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Enhanced hydrogen fermentation by zero valent iron addition

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ABSTRACT

In this study, zero valent iron (ZVI) was applied to enhance hydrogen fermentation (H_2). Glucose was used as a substrate at various concentrations of 5–40 g COD/L, and ZVI concentration was adjusted at 0–10 g/L. Without ZVI addition, H_2 yield decreased as substrate concentration increased, due to organic acids accumulation as substrate concentration increased. At low substrate concentration of 5 g COD/L, there was a slight increase of H_2 yield from 1.4 to 1.5 mol H_2/mol glucose added by ZVI addition. However, the increment was substantial at higher substrate range. For example, H_2 yield increased from 0.9 to 1.2–1.5 mol H_2/mol hexoseadded by ZVI addition at 20 g COD/L. The results indicated that ZVI may create a more favorable environment for anaerobic microbial acidogenesis with providing buffering effect to prevent pH drop due to produced hydroxyl ions as it dissolved. These changes of environmental conditions also brought differences on organic acids profile and microbial community, and those may affect to overall H_2 fermentation performances.

Introduction

Zero valent iron (ZVI) is a well-known material for environmental remediation, which is non-toxic, abundant, cheap, and easy to manufacture [1–3]. It is readily oxidized by reaction with water or oxygen under ambient condition and turned to iron oxide or iron hydroxide as reaction product. While it is being oxidized, the other reactant can be reduced by the electron transfer between ZVI and the other reactants. Therefore, reducible compounds, such as halogenated ethenes and ethanes, and some metals/metalloids (Chromium (VI), Arsenic, and Uranium), can readily react with ZVI, which reduced to less harmful products [1]. ZVI is widely used for permeable reactive barrier, which is a subsurface emplacement of reactive materials, due to its high efficiency and simple manipulation [2].

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The application of ZVI has attracted the interest because of its low cost and environmentally benign character. Full scale applications were well reported in the field of groundwater remediation and wastewater treatment [4,5]. Especially, waste iron scrap or iron shaving could be used as alternative to ZVI, which can reduce the overall cost of application significantly. In specific case of applying iron shaving in full scale wastewater treatment plant, the price of iron shaving was estimated as ~$0.25/kg [5].

Application of ZVI as reductant for environmental remediation can lead to change of redox condition of applying environment. The redox potential of ZVI lead to decrease oxidation-reduction potential (ORP) up to negative few hundreds millivolts, indicating strong reductive environment [3]. Sometimes, cathodic H_2 can be produced during the anoxic corrosion process [6]. Some specific microorganisms can be stimulated through the affected environmental conditions. Iron reducing bacteria could be stimulated due to produced soluble iron species from ZVI oxidation [7]. The cathodic H_2 that is produced during the anoxic corrosion process may biostimulate bacterial growth [8].

In addition, the application of ZVI could stimulate aerobic and anaerobic granulation due to Fe^{2+} leaching from the ZVI [9,10]. It is widely accepted that ZVI can be oxidized by reaction with water as shown in below reaction equation [6]. As a by-product of ZVI oxidation, hydroxyl ion is also produced, which increase overall solution pH.

$$Fe + 2H_2O \rightarrow Fe^{2+} + H_2 + 2OH^- \quad (1)$$

On the other hand, the most prominent field of ZVI application interacting with microbial communities could be anaerobic digestion (AD) process. AD is a technology for bioenergy production from organic materials through series of microbial process in an absence of oxygen. It is mostly implemented as waste sludge stabilization method in wastewater treatment plants, as well as for direct bioenergy production from energy crops and solid wastes [11]. Overall AD process can be classified as four main reaction steps, such as hydrolysis, acidogenesis, acetogenesis and methanogenesis, and different microbial communities are functioning in each stages [12]. Therefore, it is very important to control microbial communities to maximize productivity and process efficiency.

Application of ZVI can be a technology to control microbial communities to be suitable for AD. ZVI decreases oxidative–reductive potential (ORP) of the media, and therefore, provides a more favorable environment for AD [13,14], and it could significantly improve the hydrolysis of organic wastes [15]. The H_2 generated through reaction with water is also another influencing factor for enhanced AD process. Generated H_2 can be used by hydrogenotrophic methanogens, resulting in an increased methane production [16]. Moreover, hydroxyl ion produced in the corrosion process of ZVI can act as a buffer of the acid produced by acidogens, which is a crucial step to maintain a stable and favorable condition for methanogenesis [17]. The change of above mentioned reaction conditions were mainly due to the oxidation reaction of ZVI in the presence of water and acid. The ZVI oxidation leads to the overall change of environment for microbial communities in the reactor, therefore, it has direct impact on reactor performance such as organic acid and biogas production as well as microbial communities.

While various studies about effect of ZVI on AD process have been reported, application of ZVI for dark fermentative H_2 production (in short, hydrogen fermentation) has not been intensively studied. Hydrogen fermentation proceeds during the acidogenesis step of AD, where various acids production reactions are involved. In general, the production of acetate and butyrate is known to be related with H_2 production, while the production of other acids is not. Therefore, it is important to maintain the environment condition favorable for acetate and butyrate production for enhanced hydrogen fermentation, which could be affected by ZVI addition.

Based on the previous researches mentioned above, it is hypothesized that applications of ZVI make favorable condition for hydrogen fermentation. To verify the hypothesis, anaerobic batch tests were performed using glucose as a substrate at various doses of ZVI (0–10 g/L). The change of reaction condition was evaluated by monitoring pH and soluble iron concentration in the supernatant during fermentation. The reactor performance indicators, the H_2 production as well as organic acids production were also monitored to investigate the effect of ZVI addition. Furthermore, the change of microbial composition was monitored using 454 next generation pyro sequencing method.

Materials and methods

Materials

Anaerobic digester sludge taken from the local wastewater treatment plant in Incheon, Korea, was used as a seeding inoculum. It was filtered through 2.0 mm sieve to remove large particles, and heat-shock (90 °C for 30 min) was applied to inactive methanogenic activity. pH, concentrations of total suspended solids (TSS) and volatile suspended solids (VSS) were 7.6, 6.7 g/L and 5.0 g/L, respectively. ZVI powders were obtained from Avention Co. (Korea) as a form of reduced iron powder (400 mesh, 98%).

Experimental set-up

Batch experiments were conducted using serum bottles with a working volume of 100 mL (total volume 280 mL). The pretreated sludge was inoculated in the bottles at a VSS concentration of 5.0 g/L. The glucose was used as a substrate and the concentrations were adjusted to 5.0, 10.0, 20.0, and 40.0 g chemical oxygen demand (COD)/L, respectively. The composition and concentrations of trace metals solution (in mg L^{-1}) were: Na_2MoO_4 $4H_2O$, 5; H_3BO_3, 50, $MnCl_2$ $4H_2O$, 50; $ZnCl_2$, 50; $CuCl_2$, 30; $NiCl_2$ $6H_2O$, 92; $CoCl_2$ $6H_2O$, 50; Na_2SeO_3, 50 [18]. After inoculation, initial pH of each bottle was adjusted to 8.0 by 3 N KOH and 3N HCl. The pH was not further artificially controlled during fermentation. ZVI was also added into serum bottles at various concentrations of 0.0, 1.0, 2.5, 5.0 and 10 g/L. All bottles were purged by N_2 gas (99.99%) for 5 min to establish an anaerobic condition and were capped with butyl rubber stoppers. The bottles were placed in a shaking

incubator controlled at 35 °C and 150 rpm. During the experiments, the liquid sample from the bottles were acquired at 1–3 times a day to analyze pH data and organic acids. The tests were carried out in duplicate, and the results were averaged.

Analysis

The concentrations of TSS, VSS, COD, and alkalinity were measured by Standard Methods [19]. The glucose concentration was determined by the colorimetric method [20]. A produced biogas from the bottles was measured with glass syringe and was adjusted to the standard conditions of temperature (0 °C) and pressure (760 mmHg) (STP). The H_2 and CO_2 content in the biogas was measured by gas chromatography (GC, Gow Mac series 580) equipped with a thermal conductivity detector (TCD) using mole-sieve 5A and porapack Q (80/100 mesh) as a separation column. N_2 gas (99.999%) was used as a carrier gas with a flow rate of 30 mL/min and the temperatures of injector, detector, and column were kept at 70, 50, and 80 °C, respectively.

Liquid samples obtained from bottles were immediately diluted 10 times with distilled water and filtered through 0.45 μm pore size syringe filter to analyze pH, soluble iron concentration, and OAs. The pH was measured with a pH meter (pH METER F-71, LAQUA). Soluble iron concentration was measured by Thermo Scientific Elemental Solaar M6 atomic absorption spectroscopy. OAs such as lactate, acetate, propionate, and butyrate analyzed by a high-performance liquid chromatograph (HPLC) (LC-20A series, SHIMADZU Co.) with an ultraviolet (215 nm) detector (UV1000, SHIMADZU) and an Aminex fast acid analysis column (HPX-87H, Bio-Rad Lab.). The mobile phase was 0.005 M H_2SO_4 applied at a 0.6 ml/min flow rate and the temperature of detector, oven, and column were 40, 35, and 90 °C, respectively.

Microbial community analysis

To determine bacterial communities and their population change by adding ZVI powder, sludge samples were obtained for Deoxyribonucleic acids (DNAs) extraction after fermentative H_2 production process. DNAs in the samples was extracted using an Ultraclean Soil DNA Kit (Cat #12800-50; Mo Bio Laboratories, lnc., USA) and purified with an UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories, CA, USA). Then the preparation of libraries and next procedure for emPCR were performed as previously described method [21]. The 16S universal primers 27F (5′GAGTTTGATCMTGGCTCAG3′) and 800R (5′TACCAGGGTATCTAATCC3′) were used for amplifying the 16s rRNA genes [22].

After the PCR products were purified and quantified, sequencing was performed using a 454 pyrosequencing Genome Sequencer FLX Titanium (Life Sciences, CT, USA), according to the manufacturer's instructions, by a commercial sequencing facility (Macrogen, Seoul, South Korea). Identification of operational taxonomic units (OTRs), taxonomic assignment, community comparison, and statistical analysis were obtained by using the software MOTHUR with the sequences generated from pyrosequencing. To minimize the effects of poor sequence quality and sequencing errors, sequences were filtered and removed in part according to the previous study [21]. Then the sequences spanning the same region were realigned with the NCBI BLAST database (www.ncbi.nlm.nih.gov). In the database screening with the BLAST

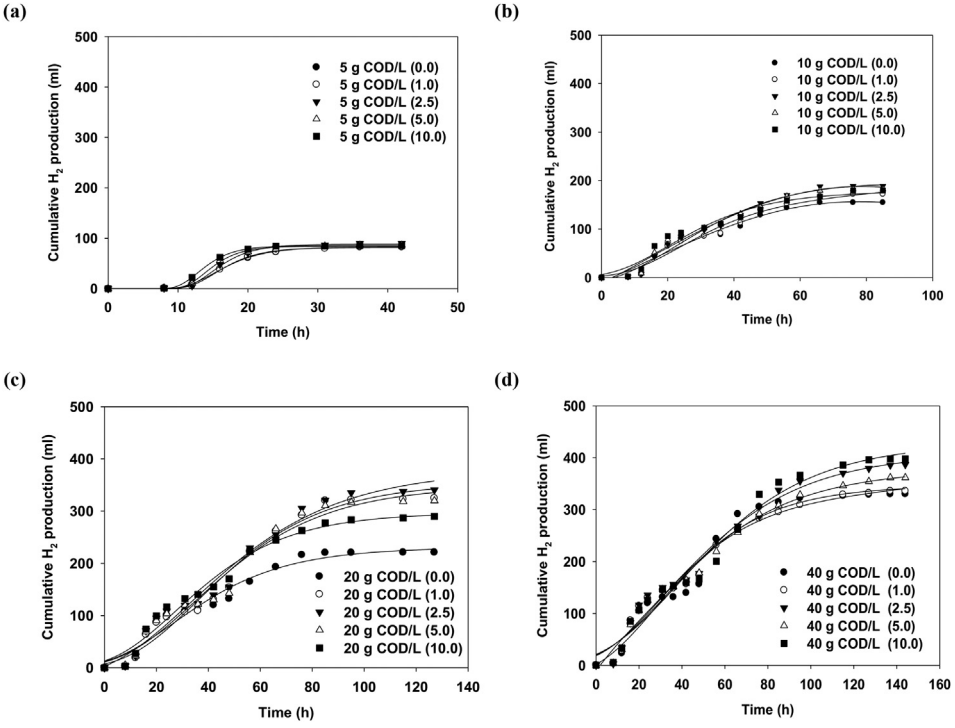

Fig. 1 – Cumulative H_2 production on different substrate concentration (glucose) with different ZVI addition, (a) 5 g COD/L, (b) 10 g COD/L, (c) 20 g COD/L, and (d) 40 g COD/L.

program, the threshold E-value to include a sequence in the next iteration was 0.001. A distance matrix was calculated from the aligned sequences, and operational taxonomical units (OTUs; 95–100% sequence similarity) were assigned using the furthest-neighbor clustering algorithm.

Results and discussion

Hydrogen production

Fig. 1 illustrates the cumulative H_2 production at various glucose and ZVI concentrations. It seemed that the effect of ZVI addition on H_2 production was affected by substrate concentration. At 5 g COD/L, the total amount of H_2 production reached around 90 mL at all concentrations of ZVI, while it widely ranged 200–350 mL at 20 g COD/L. Also, there seemed to be a substrate inhibition, based on the observation that the amount of H_2 production was not proportionally increased with substrate concentration increase. In terms of H_2 yield, obtained by using the modified Gompertz model [23], it was 1.42, 1.33, 0.95, and 0.71 mol H_2/mol glucose at 5.0, 10.0, 20.0, and 40.0 g COD/L without ZVI addition, respectively (Fig. 2). The decreased H_2 yield in this case might be due to change of pH during fermentation, which would be further discussed in section pH variation.

When ZVI was added into batch reactors, H_2 yield was increased to some extent. With an increase of ZVI addition from 0 to 2.5 g/L, the H_2 yield was increased at all substrate concentrations. Compared to control group (no ZVI addition), it was increased up to 54% at substrate concentration of 20 g COD/L. The maximum H_2 yield observed was 1.62 mol H_2/mol glucose at substrate concentration of 10 g COD/L with dosage of ZVI 2.5 g/L. In contrast, H_2 yield was decreased when the dosage of ZVI was higher than 2.5 g/L. For example, the H_2 yield at the substrate concentration of 20 g COD/L with 5.0 g/L and 10 g/L of ZVI addition were 1.37 and 1.27 mol H_2/mol glucose, respectively. These values were higher than the control value, but were not higher as 1.46 mol H_2/mol glucose, which was obtained at 2.5 g/L of ZVI addition. This result clearly showed that addition of ZVI is helpful for enhanced hydrogen fermentation, but excessive ZVI can cause inhibition or toxicity [24]. On the other hand, if the substrate concentration was too high or too low, such as 40 g COD/L or 5 g COD/L, the addition of ZVI did not significantly improve H_2 yield. From this point of view, the optimal substrate concentration and dosage of ZVI were found to be 20.0 g COD/L at 2.5 g ZVI/L.

Hydrogen production rate also showed a similar behavior as H_2 yield. The H_2 production rate without ZVI addition was 7.6, 6.8, 3.5, and 4.9 mL/h at 5, 10, 20 and 40 g COD/L, respectively. This value was significantly increased by addition of ZVI in case of 5–10 g COD/L. The maximum increase of H_2 production rate was observed as 72% at 10 g COD/L with 10 g ZVI/L. However, the increase was not significant with higher concentration of substrate. Only 29% increase of H_2 production rate was observed at 20 g COD/L with 10 g ZVI/L while increment was not observed in case of 40 g COD/L.

Therefore, it is concluded that the addition of adequate amount ZVI caused positive condition to achieve faster H_2 production as well as more production of H_2. This result is along with the previous studies on addition of ZVI into anaerobic fermentation processes [15,25]. The supplementation of ZVI provided a suitable redox environment and resulted in an enhanced H_2 fermentation than the control test. The observed improved H_2 yield obtained at all the tested substrate concentrations might be attributed by the enhanced activity of the ZVI particles, which triggered the efficient electron transfer and resulted in better activity of the hydrogenase enzyme and ferredoxin electron transfer shuttle [26,27]. To further clarify the reason for better hydrogen production, we have investigated pH variation, organic acid production as well as microbial analysis.

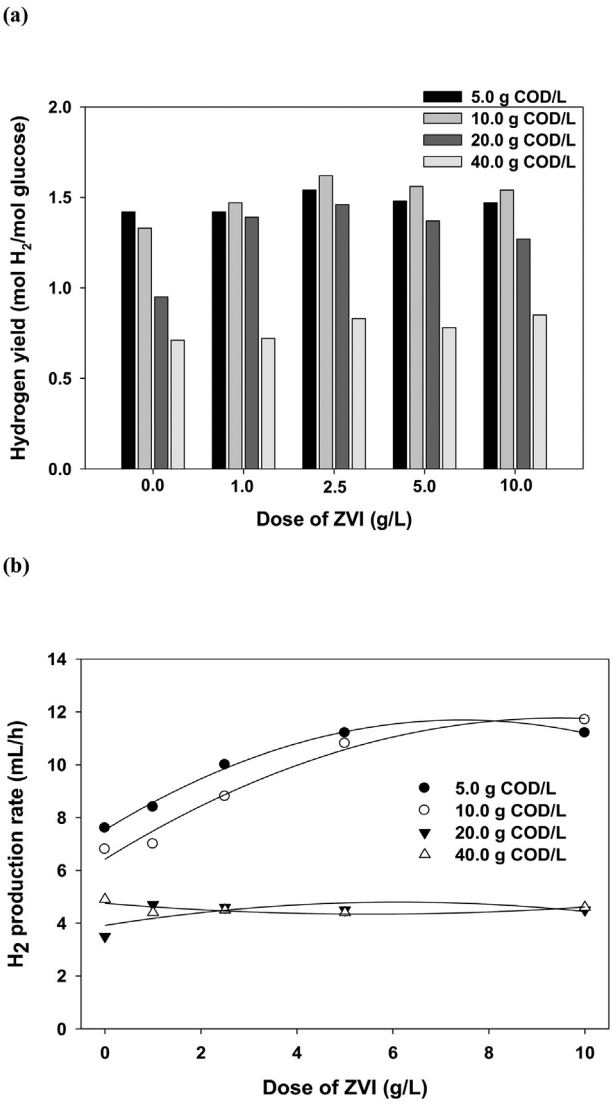

Fig. 2 – (a) H_2 yield and (b) H_2 production rate with various dosages of ZVI and substrate concentrations.

pH variation

pH is the crucial factor governing the hydrogen fermentation performance, as it is known to influence enzyme activity,

population dynamics, and product inhibition [26]. As shown in Fig. 3, the final pH value with ZVI addition was slightly higher than the control conditions (no ZVI addition) in all the tested substrate concentration ranges. The impact of ZVI concentrations (1–10 g/L) at low substrate concentration of 5 g COD/L had a slight variation in the final pH value at the end of the fermentation and lied in the range of 4.53–4.61. The control experiment showed a final pH value of 4.53 at 5 g COD/L. Whereas the other substrate concentrations of 10, 20 and 40 g COD/L resulted in the final pH values of 4.25, 3.84, and 3.76, respectively. At higher substrate concentrations (10–40 g COD/L) ZVI addition had significant effects on maintaining the final pH better than the control experiments. At substrate concentration of 40 g COD/L, the final pH value was maintained more than 4.17 and showed a significant improvement on the hydrogen yield from 0.55 (control) to 0.75 mol H_2/mol hexose with 10 g/L ZVI supplementation.

The hydrogen fermentation reaction is accompanied by the initial drop in the pH value followed by the production of organic acids. The hydrogen formation reactions have been effectively progressed at a pH value closer to the value of pH 4.0, below this range the pH had a negative influence on the hydrogen fermentation as well as the microbial growth. There has been some strategies like supplementation of the alkali reagents in continuous or sequential mode to maintain the appropriate pH range suitable for hydrogen fermentation preferably pH over 4.0, the addition of ZVI particles provides an alternative option for maintaining the pH in the desirable range for the hydrogen fermentation, without adding any external chemical agents [29]. The outcomes showed that the addition of ZVI provides a suitable buffering environment favoured for the growth of hydrogen producers and resulted in improved hydrogen production performance.

The soluble iron concentration in the supernatant could be another evidence for buffering effect of ZVI. ZVI, elemental iron, can be readily oxidized in acidic condition and it produce soluble ferrous ion as its oxidation product in aerobic condition (Eq. 1). Hydroxyl ion is also produced as reaction by-product, which lead to pH increase. In unbuffered condition, pH can easily increase up to 10–11 as shown in previous reports [30]. However, pH was quite stable in the range of 3.7–4.6, which is acidic condition. This is because of production of organic acid during fermentation process. The low pH can promote iron dissolution, therefore, the buffering effect caused by organic acid production is one of the reason for better H_2 production. The higher Fe^{2+} generation under higher organic loading can be a good evidence on this buffering effect as shown in Fig. 4. The higher soluble iron concentration directly indicates more hydroxyl iron production, which can act as a buffering agent.

Organic acids production

The organic acids production profile was monitored at the optimal substrate concentration of 10 g/L to assess the effect of ZVI addition on the distribution of the organic acids, and the results are shown in Fig. 5. As it could be seen from Fig. 5, the organic acid profile varied significantly when compared with the control experiment. The total organic acids production of 5.67 g COD/L was attained with control experiments,

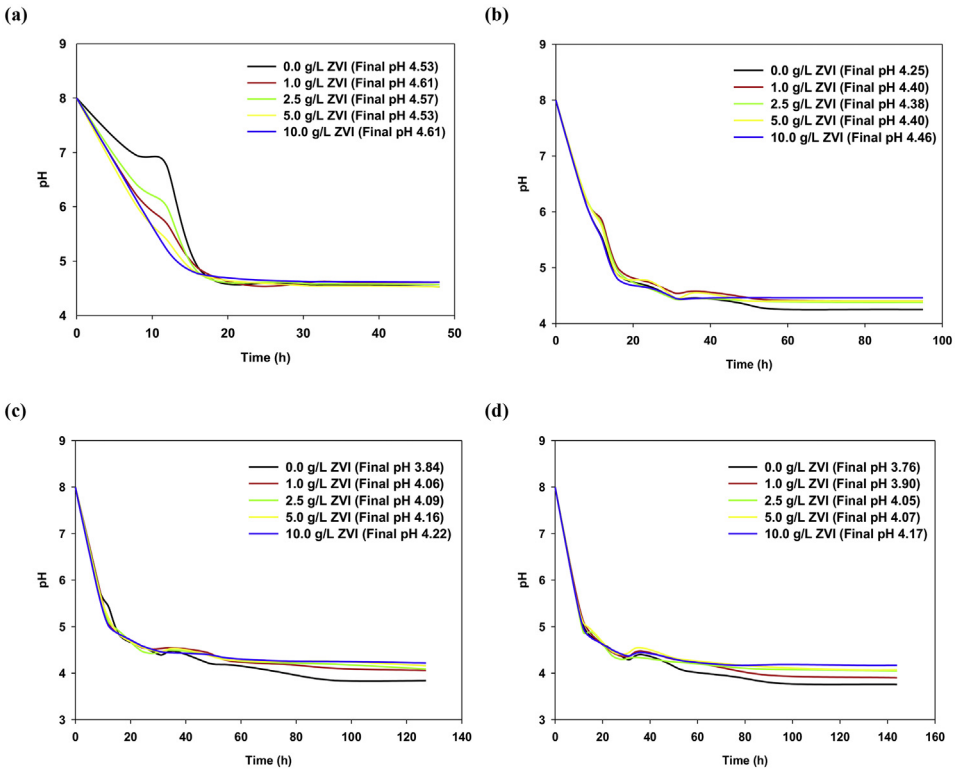

Fig. 3 – Effect of ZVI addtion on pH values during hydrogen fermentation at various glucose concentrations, (a) 5 g COD/L, (b) 10 g COD/L, (c) 20 g COD/L, and (d) 40 g COD/L.

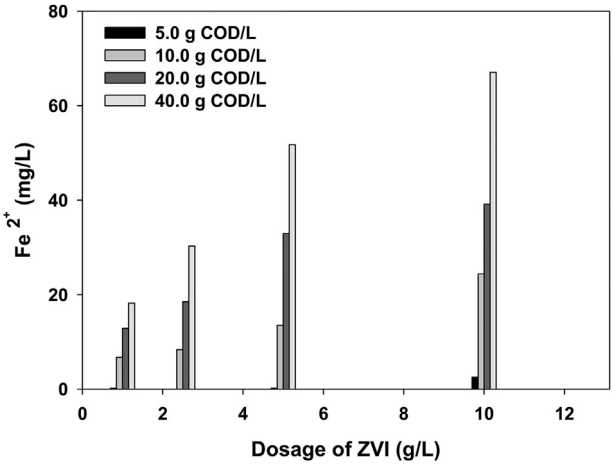

Fig. 4 – Effect of ZVI addtion on soluble iron concentration at various glucose concentrations.

whereas the addition of ZVI 10 g/L resulted in a drastic improvement in the organic acids production with a total value of 9.44 g COD/L.

Acetate and butyrate were the dominant soluble metabolic products formed during the fermentation account for 80% of the total organic acids at control test and 65% with ZVI addition. The butyrate concentration was observed as 3.35 and 3.16 g COD/L for the control and ZVI added batch. The acetate accumulation was greatly improved by the presence of ZVI with a high value of 2.41 g COD/L, the control experiments provided a value of 1.13 g COD/L, respectively. This showed that the addition of ZVI improved the hydrogen fermentation by altering the metabolic pathway favoured for the production of acetate and butyrate. This result is along with previous report about positive effect of ZVI on methane production by shifting organic acids composition to more acetate and butyrate [15].

The lactate concentration was remained lower than 0.14 g COD/L and maintained less than 3% in both conditions. The propionate distribution was maintained over 18% at control and 33% with the ZVI addition conditions. In general, the production of acetate and butyrate favoured the H_2 production, the accumulation of lactate and propionate negatively affected the hydrogen fermentation, as the lactate and propionate involved in the hydrogen consumption pathway [31,32].

The distribution of organic acids by the anaerobic microbial communities is a spontaneous reaction and is limited by biological regulations and interspecies interaction within the microbial community. Hence, monitoring of microbial communities is essential for understanding the key role of microbial consortia and developing a suitable appropriate biocontrol strategy to retain the population growth and stable hydrogen production and the summary of the microbial analysis are discussed below.

Microbial analysis

The microbial community dynamics was performed using 454 next generation sequencing method to identify the role of ZVI

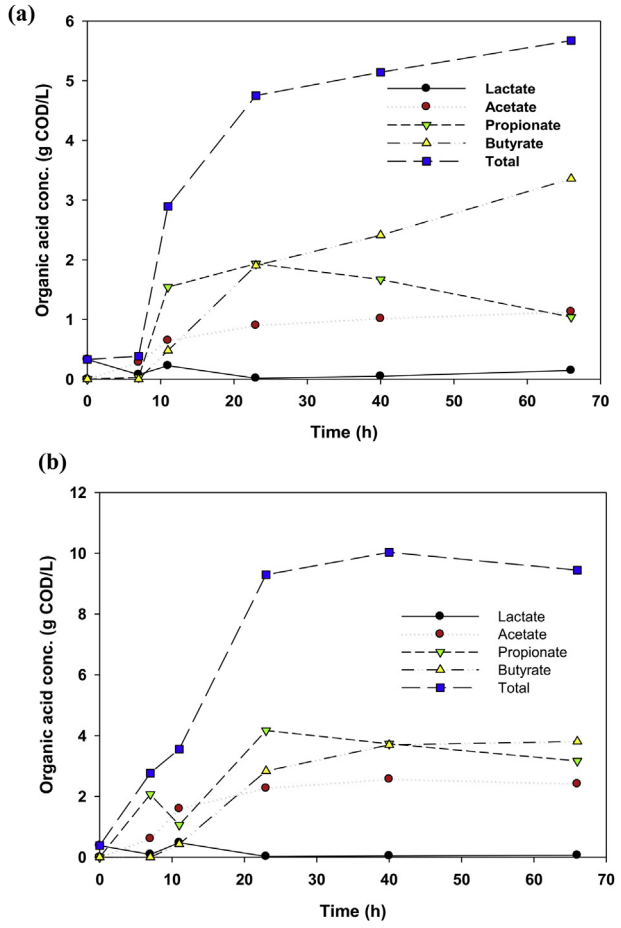

Fig. 5 – Organic acid profile at substrate concenration of 10 g/L, (a) Control (no ZVI), and (b) ZVI 10 g/L.

supplementation on the changes in species level abundance of H_2 producing microbial consortia. To better understand the role of the dominant bacterial populations involved in the hydrogen fermentation of ZVI added, the samples obtained at the end of the fermentation from the control and ZVI added samples were used for the analysis and the results are summarized in Fig. 6.

As seen from the Fig. 6, the microbial community structure varied greatly between the control and the ZVI added inoculum. The Genus *Clostridium* and *Lactobaciillus* was the major abundance bacterial community. The control experiment had a high proportion of the *Clostridium* with a relative abundance of over 68.3%, whereas the ZVI added inoculum showed an abundance percentage of 64.2%. The *Lactobacillus* community showed a different pattern with a low proportion of 26.8% obtained from control and 31.3% obtained from the ZVI added inoculum.

At the species level, the *Clostridium chromiireducens* and *Clostridium acidisoli* ratio was reduced from 19.9% to 12.5% and 20.9%–8.2% at control and ZVI added culture. The *Clostridium perfringens*, *Paraclostridium benzoelyticum*, and *Lactobacillus mucosae* populations increased when adding the ZVI from 0.2 to 1.5%, 27%–41%, and 21.4% to 31.3% than the control

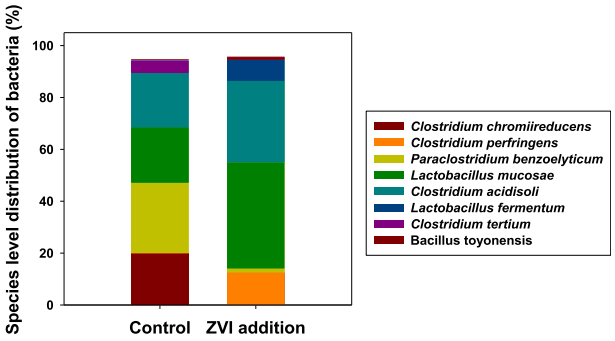

Fig. 6 – Microbial community analysis (Left: control, right: ZVI 10 g/L).

populations. The observed changes in the microbial community pattern reflects the changes in the metabolic products distribution, pH changes and overall H_2 production efficiency. When the culture was exposed to ZVI, it promotes the enrichment of dominant acid producers and it has been also noted from the organic acids analysis where the peak organic acid production of 9.44 g COD/L was observed.

Although the increased production of propionate about 3.16 g COD/L was observed during the ZVI added culture, the hydrogen fermentation was not affected with an increase in the acetate and butyrate concentration of 2.41 g COD/L and 3.80 g COD/L. These discrepancy in results was attributed by the changes in the microbial community structure pattern. The *Lactobacillus mucosae* had been earlier reported as a potential bacteria to improve the biogas and volatile fatty acids production from dried brewers grain [33]. However the role of *Lactobacillus mucosae* bacteria on the improvement of hydrogen production has not been reported earlier. The enrichment of *Lactobacillus mucosae* after the ZVI addition coincided with the increased production of propionate, acetate and butyrate and resulted in improved H_2 production performances.

The *Paraclostridium benzoelyticum* populations also enriched greatly after the ZVI addition. The *Clostridium chromiireducens* abundance was reduced from 19.9 to 12.5% after the ZVI supplementation showed that the reductive nature of the ZVI affected the population dynamics within the *Clostridium* sp. The strain *Clostridium chromiireducens* was reported to be involved in mixed acid fermentation pathway from glucose with acetate, butyrate, formate and lactate as the major organic acids accumulation during fermentation [34].

The *Clostridium acidisoli* culture was dominant at control batch experiments with a abundance over 20.8%, whereas with ZVI supplemented culture the abundance was reduced to 8.2% showed that the acid tolerant nature of the *Clostridium acidisoli* was involved in the major hydrogen fermentation reaction from control batch culture. *Clostridium acidisoli* can able to grown in a wide range of pH from 3.7 to 6.9 and produce H_2, CO_2 reactions with the formation of butyrate, lactate, acetate, and formate [35].

These results indicated that most members belonging to the *Clostridium* sp. were strict anaerobes and it requires a suitable redox environment to thrive their metabolic reactions, changes in the initial growth conditions might affect the cell growth and hydrogen fermentation ability. The addition of ZVI accelerates the hydrogen fermentation reaction by enriching the selective bacterial populations and facilitated the active metabolism favoured for hydrogen production from glucose substrate.

Conclusion

The effect of ZVI addition on hydrogen fermentation was affected by substrate concentration and its dose. Compared to control group (no ZVI addition), H_2 yield was increased up to 54% by addition of ZVI. The maximum H_2 yield observed was 1.62 mol H_2/mol glucose at substrate concentration of 10 g COD/L with dosage of 2.5 g ZVI/L. The results indicated that ZVI creates a more favorable environment for anaerobic microbial acidogenesis with providing buffering effect to prevent pH drop by hydroxyl ions as it dissolved. Also, the composition of organic acid was changed to acetate and butyrate. Minor changes in the bacterial (*Clostridium* and *Lactobacillus*) populations within the functional consortium after the ZVI addition was also identified.

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