Riboflavin Boosts Fermentative Valeric Acid Generation from Waste Activated Sludge

Binfang Shi,[a] Jingang Huang,[a,c,*] Zhenjiang Yin,[b] Wei Han,[a,c] Shanshan Qiu,[a,c] Junhong Tang,[a] and Pingzhi Hou [c]

Fermentative valeric acid production is a promising way to recycle valuable resources from waste activated sludge (WAS). This study investigated the feasibility of using riboflavin (RF) to enhance volatile fatty acids (VFAs) production, especially valeric acid production from WAS coupled with solid reduction. The results indicated that RF (0.5 mM) promoted the VFAs production by up to 41.0%. Valeric acid accounted for the most abundance within the VFAs components. When RF dosages were 0.05 to 5.0 mM in the WAS fermentation systems, the chemical oxygen demand fractions of valeric acid to the total VFAs were 41.0% to 62.8%, which were much higher than those using other chemical supplements. Moreover, RF enhanced the reduction of mixed liquor volatile suspended solids (MLVSS). When RF dosage was 0.2 mM, MLVSS reduction achieved a maximum at 47.4%, compared to that in the RF-free control (33.9% reduction). Riboflavin in this study was considered as a feasible chemical to enhance the fermentative valeric acid generation coupled to MLVSS reduction, realizing the reduction of solids and the reutilization of valuable resources from WAS.

Keywords: Riboflavin; Waste activated sludge; Fermentation; Volatile fatty acid (VFA); Valeric acid

Contact information: a: College of Materials and Environmental Science, Hangzhou Dianzi University, Hangzhou 310018, PR China; b: Binzhou University, Binzhou 256600, PR China; c: The Belt and Road Information Research Institute, Hangzhou Dianzi University, Hangzhou 310018, PR China;
** Corresponding author: hjg@hdu.edu.cn*

INTRODUCTION

Waste activated sludge (WAS) contains a large amount of organic matter including protein, carbohydrate, and so on, which can be converted into volatile fatty acids (VFAs) through fermentation treatment. VFAs are important intermediates during the fermentation/ digestion processes. They are mainly composed of six short-chain fatty acids: acetic acid, propionic acid, *n*- and *iso*-butyric acid, and *n*- and *iso*-valeric acid. Volatile fatty acids not only can serve as a supplementary carbon source for nitrogen and phosphorus removal in sewage treatment (Feng *et al.* 2009a; Li *et al.* 2011), but they can also be used as raw materials for the synthesis of biodegradable plastics such as polyhydroxyalkanoates (PHAs) (Cai *et al.* 2009; Chen *et al.* 2013). Therefore, fermentative VFAs generation is an attractive strategy to reuse the WAS solids (Li *et al.* 2011; Yin *et al.* 2019). Due to the difficulty in breaking down the crosslinked polymers in cell walls, pretreatments, including mechanical, ultrasonic, microwave, heating, and alkalic methods, have been applied to improve the hydrolysis rate (He *et al.* 2011; Yang *et al.* 2013; Li *et al.* 2018; Stachowiak-Wencek *et al.* 2019). However, the large consumption of energy and chemical supplements limited their industrial applications.

Among the VFAs components, valeric acid has the potential usage for industrial applications in producing biofuels and chemical intermediates such as flavoring/fragrance agents, plasticizers, and bio-solvents (Bhanuchander *et al.* 2019; Ganigue *et al.* 2019). Additionally, Hao *et al.* (2016) reported that valeric acid-dominant hydrolysate was a more

efficient substrate for PHAs synthesis. Thus, enhancing the valeric acid fraction in VFAs has a positive impact on the fermentative reutilization of WAS.

Riboflavin (RF) is one of the necessary co-enzymes for biological metabolism. It has been reported as a good candidate to accelerate the hydrolysis processes and fermentative VFAs generation from protein-rich substrate of WAS without any pretreatment (Huang *et al.* 2019). However, its further impact on valeric acid generation upon different RF dosages has not been illustrated in detail. In this study, the effect of RF dosage on VFAs production, suspended solid (SS) reduction, and valeric acid fraction within VFAs components was further investigated. The current study aims to provide some new insights into RF's effect on the valeric acid production from raw WAS.

EXPERIMENTAL

Materials

Sources of WAS and chemical riboflavin

The WAS was originally sampled from a sludge thickener, which followed a secondary sedimentation tank, in Qige Sewage Treatment Plant in Hangzhou, China. Impurities in WAS were immediately removed using a 1-mm screen, then washed with deionized water three times. After that, WAS was settled for 24 h at 4 °C in a refrigerator. Then, the supernatant was removed to obtain the raw WAS for the following study. The mixed liquor volatile suspended solids (MLVSS) of this prepared WAS was 27.1 g/L.

Riboflavin (RF, $C_{17}H_{20}N_4O_6$,) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Riboflavin (18.818 g) was dissolved into 1.0 L of alkalic-adjusted deionized water to obtain a stocked RF solution with a concentration of 50 mM.

Experimental Set-up

Nine experimental series with different RF dosages were set up, *i.e.*, 0.0, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0, and 5.0 mM, respectively. A dosage at 0.0 mM was set as a control without RF supplement. All experimental series were conducted in 250-mL serum bottles. In each serum bottle, 123.6 mL of prepared raw WAS, 50 mL of $NaHCO_3$-Na_2CO_3 buffer solution (0.3 M in stock, pH 9.6), and the designed RF dosage were added. Additionally, $MgCl_2 \cdot 6H_2O$ (40.6 mg/L), $CaCl_2 \cdot 2H_2O$ (29.4 mg/L), $FeSO_4 \cdot 7H_2O$ (4.56 mg/L), NH_4Cl (150 mg/L), KH_2PO_4 (30 mg/L), and stocked trace metals and vitamins (1.0 mL/L) were added according to the previous publication (Huang *et al.* 2015a). Then, deionized water was supplemented to the final volume of 250 mL. Thus the MLVSS in the fermentation system was 13.4 g/L. The remaining oxygen in the mixture was flushed with high purity nitrogen to ensure that the fermentation system was in an anaerobic condition. Finally, all serum bottles were placed in an air-bath shaker (LHS-250SC; Yiheng Scientific Instruments Co. Ltd., Shanghai, China) (at 35 °C, 150 RPM) for 21 days fermentation.–

Chemical Analysis

After 21 days of fermentation, mixtures were collected from the serum bottles and centrifugated at 8000 *g* for 20 min. The supernatant was used for the analysis of soluble chemical oxygen demand (COD), ammonia nitrogen (NH_4^+-N), and MLVSS, which were determined according to the Standard Methods (APHA/AWWA/WEF 2005). Total carbohydrate was measured by the anthrone–sulfuric acid colorimetric method with glucose as standard. Protein was determined according to the Bradford method with bovine serum albumin as the standard. Volatile fatty acid components, including acetic acid, propionic acid, butyric acid, and valeric acid, were measured *via* a high performance liquid chromatography unit (HPLC, Agilent 1200; Agilent Technologies, Santa Clara, CA, USA)

equipped with an ultraviolet (UV) detector at 210 nm. A Shodex RSpak KC-811 analytical column following a Shodex RSpak KC-G guard column was used. The mobile phase was 0.05% H_3PO_4 solution. The detailed analytical methods can be found in the previous report (Huang *et al.* 2015a). To make the results be more comparable, the concentrations of all individual VFAs were converted into the equivalent amounts of COD with special coefficients as following: acetic acid (1.067), propionic acid (1.512), butyric acid (1.818), and valeric acid (2.039).

Statistical Analysis

All experiments were conducted in triplicate, and the results were expressed as mean ± one standard deviation. Error bar means standard deviations of triplicate measurements. To evaluate the significance of results, IBM® SPSS® Statistics 25 was used and $P < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Riboflavin Enhanced Valeric Acid Production from WAS

During the acidification process of WAS, polysaccharides and proteins were first hydrolyzed to monosaccharides and amino acids, and then fermented to VFAs (He *et al.* 2011). With the supplement of different RF dosages, the concentrations of VFAs components with 2 to 5 carbon atoms, and the COD fraction of valeric acid are shown in Fig. 1. The ambient pH was kept at nearly 9 with a $NaHCO_3$-Na_2CO_3 buffer, which was suitable for the fermentation process (Feng *et al.* 2009b). The results indicated that VFAs accumulation first increased and was stabilized after RF dosage above 0.5 mM. In the RF-free control, the net accumulation of VFAs was 3376 ± 597 mg COD/L. In the series with different RF dosages from 0.05 mM to 5.0 mM, the net VFAs yields increased by 24.4%, 32.1%, 21.4%, 41.0%, 39.0%, 32.6%, 28.9%, and 39.4%, respectively. The results indicated that RF promoted the VFAs production from WAS fermentation. However, RF concentrations higher than 0.5 mM seem to make no remarkable impact on VFAs generation due to the limited role of higher RF on metabolism (Wang *et al.* 2015).

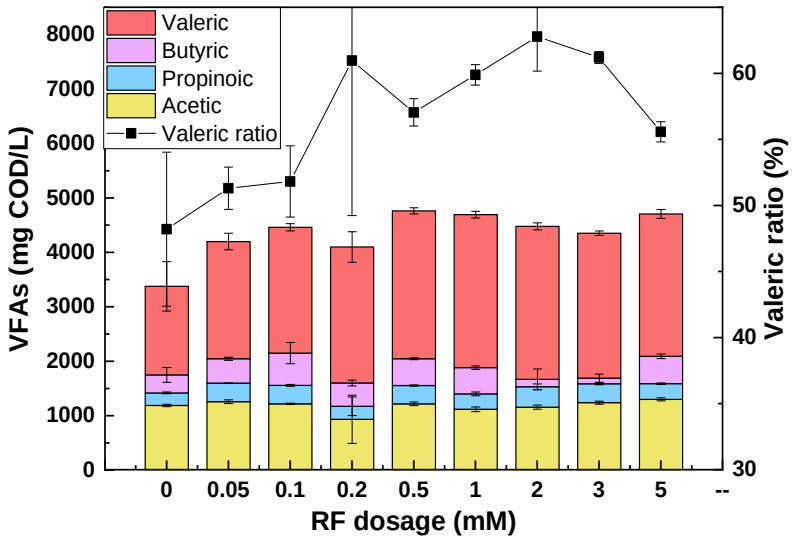

Fig. 1. Total VFAs generation, individual component distribution, and the COD fraction of valeric acid with different RF dosages

Furthermore, it was found that valeric acid was the dominant VFA component, followed by acetic acid. The COD fraction of valeric acid showed an overall increasing trend according to the RF dosage, and slightly decreased when the dosage was above 3 mM. The net productions of valeric acid with the RF addition (0.05 to 5.0 mM) were 1.32-, 1.42-, 1.54-, 1.67-, 1.73-, 1.73-, 1.64-, and 1.61-fold that of the control (1630 mg COD/L), respectively. Although acetic acid and propionic acid were always reported as the dominant fermentative products, RF promoted the valeric acid generation from WAS in this study. This might be attributed to the inhibition effect of RF on the methanogenic process (Huang *et al.* 2019), thermodynamically preventing higher-molecular-weight valeric acid from converting into lower-molecular-weight acetic acid and propionic acid. Moreover, under some conditions, the C2 (acetic acid) and C3 (propionic acid) VFAs would be formed into valeric acid through chain elongation (Ganigue *et al.* 2019).

In this study, soluble protein and the released NH_4^+-N from hydrolyzed amino acids were found as shown in Fig. 2. When compared to the RF-free control, soluble protein increased 26.7% with the supplied RF of 5.0 mM ($P < 0.05$), achieving the maximum concentration of 185 ± 12 mg/L (Fig. 2A). Meanwhile, the measured NH_4^+-N also increased up to 81.8% ($P < 0.05$), achieving 610 ± 44 mg/L (Fig. 2B). Thus, RF could promote the hydrolysis process of protein to amino acids, releasing more NH_4^+-N *via* oxidative/reductive deamination, and then promote the fermentative valeric acid generation. Rughoonundun *et al.* (2012) has also reported that the metabolic process of valeric acid production was related to the protein-dependent fermentation *via* reductive deamination of single amino acids or Stickland reaction.

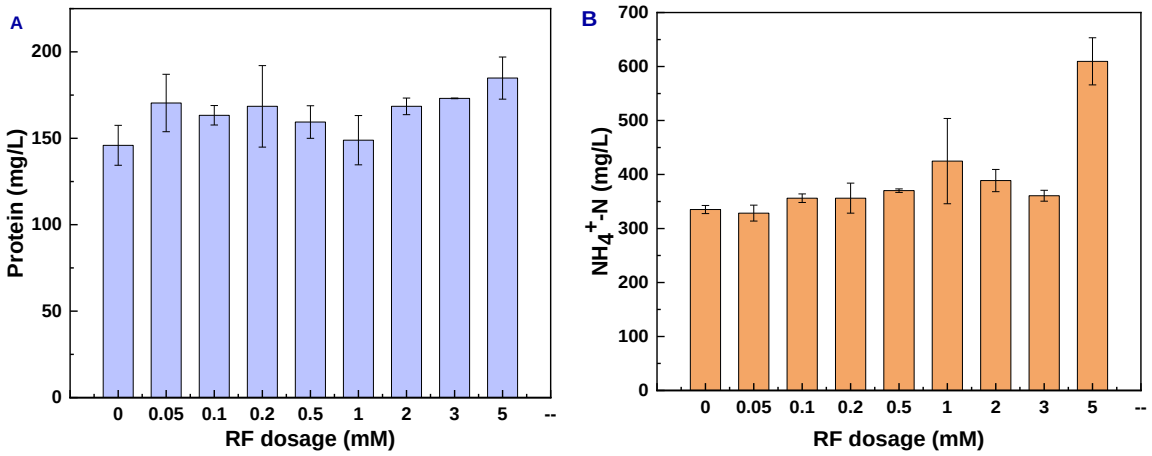

Fig. 2. The concentrations of soluble protein (A) and NH_4^+-N (B) with different RF dosages

The comparison of VFA production and COD fraction of valeric acid to the total VFAs upon different chemical supplements are shown in Table 1. It has been suggested that the total VFA generation ranged from 194 to 346 mg COD/g VSS; and the COD fraction of valeric acid ranged from 20% to 40% with the extra addition of some other chemicals, such as tetrakis hydroxymethyl phosphonium sulfate, sodium dodecylbenzene-sulfonate, nitrous (NO_2^--N), and saponin, to WAS fermentation systems. However, in this study RF supplement (0.05 to 5.0 mM) in the WAS fermentation resulted in a higher VFA concentration (355 mg COD/g VSS) and the COD fractions of valeric acid increasing up to 41.0% to 62.8%, which were much higher than others. Thus, RF could be used as a prospect exogenous chemical to produce valeric acid efficiently from WAS, enabling the recycle of WAS.

Table 1. Comparison of VFA production and COD Fraction of Valeric Acid Upon Different Chemical Supplies

Supplied Chemicals	Pretreatment	VFA production (mg COD/g VSS)	COD Fraction of Valeric Acid (%)	References
Tetrakis hydroxymethyl phosphonium sulfate	None	194	37.5	Wu *et al.* (2017)
Tetrakis hydroxymethyl phosphonium sulfate + Sodium dodecylbenzenesulfonate	None	346	< 20	Zhao *et al.* (2016)
Free nitrous acid (NO_2-N)	None	196	< 25	Li *et al.* (2016)
Saponin	None	292	< 40	Huang *et al.* (2015b)
Riboflavin	None	355	41.0 to 62.8	This study

Effect of Riboflavin on WAS Solid Reduction

To investigate the effect of different RF dosages on WAS solid reduction coupled to VFAs production, MLVSS values in each experimental series were measured at the end of fermentation (Fig. 3). It was observed that the MLVSS showed a wave-like trend upon treatment of different RF dosages. When the RF dosage was added at 0.2 mM, MLVSS achieved the minimum of 7.05 ± 1.64 g/L, and the solid reduction achieved the maximum at 47.4% because the original MLVSS of raw WAS was 13.4 g/L. Solid reduction with RF amended systems were much higher than that in the RF-free control (10.66 ± 1.63 g/L, a 33.9% reduction, $P < 0.05$). However, when RF dosage was added at 1, 2, and 3 mM, MLVSS in the fermentation liquid was increased, resulting in a sudden decline of COD to the minimum of 3058 ± 179 mg/L. This might have been attributed to the toxic inhibition of the fermentation process by higher RF concentration. Li *et al.* (2016) suggested that the destruction of cell membrane and extracellular polymeric substances (EPS) is closely related to the MLVSS reduction of WAS. Moreover, it was reported that the intracellular substance in WAS was negatively charged due to the ionization of functional groups, increasing the negative charges and forming a more stable colloidal system (Wang *et al.* 2018; Li *et al.* 2020). Thus, the greater negative charge of higher supplemented RF dosages will prevent the promotive bioreaction of protein dependent fermentation. Interestingly, it was found that RF concentration higher than 5.0 mM again reduced the MLVSS. The behind mechanism is not clear and need further study.

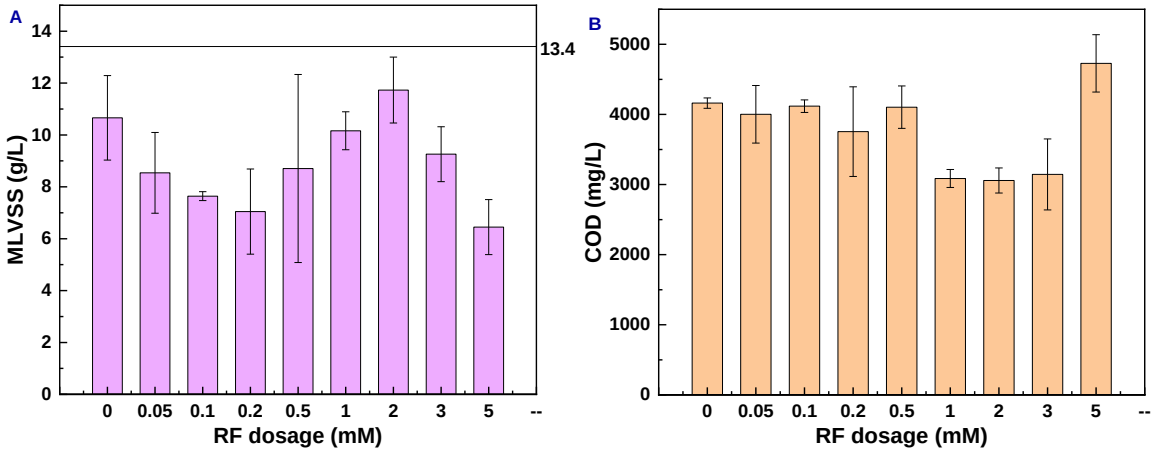

Fig. 3. The concentrations of MLVSS (A) and COD (B) upon different RF dosages

The economic benefit of this technology was calculated as follows. The cost of RF supplement was calculated at 0.015 USD/kg VSS, upon the optimum RF dosage of 0.2 mM and the industrial RF price of 2.5 USD/kg; while the return rate of valeric acid production was calculated at 0.067 USD/kg VSS, based on valeric acid yield of 0.274 g/g VSS and its price of 0.24 USD/kg. Therefore, RF supplement can be regarded as a cost-effective technology to enhance the valeric acid production from raw WAS.

CONCLUSIONS

1. Riboflavin (RF, 0.5 mM) promoted the VFAs production from WAS by up to 41.0%, compared to 24.4% in the RF-free control.
2. Valeric acid was the main VFAs component, and its COD fractions to the total VFAs were 41.0% to 62.8%, which were much higher than those with other chemical supplements.
3. Riboflavin enhanced the MLVSS reduction of WAS solids by up to 47.4%, while that in the RF-free control was only a 33.9% reduction.
4. Riboflavin can be regarded as a cost-effective chemical to enhance the fermentative valeric acid production from WAS coupled to solid reduction.

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