Optimization of VFAs and ethanol production with waste sludge used as the denitrification carbon source
Liang Guo, Jiawen Zhang, Li Yin, Yangguo Zhao, Mengchun Gao and Zonglian She

ABSTRACT
An acidification metabolite such as volatile fatty acids (VFAs) and ethanol could be used as denitrification carbon sources for solving the difficult problem of carbon source shortages and low nitrogen removal efficiency. A proper control of environmental factors could be essential for obtaining the optimal contents of VFAs and ethanol. In this study, suspended solids (SS), oxidation reduction potential (ORP) and shaking rate were chosen to investigate the interactive effects on VFAs and ethanol production with waste sludge. It was indicated that T-VFA yield could be enhanced at lower ORP and shaking rate. Changing the SS, ORP and shaking rate could influence the distribution of acetic, propionic, butyric, valeric acids and ethanol. The optimal conditions for VFAs and ethanol production used as a denitrification carbon source were predicted by analyzing response surface methodology (RSM).

INTRODUCTION
Waste sludge from wastewater treatment plants (WWTPs) contains considerable amounts of organic matter which could be converted into volatile fatty acids (VFAs) and other easily biodegradable organic polymers through anaerobic acidification. The VFAs and ethanol can be used as a carbon source for nitrogen and phosphorus removal to solve the difficult problem of carbon source shortages and the low nitrogen removal efficiency of denitrification (Ucisik & Henze 2004; Gao et al. 2011). However, the reliability of VFA generation is often not adequate, and it exits many factors that affect VFA production, including pH, temperature, stirring speed, solids retention time (SRT), sludge characteristics and the pretreatment process (Yuan et al. 2009). It was reported that VFA yields increased with the mixed liquor volatile suspended solids (MLVSS) in the range of 4.6–23.8 g/L (Xiong et al. 2012). Massive sludge flocs and cell walls could be broken by shaking and stirring steel beads, which could enhance the acidogenic fermentation process. It was investigated whether VFA yields increased with SRT from 5 to 10 days (Yuan et al. 2009). Sludge fermentation was highly temperature dependent, and the highest VFA production was achieved at 24.6 °C compared with 4 and 14 °C (Yuan et al. 2011). In addition, different combinations of pretreatment methods have been proved to be useful for promoting hydrolysis and acidification of waste sludge, such as sonolysis and ozonation (Cesaro & Belgiorno 2013), alkali-enhanced microwave and ultrasonic (Tyagi et al. 2014), wet air oxidation and thermal hydrolysis (Hii et al. 2014).

Furthermore, the diversity of VFAs could also influence utilization as organic carbon source. A proper control of environmental factors could be important for VFA optimization. It was found that propionic acid-enriched VFAs as a carbon source were more suitable for nitrate removal than acetic acid-enriched VFAs (Zheng et al. 2013; Chen et al. 2011). The pH value for VFA accumulation is widely studied by many researchers, which affects the production and type of VFAs in the fermentation (Lee et al. 2014; Wang et al. 2014; Yang et al. 2014). It is known that oxidation reduction potential (ORP) is closely associated with pH in solution, which is usually used to characterize the solution with oxidative or reductive state. The types of anaerobic fermentation and VFA compositions could be affected by ORP. Some studies found that propionic acid fermentation occurred more easily when the ORP was −200 to 100 mV,
and ORP of −350 to −200 mV was useful for ethanol fermentation (Kukec et al. 2002; Jeon & Park 2010).

Response surface methodology (RSM) is a collection of statistical techniques for designing multi-factorial experiments and building fitting models to evaluate the relative significance of several variables and to find the optimal condition for desirable responses. It has been widely used for optimization studies in many biotechnological and industrial processes (Karthic et al. 2013; Pham et al. 2015). In contrast, orthodox single-variable optimization methods are not only tedious but also can lead to misinterpretation of results, especially because the interaction between different factors is overlooked (Weuster 2000). Until recently, few studies were conducted for the interaction of suspended solids (SS), ORP and shaking rate on VFAs and ethanol production with waste sludge using RSM. The results obtained from this study are expected to provide valuable information for further utilization of VFAs and ethanol as carbon sources.

**MATERIALS AND METHODS**

**Characteristics of raw sludge and pretreatment**

Waste sludge used in this study was obtained from the secondary sedimentation tank of a municipal WWTP. Prior to use, waste sludge was sieved by grid size 2.0 mm to remove coarse matter, and then stored at 4°C. The main characteristics of raw sludge are presented in Table 1. Raw sludge was concentrated to different SS (8.0, 14.0 and 20.0 g/L) before the pretreatment process.

To enhance the VFA production, the sludge was solubilized by solubilization by thermophilic enzyme (S-TE) pretreatment. The organic substances in sludge cells could be released by both thermal and TE effect (Foladori et al. 2013). In this study, the sludge was inoculated with thermophilic bacteria (Bacillus sp. AT07-1, FJ231108) in the volume ratio of 1:20 (v/v) at 65°C for 12 h (Guo et al. 2015). The characteristics of S-TE pretreated sludge are shown in Table 2.

**Experimental design and procedure of anaerobic acidification**

Box-Behnken design under RSM was chosen to design the experiment, it has three levels (low, medium and high, coded as −1, 0 and 1) and needs fewer experiments. Box-Behnken design is more efficient and easier to arrange and interpret in comparison with central composite design and one factor design (Bosque et al. 2001). The interactive effects of SS, initial ORP and shaking rate on optimal VFAs and ethanol production were analyzed by Design Expert software (Version 8.0.6). A total of 17 runs were used to optimize the conditions of SS, ORP and shaking rate. The level and code of variables considered in this experiment are shown in Table 3. The experimental design is presented in Table 4.

The anaerobic acidification was conducted in a 250 mL conical flask containing 100 mL S-TE pretreated sludge. After the sludge was adjusted to the designed ORP value with 4 M FeCl₃, oxygen in the conical flask was removed from the headspace by sparging pure nitrogen gas for 2 min. The flask was sealed with a rubber stopper and then placed in a water-bath shaker at 35°C. The samples were taken every 2 h at each run. The obtained samples were immediately measured soluble chemical oxygen demand (SCOD), VFAs and ethanol.

**Analytical methods**

The obtained samples were centrifuged for the detection of VFAs and ethanol at 5,000 rpm for 10 min. The supernatant
was filtered through a 0.45 μm membrane and acidified by 3% hydrochloric acid to adjust pH below 3.0, and then transferred to a 1 mL gas chromatograph (GC) vial. The amount of VFAs, including ethanol, acetic, propionic, butyric and valeric acids were analyzed by GC (GC-2010, Shimadzu Co., Kyoto, Japan) equipped with a flame ionization detector and a capillary column (DB-FFAP, 30 m × 0.25 mm × 0.25 μm). The initial temperature of the GC column was 70 °C and it then increased by 10 °C/min to a final temperature of 180 °C, and was held for 5 min. Temperatures of the injection port and the detector were set at 250 °C and 300 °C, respectively, with nitrogen gas at 28 mL/min, hydrogen at 30 mL/min and air at 400 mL/min as carrier gases. The T-VFAs concentration was calculated by summing up individual acetic, propionic, butyric and valeric acids. The pH was measured with a digital pH-meter (PHB-5, Aolilong, Hangzhou, China). The ORP was monitored by an ORP-meter (ORP-260, Dapu, Shanghai, China). The concentrations of TCOD (total chemical oxygen demand) and SCOD were tested by a microwave digestion method (KDB-III Microwave Digestion System, Kedibo, Qingdao, China). SS and VSS (volatile suspended solids) were determined by the standard methods (He 1998).

### RESULTS AND DISCUSSION

#### Effect of SS, ORP and shaking rate on T-VFA yields

The T-VFA yields at different runs are shown in Figure 1. According to Table 4, 17 runs were divided into three groups based on the SS concentration. When the SS was 8.0 g/L (Figure 1(a)), T-VFA yields increased with time and the maximum concentration was achieved at 1,400.8 mg/L for 12 h with an initial ORP of 0 and a shaking rate of 60 r/min. It enhanced by 29-fold compared with the raw sludge or 3.6-fold S-TE pretreated sludge. As shown in Figure 1(b), the maximal T-VFA yield with 20.0 g/L SS was lower than that of 8 g/L SS, which might be due to low SS creating better mixing conditions and then improving the hydrolysis and acidification of organic matter (Yuan et al. 2009). The T-VFA production decreased in the first 2 h and then remained constant in the low range of 200–250 mg/L at an ORP of 200 mV and a shaking rate of 105 r/min, which were mainly caused by the inhibition of high ORP on the growth and activities of anaerobic bacteria during the acidification process. However, this general trend was not coincident with the SS of 14.0 mg/L. As shown in Figure 1 (c), T-VFA yields with 14.0 mg/L SS were all below 550 mg/L, which was less than that of 8.0 mg/L SS. It indicated that T-VFA yields did not necessarily increase with SS, which could also relate to the interaction of initial the ORP and shaking rate. In the conditions of 0 and 60 r/min, T-VFA yield was highest at both 8.0 mg/L SS and 20.0 mg/L SS. Meanwhile, T-VFA yield was lowest at the condition of 200 mV and 105 r/min. It was implied that when the SS was constant, the low shaking rate of 60 r/min was advantageous for VFA accumulation, and ORP higher than 200 mV could have an inhibiting effect.

#### Effect of SS, ORP and shaking rate on acetic acid production

The response surface contour plots and the model equations are analyzed using RSM to provide a distinct indication for

---

### Table 3  | Level and code of variables for Box-Behnken design

<table>
<thead>
<tr>
<th>Variables</th>
<th>Symbols</th>
<th>Coded levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS (g/L)</td>
<td>$X_1$</td>
<td>–1 1 20</td>
</tr>
<tr>
<td>Initial ORP (mV)</td>
<td>$X_2$</td>
<td>–200 0 200</td>
</tr>
<tr>
<td>Shaking rate (r/min)</td>
<td>$X_3$</td>
<td>60 105 150</td>
</tr>
</tbody>
</table>

### Table 4  | Box-Behnken experimental design of 17 runs

<table>
<thead>
<tr>
<th>Std</th>
<th>Coded $X_1$</th>
<th>Coded $X_2$</th>
<th>Coded $X_3$</th>
<th>SS ($X_1$)</th>
<th>Initial ORP ($X_2$)</th>
<th>Shaking rate ($X_3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>–1</td>
<td>–1</td>
<td>0</td>
<td>8</td>
<td>–200</td>
<td>105</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>–1</td>
<td>0</td>
<td>20</td>
<td>–200</td>
<td>105</td>
</tr>
<tr>
<td>3</td>
<td>–1</td>
<td>1</td>
<td>0</td>
<td>8</td>
<td>200</td>
<td>105</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>0</td>
<td>–1</td>
<td>8</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>–1</td>
<td>0</td>
<td>–1</td>
<td>20</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>0</td>
<td>–1</td>
<td>20</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>7</td>
<td>–1</td>
<td>0</td>
<td>1</td>
<td>8</td>
<td>0</td>
<td>150</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>20</td>
<td>0</td>
<td>150</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>–1</td>
<td>–1</td>
<td>14</td>
<td>–200</td>
<td>60</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>1</td>
<td>–1</td>
<td>14</td>
<td>200</td>
<td>60</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>–1</td>
<td>1</td>
<td>14</td>
<td>–200</td>
<td>150</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>14</td>
<td>200</td>
<td>150</td>
</tr>
<tr>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>105</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>105</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>105</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>105</td>
</tr>
<tr>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>0</td>
<td>105</td>
</tr>
</tbody>
</table>
understanding the interaction effects of SS, ORP and shaking rate on acetic acid production. Both linear and quadratic equations were used to model the acetic acid data obtained from the experiments. In addition, the significant models (2 h) of linear equation (Equation (1)) or maximal VFA yield (12 h) of the second order polynomial quadratic equation (Equation (2)) were selected for RSM analysis, which could explain the experimental data well.

\[
Y = 712.87437 + 10.28449X_1 - 0.91299X_2 - 5.87805X_3
\]

\[
Y = 3741.94322 - 308.62596X_1 - 1.12839X_2 - 23.30572X_3 + 0.025943X_1X_2 + 0.019009X_1X_3 + 0.000157X_2X_3 + 10.87452X_1^2 - 0.000365X_2^2 + 0.083466X_3^2
\]

where \( Y \) is the predicted acetic acid; \( X_1, X_2 \) and \( X_3 \) are the actual values of SS, initial ORP and shaking rate. Analysis of variance analysis of the fitting model with the \( P \)-value of <0.05 shows that the model is significant (Karthic et al. 2015). In this study, the Model \( F \)-value of 3.6 and the \( P \)-value of 0.0433 in Equation (1) implied the linear model was significant. As the maximal production of acetic acid appeared at 12 h, Equation (2) was used for the interactions of SS, initial ORP and shaking rate on acetic acid at this time.

The shape of the contour plots explains the presence of interactions. The higher the elliptical nature of the contour plot, the higher the significant effect on the response. The interactive effect is negligible if the contour shape is circular in appearance (Muralidhar et al. 2001; Mannan et al. 2007). In this study, the influence of SS, ORP and shaking rate on acetic acid with RSM is shown in Figure 2. Each RSM figure represented the effect of two independent factors. It was illustrated that the variables of SS and shaking rate had a strong interdependent and interaction effect on the

Figure 1 | T-VFA yields during the anaerobic acidification at different design runs (a) SS = 8 g/L; (b) SS = 20 g/L; (c) SS = 14 mg/L.
acetic acid. According to Equation (2), the predicted maximum of acetic acid was 937.1 mg/L at the condition of 8.0 g/L, −124.8 mV and 60 r/min. And it was close to the actual experimental maximum of 1,103.5 mg/L at SS 8.0 g/L, initial ORP 0 mV and shaking rate 60 r/min. As shown in Figure 2, the predicted maximum was 748.5 mg/L at the conditions of SS 20.0 g/L, initial ORP 200 mV and shaking rate 60 r/min. In addition, when the shaking rate was 60–75 r/min, acetic acid decreased with the increase of initial ORP (−200 to 100 mV). From the analysis above, it was seen that the acetic acid could be accumulated at a low initial ORP of −200 mV and a shaking rate of 60 r/min, which played an interaction role for enhancing acetic acid production.

Effect of SS, ORP and shaking rate on propionic acid production

The propionic acid was suitable for the quadratic model, and the experimental data of 2 h were more significant
than others. The following second-order polynomial quadratic equation of Equation (3) was obtained to express the propionic acid.

\[
Y = -1329.65547 + 118.98012X_1 + 0.31739X_2 + 17.54555X_3
- 0.000629X_1X_2 - 0.088778X_1X_3 - 0.003808X_2X_3
+ 3.95055X_1^2 - 0.00406X_2^2 - 0.077253X_3^2
\] (3)

where \( Y \) is the predicted propionic acid. The calculated \( R^2 \) was found to be 0.9686, which indicated a high degree of correlation between predicted and actual values. The model \( F \)-value of 24.02 and the \( P \)-value of 0.0002 both implied the quadratic model was highly significant. These values all demonstrated that the interactions of SS, initial ORP and shaking rate had a significant influence on propionic acid.

The influence of three factors on propionic acid using RSM is shown in Figure 3. It was apparent that the elliptical shape of contours indicated significant interactions of SS, initial ORP and shaking rate on the production of propionic acid. The predicted maximum of propionic acid was 422.5 mg/L at SS of 13.9 g/L, ORP of −11.6 mV and shaking rate of 105.9 r/min, which was consistent with the experimental maximum of 442.4 mg/L at SS 14.0 g/L, ORP 0 and shaking rate 105 r/min. Compared with acetic production, the maximal propionic acid was obtained at a different SS, ORP and shaking rate. The result proved that the fermentative type would be transformed to propionic acid fermentation when environmental parameters were changed.

**Effect of SS, ORP and shaking rate on butyric acid production**

The model equation of Equation (4) and RSM (Figure 4) at 6 h were selected to analyze the influence of three variables on butyric acid.

\[
Y = 447.703 - 37.58877X_1 - 0.15637X_2 - 3.26023X_3
+ 0.009062X_1X_2 + 0.037175X_1X_3 + 0.000018X_2X_3
+ 1.23706X_1^2 - 0.000728X_2^2 + 0.015296X_3^2
\] (4)

where \( Y \) is the predicted butyric acid. The model \( F \)-value of 229.64 and the \( P \)-value of <0.0001 implied the significance.
of the model. The coefficient $R^2$ was 0.9966. The adjusted $R^2$ (0.9923) suggested that the total variation of 99.23% for butyric acid was attributed to the independent variables. The predicted $R^2$ of 0.9477 was in reasonable agreement with the adjusted $R^2$ between the experimental and predicted values of butyric acid.

As shown in Figure 4, the SS and shaking rate had a strong interaction on butyric acid. The predicted maximum was 112.7 mg/L at SS of 20.0 g/L and shaking rate of 150.0 r/min, which was very close to the actual experimental value of 118.1 mg/L, while the predicted minimum butyric acid of 106.4 r/min was obtained at SS of 15.0 g/L and shaking rate of 106.4 r/min. The butyric acid yield increased with SS at an initial ORP of 0 mV and shaking rate of 150.0 r/min. At the optimal condition for butyric production, the propionic acid was less than 70.0 mg/L. It was implied that butyric acid fermentation would occur at a high shaking rate, which leads to the increase of butyric acid and a decrease of propionic acid.

**Effect of SS, ORP and shaking rate on valeric acid production**

The maximal predicted and experimental value of valeric acid was obtained at 6 h. So the model equation of Equation (5) and RSM (Figure 5) at 6 h is shown as follows.

$$
Y = 22.64207 - 2.15713X_1 + 0.019052X_2 + 0.26163X_3
- 0.000965X_1X_2 + 0.000017X_1X_3 - 0.000083X_2X_3
+ 0.099657X_1^2 + 0.000104X_2^2 - 0.001239X_3^2
$$  \(5\)

where $Y$ is the predicted valeric acid. The model $F$-value of 2.12 implied the model was not significant for valeric acid.

Figure 5 showed that SS and initial ORP had an interdependent effect on valeric acid. The predicted maximum of valeric acid was 39.2 mg/L at 20.0 g/L SS and –200 mV ORP. In this condition (Run 2), the actual production of valeric acid was 42.5 mg/L and the proportion of acetic acid was 74.4%, which indicated the fermentative type was also
butyric acid fermentation. The valeric acid also increased with SS at an ORP of −200 mV. It was seen that SS had a stronger influence on the formation of valeric acid than ORP.

**Effect of SS, ORP and shaking rate on ethanol production**

The ethanol yields in the whole anaerobic acidification were no more than 40 mg/L. The data of ethanol fitted well to the quadratic model at 2 h, and the model equation is shown below.

\[
Y = 75.93158 - 3.48463X_1 - 0.007278X_2 - 0.46556X_3 \\
+ 0.001049X_1X_2 + 0.009051X_1X_3 - 0.000048X_2X_3 \\
+ 0.086931X_2^2 - 0.000147X_3^2 + 0.001553X_3^3
\] (6)

where \( Y \) is the predicted ethanol content. The model \( F \)-value of 14.02 and the \( P \)-value of 0.0011 in Equation (6) both implied the significance of the quadratic model.

The RSM of the ethanol yield is presented in Figure 6. The SS and shaking rate had a high interaction with ethanol. The predicted minimum of ethanol was 20.4 mg/L at SS of 15.7 mg/L, an ORP of −200 mV and shaking rate of 96.7 r/min, and the predicted maximum of ethanol was 36.1 mg/L at SS of 8.0 mg/L, an ORP of −6.2 mV and shaking rate of 60 r/min. It was implied that a proper controlling of SS, ORP and shaking rate could enhance ethanol production.

**Optimization of conditions on VFA and ethanol production used as carbon sources**

The metabolite of waste sludge fermentation can be used as carbon sources for the denitrification process to improve the removal efficiency of nitrate in wastewater. In particular, denitrification rates were strongly dependent on the type of organic carbon source (Elefsiniotis et al. 2004). It was reported that acetic acid could be directly inserted into the metabolic process of anaerobic bacteria without modification. When the acetic acid began to decline, butyric and valeric acid were consumed, which required some enzyme
conversion. Propionic acid was the least effective VFA species for denitrification as presumably requiring the most modification (Xu 1996). In terms of ethanol, it had to be first changed into acetic acid before being metabolized (Constantin & Fick 1997). Therefore, the importance of each carbon source was established as follows: acetic > butyric > valeric > propionic > ethanol. Considering the importance of carbon sources for denitrification, the predicted optimal values of acetic, propionic, butyric and valeric acid and ethanol were 864.7 mg/L, 127.8 mg/L, 78.6 mg/L, 32.9 mg/L and 31.2 mg/L, respectively, at the conditions of 20 g/L SS, −33.6 mV initial ORP and 64.2 r/min shaking rate.

CONCLUSIONS

The interactive effects of SS, ORP and shaking rate were conducted to investigate the VFA and ethanol production using RSM. The RSM model obtained from this study could predict the VFA and ethanol distribution well. The T-VFA yield could be enhanced at an ORP of 0 mV and shaking rate of 60 r/min. Fermentative type could be changed at different conditions of SS, ORP and shaking rate, which reduced the diversity of VFAs and ethanol content. The acetic acid would be accumulated at a low ORP of −200 mV and shaking rate of 60 r/min. Enhancing the ORP and shaking rate to 0 mV and 105 r/min, the propionic acid accumulation could occur. The butyric acid would be enhanced at a high shaking rate, and SS had strongly influenced on the formation of valeric acid. Proper control of SS, ORP and shaking rate could accelerate the ethanol production. Considering the importance of VFAs and ethanol as a denitrification carbon source, the following sequence was established: acetic > butyric > valeric > propionic > ethanol. From the RSM model, the predicted optimal conditions were 20 g/L SS, −33.6 mV ORP and 64.2 r/min shaking rate.
ACKNOWLEDGEMENTS

The study was supported by the Natural Science Foundation of China (grant number: 51208481) and the Science and Technology Planning Project of Qingdao (grant number: 12-1-4-1-(13)-jch).

REFERENCES


First received 16 March 2015; accepted in revised form 19 June 2015. Available online 7 July 2015