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Free ammonia enhances dark fermentative hydrogen production from waste activated sludge

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ABSTRACT

Ammonium and/or free ammonia (the unionized form of ammonium) are generally thought to inhibit the activities of microbes involved in anaerobic digestion of waste activated sludge. It was found in this work, however, that the presence of ammonium (NH_4^+-N) largely enhanced dark fermentative hydrogen production from alkaline pretreated-sludge. With the increase of initial NH_4^+-N level from 36 to 266 mg/L, the maximal hydrogen production from alkaline (pH 9.5) pretreated-sludge increased from 7.3 to 15.6 mL per gram volatile suspended solids (VSS) under the standard condition. Further increase of NH_4^+-N to 308 mg/L caused a slight decrease of hydrogen yield (15.0 mL/g VSS). Experimental results demonstrated that free ammonia instead of NH_4^+-N was the true contributor to the enhancement of hydrogen production. It was found that the presence of free ammonia facilitated the releases of both extracellular and intracellular constituents, which thereby provided more substrates for subsequent hydrogen production. The free ammonia at the tested levels (i.e., 0–444 mg/L) did not affect acetogenesis significantly. Although free ammonia inhibited all other bio-processes, its inhibition to the hydrogen consumption processes (i.e., homoacetogenesis, methanogenesis, and sulfate-reducing process) was much severer than that to the hydrolysis and acidogenesis processes. Further investigations with enzyme analyses showed that free ammonia posed slight impacts on protease, butyrate kinase, acetate kinase, CoA-transferase, and [FeFe] hydrogenase activities but largely suppressed the activities of coenzyme F420, carbon monoxide dehydrogenase, and adenylyl sulfate reductase, which were consistent with the chemical analyses performed above.

1. Introduction

Hydrogen is widely considered the most promising alternative to fossil fuels, as it has a high energy yield (142.35 kJ/g) with at least 2.75 times that of any hydrocarbons and produces water instead of greenhouse gases when it is combusted (Cai et al., 2004). As the major byproduct of biological wastewater treatment, waste activated sludge is produced in huge quantities (Feng et al., 2015; Li et al., 2016; Wang et al., 2017a, 2017b). Treatment and disposal of the sludge are costly, accounting for up to 60% of the total operation costs of wastewater treatment plants (WWTPs) (Zhao et al., 2017; Wang et al., 2017c). On the other hand, sludge contains high levels of organic constituents such as protein and carbohydrate (Zhao et al., 2016; Xu et al., 2017; Xie et al., 2016; Chen et al., 2018; Wang et al., 2018), which can be used as substrates for hydrogen production. Therefore, biological production of hydrogen from waste activated sludge has recently attracted much attention (Zhao et al., 2010; Gioannis et al., 2013; Li et al., 2009), by which sludge is reduced and reused, fossil fuels are saved, and greenhouse gas productions are reduced.

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The hydrogen yield of dark fermentation of waste activated sludge is usually low due to rapid hydrogen consumption in comparison to sludge disintegration. Most previous investigations to enhance hydrogen production, therefore, focused on optimizing sludge-pretreatment methods (Yang et al., 2012; Assawamongkholsiri et al., 2013; Kim et al., 2013), operational conditions (Zhao et al., 2010; Zhou et al., 2013; Jung et al., 2011), or sludge composition (Kim et al., 2012; Chen et al., 2012a; Wang et al., 2015). For instance, Cai et al. (2004) found that sludge pretreated by alkaline (pH 11) for 24 h could increase hydrogen yield from 9.1 to 16.6 mL of H_2/g of dry solids. The bioconversion of sludge proteins and hydrogen production were found to be largely enhanced by co-fermentation of sewage sludge and carbohydrate-rich substrates, such as food wastes and agricultural wastes (Kim et al., 2012; Chen et al., 2012b; Liu et al., 2013). By elevating the content of sludge polyhydroxyalkanoates from 25 to 178 mg/g volatile suspended solids (VSS), hydrogen production from alkaline anaerobic fermentation of sludge increased from 26.5 to 58.7 mL/g VSS (Wang et al., 2015). Apart from these parameters, byproducts that are *in situ* generated in the anaerobic fermentation process may also affect hydrogen yield. To date, however, little information is available to this field.

NH_4^+-N, which is produced from the disintegration of nitrogen-rich compounds such as proteins, urea, and nucleic acids, could be accumulated at high concentrations in the sludge digestion or fermentation process (Yan et al., 2010; González-Fernández et al., 2009). The NH_4^+-N concentration in the sludge fermentation liquid is usually at ~300 mg/L (Chen et al., 2007; Zhao et al., 2015), and this value could be up to 1500 mg/L in the sludge digestion liquid (Wang et al., 2014). Almost all previous studies demonstrated that the presence of NH_4^+-N especially at high levels showed inhibitory effects on bio-gas production from anaerobic digestion. For example, Sung and Liu (2003) found that compared with the control (0.4 g/L), 5.0 and 5.8 g/L of NH_4^+-N resulted in 39% and 64% decreases in methane production, respectively. Nakakubo et al. (2008) reported that a 50% reduction in methane yield under thermophilic anaerobic digestion was observed at NH_4^+-N concentration of 11.0 g/L, as compared with 4.6 g/L NH_4^+-N. These findings suggested that it was essential to remove or reduce NH_4^+-N levels in the anaerobic digestion process to ensure bio-gas production.

Nevertheless, it was found in the current work that when 63–272 mg/L NH_4^+-N was added into the sludge mixture, hydrogen yield from alkaline pretreated-sludge was enhanced rather than reduced, as compared to the case without NH_4^+-N addition. There were two varying parameters in these fermentation systems. One is the NH_4^+-N added. As pH variation is similar among these fermentation systems, thus the other varying parameter is free ammonia (FA), the unionized form of ammonium, due to the high concentrations of ammonium added and alkaline condition maintained. FA can diffuse through cell membrane, shuttle protons between the two sides, and result in cell inactivation (Kayhanian, 1999). This could cause variations in the activities of function microbes involved in sludge anaerobic fermentation, which thereby may affect hydrogen production. However, the role of FA on hydrogen production from dark fermentation of sludge has not been clarified so far. It is unknown which one (NH_4^+-N or FA) is the main contributor to the enhanced hydrogen production.

The aim of this study was to identify whether and how FA enhances dark fermentative hydrogen production. First, hydrogen production from sludge with alkaline pretreatment (pH 9.5) in the presence of NH_4^+-N at different levels (36–308 mg/L) was compared. To identify the potential contribution of NH_4^+-N to hydrogen production, hydrogen yield from acidic fermentation of sludge (pH 5.5) was also compared at the same NH_4^+-N levels. It should be emphasized that the variations of FA level among these acidic fermenters were negligible, thus the results obtained could be used to indicate the impact of NH_4^+-N on hydrogen production. Finally, the mechanisms for FA enhancing hydrogen production were explored. The findings reported in this work reveal the details of how FA enhances hydrogen production for the first time, erase the concern regarding the inhibitory effect of NH_4^+-N or FA produced in the fermentation process on dark fermentation, and may guide engineers to develop more economic strategies for hydrogen production from sludge fermentation.

2. Materials and methods

2.1. The sources and characteristics of waste activated sludge

The sludge employed in this work was withdrawn from the secondary sedimentation tank of a municipal WWTP in Changsha, China. The raw sludge was filtrated by a stainless steel mesh (2.0 mm) and concentrated by setting at 4 °C for 24 h before use. The major characteristics of the concentrated sludge are as follows: pH 6.8 ± 0.1, total suspended soils (TSS) 14920 ± 260 mg/L, VSS 12140 ± 170 mg/L, total chemical oxygen demand (COD) 14780 ± 290 mg/L, total carbohydrate 1650 ± 230 mg COD/L, total protein 7780 ± 310 mg COD/L, lipid and oil 170 ± 20 mg COD/L, and NH_4^+-N 36 ± 4 mg/L. It can be seen that protein and carbohydrate are the top two organics in the sludge, accounting for about 64% of total sludge COD.

2.2. Hydrogen production from sludge with alkaline pretreatment in the presence of NH_4^+-N at different levels

This batch test was conducted in eight serum bottles with a working volume of 1 L each. Each serum bottle was first fed with 500 mL concentrated sludge, as mentioned above. Then different volumes of NH_4Cl stock solution (4.0 M) were added at the beginning of the test, which resulted in the initial NH_4^+-N concentration of 36, 36, 99, 141, 182, 224, 266, or 308 mg/L. It should be noted that NH_4^+-N was not added to the first two bottles, and 36 mg/L NH_4^+-N was the background ammonium concentration. Except one serum bottle with NH_4^+-N concentration at 36 mg/L (set as the blank), the tested sludge in all other serum bottles was pretreated under alkaline condition (pH 9.5) at 35 °C for 24 h before fermentation, as alkaline pretreatment was demonstrated to be an effective method for enhancing biohydrogen production from waste activated sludge (Cai et al., 2004).

The NH_4^+-N concentration, temperature, and pH applied gave rise to initial FA concentrations of 0.3 (the blank), 34, 95, 135, 174, 214, 254, or 294 mg/L. FA concentration was determined by the formula $S_{(NH3\text{-}N+NH4\text{-}N)} \times 10^{pH}/(K_b/K_w+10^{pH})$, where $S_{(NH3\text{-}N+NH4\text{-}N)}$ represents the concentration of NH_3-N + NH_4^+-N, K_b represents the ionization constant of the ammonia equilibrium equation, and K_w represents the ionization constant of water (Anthonisen et al., 1976). The value of K_b/K_w was calculated via the formula of $K_b/K_w = e^{6,344/(273+T)}$ (Anthonisen et al., 1976). After pretreatment, the serum bottles were sparged with nitrogen gas for 5 min to ensure anaerobic condition. Finally, all serum bottles were capped with rubber stoppers, sealed, and placed in an air-bath shaker (150 rpm). No extra inoculum was added into these reactors, and hence the sludge was used as both fermentation substrate and inoculum. In the fermentation process, the pH value in all bottles was not controlled but was recorded periodically. The total gas volume was measured by releasing the pressure in the bottle using a glass syringe (300 mL) to equilibrate with the atmospheric pressure (Oh et al., 2003). The cumulative volume of hydrogen gas was calculated as:

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

Where, $V_{H,i}$ and $V_{H,i-1}$ are respectively the cumulative volumes of hydrogen gas in the current (i) and previous (i-1) time intervals, $V_{G,i}$ and $V_{G,i-1}$ are respectively the total gas volumes in the current and previous time intervals, and $C_{H,i}$ and $C_{H,i-1}$ are the fractions of hydrogen gas measured by gas chromatography in the current and previous time intervals, respectively.

2.3. Hydrogen production from acidic fermentation of sludge in the presence of NH_4^+-N at different levels

This set of batch experiments was utilized to assess the impact of NH_4^+-N on hydrogen production. To minimize the impact of FA, acidic fermentation was employed. Eight serum bottles with a working volume of 1 L each were operated in this experiment. The experimental procedure was identical to that described above except that acidic condition (pH 5.5) was employed to both sludge pretreatment and dark fermentation. FA concentration in these reactors varied only between 0.02 and 0.36 mg/L, thus the impact of FA on hydrogen yield in this set of experiments is expected to be negligible.

2.4. Assessing the impacts of FA on hydrolysis, acidogenesis, acetogenesis, homoacetogenesis, methonogenesis, and sulfate-reducing processes

Sludge dark fermentation generally contains several processes. The rate of sludge disintegration, the major rate-limiting process in sludge dark fermentation, is often assessed by measuring the concentrations of soluble COD and soluble proteins released in fermentation liquor (Zhao et al., 2017; Xu et al., 2017; Li et al., 2009). Besides, several other bio-reactions such as hydrolysis, acidogenesis, acetogenesis, homoacetogenesis, methonogenesis, and sulfate-reducing processes are also involved in sludge dark fermentation, which affect hydrogen production significantly. The rates of these processes are generally evaluated by batch tests using model substrates in the literature (Zhao et al., 2010, 2016; Li et al., 2009). In this set of tests, 20 replicate serum bottles with a working volume of 1.0 L each were performed. These bottles were divided into five groups (namely, Dextran Test, Glucose Test, Butyrate Test, H_2-CO_2 Test, and H_2-SO_4^{2-} Test) with four in each group.

Dextran Test: All bottles received 450 mL of synthetic wastewater and 50 mL of same inoculum. Inoculum used in this test was withdrawn from an anaerobic sludge fermenter in our laboratory. The synthetic wastewater contains 0.5 g dextran (average molecular weight 23800, a model polysaccharide compound)/L. pH in all the bottles was controlled at 9.0 ± 0.1, which was close to the average pH value in the fermenters operated in the "Hydrogen Production from Sludge with Alkaline Pretreatment in the Presence of NH_4^+-N at Different Levels" Section. Different volumes of NH_4Cl stock solution (4.0 M) were added into the four serum bottles, resulting in an initial concentration of NH_4^+-N at 0, 540, 648, or 678 mg/L. All other fermentation conditions were the same as those described above. The NH_4^+-N, temperature, and pH applied gave rise to FA concentrations at 0, 354, 424, or 444 mg/L. The FA concentrations used in this test were respectively equal to the average FA concentrations measured from the fermenters fed with 141, 266, and 308 mg/L of initial NH_4^+-N treated sludges. By analyzing the degradation efficiency of dextran, the effect of FA on hydrolysis process could be evaluated.

Glucose Test: The operation of this test was operated with the same approach described in Dextran Test except that the fermentation substrate (i.e., dextran) was replaced with glucose. By determining the glucose degradation, the effect of FA on acidogenesis could be assessed.

Butyrate Test: The operation of this experiment was carried out the same as that depicted in Dextran Test except that the fermentation substrate was replaced with 10 g/L sodium butyrate. The effect of FA on acetogenesis could be indicated by analyzing the degradation efficiency of sodium butyrate.

H_2-CO_2 Test: Each serum bottle first received 50 mL of fermented sludge mixture as inoculums and 450 mL tap water. The pH and NH_4^+-N levels in the four serum bottles were the same as those operated in Dextran Test. All these bottles were then flushed with a combined gas (40% hydrogen, 10% carbon dioxide, and 50% nitrogen) for 5 min to guarantee that they were filled with the synthetic hydrogen-containing gas. Finally, all bottles were capped with rubber stoppers, sealed, and placed in an air-bath shaker at stirring speed of 150 rpm. The effect of FA on homoacetogenesis and methonogenesis could be assessed by comparing the consumption of hydrogen and the productions of acetic acid and methane among these fermenters.

H_2-SO_4^{2-} Test: Each bottle received 450 mL of synthetic wastewater and 50 mL of fermented sludge mixture. The synthetic wastewater contains 1.18 g potassium sulfate/L. Each bottle was flushed with a combined gas (45% hydrogen and 50% nitrogen) for 5 min to ensure that they were filled with the synthetic hydrogen-containing gas. All other operations were the same as those depicted in H_2-CO_2 Test. The effect of FA on sulfate-reducing process could be indicated by comparing the hydrogen consumption and sulfate degradation among these fermenters.

2.5. Long-term semi-continuous reactor operation for the analysis of key enzymes

Two long-term semi-continuous reactors were operated to further explore the impact of different FA levels on sludge anaerobic fermentation from the aspect of microbial analyses. The two reactors were replicate with a working volume of 1 L each and fed with alkaline (pH 9.5) pretreated sludge with either 36 or 266 mg/L initial NH_4^+-N concentration. In this experiment, two typical sludge with either 36 or 266 mg/L initial NH_4^+-N concentration was tested, as 36 mg/L NH_4^+-N was the background ammonium concentration and maximum hydrogen was achieved at 266 mg/L NH_4^+-N according to the batch test above. The fermentation conditions were the same as described in Section 2.2. According to the results obtained from the batch tests, the sludge retention time in the reactors was maintained at 7 d. Thus, ~70 mL of fermentation mixture was withdrawn daily from each reactor and replaced with the same amount of new alkaline-pretreated sludge. Afterwards, both the reactors were sparged with nitrogen gas for 5 min to remove oxygen before they were re-capped and re-sealed. Hydrogen yield reached a stable level after approximately 50 d operation, and then the assay of key enzyme activities was performed.

2.6. Analytical methods

Hydrogen and methane fractions in the generated gas were determined at normal pressure via a gas chromatograph (GC112A, China) equipped with a thermal conductivity detector, a 2-m stainless column (activated carbon, 60–80 mesh) and with nitrogen as the carrier gas. The temperatures of the injection, column, and detector were set at 70, 140, and 140 °C, respectively. The measurements of COD, NH_4^+-N, VSS, and TSS were performed in accordance with standard methods (APHA, 1998). Proteins, carbohydrates, lipids, and short-chain fatty acids were measured as previously described (Wang et al., 2015). A heat extraction method was used to extract different fractions of extracellular polymeric substances (EPS) from sludge (Xu et al., 2017). Excitation emission

matrix (EEM) fluorescence spectroscopy ((Fluoromax-4 Spectrofluorometer, HORIBA Scientific, France) with a 450 W Xe arc lamp) was applied to characterize the changes of fermentation liquid (Luo et al., 2013). Sulfide concentration was determined according to the method depicted in the literature (Aiking et al., 1982). Cell membrane integrity was indicated by the lactate dehydrogenase (LDH) release assay using a LDH-Cytotoxicity Assay Kit (BioVision, USA) according to the manufacturer's instructions and the procedure documented in our previous study (Chen et al., 2012b). The measurement of DNA released from sludge cells was conducted according to a previously established protocol (Liang et al., 2015).

For measuring the activities of key enzymes, 25 mL the fermentation mixture was collected form the long-term reactors, cleaned using 100 mM sodium phosphate buffer (pH = 7.4), sonicated at 20kHa at 4 °C for 10 min, and finally centrifuged at 12000 rpm at 4 °C for 15 min to remove the waste debris. The extracts were kept on ice before analyzing. The activities of the following eight key enzymes, i.e., protease, acetate kinase, butyrate kinase, CoA-transferase, carbon monoxide dehydrogenase, adenosine-5′-phosphosulfate reductase, coenzyme F_{420}, and [FeFe] hydrogenase were measured in this study, with the procedures detailed in Supporting Information.

2.7. Statistical analysis

All tests were conducted in triplicate. An analysis of variance was used to evaluate the significance of results, and $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Hydrogen production from sludge with alkaline pretreatment in the presence of NH_4^+-N at different levels

Fig. 1 shows the cumulative hydrogen production from dark fermentation of sludge with alkaline pretreatment (pH 9.5) at different NH_4^+-N levels. Hydrogen yield from the blank (without pH and NH_4^+-N adjustments) was low (<2 mL/g VSS). Alkaline pretreatment of sludge effectively enhanced hydrogen production, consistent with previous results (Cai et al., 2004). In the initial 168 h, hydrogen production from alkaline pretreated-sludge without addition of NH_4^+-N (i.e., 36 mg/L NH_4^+-N sludge) increased gradually, and no further significant increase was found after that time ($p > 0.05$, see Table S1 for statistical analysis). The maximal hydrogen production and optimal fermentation time were 7.3 ± 0.3 mL/g VSS and 168 h for alkaline pretreated-sludge without NH_4^+ addition. Similar trend of hydrogen production was also observed for other alkaline pretreated-sludge.

Several previous publications reported that NH_4^+-N had inhibitory effects on biogas production from anaerobic digestion of sludge. However, Fig. 1 shows that hydrogen production from alkaline pretreated-sludge was not inhibited but promoted by the increase of NH_4^+-N. With the increase of initial NH_4^+-N content from 36 to 266 mg/L, the maximal hydrogen yield increased from 7.3 ± 0.3 to 15.6 ± 0.4 mL/g VSS. Although further increase of initial NH_4^+-N to 308 mg/L caused a slight decrease of hydrogen yield to 15.0 ± 0.3 mL/g VSS, this value was still ~2 folds of that from 36 mg/L NH_4^+-N sludge. It should be noted that this decrease was considered to be statistically insignificant ($p > 0.05$). The presence of NH_4^+-N at pertinent levels clearly enhanced dark fermentative hydrogen production from alkaline pretreated-sludge.

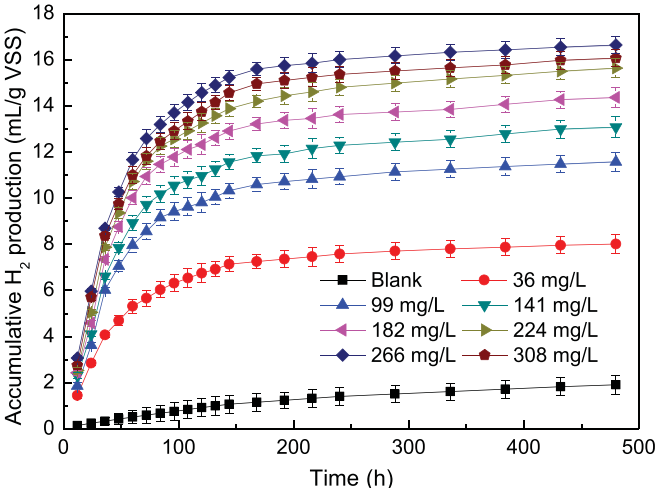


Fig. 1. Hydrogen production from dark fermentation of sludge with alkaline pretreatment in the presence of NH_4^+-N at different levels. Error bars represent standard deviations of triplicate tests.

3.2. Identification of the true contributor to the increased hydrogen production

Two major differences among the above fermenters fed with alkaline pretreated-sludge are (1) the NH_4^+-N level and (2) the unionized form of ammonium, FA level, due to the alkaline pretreatment applied. The initial NH_4^+-N level varied from 36 to 308 mg/L among the fermenters while the initial FA level varied from 34 to 294 mg/L according to the calculation. Both the two parameters correlated well with the hydrogen production from dark fermentation of alkaline pretreated-sludge (Fig. S1). Using food waste as the fermentation substrate, Pan et al. (2013). also reported that the suitable addition of NH_4^+-N (e.g., 1.5–2.5 g/L) could enhance biohydrogen production from anaerobic fermentation. To date, however, the potential impact of FA on hydrogen production has not yet been documented. Thus, the roles of these two potential contributors require further clarification.

To assess the contribution of NH_4^+-N, the variation of FA should be controlled in a negligible range. An acidic fermentation test (pH 5.5) was employed to provide such support. In this situation, FA concentration in the acidic fermentation reactors varied only between 0.02 and 0.36 mg/L, therefore the impact of FA on hydrogen yield in this test could be neglected. Although NH_4^+-N concentration in these acidic fermentation reactors still varied from 36 to 308 mg/L (as in the previous set of tests), hydrogen yield in these reactors was almost the same (Table S2), suggesting that FA rather than NH_4^+-N was the true contributor to the enhancement of hydrogen production.

3.3. How does FA enhance hydrogen production?

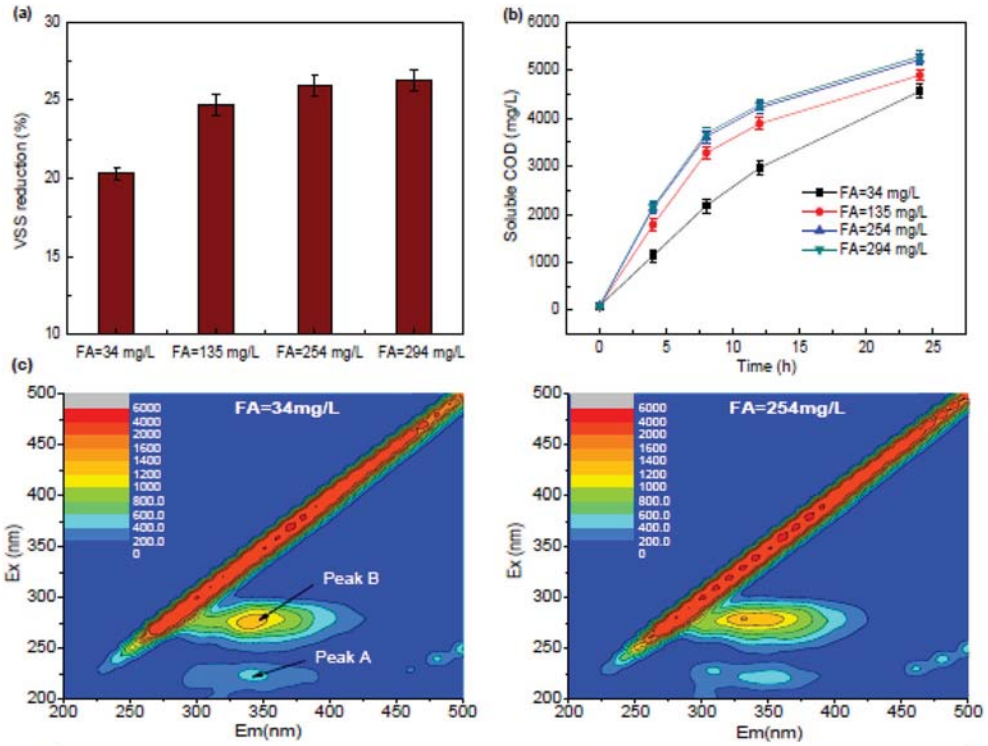
Sludge disintegration is generally considered the major rate-limiting process in anaerobic fermentation or digestion of sludge, thus the potential impact of FA on sludge disintegration was first assessed. The rate of sludge disintegration is generally indicated by VSS reduction and soluble COD release in fermentation liquor (Zhao et al., 2017; Xu et al., 2017; Wang et al., 2013, 2015).

VSS reduction after 1 d alkaline pretreatment increased from $20.3 \pm 0.5\%$ to $25.9 \pm 1.7\%$ when the initial FA concentration increased from 34 to 254 mg/L (Fig. 2a). No significant increase in VSS reduction was observed when initial FA concentration further increased to 294 mg/L ($25.9 \pm 1.7\%$ vs $26.3 \pm 1.9\%$, $p > 0.05$). With

the increase of initial FA from 34 to 254 mg/L, soluble COD after 24 h pretreatment increased from 4560 ± 140 to 5220 ± 110 mg/L, which further slightly increased to 5290 ± 120 mg/L (Fig. 2b) when the initial FA was 294 mg/L. Similar observations were also made for other pretreatment times. These data were further supported by EEM profiles of fermentation liquid (Fig. 2c). The EEM fluorescence spectroscopy is widely employed to characterize the structure changes in either EPS or fermentation liquid (Zhao et al., 2016; Xu et al., 2017; Sheng and Yu, 2006). A red-shift (blue-shift) of emission wavelength is usually relevant to an increase (decrease) of particular functional groups such as carbonyl, hydroxyl, alkoxyl, amino, and carboxyl groups, while an increase (decrease) of fluorescence intensity of characteristic peaks is often related to an increase (decrease) of fluorophores concentration (Zhao et al., 2016; Xu et al., 2017; Sheng and Yu, 2006). As shown in Fig. 2c, two major peaks (signed as Peak A and Peak B in this work) were identified from fluorescence spectra. Peak A and Peak B located at the Ex/Em of 220/340–350 and 270–280/340-350 nm in the spectra, respectively, which was reported to belong to aromatic proteins and tryptophan protein-like substances, respectively (Luo et al., 2013; Sheng and Yu, 2006). Compared with 34 mg/L FA, 254 mg/L FA caused not only red-shifts of emission wavelength (from 340 to 350 nm) but also increases of fluorescence intensity (from 499 to 571 in Peak A and from 1308 to 1460 in Peak B), suggesting that 254 mg/L FA led to more substrates released in the fermentation liquid than 34 mg/L FA. All the results revealed that the increase of FA improved sludge disintegration, resulting in more soluble substrates for subsequent hydrogen production.

Fig. 3 presents the protein and carbohydrate contents extracted from loosely bound EPS and tightly bound EPS of sludge cells at 2 h pretreatment under different FA levels. The measurements of LDH (cell membrane integrity marker) and DNA releases suggested that the increase of FA did not cause extra leakage of intracellular substrates at 2 h pretreatment time (Fig. S2), thus the data shown in Fig. 3 could be utilized to indicate the impact of FA on disruption of loosely bound EPS or tightly bound EPS. It can be seen that the increase of FA resulted in less substrates remained in both loosely and tightly bound EPS. For example, 49.2 and 46.1 mg/g VSS proteins were respectively measured in loosely bound EPS and tightly bound EPS from the sludge cells exposed to 34 mg/L FA, whereas the corresponding values were 42.5 and 37.0 mg/g VSS in loosely bound EPS and tightly bound EPS from the sludge cells exposed to 254 mg/L FA, respectively.

Fig. 4 shows the variations in LDH and DNA releases after 24 h pretreatment under different FA levels. It is known that LDH is a cell membrane integrity marker while DNA is only located in the cytoplasm of prokaryotes (i.e., bacteria and archaea). Their releases can therefore indicate the degree of the leakage of intracellular substrates. In Fig. 4, the releases of both LDH and DNA increased with the increase of FA level. Compared with 34 mg/L FA, 254 mg/L FA enhanced LDH release by 13.5 ± 3.6% and DNA release by 10.3 ± 3.6%. The above results suggested that the increase of FA



FA (mg/L)	Peak A		Peak B	
	Ex/Em (nm)	Intensity (a.u.)	Ex/Em (nm)	Intensity (a.u.)
34	220/340	499.0	270/340	1308
254	220/350	571.4	280/350	1460

Fig. 2. Effect of FA level on sludge disintegration. (a) The VSS reduction rate after 24 h alkaline pretreatment, (b) the release of soluble COD after 24 h alkaline pretreatment, and (c) EEM profiles of liquid after 24 h alkaline pretreatment. Error bars represent standard deviations of triplicate tests.

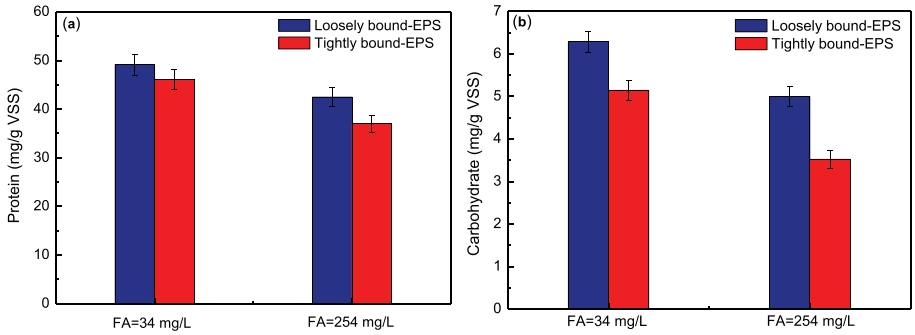


Fig. 3. The protein and carbohydrate contents extracted from loosely bound EPS and tightly bound EPS of sludge cells at 2 h pretreatment time under different FA levels. Error bars represent standard deviations of triplicate tests.

accelerated the disruption of EPS and the leakage of intracellular substrates, thereby benefiting sludge disintegration. In the literature, Wei et al. (2017) also demonstrated that the sludge biodegradability was enhanced after FA treatment.

After sludge disintegration, several biological processes involved in the anaerobic fermentation are relevant to the accumulation of hydrogen (Fig. 5A). The solubilized substrates with large molecule weight need to be hydrolyzed in the hydrolysis process before their use for hydrogen production. Hydrogen is directly produced in the acidogenesis and acetogenesis processes. However, hydrogen can serve as substrate for the production of acetate, methane, and hydrogen sulfide in the homoacetogenesis, methanogenesis, and sulfate-reducing process, respectively. All these processes are highly relevant to the yield of hydrogen production.

After alkaline pretreatment, pH in the fermenters was not controlled but recorded periodically. Although pH in these fermenters decreased during fermentation, it remained alkaline (>8.4). In addition, substantial amounts of NH_4^+-N were released due to decomposition of nitrogen-rich substrates such as proteins, urea, and nucleic acids (see Fig. S3 for cyclic variations of pH, NH_4^+-N, and FA). The average FA levels in the fermenters varied from 234 to 496 mg/L. Thus, the effect of FA in this range on these processes was also evaluated.

Table 1 summarizes the effect of FA on hydrolysis, acidogenesis, acetogenesis, homoacetogenesis, methanogenesis, and sulfate-reducing processes via a series of batch tests (i.e., Dextran Test, Glucose Test, Butyrate Test, H_2-CO_2 Test, and H_2-SO_4^{2-} Test). It was observed that the presence of FA decreased the degradation ratios of dextran and glucose. The higher the FA level, the lower the degradations of dextran and glucose. For example, when FA level increased from 0 to 424 mg/L, the degradation ratios of dextran (glucose) at 1 d fermentation decreased from 74.1 ± 4.1% (90.2 ± 5.5%) to 45.8 ± 2.8% (86.4 ± 5.2%). Similar results were also made at 2 d fermentation time. It should be emphasized that at 2 d fermentation, >83% of dextran and glucose were degraded even at the highest FA level, suggesting that although FA could inhibit the hydrolysis and acidogenesis processes, this inhibition did not affect the degradation of substrates substantially.

Apart from acidogenesis, acetogenesis can also produce hydrogen. In this work, the degradation of sodium butyrate was employed to indicate the effect of FA on this process. It was found from Table 1 that with the FA level increasing from 0 to 444 mg/L the degradation of butyrate varied from 20.9 ± 1.9% and 23.6 ± 1.2% at 1 d fermentation and from 25.3 ± 1.7% and 28.7 ± 1.5% at 2 d fermentation (Table 1). Statistical analysis showed that compared with the control (i.e., FA 0 mg/L) all the variations caused by FA at any level investigated were insignificant ($p > 0.05$), indicating that the presence of FA did not affect the acetogenesis process.

As metabolic intermediates, hydrogen produced can be further consumed by several anaerobes including methanogens, acetogens, and sulfate reducers to produce methane, acetic acid, and hydrogen sulfide via methanogenesis, homoacetogenesis, and sulfate-reducing processes (Fig. 5A). Hydrogen and carbon dioxide could be bio-converted into methane and acetate through methanogenesis and homoacetogenesis, respectively, while hydrogen and sulfate could be consumed through sulfate-reducing process to generate hydrogen sulfide. Thus, two hydrogen consumption tests (i.e., H_2-CO_2 Test and H_2-SO_4^{2-} Test) were performed to assess the impact of FA on these three processes.

The presence of FA largely reduced the consumption of hydrogen in the H_2-CO_2 Test (Table 1). For instance, when FA concentration increased from 0 to 354 mg/L, hydrogen consumption at 2 d fermentation decreased from 68.5 ± 3.1% to 9.5 ± 1.0%. Further increase of FA concentration to 444 mg/L led to a further slight decrease of hydrogen consumption (4.5 ± 0.5% at 2 d fermentation). Furthermore, methane was at a non-detectable level in all the FA present fermenters, suggesting that methanogenesis was completely inhibited by FA. Although acetic acid was detected in all the fermenters, the amount of acetic acid produced in the FA present fermenters was much less than that in the control. At 2 d fermentation, 92.7 ± 3.3 mg/L acetic acid was produced in the control whereas the corresponding data were 34.3 ± 1.2 mg/L in the 354 mg/L FA fermenter, 18.4 ± 1.1 mg/L in the 424 mg/L FA fermenter, and 15.4 ± 1.2 mg/L in the 444 mg/L FA fermenter. The

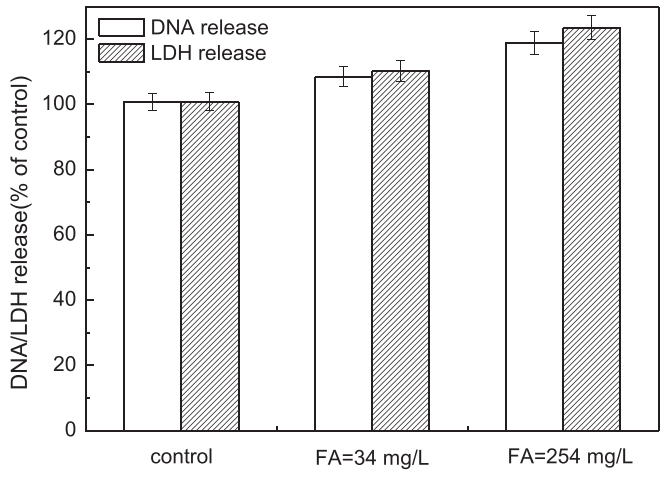


Fig. 4. The releases of LDH and DNA after 24 h pretreatment under different FA levels. Error bars represent standard deviations of triplicate tests.

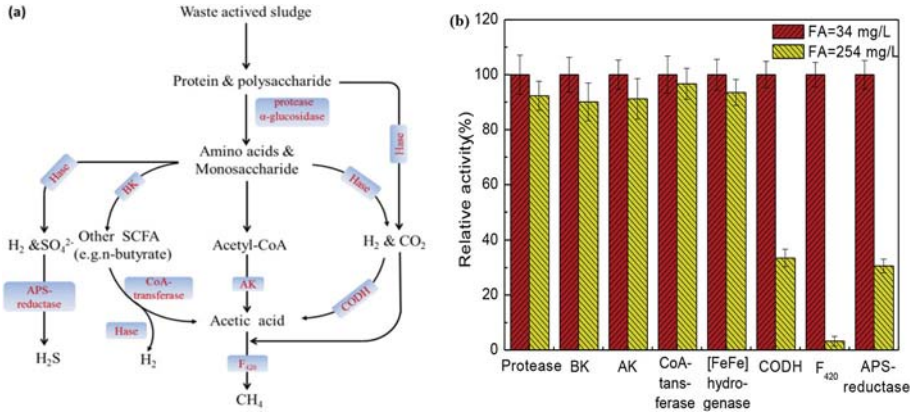


Fig. 5. The schematic diagram of metabolic pathways relevant to hydrogen accumulation (a) and relative activities of key enzymes in the long-term operated reactors (b). (AK: acetate kinase; BK: butyrate kinase; CODH: carbon monoxide dehydrogenase; F420, coenzyme F420; APS-reductase: adenosine-5′-phosphosulfate reductase; Hase: [FeFe] hydrogenase). Error bars represent standard deviations of triplicate measurements.

Table 1
The effects of FA on hydrolysis, acidogenesis, acetogenesis, homoacetogenesis, and methanogenesis processes.[a]

time (d)	FA (mg/L)[b]	Hydrolysis	Acidification	Acetogenesis	Homoacetogenesis/Methanogenesis			Sulfate-reduction	
		Dextran degradation (%)	Glucose degradation (%)	Butyrate degradation (%)	Hydrogen consumption (%)	Acetic acid production (mg/L)	Methane production (mL)	Hydrogen consumption (%)	Sulfate degradation (%)
1	0	74.1 ± 4.1	90.2 ± 5.5	23.6 ± 1.2	12.7 ± 1.0	25.7 ± 1.6	2.2 ± 0.5	14.9 ± 1.3	4.5 ± 0.3
	354	52.5 ± 3.1	87.1 ± 5.1	22.5 ± 1.7	3.3 ± 0.4	10.3 ± 0.7	ND[c]	6.0 ± 0.7	1.9 ± 0.2
	424	45.8 ± 2.8	86.4 ± 5.2	21.3 ± 1.4	2.3 ± 0.3	7.6 ± 0.6	ND	5.2 ± 0.4	1.6 ± 0.3
	444	44.0 ± 2.7	86.1 ± 4.9	20.9 ± 1.9	2.0 ± 0.3	6.4 ± 0.5	ND	4.9 ± 0.4	1.5 ± 0.2
2	0	96.4 ± 5.2	96.9 ± 5.8	28.7 ± 1.5	68.5 ± 3.1	92.7 ± 3.3	18.8 ± 1.3	34.4 ± 2.2	10.5 ± 1.2
	354	87.3 ± 4.9	93.7 ± 4.8	26.8 ± 1.8	9.5 ± 1.0	34.3 ± 1.2	ND	12.4 ± 0.8	3.5 ± 0.5
	424	84.8 ± 4.9	93.1 ± 5.1	25.9 ± 1.6	5.3 ± 0.6	18.4 ± 1.1	ND	10.7 ± 0.6	3.2 ± 0.3
	444	83.4 ± 4.8	92.8 ± 4.9	25.3 ± 1.7	4.5 ± 0.5	15.4 ± 1.2	ND	10.6 ± 0.7	3.1 ± 0.2

[a] Results are the averages and standard deviations of triplicate tests.
[b] The FA concentrations used in this batch test, i.e., 354, 424, and 444 mg/L, were respectively equal to the average FA concentrations measured from the fermenters initially fed with 141, 266, and 308 mg/L of NH_4^+-N treated sludge.
[c] ND = Non-detected.

results indicated that FA severely inhibited homoacetogenesis (Table 1).

Table 1 also shows that the addition of FA significantly inhibited the sulfate-reducing process as well since FA decreased both the hydrogen consumption and sulfate degradation (H_2-SO_4^{2-} Test). With the increase of FA level from 0 to 444 mg/L, hydrogen and sulfate consumption rates at 1 d fermentation decreased from 14.9 ± 1.3% to 4.9 ± 0.4% and from 4.5 ± 0.3% to 1.5 ± 0.2%, respectively. Similar observations were also made at 2 d fermentation.

The analysis above clarified the role of FA in the anaerobic fermentation of sludge. All processes relevant to the accumulation of hydrogen, with the exception of acetogenesis, were affected by FA. FA promoted sludge disintegration process, which provided more soluble substrates for the subsequent hydrolysis, acidogenesis, and acetogenesis processes. Although FA inhibited the hydrolysis and acidogenesis processes, the inhibitions did not affect the degradation of substrates such as dextran and glucose to a great extent. Compared with the hydrolysis and acidogenesis processes, the inhibition of FA to the three hydrogen consumption processes (i.e., methanogenesis, homoacetogenesis, and sulfate-reducing processes), however, were much severer. These explain why FA promoted hydrogen accumulation. Additionally, as soluble COD measured at 294 mg/L of FA pretreatment (i.e., the initial NH_4^+-N concentration of 308 mg/L) was not significantly higher than that measured at 254 mg/L of FA pretreatment (i.e., the initial NH_4^+-N concentration of 266 mg/L) while the former level of FA caused severer inhibitions to hydrolysis and acidogenesis processes than the latter level of FA. It can be understood why increasing the initial NH_4^+-N concentration from 266 to 308 mg/L did not enhance hydrogen production (Fig. 1).

No significant increase in VSS reduction was observed when initial FA concentration further increased to 294 mg/L (25.9 ± 1.7% vs 26.3 ± 1.9%, $p > 0.05$). With the increase of initial FA from 34 to 254 mg/L, soluble COD after 24 h pretreatment increased from 4560 ± 140 to 5220 ± 110 mg/L, which further slightly increased to 5290 ± 120 mg/L (Fig. 2b) when the initial FA was.

3.4. Comparison of key enzyme activities in the two long-term semi-continuous reactors

The generation of hydrogen in sludge anaerobic fermentation is directly related to the activities of some key enzymes (Fig. 5a), thus we finally compared them in the two long-term reactors fed with alkaline (pH 9.5) pretreated-sludge with 0 or 266 mg/L NH_4^+-N addition. Protease, butyrate kinase, acetate kinase, CoA-transferase, carbon monoxide dehydrogenase, coenzyme F_{420}, adenosine-5′-phosphosulfate reductase, and [FeFe] hydrogenase, which are key enzymes for hydrolysis, acidogenesis, acetogenesis, methanogenesis, homoacetogenesis, sulfate-reducing, and hydrogen production, respectively, were selected to be measured in this work.

Fig. 5b shows that FA inhibited the activities of all the enzymes measured to some extent. It was reported that FA could diffuse through cell membrane, shuttle protons between the two sides, and therefore lead to cell inactivation (Kayhanian, 1999). Wang

et al. (2017d) also found that FA treatment at 210 mg/L for 24 h decreased the activities of both ammonium oxidizing bacteria and nitrite oxidizing bacteria, which was in accord with our results. However, the inhibition of FA to the enzymes relevant to hydrogen production was much lighter than that to the enzymes relevant to hydrogen consumption. For example, FA caused only 7% of inhibition to [FeFe] hydrogenase but 97% of inhibition to coenzyme F_{420}. The observations were consistent with the chemical analyses results.

3.5. Implication to sludge anaerobic treatment

To date, almost all previous studies reported that NH_4^+-N would inhibit bio-gas (methane) production from anaerobic digestion of sludge. Pan et al. (2013) showed that the addition of NH_4^+-N (e.g., 1.5–2.5 g/L) enhanced biohydrogen production from anaerobic fermentation of food waste. The findings obtained in this work significantly advanced the understanding of the impact NH_4^+-N on fermentation, because they did not identify the impact of FA on each step of anaerobic fermentation of sludge. In this work, we found that FA promoted sludge disintegration and caused severe inhibitions to the three hydrogen consumption processes (i.e., methanogenesis, homoacetogenesis, and sulfate-reducing processes). Thus, this work revealed for the first time that the presence of FA in a suitable range could enhance hydrogen accumulation from sludge anaerobic fermentation, which was previously existed in sludge fermentation systems but unrecognized before. This was experimentally demonstrated through the use of chemical analysis on a series of batch tests using either real sludge or model substrates and microbial analysis on the long-term semi-continuous experiment.

With the increasing demand for energy worldwide, energy recovery from WWTPs attracts growing attention (Wang et al., 2017e; Li et al., 2014; Li et al., 2015). Compared with methane, hydrogen is a more valuable product due to its higher energy yield and lower carbon footprint. The findings achieved here may enlighten engineers to develop more economic strategies for hydrogen production from sludge fermentation. In the past, extensive efforts such as acid, alkaline, heat, and ozone/ultrasound pretreatment were dedicated to enhancing hydrogen yield. These strategies require high input of either chemicals or energy. In comparison, FA is a waste-generated, renewable chemical that can be generated *in situ* in WWTPs as a byproduct of sludge treatment (i.e., FA can be attained directly from anaerobic digester/fermenter effluent), thus the FA-based strategy does not have these limitations.

Fig. 6 presents an enhanced hydrogen production concept of a sludge fermentation system with the economical FA-based pretreatment method applied. The essential chemical for this method, i.e., FA, can be directly obtained from the anaerobic fermenter effluent. The sludge fermentation liquid, which is required to be returned to the head of WWTPs for treatment, usually contains ~300 mg/L NH_4^+-N (Chen et al., 2007; Zhao et al., 2015). By recycling a part of fermentation liquid into the pretreatment unit and controlling pH and temperature at suitable levels, the desirable FA concentration can be readily achieved. In such a sludge fermentation concept, with FA-supported sludge pretreatment, both sludge reduction and hydrogen yield are achieved in an economic way. Based on the results obtained in this work, it is calculated that this FA-supported strategy can cause ~0.8×10^3 t more in sludge reduction and ~7.6×10^4 m^3 more in hydrogen production annually in a WWTP ($Q = 10^5$ m^3/day), as compared with the alkaline (pH 9.5) pretreatment method only (Table S3). Considering the large number of WWTPs worldwide, this FA-based pretreatment method could have significant benefits.

It should be emphasized that this work mainly aimed to identify the contribution of FA to the enhanced hydrogen production and to reveal details of how FA enhances hydrogen production, thus technical optimization was not carried out. For example, only seven NH_4^+-N concentrations (i.e., 36, 99, 141, 182, 224, 266, and 308 mg/L), one pretreatment time (i.e., 24 h), and one pH value (i.e., 9.5)

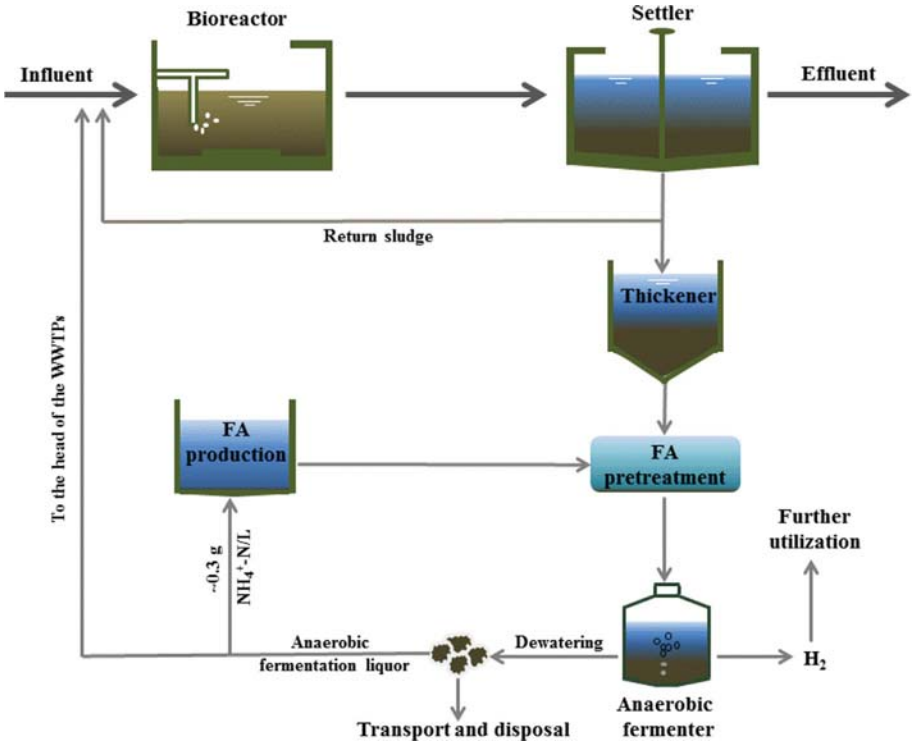


Fig. 6. The conceptual sludge fermentation system for hydrogen production, with an FA-based pretreatment method applied for enhancing hydrogen yield.

were tested in this work. Although one hydrogen consumption process (i.e., methanogenesis) was completely inhibited, hydrogen was still consumed by two other hydrogen consumption processes (i.e., homoacetogenesis and sulfate-reducing processes) (Table 1). The hydrogen yield would be further enhanced if these two processes can be further inhibited via optimizing both the FA concentration and pretreatment time. A comprehensive optimization study (e.g., a mathematical model study or a response surface study) is therefore required in the future. Based on the optimization study, scaling up this FA-supported hydrogen production method to pilot-scale or full-scale levels is also required in the future to further evaluate its technical and economic feasibilities under real-world situations.

4. Conclusion

In this study, the contribution of FA to the increased hydrogen production from anaerobic sludge fermentation was identified and the underlying mechanism of how FA enhanced hydrogen production was investigated for the first time. The results showed that with the increase of initial NH_4^+-N level from 36 to 266 mg/L, the maximal hydrogen production from alkaline pretreated-sludge increased from 7.3 to 15.6 mL/VSS. Further increase of NH_4^+-N to 308 mg/L caused a slight decrease of hydrogen yield (15.0 mL/g VSS). FA instead of NH_4^+-N was demonstrated to be the true contributor. It was found that the presence of free ammonia facilitated the releases of both extracellular and intracellular constituents. Except for acetogenesis, FA inhibited all other bio-processes, but its inhibition to the hydrogen consumption processes (i.e., homoacetogenesis, methanogenesis, and sulfate-reducing process) was much severer than that to the hydrolysis and acidogenesis processes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.watres.2018.01.051.

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