Volatile fatty acids produced by co-fermentation of waste activated sludge and henna plant biomass

Jingang Huang ^{a,*}, Rongbing Zhou ^a, Jianjun Chen ^a, Wei Han ^a, Yi Chen ^b, Yue Wen ^c,

Junhong Tang ^a

^a *Institute of Environmental Science and Engineering, Hangzhou Dianzi University, Hangzhou 310018, PR China.*

^b *Department of Applied Ecology, Faculty of Environmental Sciences, Czech University of Life Sciences Prague, 16521, Prague 6, Czech Republic.*

^c *State Key Laboratory of Pollution Control and Resource Reuse, College of Environmental Science and Engineering, Tongji University, Shanghai 200092, PR China.*

* Corresponding author. Address: Room 501, The 6th Building, Institute of Environmental Science and Engineering, Hangzhou Dianzi University, Hangzhou 310018, PR China; Tel./fax: +86-571-86919158; E-mail address: hjg@hdu.edu.cn (J. Huang).

Abstract

Anaerobic co-fermentation of waste activated sludge (WAS) and henna plant biomass (HPB) for the enhanced production of volatile fatty acids (VFAs) was investigated. The results indicated that VFAs was the main constituents of the released organics; the accumulation of VFAs was much higher than that of soluble carbohydrates and proteins. HPB was an advantageous substrate compared to WAS for VFAs production; and the maximum VFAs concentration in an HPB mono-fermentation system was about 2.6-fold that in a WAS mono-fermentation system. In co-fermentation systems, VFAs accumulation was positively related to the proportion of HPB in the mixed substrate, and the accumulated VFAs concentrations doubled when HPB was increased from 25% to 75%. HPB not only adjust the C/N ratio; the associated and/or released lawsone might also have a positive electron-shuttling effect on VFAs production.

Keywords: Co-fermentation; Henna plant biomass; Waste activated sludge; Volatile fatty acids; Lawsone

1. Introduction

During the past few decades, many wastewater treatment plants (WWTPs) have been constructed in China. At the end of 2014, there were more than 3700 WWTPs in operation treating ~160 million tonnes of municipal wastewater per year. Waste activated sludge (WAS) is the main solid by-product in WWTPs, and it is generated at a yield of 0.032 kg per m³ of treated wastewater (Ng et al., 2014). To date, the production rate of WAS has surpassed 10% in China. Thus, WAS needs to be properly treated because of the vast quantities that are produced, which can cause secondary pollution and are highly costly. Anaerobic digestion (AD) is not only the most conventional but also an attractive way to treat WAS as it is a harmless process with high energy recovery. The AD process includes three steps: hydrolysis, acidification and methanogenesis. Among these, hydrolysis and/or acidification are the key steps due to the slow rate at which particle sludge is transformed to soluble organic compounds such as volatile fatty acids (VFAs) (Feng et al., 2014; Tiehm et al., 1997). Besides, insufficient carbohydrate content in the raw WAS results in a low carbon-to-nitrogen mass ratio (C/N), which also limits VFAs production from WAS fermentation. VFAs are very useful in applications such as biogas (methane or hydrogen) recovery, degradable plastic synthesis, electricity generation, and the enhanced removals of nitrate, phosphorus, sulfate, azo dyes and disinfection byproducts from wastewaters (Chen et al., 2014; Lee et al., 2014). Therefore, much research has been focused on the enhanced production of VFAs from WAS. Ultrasonic, thermal, hydrothermal, and added alkaline, surfactant or zero valent iron are the usual pretreatment methods used to promote the release of organic compounds from particle sludge and enhance the related enzymatic activities, with the aim of improving VFAs production (Feng et al., 2014; Tiehm et al., 1997; Yang et al.,

2014; Yin et al., 2014; Zhou et al., 2015). Furthermore, carbohydrate-enriched wastes including primary sludge, food waste, agricultural and forestry residues, pulp and paper waste, organic-rich industrial wastewaters have been eco-friendly added to the raw WAS to adjust C/N ratio for the enhanced production of VFAs during the fermentation process (Bayr and Rintala, 2012; Chen et al., 2013; Feng et al., 2009; Jiang et al., 2013; Luste and Luostarinen, 2010; Zhou et al., 2014). This is referred to as co-fermentation process, which can be affected by pH, temperature, oxidation-reduction potential (ORP), feedstock proportion, sludge retention time (SRT) and so on. In this way, both WAS and organic wastes can be efficiently reduced due to the balanced C/N ratio and better pH buffering capacity (Wang et al., 2011).

Many natural plant biomass substances, such as rice straw, corn stover and bagasse, have been popularly used as co-substrates to adjust the C/N ratio during WAS digestion (Rughoonundun et al., 2012; Zhou et al., 2013). Plant biomass, which is an abundant carbohydrate sink, is mainly composed of cellulose, hemi-cellulose and lignocelluloses. Because of the complex structure and a caused enzymatic attack of these substances, the hydrolysis is quite slow and is the rate-limiting step of the overall fermentation process (Noike et al., 1985; Wang et al., 2014a). Therefore, natural plant biomass usually needs to undergo mechanical, thermal, and/or alkaline pretreatment before it is introduced to a co-fermentation system. However, the high energy consumption of these pretreatments currently restricts their broad application. As a solution, extracellular redox mediators (RMs), such as anthraquinone-2,6-disulfonate (AQDS) and humic acid (HA) associated with a quinone group, have been reported to act as electron-shuttling systems to improve the related enzymatic activities, and thus to enhance VFAs production during WAS fermentation (Liu et al., 2015; Yang et al., 2012). Henna (*Lawsonia inermis*) is a

carbohydrate-enriched plant biomass containing abundant lawsone, which is a typical quinone group-based RM that has been reported to improve hydrogen production in a co-cultured fermentation system (Zhang et al., 2013). Therefore, using henna plant biomass (HPB) as a co-substrate during WAS fermentation could balance the C/N ratio, plus the associated and/or released lawsone might have a positive effect on VFAs production.

The aim of this study was to investigate the feasibility of using WAS and HPB as substrates in a co-fermentation process to enhance VFAs production. Under different feedstock proportions of WAS to HPB, the concentrations of various soluble organics and key enzymatic activities in the fermentation systems were evaluated to aid in understanding the mechanism of improved VFAs production.

2. Materials and methods

2.1. Sources of WAS and HPB

The WAS was obtained from a secondary sedimentation tank of a WWTP in Hangzhou, China. After sampling, the sludge was firstly screened with a 1 mm sieve to remove the impurities, and then was washed with deionized water for three times. After pretreatment, the WAS was concentrated by settling at 4 °C for 24 h before use. The main characteristics of the concentrated WAS were shown in Table 1. Commercial HPB (leaves powder) was purchased from Kaihangzhongyi Trading Co. Ltd, in Xinjiang, China. HPB powder was dried at 45 °C for 8 h before usage. The HPB particle size distributed between 0.02 to 355 μm, while around 80% of them were in the range of 3 to 25 μm (Fig. S1, Supplementary Materials). To analyze the functional group of HPB, FTIR spectrum was recorded, showing two specific bands of transmission at 1625 cm⁻¹

and 1725 cm^{-1} , respectively (Fig. S2, Supplementary Materials). This functional group characteristic indicates the presence of lawsone (Nik et al., 2012).

2.2. Experimental set-up

The fermentation experiments were conducted in plexiglass anaerobic reactors with a working volume of 5.0 L. The water seals were located at the top of each reactor to ensure that the reactors were airtight. During the fermentation process, the reactors were mechanically stirred (120 rpm) using a power-driven force mixer (HD 2004W, Sile Co., China), and feedstock was mesophilically fermented in a water bath at $35 \pm 1\text{ }^{\circ}\text{C}$.

To investigate the effect of feedstock proportion ($\text{TS}_{\text{WAS}} : \text{TS}_{\text{HPB}}$) on the fermentation process, five sets of reactors (R1, R2, R3, R4 and R5), each with a duplication, were established in this study. The concentrated WAS and dried HPB were added to the reactors to achieve a total solids (TS) content (WAS + HPB) of 30 g L^{-1} . In these reactors, the HPB proportions in the mixed substrate were as follows: R1 (0%), R2 (25%), R3 (50%), R4 (75%) and R5 (100%). R1 and R5 were the control systems (mono-fermentation), in which a mono-substrate, i.e., only WAS or HPB, was supplied; while R2, R3 and R4 were the co-fermentation systems with different feedstock proportions ($\text{TS}_{\text{WAS}} : \text{TS}_{\text{HPB}}$). The inoculum in the above systems was originally obtained from an anaerobic reactor in a starch WWTP in Hangzhou, China. During this study, 50 mL of the inoculated sludge was supplemented, resulting in a final inoculum concentration in each reactor of 1.0 g VSS L^{-1} . The initial pH was adjusted to 8.0 using a stocked NaHCO_3 solution (50 g L^{-1}). NH_4Cl (80 mg L^{-1}), KH_2PO_4 (20 mg L^{-1}), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (20.3 mg L^{-1}), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (14.7 mg L^{-1}), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (2.8 mg L^{-1}), and stocked trace metals and vitamins (1.0 mL/L) were supplied as the previous study (Huang et al., 2015). After preparation, the reactors ran continuously for 21 days.

During the entire operating period, pH and ORP in the systems were measured daily. Mixture samples were withdrawn from each reactor at appropriate time intervals (1–3 days) for the following chemical and enzymatic activity analysis.

2.3. Chemical analysis

pH and ORP in the reactors were measured by a portable pH/ORP meter (SANXIN, SX751, China). Suspended mixture samples, withdrawn at days 1, 2, 4, 6, 8, 10, 12, 15, 18 and 21 from each reactor, were firstly centrifuged at $8000 \times g$ for 20 min, and then be filtered through 0.45 μm membrane filters (Anpel Co., Shanghai, China). The filtered supernatant was analyzed for soluble chemical oxygen demand (SCOD), soluble carbohydrate, soluble protein, VFAs and the released lawsone.

Soluble carbohydrates was measured by the anthrone-sulfuric acid colorimetric method with glucose as standard. Soluble protein was determined according to the Bradford method with bovine serum albumin as the standard. VFAs were composed of acetate, propionate, *n*-butyrate, *n*-valerate and their isomers (*iso*-butyrate and *iso*-valerate). VFAs components and soluble lawsone were measured by high performance liquid chromatography unit (HPLC, Agilent 1200, Agilent Technologies, CA, USA) equipped with an UV detector. The detailed procedure of HPLC analysis was the same as described in the previous publication (Huang et al., 2015). SCOD, TS, VSS were determined according to *Standard Methods*. To compare the concentrations of different soluble fermentative intermediates in the mixture, carbohydrates, proteins and VFAs were all unified as COD. The COD coefficient of carbohydrate and protein were 1.070 and 1.500, respectively; and the COD coefficient of VFAs components, i.e., acetate, propionate, butyrate, valerate, were 1.067, 1.512, 1.818 and 2.039, respectively. The particle size distribution was determined by light scattering using a Malvern Mastersizer

2000 system. The FTIR spectrum was recorded by a Nicolet 6700 FTIR spectrometer.

2.4. Enzymatic activity assay

During the fermentation process, the mixture from each reactor at appropriate time intervals were sampled and used for the determination of extracellular hydrolysis enzymatic activities of α -glucosidase and protease. The detailed procedures in determining these enzymatic activities can be found in the previous publication (Goel et al., 1998). After the colorimetric measurements, the absorbency differences between the incubation assays and the controls were obtained. The conversion factors from above absorbency differences to enzymatic activities (α -glucosidase and protease) were 0.299 and 2.22, respectively.

2.5. Statistical analysis

All experimental reactors were established in duplicate and the results were expressed as mean $\pm$ standard deviation. To compare the average levels of SCOD, carbohydrate, protein and VFAs during the entire fermentation time (days 1, 2, 4, 6, 8, 10, 12, 15, 18, 21) between two experimental sets with different feedstock proportions, an analysis of Student's unpaired *t*-test was used and $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. SCOD concentrations

Anaerobic fermentation are usually composed of the following steps: hydrolysis, acidification and methanogenesis. Among these the hydrolysis of solid WAS and/or HPB is the first and also the rate-limiting step. SCOD is a key parameter associated with the release of organics from the particulate WAS and HPB (Chen et al., 2012). The

SCOD of mixtures with different feedstock proportions in each reactor are shown in Fig. 1. As indicated in Fig. 1, the feedstock proportion ($TS_{WAS} : TS_{HPB}$) and retention time were both found to affect the release of organics from the substrates. Within the mono-fermentation systems, it is apparent that SCOD in R5 (with only HPB as substrate) at any fermentation time was significantly higher than that in R1 (with only WAS as substrate) ($P < 0.05$). On day 6, the maximum SCOD in R5 was $11083 \pm 707 \text{ mg L}^{-1}$, while that in R1 was only $4022 \pm 334 \text{ mg L}^{-1}$. This indicates that less organic matter was released from the WAS. The complex structure of the microbial cell wall, which contains glycan cross-linked by peptide chains, presumably leads to the resistance to hydrolysis of WAS in this study (Feng et al., 2014); however, organic matter can be abiotically released from the plant biomass (Chen et al., 2012). In the co-fermentation systems (R2, R3 and R4), the SCOD concentration was found to be dependent on the feedstock proportion. With increasing HPB content in the co-substrate, higher SCOD levels could be observed except on days 12, 15, 18 and 21. In R4 ($TS_{WAS} : TS_{HPB} = 25\% : 75\%$), the maximum SCOD ($8790 \pm 728 \text{ mg L}^{-1}$) was observed on day 10, and it was significantly higher than those in R3 ($TS_{WAS} : TS_{HPB} = 50\% : 50\%$) and R2 ($TS_{WAS} : TS_{HPB} = 75\% : 25\%$) ($P < 0.05$). As a plant biomass, HPB can therefore improve the release of organics by adjusting the C/N ratio. Previous studies have reported that certain types of plant biomass, such as corn straw, bagasse and tall fescue, co-fermented with WAS can enhance the yield of carbohydrates, proteins, VFAs, biogas and so on (Rughoonundun et al., 2012; Zhang et al., 2015; Zhou et al., 2013). The detailed results for these organic components will be discussed in the following sections. Furthermore, it was observed that the net SCOD accumulation with fermentation time in each reactor increased substantially during the initial stage, and then slightly decreased or remained

steady during the latter stage. This could be attributed to a balance between the release and consumption of organics (Chen et al., 2007; Chen et al., 2012). When the release rate exceeded the consumption rate, organics could be accumulated. Therefore, the much higher and more stable accumulation of SCOD in R4 and R5 implies the greater organic-release potential of HPB fermentation.

3.2. Carbohydrate and protein solubilization

Polysaccharides and proteins are the main constituents of natural plant biomass (HPB) and WAS, respectively (Zhang et al., 2015). During the hydrolysis process, polysaccharides and proteins are hydrolyzed to the intermediates glucose and amino acids by the key enzymes of α -glucosidase and protease, respectively (Chen et al., 2007; Luo et al., 2014). These two intermediates are the key substrates for the following acidification process of VFAs production. The concentrations of soluble carbohydrate and protein at different feedstock proportions ($TS_{WAS} : TS_{HPB}$) are shown in Fig. 2; and the enzymatic activities of α -glucosidase and protease are shown in Fig. S3 (Supplementary Materials).

In the initial few days soluble carbohydrate was in higher level when it with more HPB in the substrate (Fig. 2A). On day 1, the carbohydrate concentrations in R4 and R5 were highest at 402 ± 27 mg COD L⁻¹ and 417 ± 31 mg COD L⁻¹, respectively. On day 6, the carbohydrate concentrations in these two reactors had sharply decreased to 136 ± 12 and 158 ± 16 mg COD L⁻¹, respectively. Interestingly, it was found that the carbohydrate concentrations in R4 and R5 on subsequent fermentation days were overturned to be lower than those in reactors with less HPB. In contrast, it was observed that the carbohydrate levels in R1, R2 and R3 slightly increased from day 1 to day 4, and then remained steady, although a small amount of fluctuation occurred on the

1 following days. Therefore, these results indicated that more carbohydrate was released
2 and then consumed in reactors with higher HPB content (R4 and R5). This might be due
3 to the adjustment in C/N ratio caused by the addition of carbohydrate-enriched substrate
4 HPB. There was no statistical significant difference in soluble protein concentration
5 among R1, R2, R3 and R4 ($P > 0.05$), which slightly increased from ~200 to the steady
6 value of ~280 mg L⁻¹ (Fig. 2B). Interestingly, the soluble protein in R5 (mono-HPB
7 control) was the lowest at any SRT among the reactors. The maximum concentration of
8 protein appeared in R2 on day 18, achieving 309 ± 16 mg COD L⁻¹. Because the protein
9 content in HPB was lower than that in WAS, the lowest accumulation of soluble protein
10 was observed in R5.

11 The results for enzymatic activity (Fig. S3A, Supplementary Materials) indicate that
12 α -glucosidase in the co-fermentation systems, especially in R3 and R4, were mostly
13 higher than those in the mono-fermentation systems (R1 and R5). With the fermentation
14 time expanding, the α -glucosidase activity varied, and the maximum appeared in the
15 middle stage (days 6–12). On day 12, R4 (TS_{WAS} : TS_{HPB} = 25% : 75%) displayed the
16 maximum α -glucosidase activity, achieving 0.583 U, which is about 8.7-fold and
17 2.6-fold higher than the activities in R1 and in R5, respectively. This suggests that more
18 hydrolyzed glucose would be produced in the co-fermentation systems than in the
19 mono-fermentation systems. However, reducing sugar did not accumulated during the
20 entire fermentation period (data not shown), implying that the hydrolysis rather than the
21 acidification was the rate-limiting step. The glucose could have been immediately
22 acidified to VFAs, resulting in the higher VFAs production and greater decrease in
23 soluble carbohydrate observed in R4 (Fig. 2A and Fig. 3). In R1, the α -glucosidase
24 activity gradually decreased due to the lack of soluble carbohydrate when WAS was the

mono-substrate. Furthermore, it was interesting to found that the protease activities in systems with less HPB (such as in R1 and R2) were higher in some cases than those in systems with more HPB (such as in R5). This was attributed to the fact that R1 and R2 had more protein than R5, resulting in changes in the protease activities (Fig. S3B, Supplementary Materials). The protease activities in this study also displayed wave-like variations. However, these were in contrast with the variations in α -glucosidase. The protease displayed relatively higher activities in the initial and latter stages, whereas the protease activities were at minimum during the middle stage for each reactor. These differences between α -glucosidase and protease activities were attributed to the fact that glucose is the metabolic product of α -glucosidase, while protein is the substrate of the protease (Goel et al., 1998).

In this study, the protein and carbohydrate concentrations at different feedstock proportions were comparable with a previous study, and their concentrations along with the fermentation time varied little (Luo et al., 2014). This was due to a net balance between the release and consumption of organics from both WAS and HPB. The consumed carbohydrate and protein were believed to have been at least partially responsible for the VFAs production, as discussed below.

3.3. Total VFAs (TVFAs) production

During the acidification process in this study, the glucose hydrolyzed from polysaccharides and the amino acids hydrolyzed from proteins would be rapidly fermented to VFAs via pyruvate or lactate (Chen et al., 2013; Wang et al., 2014b). Besides, the amino acids could also be acidified to VFAs by the redox reaction between pairs of amino acids (Stickland reaction) and/or the reductive deamination of single amino acid molecules (Ramsay and Pullammanappallil, 2001).

VFAs concentrations (in terms of COD) under different feedstock proportions during the entire fermentation period (21 days) are shown in [Fig. 3](#). As mentioned for the mono-fermentation systems, significantly higher levels of VFAs accumulation were observed in R5 (HPB as mono-substrate) compared to that in R1 (WAS as mono-substrate) ($P < 0.05$). The maximum VFAs concentration in R5 was 7891 ± 411 mg COD L⁻¹ on day 6, which was about 2.6-fold that of the maximum (3022 ± 75 mg L⁻¹) in R1 on day 12. As mentioned for the co-fermentation systems with mixed substrates (WAS and HPB) in R2, R3 and R4, VFAs accumulation at any operating time (except on day 10) was positively correlated with the HPB content in the feedstock. In R4 (TS_{WAS} : TS_{HPB} = 25% : 75%), VFAs accumulated to a maximum concentration of 7875 ± 787 mg COD L⁻¹, which was 2.0-fold and 1.5-fold of that in R2 (3910 ± 721 mg COD L⁻¹) and R3 (5326 ± 452 mg COD L⁻¹), respectively. The above results indicated that HPB was an advantageous substrate compared to WAS for VFAs production, resulting in lower pH levels in the systems with higher HPB proportions such as in R4 and R5 ([Fig. S4A, Supplementary Materials](#)). In this case, more hydrolyzed carbohydrate and protein were consumed during the subsequent acidogenesis process ([Fig. 2](#)), creating lower ORP conditions and, in turn, a synergistic effect that enhanced VFAs production ([Fig. S4B, Supplementary Materials](#)).

Furthermore, it was observed that VFAs concentrations in R1, R2, R3 and R4 gradually increased during the initial 10 days and then decreased during subsequent fermentation times. This result was consistent with a previous study reporting improved VFAs production from WAS fermentation upon carbohydrate addition ([Luo et al., 2014](#)). The net accumulation of VFAs was a balance between acidification and methanation. When the fermentation time was shorter than 8 days the acidogenic bacteria were

dominant, whereas methanogenesis activity might outcompete the hydrolysis and acidification processes when the retention time was longer than 10 days, and the net VFAs concentrations in R1 were even observed to decrease to zero. However, the net accumulation of VFAs in R5 always remained at a stable level after it achieved a maximum ($7891 \pm 411 \text{ mg COD L}^{-1}$) on day 6. As shown throughout Figs. 1, 2 and 3, the concentrations of VFAs were much higher than those of carbohydrates and proteins, accounting at its maximum for 93% of the total SCOD (R5, day 21). Thus, VFAs were the main constituents of the released organics in this study, and the above variations in VFAs concentrations were consistent with those of COD. This also proved that the hydrolysis process of solid HPB and WAS that produced the soluble intermediates of carbohydrates and proteins is the rate-limiting step. As mentioned in section 3.1, the organics in HPB were much more easily hydrolyzed than those in WAS, producing more soluble intermediates for the subsequent acidification process. Thus, the addition of HPB to WAS could enhance the stable production of VFAs, which then served as efficient electron donors for the improved denitrification, phosphorous removal and reduction removal of oxidized pollutants (Lee et al., 2014).

3.4. Individual VFA distributions

The distributions of individual VFAs (acetate, propionate, *n*-butyrate, *n*-valerate and their isomers) and their percentages of the total VFAs are shown in Fig. 4. During the initial and middle stages of the fermentation period, acetate and propionate were always most abundant in the systems with relative lower HPB proportions such as in R1, R2 and R3. These results agreed with previous studies for mono-fermentation systems with WAS or plant biomass as substrates and other co-fermentation systems by mixing these substrates (Chen et al., 2007; Chen et al., 2012; Zhou et al., 2013). Furthermore, the

percentages of acetate and propionate in systems with less HPB (R1, R2 and R3) displayed a declining trend with fermentation time, whereas those of butyrate (*iso + n*) and valerate (*iso + n*) increased. At the end of the fermentation tests, the percentages of acetate and propionate in R1, R2 and R3 had decreased to 0%, 0% and 19.5%, from 82.3%, 68.6% and 61.4% at day 1, respectively. However, those percentages in systems with higher HPB proportions (R4 and R5) displayed stable and even increasing trends along with the fermentation time. At the end of the fermentation tests, the percentages of acetate and propionate in R4 and R5 increased to 78.1% and 76.2%, from 62.8% and 40.5% at day 1, respectively. Therefore, the addition of the carbohydrate-enriched substrate HPB can condition the WAS fermentation for the stable production of acetate, which is beneficial for the following methanation process (Xiong et al., 2012; Zhou et al., 2013). Because the main contents of WAS and HPB are protein and carbohydrate, respectively, more acetate and propionate accumulated in systems with higher HPB proportions (R4 and R5). In addition to the enriched carbohydrate, HPB also contains abundant lawsone, which is a typical quinone group-based RM. Ye et al. (2011) have indicated that quinone group associated RMs such as AQDS can shift the butyric acid pathway to the acetic acid pathway due to changes in the intracellular NADH/NAD⁺ ratio in the presence of external RM. Besides, a previous study has also indicated that acetate production would be enhanced by adding HA (quinone group-based RM) to the fermentation system (Liu et al., 2015). Therefore, the lawsone in HPB could also potentially improve the production of acetate, leading to greater accumulation of acetate in R5 as it has the highest HPB proportion (Fig. 4).

3.5. Redox mediator (lawsone)

HPB contains abundant lawsone, accounting for up to 1.8% of its dry weight

(Almeida et al., 2012). The FTIR spectrum of HPB (Fig. S2) shows two specific bands of transmission at 1625 cm^{-1} and 1725 cm^{-1} , respectively, suggesting that 2-hydroxy-1,4-naphthoquinone (lawsone) was associated with HPB (Nik et al., 2012). During the entire fermentation period, the soluble lawsone concentrations in each system are shown in Fig. 5. The results show that no lawsone was found in R1 (mono-WAS control), while it was found in the other systems, indicating that the associated lawsone could be released from solid HPB to the liquid phase. Generally speaking, the soluble lawsone (except that in R4 after day 10) was positively correlated with the HPB proportion at all fermentation times. The maximum concentration was found to be $67.7 \pm 6.5\text{ mg L}^{-1}$ in R5 on day 1, implying that the associated lawsone can be quickly released to the liquid phase. Subsequently, the soluble lawsone in R5 sharply decreased along with retention time. At the end of the fermentation tests, the soluble lawsone concentration was only $5.9 \pm 0.8\text{ mg L}^{-1}$. In R2, R3 and R4, soluble lawsone slightly increased at first and then decreased with further fermentation. The above results suggest that the released lawsone might be degraded or adsorbed in all of the systems, resulting in a decrease in its soluble form. The detailed mechanism needs further study. Quinone group-based RMs including lawsone not only act as an effective electron shuttling system for the enhanced removal of various oxidized pollutants (van der Zee and Cervantes, 2009), but also promote the acidification process and even inhibit methanogenic activities in the fermentation systems, resulting in the enhanced production of VFAs and H_2 (Liu et al., 2015; Yang et al., 2012; Zhang et al., 2013). The nearly neutral pH conditions in this study (Fig. S4A) were likely suitable for shuttling electrons by lawsone (Huang et al., 2015). In addition, it was noticed that the VFAs accumulations were not always positively correlated with the soluble lawsone

1 concentrations. In R5, although the highest soluble lawsone concentration was observed
2 in the initial stages, the accumulated VFAs was at a low level. Yang et al. (2012) have
3 demonstrated that the enhanced VFAs production by AQDS was mainly attributed to
4 protein decomposition associated with amino acid degradation, not to carbohydrate. As
5 mentioned above, WAS was mainly composed of protein while HPB was mainly
6 carbohydrate. Therefore, the soluble lawsone might take part in the WAS fermentation
7 to enhance VFAs production in the co-fermentation systems (R2, R3 and R4).

8 **4. Environmental significance**

9 VFAs production from WAS fermentation was mainly restricted by its low C/N ratio
10 and limited electron-shuttling ability. Although many pretreatments have been applied
11 to enhance the release of organic compounds from particle sludge, high energy
12 consumption is still the bottleneck that impedes their wide application. HPB is enriched
13 with carbohydrate and lawsone (a quinone group-based RM). Thus, the co-fermentation
14 of HPB with WAS not only adjusted the C/N ratio, but also supplied an efficient
15 electron-shuttling mediator (lawsone) during the digestion process. According to this
16 dual role, the VFAs production was efficiently enhanced. The above co-fermentation
17 process seems a promising way to treat WAS that is energy saving, and can furthermore
18 enhance the stable performance of WAS digestion.

20 **4. Conclusions**

21 HPB is enriched with carbohydrate and lawsone (a quinone group-based RM). It
22 was an advantageous substrate compared to WAS for VFAs production, and the
23 maximum VFAs concentration in the HPB mono-fermentation system was about
24 2.6-fold that in the WAS mono-fermentation system. When HPB was co-fermented with

1 WAS, VFAs accumulation was positively correlated with the proportion of HPB in the
2 mixed substrate; the accumulated VFAs concentrations doubled when HPB proportion
3 was increased from 25% to 75%. HPB played a dual role in adjusting the C/N ratio and
4 shuttling electrons to enhance VFAs production during the fermentation process.

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Table and Figure captions

Tables

Table 1. Main characteristics of concentrated WAS.

Figures

Figure 1. SCOD concentrations of the fermentation liquid with different feedstock proportion.

Figure 2. Concentrations of soluble carbohydrate and protein in the fermentation liquid with different feedstock proportion.

Figure 3. Concentrations of total VFAs in the fermentation liquid with different feedstock proportion.

Figure 4. Distribution of individual VFA and their percentages of the total VFAs (TVFAs) with different feedstock proportions.

Figure 5. Soluble lawsone concentrations in the fermentation liquid with different feedstock proportions.

FIGURES

Fig.1. SCOD concentrations of the fermentation liquid with different feedstock proportion.

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Fig.5. Soluble lawsone concentrations in the fermentation liquid with different feedstock proportions.

TABLE

Table 1. Main characteristics of concentrated WAS

Parameters	Value ^{a,b}
pH	7.12 ± 0.11
Total solids (TS)	31680 ± 1040
Volatile suspended solids (VSS)	20592 ± 844
Soluble chemical oxygen demand (SCOD)	76 ± 15
Soluble carbohydrate (as COD)	22 ± 3
Soluble protein (as COD)	18 ± 2
VFAs in the suspended solution (as COD)	ud. ^c
Soluble lawsone	ud.

^a All values are expressed in mg L⁻¹ except pH.

^b Average value ± one standard deviation.

^c ud. represents undetectable.