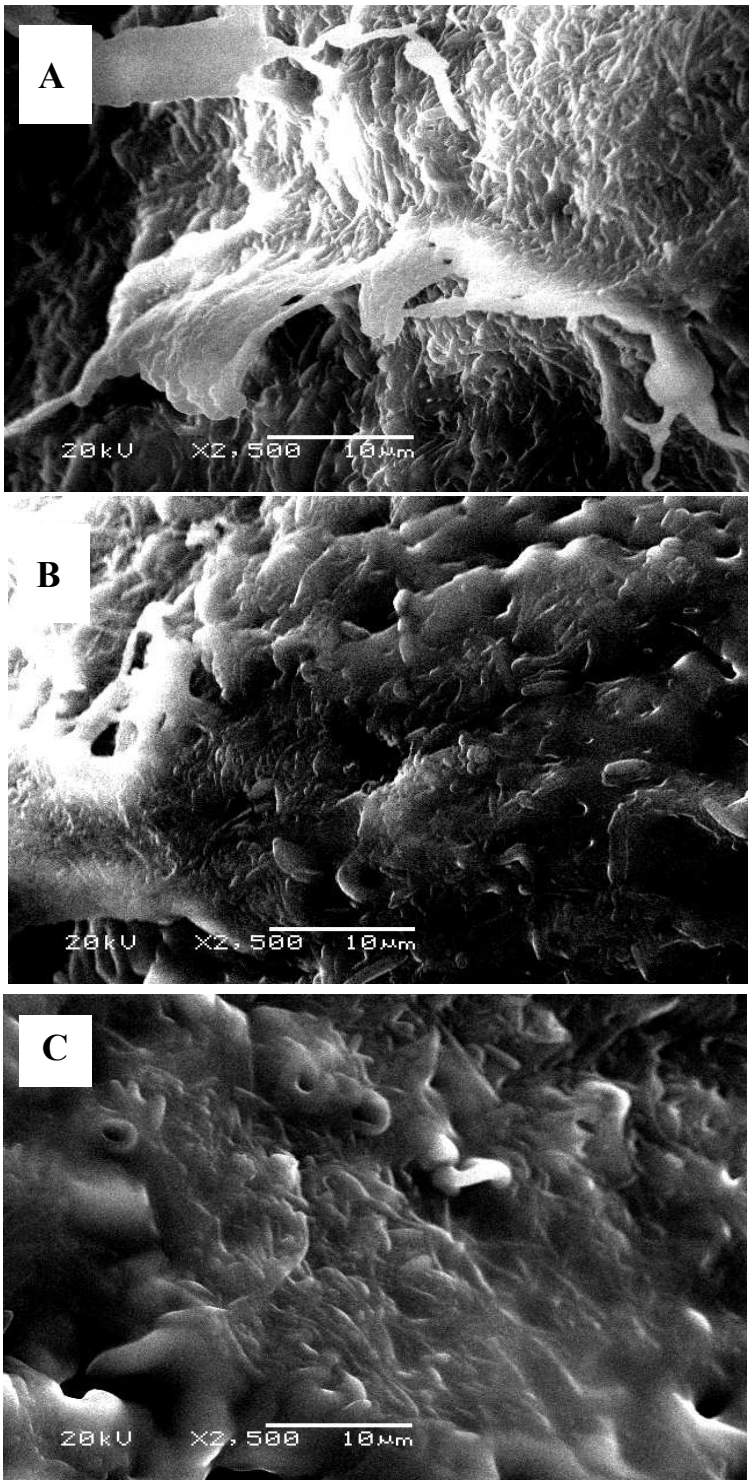

Fig. 2.9 SEM images of anaerobic microbes immobilized on the different parts of CPE suspended in the broth after semi-continuous fermentation: (A) upper; (B) center; (C) bottom, magnification 2500×.

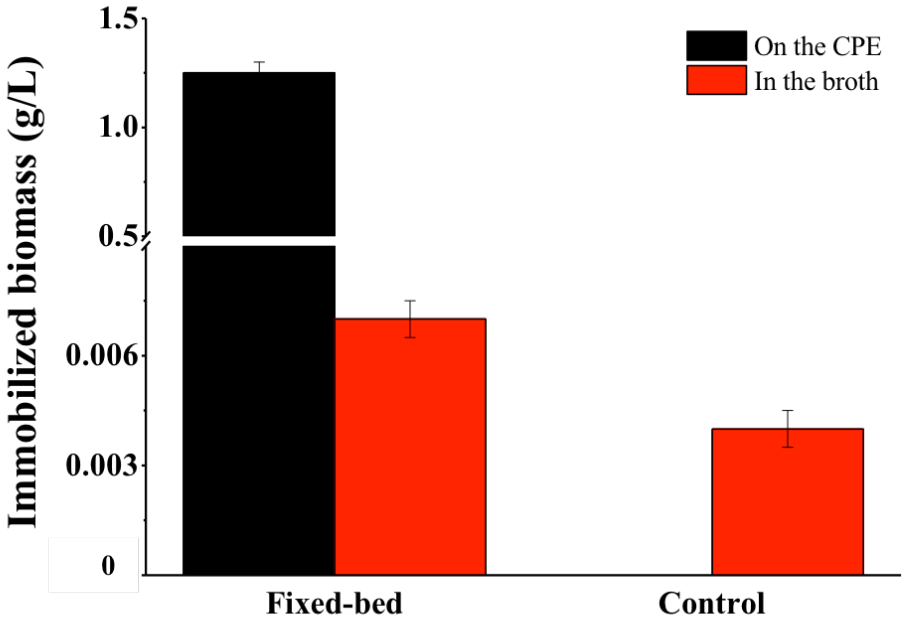

Fig. 2.10 Biomass immobilized on the CPE and in the broth of the bioreactors.

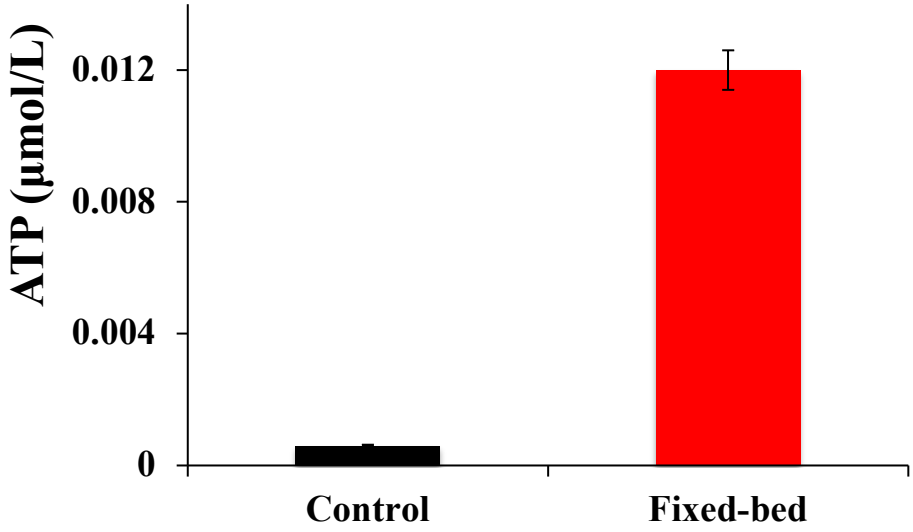

Fig. 2.11 The ATP value of bioreactors at the end of the experiment.

Chapter 5 General conclusions

In the present study, a novel fixed-bed bioreactor was developed for enhancement of biohydrogen production through hydrogen fermentation. Firstly, the optimum fixed-bed bioreactor was identified. Then, the CPE fixed-bed bioreactor combining with zeolite under batch and semi-continuous operation were conducted to investigate the effective of increasing hydrogen gas. Finally, a Fe-modified zeolite was synthesized in CPE fixed-bed bioreactor to achieve sustainable and stable improvement of hydrogen production for practical application. The following results can be concluded from previous chapters.

5.1 Study on the optimal fixed-bed bioreactor and the capacity of CPE fixed-bed bioreactor

Three different bedding materials including PN, CPE and LS were compared to identify the optimal bedding material. The capacity of optimum CPE fixed-bed bioreactor was investigated. The major conclusion as follows:

(1) CPE Fixed-bed bioreactor could provide an appropriate condition for immobilization of microorganisms and could also improve the activity of the microbes, contributing to higher hydrogen production.

(2) The stable and non-toxic CPE fixed-bed bioreactor has ability to operate under high substrate concentration for bio-hydrogen production.

(3) The long-term semi-continuous operation verified the practical application of the CPE fixed-bed bioreactor. This work could provide a new viewpoint for enhancing hydrogen production by developing an efficient CPE bioreactor.

5.2 Combination of zeolite with CPE fixed-bed bioreactor for long-term operation

As zeolite could provide some essential micronutrients such as metal ions, the combination of CPE fixed-bed bioreactor and zeolite was conducted to enhance biohydrogen production continuously both under batch and semi-continuous experiments. Following conclusion were obtained:

(1) The Hybrid fixed-bed bioreactor (CPE with zeolite) showed stable and sustained ability to enhance bio-hydrogen production under batch and semi-continuous operation.

(2) In the Hybrid bioreactor, the CPE could provide a suitable condition for microbial immobilization result in enhancement of H_2 production dramatically, and zeolite not only acted as an micronutrition provider to increased microorganisms' density, but also exhibited pH buffer capacity, contributing to improve hydrogen production stable and continuously in 50 days.

5.3 Development of the iron-modified zeolite in CPE fixed-bed bioreactor for enhancing long-term of biohydrogen production

The iron-modified zeolite was synthesized to identify the effect on improving hydrogen production in the CPE fixed-bed bioreactor.

(1) The iron-modified zeolite could promote favorable fermentation pathway for high-efficient hydrogen gas generation.

(2) With addition of iron-modified zeolite in the CPE fixed-bed bioreactor, the utilization of electron transfer by hydrogenase for high biohydrogen producing was enhanced

(3) As iron is an important composition of several hydrogenases, the iron-modified zeolite supplies Fe and improves the activity of hydrogenase, contributing to the enhancement of hydrogen production.

(4) The combination of Fe-modified zeolite in the CPE fixed-bed bioreactor could enhance biohydrogen production sustainably under long-term operation.

5.4 Future research

In the present study, the Hybrid bioreactor was developed and successfully considered as a promising way to enhance biohydrogen production. And the iron-modified zeolite was synthesized to supply essential metal ions for the growth of microbes. The Hybrid bioreactor exhibited great potentiality to immobilize microbes and promoting activity of microorganisms. Further investigation is necessary to determine the potential capacity of the bioreactor. Therefore, in the future, more research as follows will be concluded:

(1) Co-supplementary micronutrients will be carried out in Hybrid bioreactor for enhancing continuous biohydrogen production.

(2) A sustainable substrate will be determined. As algae has a lot of advantages such as the wide distribution, rapid aquatic growth and high variable of chemical composition. Therefore, the algae will be investigated as the substrate in the future, and the optimal substrate to inoculum ratio will be determined using Hybrid bioreactor.

(3) Two steps of H_2/CH_4 fermentation system under illuminated condition will be established by using high efficiency of Hybrid bioreactors to achieve energy

conversion completely.

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Acknowledgement

My deepest gratitude goes first and foremost to my supervisor Professor Yang Yingnan. During my Master and PhD study, Professor Yang always provided me with constant encouragement and valuable guidance on my every stage of study. She has walked me through all the stages of this thesis. Without her consistent and illuminating instruction, this thesis would not have reached its present form. Her innovation, expertise, vigorous academic observation and passion in scientific research enlightens me deeply and encourages me in my future career. Professor Yang always support me for all the things when I was in trouble in life. She is not only a great professor but my important family and her valuable advice will continue guiding me in my future life. I wish to take this opportunity to express my great gratitude to her.

Second, I would like to express my heartfelt gratitude to Professor in my Advisory Committee: Prof. Yutaka Kitamura, Prof. Motoo Utsumi and Prof. Kosumi Yamada who have instructed and helped me a lot in the past five years and given their insightful comments both academically and morally.

Furthermore, I also appreciate the help from my lab members, Doctor Zhang Nan, Doctor Zheng Hanying and many others, who gave numerous advices concerning my research and their help of my study in University of Tsukuba. I am really greatly indebted to the Doctor Zheng Hanying, she is always patient and encourage me even when I had little progress.

Last but not least, I would like to thank my parents Zhao Guochen and Li Yongmei who have always supported my research and help me out of difficulties.