Optimizing the Conditions of Anaerobic Fermentation in Pig Manure to Produce Volatile Fatty Acids and its Efficiency in Killing Root-Knot Nematodes

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To concurrently control root-knot nematodes and treat pig manure and further clarify the mechanism of anaerobic digestion slurries on nematodes, the optimal conditions for producing volatile fatty acids (VFAs) were studied, and the relationship between the carboxyl group in anaerobic fermentation and the mortality rate of root-knot nematodes was also researched. When the fermentation condition parameters were set as initial pH=10.0, temperature of 25 °C, and total solid (TS) loading of 15%, the largest quantity of VFA production was obtained through orthogonal experiments and range analyses. When the concentration of VFA in the anaerobic fermentation slurry was enhanced to 3.2 g/L, the rate of root-knot nematode mortality was up to 43.3% and was significantly different from that of the control. However, after the concentration of VFA reached 4.3 g/L, no significant difference was observed for the treatments with even higher concentrations of VFA. This indicated that the pig manure slurry with anaerobic digestion exhibited a limited range to kill root-knot nematodes. The results of this study provide a theoretical basis for producing acid from anaerobic digestion in pig manure and for slurry applications.

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INTRODUCTION

Root-knot nematodes are among the types of plant pathogens that seriously threaten agricultural production worldwide (Janati *et al*. 2018). These nematodes have a wide range of hosts and can infect more than 3,000 plants (Jaouannet *et al*. 2013). Root-knot nematodes mainly form nodular root knots by invading wounds (Mitchum *et al*. 2013); they destroy the root structure of plants, absorb plant nutrients, and affect plant metabolism (Tileubayeva *et al*. 2021), resulting in a reduction in crop yield by 10% to 20% and even up to 75% in severe cases (Peiris 2021). Root-knot nematodes could cause 100 billion U.S. dollars economic crop losses each year worldwide (Khan and Kim 2007; Li *et al*. 2015). In China, root-knot nematodes are widely distributed and occur with a high incidence. The area of root-knot nematode disease is gradually increasing, which is an important factor that affects crop production (Dutta *et al*. 2019; Talebear *et al*. 2021). Similarly, China contains a large scale of live pig farming; the total number of pigs in 2020 was 527 million, with 385 million tons of pig manure production (Chen *et al*. 2021), which causes great harm to the environment and has become a main sources of pollution in China (Song *et al*.

2010; Shi *et al*. 2011). Both of these topics have become urgent issues that must be solved for sustainable development in the current Chinese agriculture and breeding industry.

Presently, chemical, physical and biological methods can be used for controlling nematodes (Phanie *et al*. 2021; Naz *et al*. 2021; Wang *et al*. 2021). Chemical methods have a very high nematode-killing efficiency, but polluting the environment and causing the nematode to develop resistance using chemical nematicides is unavoidable. Physical and biological methods are affordable and exhibit relatively good efficiency, yet they can only be applied on a small scale (Rajasekharan *et al*. 2020). Therefore, managing plant pests and diseases requires environmentally friendly methods that are both efficient and convenient. The development of anaerobic disinfection has the potential to be an efficient and sustainable technology for disease control (Strauss and Kluepfel 2015), in which solution is the vital factor that guarantees the efficiency of anaerobic disinfection. Studies have shown that biomass anaerobic digestion slurries exhibit preventive effects on nearly 30 kinds of diseases due to their complex active ingredients (Conn and Lazarovits 2000). Westphal *et al*. (2016) found that anaerobic digestion slurries have the ability to kill nematodes. Pig manure is an important biomass resource, and anaerobic fermentation is one of the main ways to treat pig manure. However, the residual anaerobic fermentation slurry becomes a new pollutant; therefore, it is essential to study whether the anaerobic fermentation slurry could be used for soil anaerobic disinfection and enhance the efficiency of the process.

The objective of this study was to find the optimal conditions for producing volatile fatty acids (VFAs) during anaerobic digestion and to clarify the relationship between the efficiency of killing nematodes and the ingredients in the slurry, which would provide a guide for producing VFAs and slurry application in the anaerobic disinfection of the soil.

EXPERIMENTAL

Materials

Pig manure was sampled from Modern Farm in Rugao City, Jiangsu Province, of which the total solids (TS) was 38.24% and pH=6.5. Biogas slurry was taken from the biogas treatment project of the Kaiping Livestock Farm in Pukou District of Nanjing City, Jiangsu Province, of which the total solids (TS) was 5.44% and pH=7.54. *Botrytis cinerea* for nematode feeding was preserved in the Bioenergy Laboratory of Nanjing Agricultural University. Root-knot nematodes were isolated from the agricultural soil of the facility.

The root-knot nematodes were raised on *B. cinerea* lawns that were inoculated in PDA medium. The nematodes needed 4 days to spread on the whole plates and were then harvested for further use.

An anaerobic digestion slurry of pig manure containing 14.4 g/L formic acid, 110.0 g/L acetic acid, 0.3 g/L propionic acid, 4.6 g/L butyric acid and 129.3 g/L total acid was selected to kill nematodes in this study. The slurry was fermented under the following conditions: pH=10.0 and 10% total solid (TS) loading at 45 °C for 144 h.

Methods of Anaerobic Digestion and VFA Detection

The anaerobic fermentation test referred to the VID 4630 method. Pig manure with a certain pH and TS loading was input into 500 mL blue-mouth flasks and then purged with argon gas to expel the air. The flasks were then placed in a shaker (TENSUC, Shanghai Tiancheng Experimental Instrument Manufacturing Co., Ltd., Shanghai) at a rotating speed of 100 r/min for 192 h. A 45 mL anaerobic digestion slurry was sampled every 48 h for future use.

The VFA contents were determined by high-pressure liquid chromatography (HPLC, LC-20A, Shimadzu Enterprise Management Co., Ltd., Japan) with a 250×4.6 mm C18 chromatographic column (5020-39203, Shimadzu Laboratory Equipment Co., Ltd., Shanghai), and the mobile phase was 22% methanol aqueous solution (v/v); the column temperature was 30 °C; the injection volume was 20 μL; and the running time was 20 min.

Design of the Single Factor Experiment

Single-factor experiments were carried out to explore the law of anaerobic fermentation of pig manure for volatile fatty acid production at different digestion temperatures (25 °C, 35 °C and 45 °C, initial pH was 7.0, TS loading was 15%), different initial pH values (5.0, 7.0 and 10.0, digestion temperature was 35 °C, TS loading was 15%), and different TS loadings (5%, 10%, and 15%, digestion temperature was 35 °C, initial pH was 7.0).

Design of the Orthogonal Experiment

To further identify the optimal parameters for VFA production from anaerobic pig manure, TS loading, initial pH, and digestion temperature were selected as experimental factors. Each factor had 3 different levels, and the orthogonal test was set up according to L_9 (3^4). The specific factor levels of the test are shown in Table 1.

Table 1. Factors and Levels of the Orthogonal Experiment

Level	Initial pH	Fermentation Temperature (°C)	TS (%)
1	5.0	25.0	5.0
2	7.0	35.0	10.0
3	10.0	45.0	15.0

Experiment to Kill Root-knot Nematodes with a Pig Manure Anaerobic Digestion Slurry

A total of 1.5 mL of the supernatant of the pig manure digestion slurry was adjusted to different concentrations with deionized water, and 1 mL was sampled into a small petri dish containing root-knot nematodes (50 μL deionized water approximately 30 to 35 root-knot nematodes) for 24 h. Then, the nematode activity was observed under a microscope, and the number of dead nematode deaths was recorded. The mortality rate of the nematodes in digestion slurry was calculated using the following formula,

$$D = s/t \times 100 \quad (1)$$

where D is the mortality rate of nematodes (%), s is the number of dead nematodes, and t is the total number of nematodes.

The carboxyl concentration in anaerobic fermentation slurry was calculated using the following formula,

$$Cc = a_1 \times Cv_1/Mw_1 + a_2 \times Cv_2/Mw_2 + a_3 \times Cv_3/Mw_3 + a_4 \times Cv_4/Mw_4 \quad (2)$$

where Cc is the concentration of carboxyl groups in anaerobic fermentation slurry (mol/L), a_1, a_2, a_3, and a_4 are the number of carboxyl groups in a single formic acid, acetic acid, propionic acid, and butyric acid molecule, respectively, Cv_1, Cv_2, Cv_3, and Cv_4 are the concentrations of formic acid, acetic acid, propionic acid, and butyric acid in anaerobic fermentation slurry, respectively, and Mw_1, Mw_2, Mw_3, and Mw_4 are the molecular weights of formic acid, acetic acid, propionic acid, and butyric acid, respectively.

Data Processing

All treatments were performed in triplicate, and data processing was performed with Excel 2016 (Microsoft Corporation, Redmond, WA, USA), SPSS 25.0 (IBM Corporation, New York, NY, USA) and Origin Pro 2019 (Origin Lab Corporation, Northampton, MA, USA). Significant differences were assessed using Duncan's test (α=0.05) for multiple comparisons.

RESULTS AND DISCUSSION

Effect of Environmental Conditions on VFA Production from Pig Manure Anaerobic Digestion

The anaerobic digestion process can be regarded as having four stages, including hydrolysis, acidification, acetylation, and methanogenesis (Veeken *et al*. 2000). During hydrolysis, insoluble organic matter is dissolved by enzymes that are produced by hydrolytic microorganisms. In the course of acidification, these hydrolysates are converted to organic acids, alcohols, hydrogen, and carbon dioxide. In the acetylation stage, the products of the acidogenesis are converted into acetic acid, hydrogen, and carbon dioxide. During methanogenesis, methane is produced from acetic acid, hydrogen, and carbon dioxide as well as directly from other substrates inclusive of formic acid and methanol. VFAs are obtained during these processes. The four VFAs appeared in the order of formic acid, acetic acid, propionic acid, and butyric acid according to HPLC, and the retention time of the four organic acids was 3.45, 4.27, 7.19, and 15.52 min, respectively (Fig. 1). As shown in Fig. 2A, when the digestion temperature was 35 °C, the highest total acid production was obtained, which reached 202.6 g/L, but the difference in the treatments under different temperatures was not significant for total acid production (P=0.083). The treatment with the initial pH=7.0 resulted in the highest total VFA production, which was significantly higher than that with initial pH=5.0 (P=0.001) and pH=10.0 (P=0) (Fig. 2B). The total VFA produced by the treatment with 15% TS loading reached 212.7 g/L, which was significantly higher than that of the treatment with 5% TS loading (P=0.003), while there was no significant difference between the treatments with 15% and 10% TS loading (P=0.619) (Fig. 2C). The results indicated that medium temperature, neutral pH, and high TS loading were favorable for total VFA production.

The compositions of the total acids also varied under different environmental conditions. As shown in Fig. 2A, under the relatively low temperature (25 °C), there was significantly higher propionic acid production and lower acetic acid and butyric acid production. The treatment at a higher temperature (45 °C) produced less propionic acid production, while all the composites of the total VFA in the treatments at the medium temperature contained relatively higher contents, perhaps because the anaerobic digestion reaction benefited from the medium temperature. For each composite in the total VFA except for formic acid, the treatment with initial pH=7 resulted in the largest production (Fig. 2B). Treatment with 15% TS loading produced more small VFA molecules, such as formic acid and acetic acid, while 10% TS loading produced more butyric acid (Fig. 2B).

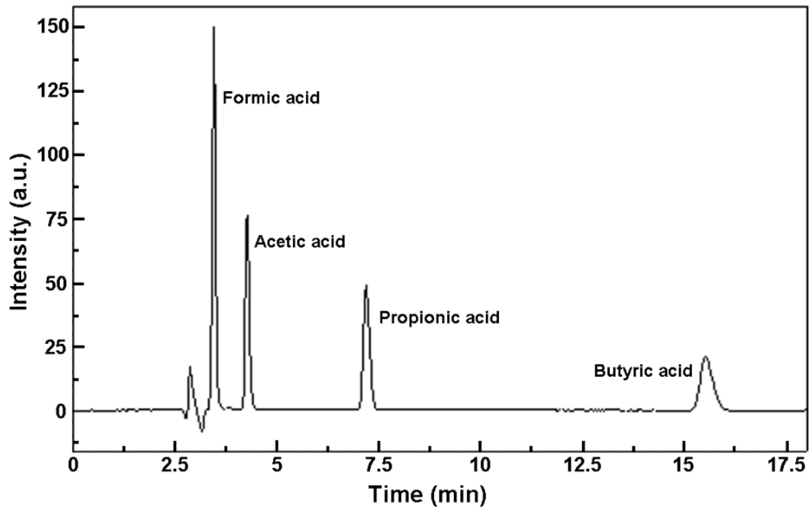

Fig. 1. The HPLC chromatogram of mixtures of four organic acids

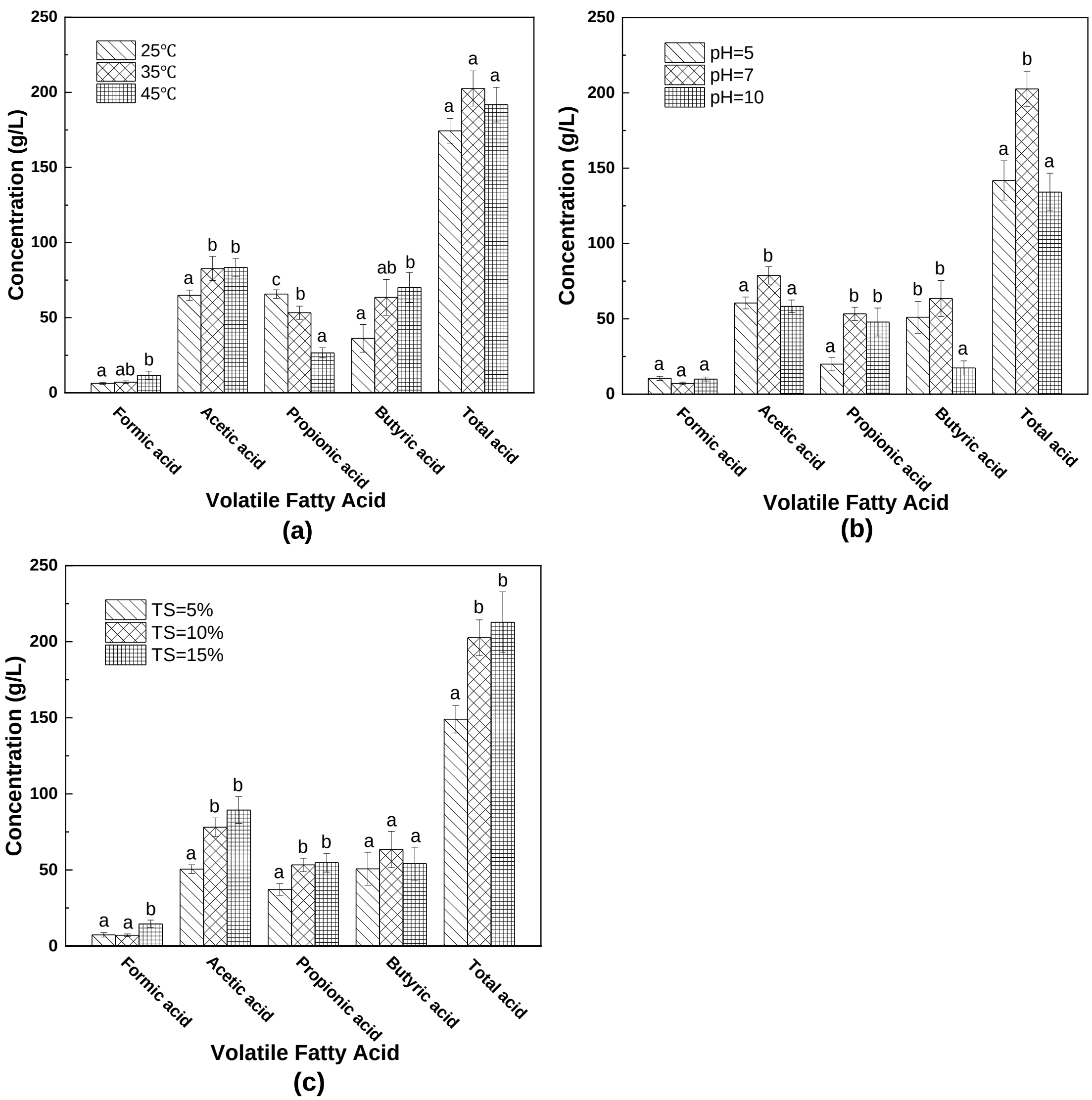

Fig. 2. Effects of the digestion temperature (a), initial pH (b) and TS (c) loading on VFA production from pig manure anaerobic digestion

The reason why the environmental factors could affect the anaerobic reaction occurrence and how they acted need to be further clarified.

The Optimal Combination of Environmental Conditions for VFA Production

To obtain the optimal processing parameters for VFA production from pig manure anaerobic digestion, an orthogonal experiment was carried out on the basis of a single factor. The results are shown in Table 2. The influence of various factors on VFA production from pig manure in descending order were TS loading, initial pH, and digestion temperature. According to the results of the range analysis, the optimal combination of pig manure for VFA was initial pH=10.0, fermentation temperature at 25 ℃, and 15% TS loading, which could produce 342.8 g total acid per liter anaerobic slurry under these combination conditions.

Table 2. Effect of the Combination of Environmental Factors on VFA Production and Results of the Significance Test for Range Analysis

Test No.	Initial pH	Fermentation Temperature (°C)	TS (%)	Total Acid
1	5.0	25.0	5.0	63.8
2	5.0	35.0	10.0	122.0
3	5.0	45.0	15.0	201.7
4	7.0	25.0	10.0	142.5
5	7.0	35.0	15.0	172.0
6	7.0	45.0	5.0	82.9
7	10.0	25.0	15.0	342.8
8	10.0	35.0	5.0	138.6
9	10.0	45.0	10.0	214.2
$\bar{K}1$	129.2	183.0	95.1	
$\bar{K}2$	132.4	144.2	159.6	
$\bar{K}3$	231.9	166.3	238.8	
R	102.7	38.8	143.8	

Note: Results The unit of total acid concentration was g/L, $\bar{K}$, respectively represents the average value of each factor at each level; R stands for range.

Killing Efficiency of the Anaerobic Digestion Slurry on Root-knot Nematodes

The killing efficiency of the anaerobic digestion slurry on root-knot nematodes is shown in Table 3. The mortality rate of root-knot nematodes in the anaerobic digestion slurry was higher than that in the sterile water control treatment. The mortality rate of nematodes increased with increasing total acid concentration. The mortality rate of root-knot nematodes in the 10-fold dilution treatment was higher than that in the 20-fold and 30-fold dilutions treatments, but the difference in the mortality of nematodes under these three treatments was not significant (P=0.057). When the concentration of VFA in the anaerobic fermentation slurry was enhanced to 3.2 g/L, the mortality rate of root-knot nematodes was up to 43.3% and was significantly different from the control. However, after the concentration of VFA reached 4.3 g/L, there was no significant difference for the treatments with even higher concentrations of VFA.

This result indicated that the anaerobic digestion in the pig manure slurry has a limited range to kill root-knot nematodes. To study the mechanism of action of anaerobic digestion in the pig manure slurry on root-knot nematodes, the correlation of the total carboxyl group concentration and the mortality rate of nematodes was analyzed. As shown in Table 3, when the carboxyl group concentration reached 0.055 mol/L, the mortality rate of nematodes was significantly different from that of the control group (P=0.01). When the concentration of carboxyl groups in the pig manure fermentation broth was increased to 0.073, 0.110, and 0.220 mol/L, the mortality rate of nematodes reached 63.12%, 77.57%, and 81.32%, respectively. The results showed that the higher the concentration of carboxyl groups in the anaerobic digestion slurry was, the better the efficiency of killing nematodes. The curve estimating the carboxyl group concentration and nematode mortality was further carried out, and it was found that the relationship of the highest degree of model fit was the following quadratic curve: Y=-20.479X^2+7.659X+0.13 (X was the carboxyl group concentration, Y was the mortality rate of nematodes, R^2=0.942, P=0.014). This indicated that the concentration of carboxyl groups in the pig manure anaerobic digestion slurry played a vital role in the efficiency of killing root knot nematodes.

Table 3. Effect of the Concentration of Total Acid and Carboxyl Groups on the Mortality Rate of Root-knot Nematodes

Dilution Times	Acid Concentration (g/L)	Carboxyl Concentration (mol/L)	Mortality (%)
contrast	0	0	18.03±0.08a
50	2.59	0.044	34.21±0.11ab
40	3.23	0.055	43.33±0.06b
30	4.31	0.073	63.12±0.07c
20	6.47	0.110	77.57±0.18c
10	12.93	0.220	81.32±0.01c

Note: Different lowercase letters in the table represent significant differences in the mortality rate of root-knot nematodes with different fermentation broth concentrations (Duncan test, α=0.05). The treatment time of nematodes with anaerobic fermentation slurry was 24 hours.

Factors Influencing Anaerobic Digestion and Acid Production from Pig Manure

Previous studies have shown that digestion temperature, pH, and TS loading affect the anaerobic digestion process in pig manure (Ameen *et al*. 2021). Temperature is an important factor affecting digestion efficiency (Devrieze *et al*. 2015; Tian *et al*. 2021). Liu *et al.* (2007) found that the gas and VFA production rates benefited from 35 °C compared to 55 °C in medium- and high-temperature anaerobic digestion tests with cow manure, which was consistent with the conclusions of this study. However, previous studies did not clarify the degree of influence of temperature on anaerobic digestion, while it was found in this work that temperature had less influence on anaerobic digestion in the range of 25-45 °C. This might be because the activity of acid-producing bacteria was not greatly affected at a certain temperature range. This result provides an important reference for whether pig manure is digested to produce acid for heating.

TS (González-Fernández and García-Encina 2009) and pH (Zhou *et al*. 2016) are also important factors affecting the anaerobic acid production of pig manure. Song *et al.* (2010) studied the effect of total solids on the gas production characteristics of manure and straw mixed fermentation. It was found that the optimal total solids mass fraction of anaerobic fermentation was 14.6% (Song *et al*. 2010). This was basically consistent with the conclusion obtained in this study that the pig manure produces more acid with a total solids mass fraction of 15%. Further research found that when the fermentation substrate was insufficient, increasing the fermentation substrate significantly increased acid production. However, when the fermentation substrate was sufficient, simply increasing the fermentation substrate did not significantly increase the acid production. Therefore, to enhance the anaerobic acid production of pig manure, an appropriate total solid mass fraction should be selected. This study found that the acid production of anaerobic fermentation of pig manure under the condition of pH=7.0 was significantly higher than that under the conditions of pH=5.0 and pH=10.0, which was consistent with some research conclusions by Li *et al*. (2011). However, in the orthogonal experiment, the optimal pH was found to be 10.0, which may be related to the interaction between the three conditions. Therefore, selecting suitable parameters for the process of anaerobic fermentation in pig manure is essential to increase acid production.

Insecticidal Effect of the Anaerobic Digestion Slurry in Pig Manure

The components of the anaerobic digestion slurry were quite complex. The mechanism of nematode control and other plant pathogen management is still unclear; Xiao *et al.* (2007) found that adding a pig manure anaerobic digestion slurry that is rich in VFAs and NH4+ to the soil could effectively inhibit the growth of soybean cyst nematode eggs (Xiao *et al*. 2007). Dutler and associates found that the combination of soil anaerobic disinfection and sun exposure could effectively control soil-borne plant pathogens and plant parasitic nematodes in vegetable production systems (Butler *et al*. 2012). López-Robles *et al.* (2013) found that adding pig manure after an anaerobic digestion slurry to the soil could significantly reduce the number of potato cyst nematodes in the soil and the hatching rate of eggs, which could achieve a good control effect. This was consistent with the conclusion found in this study that the total acid concentration of pig manure anaerobic fermentation broth reached 3.2 g/L, producing a better killing efficiency on root-knot nematodes. However, the present research further found that the concentration of carboxyl groups was significantly related to the mortality rate of root-knot nematodes; this provided a theoretical basis for the subsequent large-scale uses of pig manure anaerobic fermentation broth for controlling root-knot nematode in subsequent farms to provide a theoretical basis for popularization; however, due to the complex composition of pig manure anaerobic fermentation broth, the mechanism of disease control in soil anaerobic disinfection technology based on pig manure anaerobic fermentation broth still needs further research.

CONCLUSIONS

1. The production of volatile fatty acids in the pig manure anaerobic digestion slurry was affected by digestion temperature, pH and total solids (TS) loading. The study found that the optimal acid production process parameters were pH=10.0, 15% TS loading, and digestion temperature of 25 °C. Under these conditions, the content of volatile fatty acids in the anaerobic digestion slurry reached 342.8 g/L.
2. The pig manure anaerobic digestion slurry exhibited a good killing efficiency on root-knot nematodes, and the mortality rate of root-knot nematodes was related to the total acid concentration in the anaerobic digestion slurry. When the total acid concentration reached 3.2 g/L, the killing efficiency on root-knot nematodes was significant. However, after the concentration reached 4.3 g/L, simply increasing the total acid concentration did not significantly increase the mortality rate of root-knot nematodes.

3. The carboxyl group concentration in the anaerobic digestion slurry of pig manure and the mortality rate of root-knot nematodes showed a quadratic curve Y = -20.479X^2+7.659X+0.13, where X is the concentration of carboxyl groups and Y is the mortality rate of nematodes.

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