A study of the kinetics and the effect of trace elements on mixed anaerobic fermentative biogas production by ternary quadratic general rotary unitized design

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ABSTRACT
In this study the effect of trace elements on methanogenesis was investigated during mixed anaerobic fermentation using a single-factor experiment in the present study. The most effective concentrations of Fe⁰, Fe²⁺, Co²⁺ and Ni²⁺ that were added were 1500, 250, 0.3 and 0.6 mg/L, respectively. The optimal trace element combination was 0.58 mg/L Ni²⁺, 1200 mg/L Fe⁰ and 0.34 mg/L Co²⁺ by the ternary quadratic general rotary unitized design method. The degree of influence exerted by trace elements on the cumulative methane yields decreased in the order of Ni²⁺, Fe⁰ and Co²⁺, and the maximum CH₄ yield was 241.6 mL/g volatile solids (VS), according to a regression equation. The non-dissolved organic carbon hydrolytic process showed a good fit with the first-order kinetic model. The maximum value of CH₄ was 312.87 mL/g VS. Compared to the control, the bioconversion efficiencies of CH₄ and CO₂ production increased by 36.76% and 74.50%, respectively, at the optimal trace element combination. The obtained results provide new knowledge for improvements in the efficiency of anaerobic fermentation biogas production.

KEYWORDS
Trace elements; mixed anaerobic fermentation; methane; single-factor experiment; ternary quadratic general rotary unitized design

Introduction
With rapidly growing populations and economies, energy sources have been decreasing dramatically, and large amounts of animal dung and crop straw have been produced.[1–4] Therefore, human beings are challenged by the energy pinch and by the environmental contamination. As is known, animal dung and crop straw could be used as energy sources. Consequently, many researchers have paid much attention to resource recovery, which is the key for easing energy problems and solving environmental problems simultaneously.[5] Some of the disposal methods for animal dung and crop straw treatment are straw biogasification technology, aerobic composting technology and biohydrogen production.[6–11] There are some disadvantages with these methods. Straw biogasification leads to the production of solid impurities, such as dust particles, and biogas impurities, such as sulphur, nitrogen and chlorine compounds, which could result in secondary pollution. [12] Aerobic composting technology is very poor with regard to sanitary conditions and requires meticulous attention to key parameters.[13] Low hydrogen yield heavily constricts the use of biohydrogen production. [14] Hence, the large amount of work is done to develop new ideas for animal dung and crop straw utilization. Generally, animal dung is considered to be an excellent co-substrate because of its high nitrogen content and its wide range of nutrients, which are needed by methanogens. Furthermore, anaerobic fermentation biogas production is an efficient path for biomass regeneration for various applications, such as cooking with biogas, providing energy for cars and generating power for electricity. Thus, the co-fermentation of animal dung with crop straw seems to be promising, and anaerobic fermentation methanogenesis has attracted extensive attention worldwide.[15–18]

However, the use of anaerobic fermentation processes for methane production has been demonstrated to be quite complex. Biogas production is affected by pretreatment methods used for dung and straw and by temperature, the ratio of carbon to nitrogen, and trace elements.[19] Furthermore, it has been shown that trace elements are also essential nutrients for micro-organism activity [20,21] and for the structure of enzymes, such as methyl-coenzyme M reductase and the coenzyme M methyltransferase complex.[22–24] In addition, some trace elements could promote anaerobic fermentation
methane production, the loading of the system and the conversion rate of wastes. However, the reports published to date primarily concentrate on investigating the effect of a single element on fermentation. Also, the kinetics related to substrate hydrolysis are essentially absent from these reports.

Based on the co-fermentation of cow dung (CD) and corn straw (CS), the aims of this study were: (1) to optimize the concentrations of Fe⁰, Fe²⁺, Co²⁺ and Ni²⁺ added in the system, using a single-factor experiment and obtain the effect order of Ni²⁺, Fe⁰, Co²⁺ and the effect of the optimal combination of trace elements on the biogas production characteristics by introducing the ternary quadratic general rotary unitized design method; (2) to reflect the non-dissolved organic carbon (NDOC) hydrolysis process by hydrolysis kinetics; (3) to investigate the effect of the optimal combination on volatile fatty acid (VFA) concentrations in fermentation biogas slurry; and (4) to discuss the effect of the optimal combination on daily biogas yields. The results will provide technical support for improving the efficiency of anaerobic fermentation biogas production.

**Materials and methods**

**Substrates and seed sludge used for anaerobic fermentation processes**

The CD was collected from a dairy farm at the Hebei University of Science and Technology. The CD was collected 10 h prior to use in batch experiments. The CS was collected from Nanli farmland. The dried straw was ground into particles of less than 0.5 mm. The granule sludge (GS), which was used as the inoculum, was obtained from an anaerobic reactor from the Ningjin starch waste-water treatment plants. Iron powder, FeSO₄·7H₂O, CoCl₂·6H₂O and NiCl₂·6H₂O were all analytical grade and purchased from Tianjin Chemical Ltd (Tianjin Chemical Ltd, Tianjin, China).

**Experimental set-up and procedures**

Mixed anaerobic fermentation experiments were carried out using conical flasks (150 mL) and graduated cylinders (250 mL), which were used as fermentation flasks and biogas extractors, respectively. Biogas was collected using the water displacement method. Conical flasks were sealed by rubber plugs, in which the pH was recorded using a pH electrode. The concentrations of each trace element were recorded at the beginning of the tests. Biogas volumes and their composition were monitored daily. Three parallel experiments were conducted. All of the parameters were taken as averages.

**Mixed anaerobic fermentation with Fe⁰, Fe²⁺, Co²⁺ and Ni²⁺**

According to some reports, the best initial conditions for mixed anaerobic fermentative biogas production are total solids (TS) 8%, C/N 26 and inoculum 25%. The contents of TS, C and N in CD, CS and inoculum could be measured. Based on our previous study, the optimal loading rate (i.e. volatile solids (VS) per unit of reactor volume) was 46.67 g/L, the optimal proportion of CD and CS was 5:1 (VS), the optimal volume of inoculum was 533.33 mL/L, and the optimal particle size of the CS was less than 0.5 mm. Therefore, 30.02 g CD, 2.85 g CS, 80 mL inoculum and 37.2 mL water were mixed evenly after calculations, and different trace elements, namely, Fe⁰ (0, 100, 500, 1000, 1500 and 3000 mg/L), Fe²⁺ (0, 10, 100, 250, 500 and 1000 mg/L), Co²⁺ (0, 0.06, 0.3, 0.6 and 3 mg/L) and Ni²⁺ (0, 0.06, 0.6, 3, and 6 mg/L) were added. The mixtures were sealed into the 150 mL conical flasks, which were placed in a water bath to maintain a constant temperature (38 ± 1 °C).

Rao et al. demonstrated that the biogas production curve corresponds to a slower flat curve when complex solid organic materials are used as substrates. Hence, apart from the cumulative methane yield and methane production rate constant, the lag phase (λ) is also an important factor used to reflect the efficiency of anaerobic digestion. The lag phase can be calculated using the modified Gompertz model, which is a typical ‘S’ style curve equation, as follows:

\[
M(t) = P_m \times \exp \left\{ - \exp \left[ \frac{R_m \times e}{P_m} (\lambda - t) + 1 \right] \right\}. \tag{1}
\]

where \(M(t)\) is the cumulative methane yield at time \(t\) (mL/g VS), \(P_m\) is the methane potential maximum production (mL/g VS), \(R_m\) is the maximum methane production rate (mL/(g VS-d)), \(\lambda\) is the lag phase (d), \(t\) is the duration of the assay (d) and \(e\) is exp(1) = 2.7183.

**Optimization of the Ni²⁺, Fe⁰ and Co²⁺ concentrations by the ternary quadratic general rotary unitized design method**

The ternary quadratic general rotary unitized design was used to investigate the cooperative effect of Ni²⁺, Fe⁰ and Co²⁺ on fermentation. The concentrations of Ni²⁺ (\(X_i\)), Fe⁰ (\(X_j\) and Co²⁺ (\(X_k\)) were chosen as investigated factors, and the cumulative methane yield per unit mass material (VS) was chosen as the dependent variable (Y). The table of coded factors and levels is shown in Table 1.

**Verification experiments**

To investigate the effect of trace elements on the hydrolysis, acidification and methanation of the substrates,
The alcohols and VFA concentrations in the solution were also analyzed by a biogas chromatography (GC-7900 Techcomp, China) using a flame ionization detector equipped with a capillary column (Agilent DB-FFAP, 30 m × 0.25 mm × 0.25 μm). The temperatures of the column, oven and detector were 118, 220 and 240 °C, respectively. The carrier gas was nitrogen at a flow rate of 1.1 mL/min and a split ratio of 10:1. The TOC in the biogas slurry was measured using a TOC analyzer (TOC-Vcpn, Shimadzu, Japan). The dissolved iron, cobalt and nickel were measured by flame atomic absorption spectrometry (AA-680, Shimadzu, Japan).

Data analysis

Data presented in figures are mean values with standard deviation from three independent experiments. Statistical analysis was carried out using the Origin 8.5.1 software. The cumulative methane yields in a single-factor experiment were fitted using the modified Gompertz model, using the Curve Expert (version 1.4) software. The optimization of trace element concentrations using the ternary quadratic general rotary unitized design method was performed using the Design Expert (version 8.0) software. Statistical significance was established at a P-value less than 0.05. The first-order kinetic model for describing the hydrolysis kinetics of NDOC was obtained by the Origin 8.5.1 software.

Results and discussion

Effect and kinetics of trace elements on mixed anaerobic fermentation

The effect of different Fe$^0$, Fe$^{2+}$, Co$^{2+}$ and Ni$^{2+}$ concentrations on mixed anaerobic fermentation was studied and the modified Gompertz model was used to predict the cumulative methane yields and obtain the lag times.

Effect of trace elements on biogas production and dissolved trace elements in biogas slurry

The effect of different Fe$^0$, Fe$^{2+}$, Co$^{3+}$ and Ni$^{2+}$ concentrations on mixed anaerobic fermentation is illustrated in Figure 1. Each batch test was conducted over a period of 30 days.

Fe is an electron carrier in enzymatic reactions and an activator that enhances enzyme activity. It is antagonistic towards the toxicants produced during the fermentation process. Fe has a vital function in microbial activities, and different amounts of iron are needed by various species of microflora. As shown in Figure 1(A), the average daily biogas yield exhibited an increase, followed by a decline, when Fe$^0$ concentrations were increased from 0 to 3000 mg/L and reached the maximum (95 mL) when the Fe$^0$ concentration was 1000 mg/L. The average daily methane content variation was the same as that of the

Table 1. Coding table of experimental factors and levels.

| Factors | X1 (mg/L) Ni$^{2+}$ concentration | X2 (mg/L) Fe$^{0}$ concentration | X3 (mg/L) Co$^{2+}$ concentration |
|---------|----------------------------------|----------------------------------|----------------------------------|
| B(Z0)   | 1.2                              | 1200                             | 0.4                              |
| 1(Z0+ Zj) | 0.9                              | 900                              | 0.3                              |
| 0(Z0)   | 0.6                              | 600                              | 0.2                              |
| −1(Z0− Zj) | 0.3                              | 300                              | 0.1                              |
| −n(Z0)  | 0                                | 0                                | 0                                |

further verification tests with the optimal combination were conducted. Two sets of parallel experiments were carried out. One was for biogas yield and biogas composition measurement, and another was used for VFAs measurement by taking samples of 3 mL of slurry at 1, 2, 5, 10, 16 and 30 days. Similarly, the controls were set up without trace elements.

The carbon element (mg/L) was from CD, CS and GS at the beginning of fermentation and comprised CH$_4$, CO$_2$, organic carbon and inorganic carbon in the biogas slurry and total organic carbon (TOC) in the non-dissolved solids at the end of fermentation. Hence, the NDOC (mg/L) at time t could be calculated according to the carbon balance. The first-order kinetic model was used to describe the hydrolysis kinetics of NDOC. It was calculated as follows:

$$\ln(C_0 - C) = K \times t + a$$

where $C_0$ is the NDOC concentration (mg/L), $C$ is the NDOC concentration at time $t$ (mg/L), $K$ is the hydrolysis rate constant (d$^{-1}$), $t$ is the time (d) and $a$ is a constant.

Analytical methods

TOC in the substrate was measured using the modified Tyurin method.[35] The TS, VS, C and N were determined according to standard methods [36].

The pH was determined using a pH meter (PHS-3C, Mettler, China). Biogas composition (CH$_4$ and CO$_2$) was determined using a gas chromatography (GC) system (GC-7890 Techcomp, China) equipped with a packed column (2 m × Φ3 mm) and a thermal conductivity detector. Argon was used as the carrier gas at a flow rate of 21 mL/min. The operating temperatures of the column, injector and detector were 40, 110 and 140 °C, respectively. Before the analysis, soluble metabolites were centrifuged at 10,000 r/min for 15 min and then acidified using formic acid and filtered through a 0.22 μm membrane. The alcohols and VFA concentrations in the solution were also analyzed by a biogas chromatography unit (GC-7900 Techcomp, China) using a flame ionization detector equipped with a capillary column (Agilent DB-FFAP, 30 m × 0.25 mm × 0.25 μm). The temperatures of the column, oven and detector were 118, 220 and 240 °C, respectively. The carrier gas was nitrogen at a flow rate of 1.1 mL/min and a split ratio of 10:1. The TOC in the biogas slurry was measured using a TOC analyzer (TOC-Vcpn, Shimadzu, Japan). The dissolved iron, cobalt and nickel were measured by flame atomic absorption spectrometry (AA-680, Shimadzu, Japan).
average daily biogas yield, and the methane content reached a peak (56%) when the Fe⁰ concentration was 1500 mg/L (data not shown).

Therefore, low Fe⁰ concentrations that are too low would decrease the activity of the fermentation community, and an excessively high concentration is toxic to the micro-organisms, leading to less biogas production. Consequently, the optimal Fe⁰ concentration range was from 1000 to 1500 mg/L.

The dissolved iron concentration in the biogas slurry was investigated after fermentation. It increased first, then slightly decreased and finally increased sharply with the increase in Fe⁰ concentration from 0 to 3000 mg/L. There was 0.7 mg/L dissolved iron in the biogas slurry without Fe⁰, which most likely resulted from residual metal additives in the cattle feeds. When Fe⁰ concentrations were 500, 1000 and 1500 mg/L, the content of dissolved iron showed little difference (<1.3 mg/L). This result could be due to microbial utilization, iron salt precipitation, metal chelating and Fe⁰ deposition, which lead to stable dissolved iron concentrations. Dissolved iron still existed, although some sediment had been generated when 3000 mg/L Fe⁰ was added.

The effect of the Fe²⁺ concentration on the average daily biogas yield is shown in Figure 1(B). The results showed that the average daily biogas yield first increased and then decreased depending on the Fe²⁺ concentration. In addition, the peak value (93 mL) was

Figure 1. Effect of Fe⁰ (A), Fe²⁺ (B), Co²⁺ (C) and Ni²⁺ (D) concentrations on average daily biogas yield and dissolved trace elements.
achieved when the Fe$^{2+}$ concentration was 250 mg/L. The average daily methane content reached a maximum when the Fe$^{2+}$ concentration was 500 mg/L (data not shown). Consequently, 250–500 mg/L was determined to be the optimum concentration range. Figure 1(B) also illustrates that the dissolved iron concentration increased gradually in a Fe$^{2+}$ concentration-dependent manner. In the system with 100 mg/L Fe$^{2+}$, the percentage of dissolved iron increased from 3.8% to 21.9%, compared to that in the 100 mg/L Fe$^{0}$ system, indicating that higher solubility was obtained with less Fe$^{2+}$. As is known, there is 0.075 mg Co (0.075 mg Co/g dry cell weight) in 1 g dry cell weight of methanogens,[37] and Co plays an important role in micro-organisms. Figure 1(C) illustrates that the average daily biogas yield first increased and then decreased when the Co$^{2+}$ concentration increased from 0 to 3 mg/L. It reached its peak value (94 mL) when the Co$^{2+}$ concentration was 0.3 mg/L. The maximum average daily methane percentage of 56% was obtained at the Co$^{2+}$ concentration of 0.06 mg/L (data not shown). Too little or excess Co$^{2+}$ would reduce micro-organism activity. Hence, the optimal Co$^{2+}$ concentration range was determined to be 0.06–0.3 mg/L. Dissolved cobalt concentrations in the biogas slurry increased with increasing Co$^{2+}$ concentrations. It should be noted that 0.3 mg/L dissolved cobalt was detected even without any Co$^{2+}$ addition.

When the Ni$^{2+}$ concentration increased from 0 to 0.6 mg/L, the average daily biogas yield was on the increase, reaching a plateau in the range of 0.6–6 mg/L Ni$^{2+}$. The maximum value (98 mL) appeared when the Ni$^{2+}$ concentration was 0.6 mg/L (Figure 1(D)). The maximum average daily methane percentage of 56% was obtained at the Ni$^{2+}$ concentration of 0.06 mg/L (data not shown). Hassan et al. [38] also reported that small amounts of nickel in the medium-enhanced biogas and methane production, both of which decreased at higher Ni$^{2+}$ concentrations. Therefore, in our study, the optimal Ni$^{2+}$ concentration was determined to be within the range of 0.06–0.6 mL. Figure 1(D) shows that the dissolved nickel concentration in the biogas slurry increased parallel to the increase in Ni$^{2+}$ concentration. In comparison, 0.4 mg/L dissolved nickel was detected in the control (without the addition of Ni$^{2+}$). A possible explanation could be the heavy metal additives in the cattle feed. For example, 0.48 mg/L dissolved nickel was detected in the variant in which 0.6 mg/L of Ni$^{2+}$ was added. Ashley et al. [39] demonstrated that the methane bacteria activity is restrained in the presence of more than 1 mg/L Ni$^{2+}$ during sludge anaerobic fermentation. However, Takashima and Speece [40] found that the optimum Ni$^{2+}$ concentration range was from 0.012 to 5 mg/L, in batch-mode experiments.

**Modified Gompertz model**

During the mixed anaerobic fermentations with different Fe$^{0}$, Fe$^{2+}$, Co$^{2+}$ and Ni$^{2+}$ concentrations, the cumulative methane yields were calculated. Compared with the control, moderate concentrations of trace elements were beneficial to methane production. According to Equation (1), the maximum values of 181.76, 164.32, 166.19 and 178.04 mL/g VS (increases of 29.58%, 17.07%, 16.05% and 35.56% compared with the control) correspond to the addition of 1500 mg/L Fe$^{0}$, 250 mg/L Fe$^{2+}$, 0.3 mg/L Co$^{2+}$ and 0.6 mg/L Ni$^{2+}$, respectively. There were small discrepancies in cumulative methane production between the theoretical values in the modified Gompertz model and the experimental values. The lag times were 1.39, 1.06, 2.01 and 1.06 days, compared with the control (3.04 days). Thus, the lag time was obviously affected by the trace elements.

**Optimization of trace element concentrations using the ternary quadratic general rotary unitized design method**

The ternary quadratic general rotary unitized design method was used to investigate the effect of Ni$^{2+}$, Fe$^{0}$ and Co$^{2+}$ concentrations on anaerobic fermentation methane production and to obtain the optimal combination, where cumulative methane production (per gram VS) was used as the response value, and the concentrations of Ni$^{2+}$ ($X_1$), Fe$^{0}$ ($X_2$) and Co$^{2+}$ ($X_3$) were chosen as variables. The results of the ternary quadratic general rotary combination design are shown in Table 2, and the variance analysis is shown in Table 3.

| Table 2. Optimization of ternary quadratic general rotary unitized design experiments. |
|---|---|---|---|---|---|---|
| Number | $x_1$ | $x_2$ | $x_3$ | $X_1$ | $X_2$ | $X_3$ | Y |
| 1 | 1 | 1 | 1 | 0.9 | 900 | 0.3 | 215.6 |
| 2 | 1 | 1 | -1 | 0.3 | 900 | 0.3 | 176.4 |
| 3 | 1 | -1 | 1 | 0.9 | 900 | 0.1 | 173.6 |
| 4 | 1 | -1 | -1 | 0.3 | 900 | 0.1 | 159.6 |
| 5 | -1 | 1 | 1 | 0.9 | 300 | 0.3 | 229.6 |
| 6 | -1 | 1 | -1 | 0.3 | 300 | 0.3 | 226.8 |
| 7 | -1 | -1 | 1 | 0.9 | 300 | 0.1 | 198.6 |
| 8 | -1 | -1 | -1 | 0.3 | 300 | 0.1 | 210.2 |
| 9 | -1.682 | 0 | 0 | 0.6 | 1200 | 0.2 | 154.0 |
| 10 | -1.682 | 0 | 0 | 0.6 | 600 | 0.2 | 190.4 |
| 11 | 0 | -1.682 | 0 | 0.6 | 600 | 0.4 | 218.4 |
| 12 | 0 | 1.682 | 0 | 0.6 | 600 | 0.4 | 210.1 |
| 13 | 0 | 0 | 1.682 | 1.2 | 600 | 0.2 | 209.8 |
| 14 | 0 | 0 | -1.682 | 0 | 600 | 0.2 | 187.6 |
| 15 | 0 | 0 | 0 | 0.6 | 600 | 0.2 | 217.1 |
| 16 | 0 | 0 | 0 | 0.6 | 600 | 0.2 | 225.6 |
| 17 | 0 | 0 | 0 | 0.6 | 600 | 0.2 | 216.9 |
| 18 | 0 | 0 | 0 | 0.6 | 600 | 0.2 | 211.2 |
| 19 | 0 | 0 | 0 | 0.6 | 600 | 0.2 | 212.8 |
| 20 | 0 | 0 | 0 | 0.6 | 600 | 0.2 | 218.4 |
From Table 2, there are a total of 20 experimental points. The zero point was repeated six times to estimate the error. The regression equation at the 0.05 significant level is

\[ Y = 216.84 - 14.75X_1 + 8.8X_2 + 6.04X_3 + 1.42X_1X_2 + 7.72X_1X_3 + 4.92X_2X_3 - 14.77X_1^2 - 0.09X_2^2 - 5.35X_3^2 \]

\( F_2 \) was greater than \( F_{0.05} (9, 5) = 4.77 \), and \( P = 0.0429 < 0.05 \) (Table 3), indicating that \( F_2 \) was significant at the 0.05 level and the regression of quadratic equation was significant. The lack of fit \( (F_1) \) was smaller than \( F_{0.05} (5, 5) = 5.05 \), indicating that \( F_1 \) was insignificant at the 0.05 level (i.e. there is no significant difference, because \( P < 0.05 \)). Therefore, the model fitted well with the actual experiment.

The \( P \) values were used as a tool to check the significance of each factor. The result showed that the effects of Ni\(^{2+}\), Fe\(^0\) and Co\(^{2+}\) concentrations on the cumulative methane production were highly significant \( (P < 0.01) \). The effects of the interaction between Ni\(^{2+}\) and Fe\(^0\) concentrations and that between Fe\(^0\) and Co\(^{2+}\) concentrations on the cumulative methane production were also significant \( (P < 0.05) \). The coefficient of the equation showed that the effect order of the factors was \( X_1 > X_2 > X_3 \), in other words Ni\(^{2+}\) > Fe\(^0\) > Co\(^{2+}\). The optimal trace element combination was determined to be 0.58 mg/L Ni\(^{2+}\), 1200 mg/L Fe\(^0\) and 0.34 mg/L Co\(^{2+}\), according to the regression equation.

### Hydrolysis kinetics of non-dissolved organic carbon

Anaerobic fermentation was investigated using the optimal trace element combination, and the NDOC in the system and the control both showed a good fit with first-order kinetics (Figure 2). The NDOC concentration was calculated using the carbon conversion principle, and the variation is given in the inset. Two values for \( R^2 \) were greater than 0.93, demonstrating that the model is consistent with the hydrolytic process of NDOC in the

### Table 3. Variance analysis of the results shown in Table 2.

| Variation sources | SS   | DF | Variance | F value | Significance level (\( P \)) |
|-------------------|------|----|----------|---------|-------------------------------|
| \( x_1 \)         | 2970.59 | 1  | 2970.59  | 38.90   | 0.0002                        |
| \( x_2 \)         | 1057.21 | 1  | 1057.21  | 13.84   | <0.0001                       |
| \( x_3 \)         | 499.69  | 1  | 499.69   | 6.54    | 0.0040                        |
| \( x_1x_2 \)      | 16.24   | 1  | 16.24    | 0.21    | 0.0285                        |
| \( x_1x_3 \)      | 477.40  | 1  | 477.40   | 6.25    | 0.0514                        |
| \( x_2x_3 \)      | 194.04  | 1  | 194.04   | 2.54    | 0.0314                        |
| \( x_1^2 \)       | 3146.64 | 1  | 3146.64  | 41.21   | 0.0124                        |
| \( x_2^2 \)       | 0.12    | 1  | 0.12     | 0.0015  | 0.0001                        |
| \( x_3^2 \)       | 410.43  | 1  | 410.43   | 5.37    | 0.0694                        |
| Regression        | 8633.12 | 9  | 959.24   | 12.56   | 0.0429                        |
| Residue           | 763.61  | 10 | 76.36    |         |                               |
| Lack of fit       | 636.39  | 5  | 127.28   | 5.00    | 0.0509                        |
| Error             | 127.22  | 5  | 25.44    |         |                               |
| Sum total         | 9396.73 | 19 |          |         |                               |

Note: SS, sum of squares of deviations; DF, degrees of freedom.
anaerobic fermentation. The hydrolysis rate constant with the optimal combination was greater than the control ($K_{optimal} = 1.74 K_{control}$); therefore, the hydrolytic process in 1–15 days could be considered to be mainly accelerated by the studied trace elements.

**Effect of the optimal combination on VFA concentrations and biogas production**

**Effect of the optimal combination on VFA concentrations in the biogas slurry**

As shown in Figure 3, the effect of the optimal trace element combination on the overall trend of VFA concentration change was not considerable when compared with the control, although at some time points there were certain variations in the VFA concentrations. Acetic acid and butyric acid concentrations both decreased dramatically in the first two days and then remained below 100 mg/L (Figure 3(A) and 3(B)). However, the propionic acid concentration was influenced tremendously and accumulated to 501 mg/L in the control, while it was consumed to a concentration of less than 40 mg/L in the first five days with the optimal combination (Figure 3(C)). It is speculated that propionic acid metabolism or fermentation types could be accelerated or changed by moderate trace elements; thus, acid production (mostly propionic acid) could be avoided. The variation in the total VFA (acetic acid, butyric acid and propionic acid)
concentration was provided by converting it into COD in Figure 3(D). The concentration was lower than the control in the first 10 days, indicating that trace elements were beneficial to the fermentation.

Effect of optimal combination on biogas production

Figure 4 shows that the daily biogas yields present both decreased gradually in the system of the optimal combination and the control. The daily biogas yield for the optimal combination was greater than the control during the entire fermentation cycle. Therefore, it can be speculated that the methanation stage could be accelerated in the first 15 days with the optimum combination, and hydrolysis became the limited stage in the last 15 days, with VFA concentrations decreasing.

Methane (CH$_4$) content rapidly increased to over 60% from the first day to the seventh day and then remained stable at 60%—68% for the control and the optimal combination. The CO$_2$ content variations were similar to those of CH$_4$. They rapidly increased to over 30% from the first day to the seventh day and then decreased to less than 15%.

The maximum cumulative methane yields were 312.87 mL/g VS in the optimal combination and 228.78 mL/g VS in the control. They were fitted by the modified Gompertz model in Curve Expert, and their correlation coefficients, $R^2$, were all greater than 0.99, indicating that this model reflected the real methanogenic process well. The maximum methane production rate increased by 46.40%, the lag time was shortened by 0.24 days, i.e. the lag periods were 0.28 and 0.04 days in the optimal combination and the control (data not shown) and the fermentation cycle was shortened by 0.85 days.

The substrate utilization is reflected by the efficiency of CH$_4$ bioconversion and CO$_2$ bioconversion (the ratio of the actual and the theoretical). The theoretical yields of CH$_4$ and CO$_2$ could be calculated according to chemometrics.[41]

As shown in Table 4, the maximum cumulative CH$_4$ and CO$_2$ yields increased by 36.76% and 74.54%, respectively, compared with the control. The efficiency of CH$_4$ biotransformation and CO$_2$ biotransformation increased by 36.76% and 74.50%, respectively. Therefore, the biotransformation biogas production efficiency of the fermentation substrate can be improved using moderate concentrations of trace elements.

The results obtained in this study are consistent with those of other authors. For example, Qiang et al. [27] also found that Fe, Co and Ni could enhance the fermentation of food waste. Ma et al. [42] studied the effect of Fe$^{2+}$ concentration on some enzyme activities during methane production from blue-green algae by anaerobic digestion and the total methane yield reached 986.7 mL with 3 mg/L Fe$^{2+}$ added, which was 43 times of the control.

Since microbial populations are known to be an important factor in anaerobic fermentative biogas production, future research should focus on the microbial populations as an object of impact. Manure and the GS in the bioreactor after perennial fermentation could both be used as an inoculum. As there are much more bacteria in manure, using an inoculum from manure will also be considered in future research.

Conclusions

This study investigated the influence of trace elements on biogas production, using a single-factor experiment. The optimal concentrations for Fe$^0$, Fe$^{2+}$, Co$^{2+}$ and Ni$^{2+}$ alone were 150, 250, 0.3 and 0.6 mg/L, respectively. In addition, the ternary quadratic general rotary unitized design method was used to investigate the effect of the Ni$^{2+}$, Fe$^0$ and Co$^{2+}$ concentrations on anaerobic fermentation methane production, and the optimal trace elements combination was 0.58 mg/L Ni$^{2+}$, 1200 mg/L Fe$^0$.
and 0.34 mg/L Co^{2+}. The result showed that the effect order of the factors was Ni^{2+} > Fe^{2+} > Co^{2+}. The hydrolytic process of the NDOC showed a good fit with a first-order kinetic model, and the hydrolysis rate constant with the optimal trace element combination was 1.74 times that of the control (from 1st to 15th day). The maximum CH₄ yield of 312.87 mL/g VS was achieved under optimal conditions. Biotransformation biogas production efficiency of a fermentation substrate can be improved using moderate concentrations of trace elements.

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Therefore, this study could provide theoretical support for anaerobic fermentation biogas production.

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