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Effect of temperature on fermentative VFAs production from waste sludge stimulated by riboflavin and the shifts of microbial community

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ABSTRACT

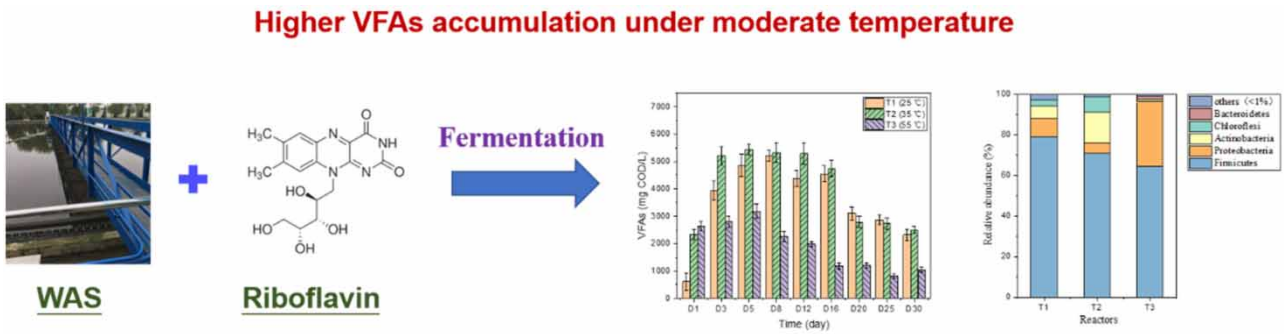
Fermentative volatile fatty acids (VFAs) production from waste activated sludge (WAS) under moderate temperature is a promising way for resource and energy regeneration in municipal wastewater treatment plants (MWTPs). In this study, the effect of temperature on VFAs production and the associated microbial community from riboflavin-assisted WAS fermentation were investigated. Three fermentative reactors under 25, 35 and 55 °C were operated for 30 days, respectively. The results indicated that riboflavin enhanced VFAs production from WAS fermentation under moderate temperatures (25 °C, 35 °C), increasing conversion of organic matters to bioavailable substrates for the subsequent acidification process. Although a small dosage of riboflavin (1.0 $\pm$ 0.05 mM) hardly inhibited the methanogenic process, it could mediate the electron sink for VFAs under lower temperatures. This in turn increased the accumulation of acetic and propionic acids (up to 234 mg/g of volatile suspended solids) and their proportions relative to the total VFAs, being efficient electron donors and carbon sources for nutrient removal in MWTPs. Furthermore, microbial communities were shifted in response to temperature, and riboflavin stimulated the special fermentative bacteria under room temperature and mesophilic conditions. The study suggested a feasible and eco-friendly method to improve VFAs production from crude WAS at a relatively lower temperature.

Key words: microbial community, riboflavin, temperature, volatile fatty acids (VFAs), waste activated sludge (WAS)

HIGHLIGHTS

- Effect of temperature on VFAs yield from riboflavin-assisted WAS were studied.
- Riboflavin increases the hydrolysis of protein and the release of ammonia.
- Riboflavin stimulates VFAs production under moderate temperature.
- Acetic and propionic acids generation improved by up to 234 mg/g of VSS.
- Thermophilic fermentation reduced the abundance of Firmicutes.

GRAPHICAL ABSTRACT



INTRODUCTION

In China, there are more than 10,000 municipal wastewater treatment plants (MWTPs) with the treatment capacity of 230 million tons per day. Waste activated sludge (WAS) is largely produced during the operation of MWTPs, achieving to 70 million tons annually (Zhang *et al.* 2021). At this time, the disposal methods for the residual WAS are digestion, incineration, landfill, agricultural utilization and ocean dumping (Zhen *et al.* 2017). These treatments not only increase the energy consumption, but also cause secondary pollution. For example, carcinogenic dioxins would be produced when incineration technology was used. Moreover, the continuous emission of carbon dioxide (CO_2) from the treatment facilities, converting from organic components, hinders the carbon neutralization target for future years (Zamri *et al.* 2021).

The main contents of solid WAS include carbohydrates and proteins, which are valuable organic-carbon materials (Appels *et al.* 2012). Thus, the resource and energy regeneration from WAS to supplement carbon sources for nutrient removal is a trend in future carbon-neutral operating MWTPs. Fermentative volatile fatty acids (VFAs) production from WAS is a common solution to cope with the above issues. VFAs serve as an effective carbon source and electron donor for denitrification (Guo *et al.* 2015). In addition, VFA is also the raw substrate to synthesis poly-β-hydroxybutyric acid (PHB) and poly-β-hydroxyvaleric acid (PHV) (Zhang *et al.* 2014). Therefore, the reutilization of VFAs from WAS saves useful industrial products such as methanol and sodium acetate to enhance the nutrients removal, indirectly reducing the CO_2 emission during the MWTP operation.

The VFAs fermentation process is affected by various parameters such as pH, temperature, organic loading rate (OLR), mass ratio of total carbon to total nitrogen (C/N ratio), and retention time (Wang *et al.* 2014). Among the above, temperature is a crucial factor affecting the microbial metabolism and community that determines the efficiency and stability of VFAs production. Thermophilic fermentation (55 °C) promotes the hydrolysis and acidification process, which benefits the VFAs production; however, tremendous energy supplementation is needed to maintain the high temperature in engineering applications (Liu *et al.* 2018; Pan *et al.* 2021). The fermentation process such as that under mesophilic conditions (35 °C) or at even room temperature (25 °C) is an energy-saving process, while disadvantages are the lower hydrolysis and fermentation rates (Gavala *et al.* 2003). This results in an unstable VFAs generation, requiring a longer retention time. Therefore, it is meaningful to enhance the VFAs generation rate under moderate temperature. Previous studies had found that high-alkalic, ultrasonic, hydrothermal and microwaving pretreatments could help to break down the crosslinked structures of proteins associated within WAS biomass, increasing the following hydrolysis rate and the VFAs production (Appels *et al.* 2012; He *et al.* 2019). However, more energy or chemicals were required, urging the change to energy-saving processes and eco-friendly replacement of additives.

Riboflavin is a biogenic biocatalyst which could be industrially produced by fermentation with waste organic substrates (Ryhan Bashandy *et al.* 2021). In biological systems, riboflavin has been reported to serve as an extracellular redox mediator to transfer electrons between redox reactions, promoting enzymatic activities, biofilm formation, and pollutants removal (Edel *et al.* 2021). Previous study had indicated that riboflavin could improve the VFAs production by 43% from the crude WAS (Huang *et al.* 2019), suggesting a great choice for the enhanced WAS fermentation in industrial applications. However, the complex effects of temperature on VFAs accumulation and the associated microbial community in this riboflavin-mediated system were still not clarified. This study aims (1) to evaluate the feasibility of effective VFAs production from riboflavin-assisted WAS fermentation system under relative lower temperature; (2) and to reveal the shifts in microbial communities responsible for the fermentation; (3) to provide new insights into the energy-saving method for VFAs production from WAS.

MATERIALS AND METHODS

Chemicals and WAS source

Analytical grade riboflavin (CAS# 83-88-5) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Before adding into the fermentation system, it was first prepared as a 50 mM stock in NaOH solution. To prepare a macro-metal stock, 10.15 g of $MgCl_2{\cdot}6H_2O$, 7.35 g of $CaCl_2{\cdot}2H_2O$, and 1.14 g of $FeSO_4{\cdot}7H_2O$ were fully dissolved into a 500 mL flask. In addition, to obtain the trace metal stock, 0.25 g of $ZnCl_2{\cdot}7H_2O$ and H_3BO_3, 0.2 g of $MnCl_2{\cdot}7H_2O$, 0.5 g of $CoCl_2{\cdot}6H_2O$, 0.025 g of $CuCl_2{\cdot}7H_2O$, $NiCl_2{\cdot}6H_2O$, $Na_2SeO_3{\cdot}7H_2O$ and $Na_2WO_4{\cdot}2H_2O$, and 0.1 g of $Na_2MoO_4{\cdot}2H_2O$ were dissolved into a 500 mL solution. All the stocks were prepared with deionized water.

The WAS used in this study was originally from a secondary sedimentation tank at the Qige sewage treatment plant in Hangzhou, China. After 24 hours of gravity sedimentation at 4 °C in a refrigerator, the supernatant was discharged and

the concentrated sludge with solid contents of 30 g MLSS/L and 16.8 g mixed liquor volatile suspended solids (MLVSS)/L was used as the fermentation substrate. Physicochemical parameters of the supernatant were as follows: pH 7.5, soluble chemical oxygen demand (COD) 24.0 mg/L, ammonia (NH_4^+-N) 2.2 mg/L, soluble carbohydrate 6.8 mg/L, soluble protein 1.2 mg/L, VFAs were not detected. The inoculated sludge was obtained from an upflow anaerobic sludge blanket (UASB) reactor in Hangzhou STARPRO Starch Co. Ltd (Hangzhou, China), and was placed in a refrigerator at 4 °C before use. The UASB reactor was designed to pretreat the starch wastewater, and the operating temperature was ranged from 35 °C to 40 °C.

Experimental set-up

The experiments were conducted in double-layer Plexiglas anaerobic reactors with the working volume of 5 liters. Three temperature series, i.e., room temperature (T1, 25 °C), mesophilic condition (T2, 35 °C), and thermophilic condition (T3, 55 °C), were set up with continuously stirring (90 RPM) reactors. The temperature was maintained by water bath in the interlayer around the Plexiglas cylinder. Before the fermentation experiment, 100 mL of stocked riboflavin, 5 mL of macro-metal and micro-metal stocks, and 50 mL of anaerobic inoculum were mixed with 5 L of concentrated WAS in the reactors. Thus, the initial VSS in the designed reactors was 16.7 g/L, and the added riboflavin was 1.0 ± 0.05 mM. The initial pH in the designed reactors was 7.5 ± 0.2 without any adjustment during the entire fermentation period (30 days). Inititally, the reactors were purged for 2 hours with high purity nitrogen gas. At different time intervals on days 0, 1, 3, 5, 8, 12, 16, 20, 25, and 30, defined as D0, D1, D3, D5, D8, D12, D16, D20, D25 and D30, 10 mL of the mixture in the reactors were sampled in triplicate for the subsequent analysis.

Chemical analysis

The collected mixture was immediately centrifuged (8,000 rpm, 20 min) after sampling, and the supernatant was used for the analysis of cumulative substances. The observed soluble COD, SS, VSS and NH_4^+-N were measured according to Standard Methods (APHA/AWWA/WEF 2005). VFAs with the components of acetic acid, propionic acid, (*n*- and *iso*)-butyric acid, (*n*- and *iso*)-valeric acid, were detected using an Agilent 1200 HPLC system equipped with an ultraviolet (UV) light detector and two connected columns Shodex RSpak KC-G and KC-811. For separating VFAs components, 0.05% H_3PO_4 solution with a flow rate of 0.7 mL/min was used as the mobile phase, and the temperature was set at 55 °C. The wavelength for detection was 210 nm. After qualification, to normalize the concentrations of different VFAs components, acetic acid, propionic acid, butyric acid, and valeric acid were converted into COD equivalents with the coefficients of 1.067, 1.512, 1.818 and 2.039, respectively.

Microbial community

After total DNA extraction from the liquid mixtures in different reactors at the end of the operation, target genomic 16S rRNA (V4 region) was then amplified by polymerase chain reaction (PCR), purified by gel recovery kit (Axygen), and quantified by microplate reader (BioTek, FLx800). The PCR primers were 520F (5′-AYTGGGYDTAAAGNG-3′) and 802R (5′-TACNVGGGTATCTAATCC-3′).

Microbial communities, diversities and their abundances associated with each designed fermentation system were analyzed by high throughput sequencing, using the Illumina® MiSeq platform. The sequencing service was provided by Personal Biotechnology Co., Ltd, Shanghai, China. After sequencing, the sequences (>150 bp) were clustered into operational taxonomic units (OTUs) at 97% similarity level using QIIME software (Version 1.8.0, http://qiime.org), which was referred on the Greengenes database (Release 13.8, http://greengenes.secondgenome.com). Afterward, α-diversity index including richness estimators of Chao1 and ACE, and evenness indexes that is Shannon–Wiener and Simpson indexes were calculated. Furthermore, taxonomic classifications at phylum and genus levels were constructed, and the specific components within each reactor under different temperatures at each classification level were obtained. To distinguish the community abundance of taxons or the similarity between samples at different classification levels, the above results were clustered and visually presented by heat map using R statistical software (Version 4.0.2).

Statistical analysis

One-way analysis of variance (ANOVA) was used to compare the difference in COD, NH_4^+-N, and VFAs accumulation between different reactors under varied operating temperatures, and $p < 0.05$ was recognized as statistically significant. Redundancy analysis (RDA) was conducted using the CANOCO 5 program to reveal the relationships between microbial communities (diversity and richness indexes) and environment variables (temperature, riboflavin, COD, VFAs, NH_4^+-N).

RESULTS AND DISCUSSION

Release of organics and ammonia

Soluble COD and ammonia (NH_4^+-N) were monitored as key parameters that could provide insights into the WAS fermentation. The higher the COD concentration in the supernatant, the more organic matter was released from the solid WAS (Im *et al.* 2016). This showed that COD concentrations varied with the fermentation temperature and time in the riboflavin-assisted system (Figure 1). Soluble COD in the thermophilic system (T3) was always higher than those in lower temperature systems (T1 and T2) ($p < 0.05$), and the time for achieving ceiling COD was the shortest in T2 (12 days) as compared to those in T1 (20 days) and T3 (16 days). Under thermophilic conditions (T3, 55 °C), soluble COD increased from the initial 3,799 ± 146 mg/L on D1 to the peak 9,770 ± 379 mg/L on D16; while that under room temperature (T1, 25 °C) was from 2,371 ± 91 mg/L on D1 to 7,044 ± 272 mg/L on D20. However, the maximum COD accumulation was in the mesophilic system (T2), which was up to 11,804 ± 458 mg/L on D12, and then it decreased with the ongoing fermentation. In this case, the maximum solubilization of sludge cell ($C_5H_7NO_2$) was calculated at 49.5%, which was about 20% higher than those with alkalic or microwave pretreatments, but was comparable with combined microwave–alkalic pretreatment (up to 46%) (Chang *et al.* 2011).

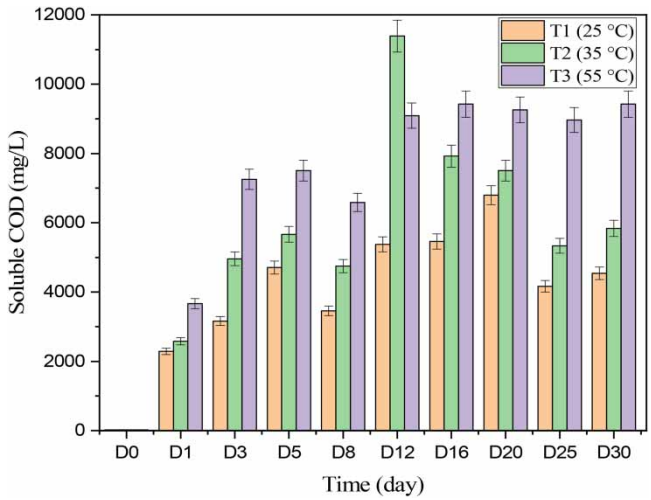


Figure 1 | Accumulation of soluble COD in riboflavin-assisted WAS fermentation systems at different temperatures. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wst.2022.020.

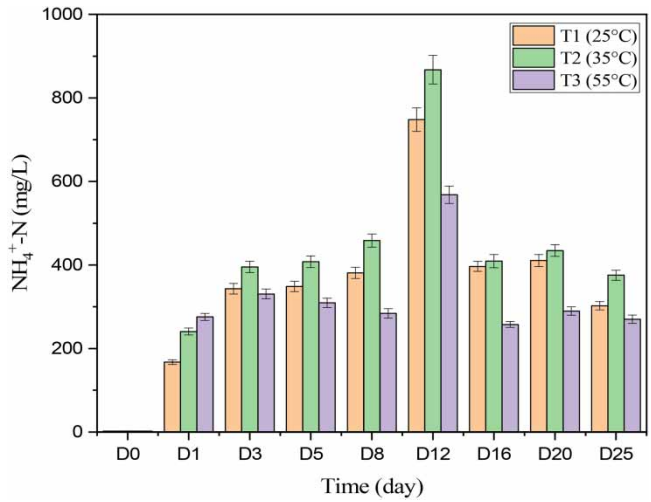


Figure 2 | Ammonia concentrations in riboflavin-assisted WAS fermentation systems at different temperatures. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wst.2022.020.

Interestingly, it was found that NH_4^+-N concentrations in T3 were always lower than found in the other two systems ($p < 0.05$), but the maximum NH_4^+-N concentration (899 ± 34 mg/L) was also observed on D12 in T2 (Figure 2), resulting in the maximum nitrogen release (44.6%) from sludge cells ($C_5H_7NO_2$). The release of NH_4^+-N is an indicator for the degradation of proteins associated with WAS (Chen *et al.* 2019). It has been reported that NH_4^+-N concentrations in the fermentation liquid were positively related to protease activity and protein degradation (Jiang *et al.* 2019). Thus, although more organics were released from solid WAS in T3, the hydrolysis of proteins in T1 and T2 was more effective. The results suggest that temperature might not be the only factor impacting the organics and nitrogen release from the WAS. Our previous study had indicated that although net COD accumulations in riboflavin-assisted systems declined by 35% as compared with riboflavin-free control, the release of protein and its hydrolysis increased by 27 and 82%, respectively (Shi *et al.* 2020). Thus, under lower temperature such as in room temperatures and mesophilic conditions, the external addition of riboflavin increased the hydrolysis of protein from WAS, converting more organic matters to bioavailable substrates for the subsequent acidification process. This could potentially shorten the WAS fermentation process for VFAs production, and save the energy input as well.

VFAs accumulation and their composition

Under different temperature conditions, the VFAs accumulation in riboflavin-added systems varied upon the fermentation time in each system (Figure 3). The ceiling VFAs concentrations in T1, T2 and T3 were 5,215.3 mg/L (D8), 5,432.9 mg/L (D5) and 3,181.0 mg/L (D5), respectively. Afterward, the net VFAs accumulation declined. Despite the relatively lower soluble COD levels under room temperature and mesophilic systems, the highest VFAs accumulations in T1 and T2 were 1.6-fold and 1.7-fold higher than that under the thermophilic system (T3). This is different from previous studies with WAS as a fermentation substrate, which suggested that higher temperatures (50 °C) favored the VFAs production by up to several folds (Huang *et al.* 2021). Thus, riboflavin could promote VFAs production from WAS fermentation under relatively lower temperatures. This benefit could be due to the following reasons.

The net VFAs accumulation in the fermentation system was the dynamic balance of its generation from hydrolyzed substrates and consumption for the followed methanogenic process. On the one hand, extracellular hydrolytic enzymes for WAS fermentation were at a stronger level under thermophilic conditions (Kim *et al.* 2012), resulting in more organics released as compared to those in T1 and T2, especially during the latter stage (Figure 1). On the other hand, the higher temperature was reported to induce stronger methanogenic activity (Guo *et al.* 2014), triggering more VFAs consumption as substrate. In this study, the results of higher soluble COD but lower VFAs accumulation in T3 (Figures 1 and 3) suggested the possible inhibition of high temperatures on the acidification process. Previous studies had verified that redox mediators could inhibit the methanation process, but this only occurred under high concentrations such as 20 mM (Liu *et al.* 2015; Huang *et al.* 2019). Thus, the methanogenic process in T3 under high temperatures was little affected by the small dosage of riboflavin

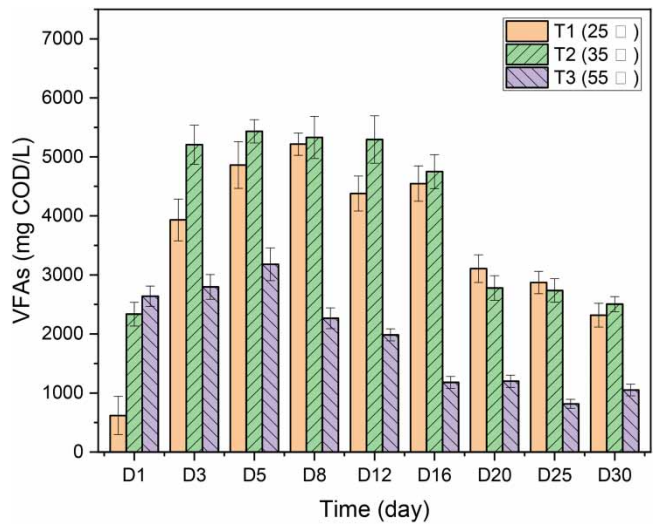


Figure 3 | Total VFAs accumulation in riboflavin-assisted fermentation systems upon different temperatures. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wst.2022.020.

(1.0 ± 0.05 mM) in this study. Moreover, it was observed that the COD fractions of VFAs (mg COD/L) decreased with the increase in fermentative temperature. In most systems, the maximum VFAs accumulation was achieved on day 5, and the COD fraction was calculated at 99, 92 and 40% (Figures 1 and 3), respectively. Afterward, this fraction in the three reactors all decreased with the fermentation time, and those in T3 were much lower than in T1 and T2. At the end of the fermentation, VFAs (mg COD/L) accounted for a total soluble COD of 49% in T1, 41% in T2, and only 10% in T3. Thus, under room temperature and mesophilic conditions, the presence of riboflavin could prevent the electron sink in the methanogenic process. This retained most chemical energy as stored by VFAs in the liquid phase but not been by methane in the gas phase. A previous study had indicated that lower temperature was not feasible for VFAs generation due to the low rates of hydrolysis and methanation (Yuan *et al.* 2011). However, the VFAs accumulation in this study was even higher with the small dosage of riboflavin (1.0 ± 0.05 mM), indicating the positive effect of riboflavin on WAS fermentation at lower temperatures.

The previous study had suggested that riboflavin could improve the valeric acid production in the WAS fermentation process and shift the degradation pathways of protein (Huang *et al.* 2019). However, the impact of temperature on the distribution of individual VFAs abundance was not investigated. In this study, the distribution of individual VFA constituents, including acetic acid, propionic acid, butyric acid and valeric acid in response to temperature are shown in Figure 4. Obviously, it was noted that the VFA constituent in T3 was mainly valeric acid; however, the COD fractions of acetic acid and propionic acid, which were the most efficient carbon sources for de-nitrification and phosphorous removal during wastewater treatment, were the lowest in

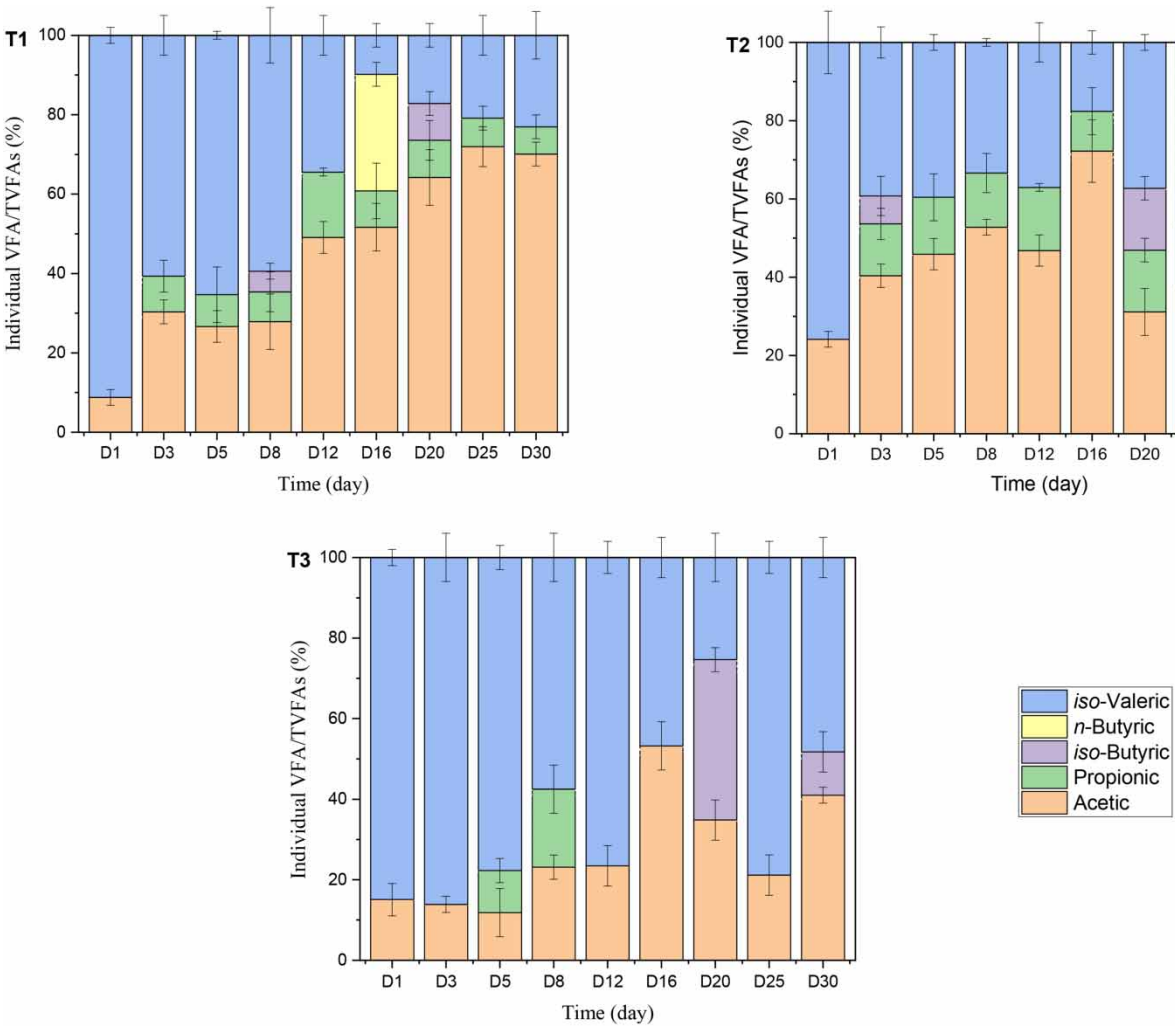


Figure 4 | VFAs components and their distribution in each riboflavin-assisted WAS fermentation system under room temperature (T1, 25 °C), mesophilic conditions (T2, 35 °C) and thermophilic conditions (T3, 55 °C). Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wst.2022.020.

T3. In T1, the maximum abundance of C2 and C3 VFAs (acetic acid + propionic acid) accounted for 79% on Day 25, with the concentration of 2,872 mg COD/L, i.e., 172 mg/g of VSS. In T2, the maximum abundance of C2 and C3 VFAs accounted for 82% on Day 16, with the highest accumulation of 3,915 mg COD/L, i.e., 234 mg/g of VSS. In T3, this maximum abundance only accounted for 53% on Day 16, with concentrations of 962 mg COD/L, i.e., 58 mg/g of VSS. The above results were similar to those of previous studies, reporting that the observed yield of acetic acid from crude WAS under 35 °C was ~1.5-fold of that under 55 °C (Komemoto *et al.* 2009; Zhang *et al.* 2019). This decreased trend at higher temperature might be attributed to the lower abundance of fermentative bacteria (*Firmicutes*) (see Figure 5 in the following section), and the more activated methanogenic process (Ding *et al.* 2017). This resulted in less accumulation of C2 and C3 VFAs in T3.

The observed concentrations of acetic acid and propionic acid and their proportions to total VFAs in riboflavin-added systems, and those in riboflavin-free systems are compared in Table 1. Compared with other studies in riboflavin-free systems, riboflavin supplement in this study increased the production of acetic acid and propionic acid by up to 167% at 25 °C and by up to 159% at 35 °C, respectively; in addition, the proportions of these two VFAs individuals increased as well. Under thermophilic conditions (55 °C), although the addition of riboflavin finitely increased the net yield of acetic acid and propionic acid, it significantly increased their proportion. Thus, the addition of riboflavin could promote the accumulation of small molecule VFAs during the WAS fermentation process, which was an eco-friendly and promising method for the carbon-neutral operation for MWTPs.

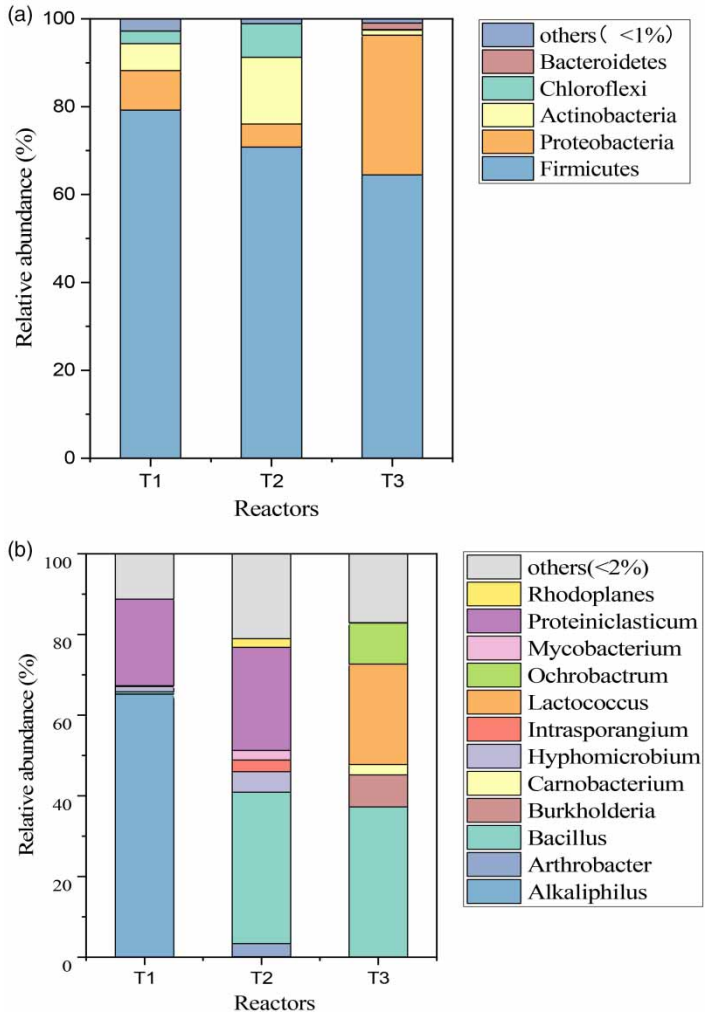


Figure 5 | Microbial communities at phylum level (a) and genus (b) level. Relative abundance lower than 1% (phylum) or 2% (genus) were grouped as 'others'. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wst.2022.020.

Table 1 | C2 and C3 VFAs production from various WAS fermentation systems

Temperature (°C)	Maximum C2 and C3 VFAs (mg COD/g VSS)	Proportion of C2 and C3 VFAs (%)	References
25	64	40%	Yu & Fang (2003)
35	91	45%	Zhang *et al.* (2019)
35	206	30%	Komemoto *et al.* (2009)
55	61	12%	Komemoto *et al.* (2009)
25	172	79%	This study
35	234	82%	This study
55	58	53%	This study

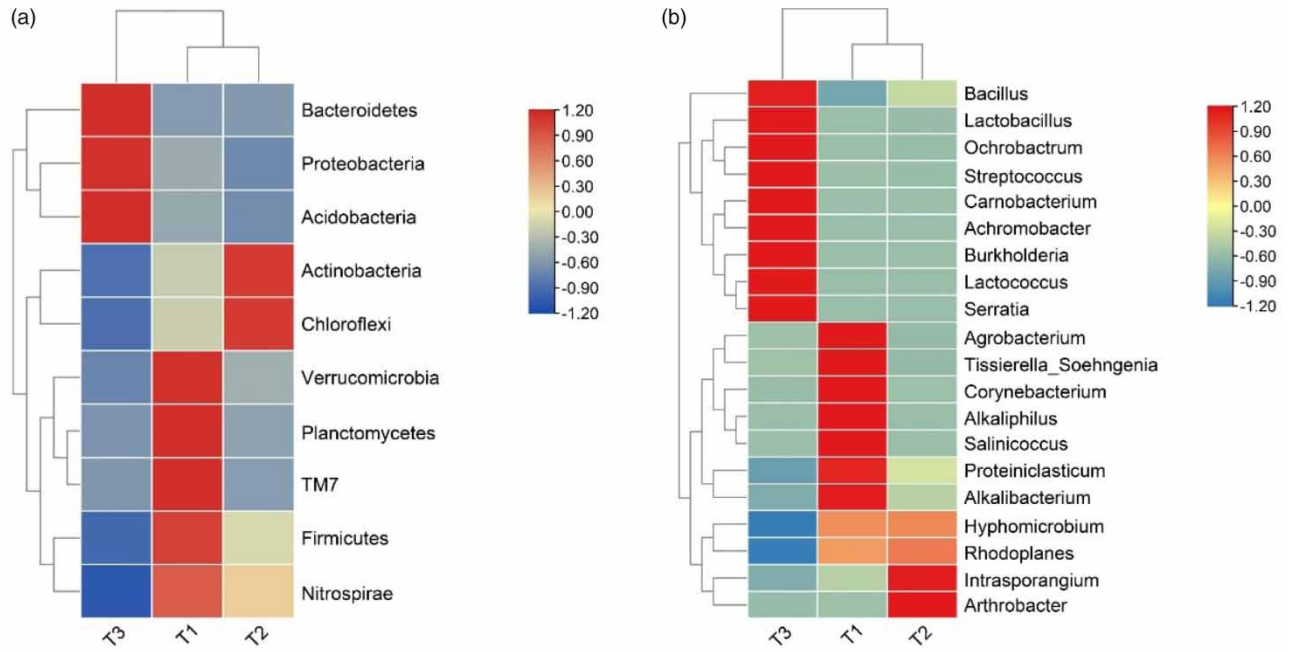


Figure 6 | Relative abundance heatmap and their clustering of the top 10 microbial genera at phylum level (a) and top 20 genera at genus level (b). Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wst.2022.020.

Microbial community in response to temperature

The microbial structures, including their diversity and relative abundance, were analyzed in response to the temperature (Figure 5). To provide more information on the microbial community, the relative abundance heatmap and their clustering of the top 10 microbial genera at phylum level and top 20 genera at genus level were analyzed in Figure 6(a) and 6(b).

The 16S rRNA sequencing results indicated that the richness estimators of OTUs in T3 (2419) were only about a half of those in T1 (4667) and T2 (4831); and the ACE index and Chao 1 index in T3 were also much lower (Table 2). This indicated that the bacterial richness in thermophilic fermentation was lower than that at room temperature and mesophilic conditions. Moreover, the Shannon index and Simpson index in T2 beyond that in T3, indicated higher microbial diversity under mesophilic conditions. A quantitative correlation between microbial communities and environment variables by RDA indicated that temperature and riboflavin both affected the microbial diversity and richness (Figure 7). The richness indexes of OTUs, Chao 1 and ACE were positively correlated with riboflavin but negatively with temperature. However, the diversity indexes of Shannon and Simpson showed a different correlation. Riboflavin could reduce the activation energies and allow the microbes to exist in a more disordered ground state (D'Amico *et al.* 2006; Paredes *et al.* 2011), stimulating microbial diversity and richness in the fermentation system (Huang *et al.* 2019). However, higher temperatures such as 55 °C in this study would cease this stimulation, resulting in lower acidification enzymatic activity for thermophilic fermentation as

Table 2 | Indexes for microbial communities in riboflavin-assisted WAS fermentation systems upon different temperatures

Sample	T1 (25 °C)	T2 (35 °C)	T3 (55 °C)
Sequencing reads	63,648	53,634	63,256
Operational taxonomic units (OTUs)	4,667	4,831	2,419
Shannon index	4.39	5.61	4.43
ACE index	1,483.87	1,580.07	583.64
Chao1	1,363.20	1,562.32	574.00
Simpson index	0.76	0.89	0.88

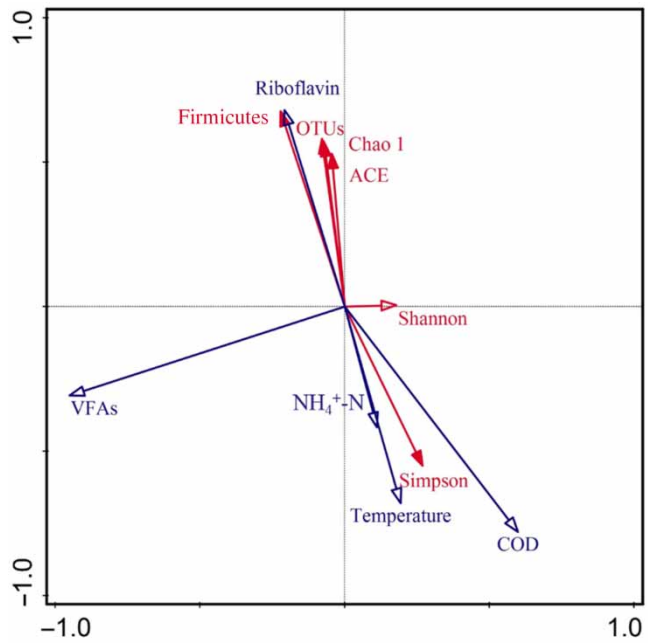


Figure 7 | Quantitative correlation between indexes for microbial communities (Firmicutes abundance, OTUs, Shannon index, Simpson index, ACE and Chao 1 index) and environment variables (temperature, riboflavin, COD, VFAs, NH_4^+-N). Correlation analysis was conducted by redundancy analysis (RDA). Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wst.2022.020.

compared to that for mesophilic fermentation at 35 °C (Zhuo *et al.* 2012). Thus, riboflavin supplement in this study would change the microbial communities associated with WAS fermentation systems at different temperature.

Under exposure to riboflavin (1.0 ± 0.05 mM), the relative bacterial abundance in T1, T2 and T3 at phylum and genus levels are shown in Figure 5(a) and 5(b). At phylum level, the main bacterial phyla (top 5) were Firmicutes (64.5–79.2%), Proteobacteria (5.3–32%), Actinobacteria (1.1–15.2%), Chloroflexi (0.2–7.7%), and Bacteroidetes (0–1.5%). Obviously, the abundance of Firmicutes trended to decrease from T1 to T3 along with the fermentation temperature (Figure 5(a)). The heatmap and clustering results showed that the color in T3 changed dramatically from that in T1 and T2; especially between T1 and T3, and was always from blue to red, indicating that the bacterial abundance at the phylum level was quite different among T1, T2 and T3. Furthermore, the bacterial structure in T1 and T2 was clustered into one group, which was far away from T3 (Figure 6). Phyla Firmicutes is vitally responsible for the extracellular hydrolysis enzymes, thus enhancing the VFAs production (Zhi *et al.* 2019). Our previous study indicated that riboflavin could increase the protease activities (Huang *et al.* 2019). RDA analysis from Figure 7 implied that Firmicutes abundance was positively correlated with riboflavin, but was negatively correlated with temperature and concentrations of NH_4^+-N and COD. To some extent, VFAs accumulation would also reduce the abundance of Firmicutes, which was the important bacteria phyla for acidification process. Thus, higher temperature might hinder the promotive effect of riboflavin on acidification process, reducing the VFAs accumulation in T3. This additionally explained the possible inhibition of high temperature on WAS fermentation in this study.

At the genus level, the bacterial structure associated with each system was quite different (Figure 5(b)). The top three microorganisms and their relative abundances were: *Alkaliphilus* (65.2%), *Proteiniclasticum* (21.5%) and *Hyphomicrobium* (1.3%) in T1; *Bacillus* (37.5%), *Proteiniclasticum* (25.5%) and *Hyphomicrobium* (5.1%) in T2; and *Bacillus* (37.2%), *Lactococcus* (24.9%) and *Ochrobactrum* (10.2%) in T3. Interestingly, an important genus of *Proteiniclasticum* with relative abundance of nearly a quarter in T1 and T2, disappeared in T3; while it was replaced by genera *Lactococcus* and *Burkholderia* (7.9%). In addition, the dominant bacteria of *Alkaliphilus* under room temperature also disappeared in the other two systems. Except for two genera (*Intrasporangium* and *Arthrobacter*) with small abundance of less than 2%, the relative abundance of most microorganisms in T1 was significantly different from that in T3 (Figure 6(b)). Interestingly, some genera shared similar abundance between the two systems but some others were quite different. As compared to the dominant microorganisms in T1 and T2, the relative abundance of *Proteiniclasticum* was similar, while those of *Alkaliphilus* and *Bacillus* were markedly different. As compared to the relative abundance in T2 and T3, the genus *Bacillus* shared similar fractions, but those of *Proteiniclasticum* and *Lactococcus* were very different. Furthermore, the bacterial structure at genus level in T1 and T2 was also clustered into one group, which was far away from T3.

The above results showed a significant shift of microbial community in response to temperature in riboflavin-assisted WAS fermentation systems. The supplementation of riboflavin is an eco-friendly option to promote the VFAs production from crude WAS under relative lower temperatures by stimulating special fermenters. In future work, the structure and diversity of the Archaea community were highly expected to be investigated to better understand the complex effects of riboflavin and temperature on methanogenic processes and the VFAs accumulation from WAS fermentation.

CONCLUSIONS

Riboflavin enhanced VFAs production from WAS fermentation under room temperature and mesophilic conditions, increasing the valid hydrolysis and conversion of organic matters to bioavailable substrates for the subsequent acidification process. In this study, although little dosage of riboflavin (1.0 ± 0.05 mM) hardly inhibited the methanogenic process, it could mediate the electron to be sank into VFAs in the liquid phase under lower temperature. In this condition, the accumulation of acetic and propionic acids (up to 234 mg/g of VSS) and their proportions to the total VFAs increased significantly, providing efficient electron donor and carbon sources for nutrient removal in MWTPs. Furthermore, microbial communities were dependent on the temperature, and riboflavin stimulated the special bacteria for VFAs production under lower temperatures (25 °C and 35 °C) in the WAS fermentation system.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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