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Co-inhibition of methanogens for methane mitigation in biodegradable wastes

ZHAO Tiantao[1,2], ZHANG Lijie[1], CHEN Haoquan[3], ZHAO Youcai[2,*]

1. Department of Chemical and Biological Engineering, Chongqing University of Technology, Chongqing 400050, China. E-mail: zhaott@cqut.edu.cn
2. State Key Laboratory of Pollution Control and Resources Reuse, Tongji University, Shanghai 200092, China
3. Shanghai Environmental Engineering Technology Co., Ltd., Shanghai 200021, China

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Abstract

The inhibition effects and mechanisms of chlorinated methane and acetylene on methanogenesis in the anaerobic digestion process of the biodegradable wastes were investigated. It was found that both chloroform and acetylene could effectively inhibit methanogens while the biodegradability of the wastes was not affected. Acetylene inhibited the activity of methanogens, while chloroform inhibited metabolic process of methanogenesis. A central composite design (CCD) and response surface regression analysis (RSREG) were employed to determine the optimum conditions and interaction effects of chloroform and acetylene in terms of inhibition efficiency, production of volatile fatty acids (VAF) and molar ratio of propionic acid to acetic acid. Chloroform had significant effect on enhancing the production of VFA ($F = 121.3$; $p < 0.01$), and acetylene promoted the inhibition efficiency ($F = 99.15$; $p < 0.05$) more effectively than chloroform ($F = 9.72$; $p > 0.05$). In addition, a maximum molar ratio of propionic acid to acetic acid of 1.208 was estimated under the optimum conditions of chloroform concentration of 9.05 mg/kg and acetylene concentration of 3.6×10^{-3} (*V/V*). Hence, methanogens in the wastes can be inhibited while the stabilization process of the biodegradable wastes can still work well, as propionic acid generated during the inhibition process could hardly be utilized by methanogens.

Key words: methanogenesis; biodegradable wastes; inhibition mechanism; response surface regression analysis; stabilization process
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Introduction

Methane (CH_4) is an important greenhouse gas as its global warming potential is 21 times over that of CO_2, while the atmospheric methane concentration has been increasing at a rate of 1%–2% per year. Meanwhile, refuse landfills have been recognized as a main source of anthropogenic methane emission because food origin wastes are consisted of the main components in the refuse and they are highly perishable, readily biodegradable and rich in organic compounds and trace elements (Smidt and Meissl, 2007). Refuse landfills emit landfill gas (LFG, which contains mainly with CH_4 and CO_2) in 3–6 months of waste placement and reach gas emission peak in about one year (Tchobanoglous *et al*., 1993). The methane generated in the landfills is generally not well collected but discharged into air directly. Even in some landfills installed the landfill gas collection and utilization facilities, the methane, which was generated before the final cover operation, has to discharge into air directly if elimination measures are not taken. Hence, the inhibition of methane is necessary for the mitigation of greenhouse gas generation at landfills. However, little literature can be traced as to the inhibition practice at landfills.

Because methanogenesis has a negative correlation with energy utilization in ruminants, many efforts have been made to inhibit its production and to rechannel hydrogen to produce more volatile fatty acids (VFAs) and microbial mass (Orskov *et al*., 1968). As methanogens are strict anaerobic and very sensitive to many chemicals, the activity of methanogens can be inhibited by the addition of toxic chemicals. Many compounds have been tested *in vitro* and *in vivo* as methane inhibitors. Some compounds such as halogenated methane analogs, alcohol-halogen derivatives, diaryliodonium derivatives, coenzyme-M analogs, anthraquinones, hydroxymethylglutaryl-S-CoA reductase inhibitors and uncouplers of proton motive force have been used to inhibit methane production in ruminal fermentations (Czerkawski and Breckenridge, 2007; Garcia-Lopez *et al*., 1996; Martin and Macy, 1985). Generally, compounds that have blocked reductive steps in methanogenesis have resulted in a reduction in acetic acid and sometimes ammonia and have caused an increase in propionate and sometimes butyrate (Garcia-Lopez *et al*., 1996). They inhibit methane generation *in vitro* and *in vivo* with varying degrees of success. However, some of these feed additives may also inhibit other biochemical reactions.

With regard to methanogens inhibition, the conventional "change-one-factor-at-a-time" method was always used for

* Corresponding author. E-mail: zhaoyoucai@tongji.edu.cn

multifactor experiment design in previous studies (Martin and Macy, 1985). Indeed, the single-dimensional research is laborious, time consuming, and incapable of reaching the true optimum due to ignoring the interaction effects among variables. To resolve this problem, response surface regression analysis (RSREG) was proposed in this study to determine the optimal value of response variable and interactive influence of dependent variable. The RSREG is a statistical technique for designing experiment, building models, evaluating the effects of several factors and searching optimum conditions for desirable responses and reducing number of experiments. With RSREG, the interactions of possible influencing parameters can be effectively evaluated. In addition, Analysis of variance (ANOVA) was proposed in this study to determine the influences of individual factors and the adequacy and significance of the quadratic model.

The object of this study was to test the inhibition efficiencies of chlorinated methane and acetylene on methane production and investigate the inhibition mechanism of inhibitors on methanogens during the food origin wastes anaerobic digestion process. In addition, the production of VFA and the molar ratio of propionic acid to acetic acid were explored, aiming at determining the effects of chlorinated methane and acetylene on acid-producing bacteria and the stabilization process of the biodegradable wastes.

1 Materials and methods

1.1 Materials

Dichloromethane, chloroform, and carbon tetrachloride, commercially available from China Sinopharm Chemical Reagent Co. Ltd., were of analytical grade. The purity of acetylene used was 99.99% as restored in steel-bottle.

Food origin wastes were used as the substrate and sampled from the student's dinning-hall at the Tongji University, mainly including cooked rice, uncooked vegetable, fruit waste and meat. The wastes and aged refuse were mechanically smashed to an average size of 1 mm in a blender. Anaerobic granular sludge from UASB tank treating cassava waste was used as inoculum. The food origin wastes and sludge were stored at 4°C in the refrigerator before use.

1.2 Experimental procedure

The reaction substrate, which has a water content of 70%, are composed of ca. 95% (kg food origin wastes/kg substrate) ca. 5% (kg sludge/kg substrate) sludge and appropriate amount of aged refuse. First, one liter reaction substrate was incubated under 37°C for an acclimatization period of about two weeks. Then, batch experiments were conducted using 40 g acclimatized reaction substrate placed a series of 300 mL serum bottles. The headspaces of the bottles were flushed with nitrogen for 1 min and the bottles were tightly sealed with rubber septa. Chlorinated methane with designated concentration and/or acetylene with assigned volume are then injected as inhibitor. Finally, all bottles were placed in a greenhouse under 37°C.

1.3 Analytical methods

Biogas composition analysis was carried out by gas chromatography separation (Shimadzu GC-14B, Japan) with a stainless steel column packed with Carbosive SII (3.2 mm in diameter and 2.0 m in length) and thermal conductivity detector (TCD). The temperatures of injector, detector and column were kept at 100, 105, and 60°C, respectively. Nitrogen was used as the carrier gas with a flow rate of 30 mL/min. The measured methane volume was adjusted to the volume at standard temperature and pressure.

VFAs were determined by a gas chromatography (GC-6890N, HP Inc., USA) equipped with a flame ionization detector (FID) and a 30 m × 0.25 mm × 0.25 μm fused-silica capillary column (DB-FFAP). The temperatures of the injector and detector were 110 and 220°C, respectively. The oven temperature was initially at 70°C for 2 min, followed with a ramp of 20°C/min for 5.5 min and held at final temperature of 220°C for 0.5 min. Nitrogen was used as the carrier gas with a flow rate of 2.6 mL/min. Samples and standards were acidified by the addition of 50 μL of H_3PO_4 (10%) to 5 mL sample, the sample injection volume was 1.0 μL. The sum of measured acetic, propionic, butyric, isobutyric, pentanoic, and isovaleric acids was recorded as the total amount of VFA.

Inhibition effect was measured by inhibition efficiency (IE), which was calculated as follows:

$$\mathrm{IE} = \left(1 - \frac{V_{\mathrm{methane,CF}}}{V_{\mathrm{methane,control}}}\right) \times 100\% \quad (1)$$

where, $V_{\mathrm{methane,CF}}$ is the methane production of experimental group and $V_{\mathrm{methane,control}}$ is the methane production of control group (without inhibitors). During the experiment, the methane production of control group was 4.67 ± 0.21 mL/g substrate. Stabilization process of food origin wastes anaerobic digestion was evaluated by the production of VFA and the molar ratio of propionic acid to acetic acid (P/A).

2 Results and discussion

2.1 Inhibitors optimization

Inhibition time optimization of chloride methane with different concentrations was studied (Fig. 1). It can be seen that when the inhibition time was over 12 h, the inhibition efficiencies did not change apparently, therefore, 12 h was chosen as the reaction time for the subsequent experiments.

The inhibition efficiencies of dichloromethane, chloroform and tetrachloromethane on food origin wastes anaerobic digestion are shown in Fig. 2. Chlorinated methane decreased the methane production, but not closely related to the number of chlorine in chlorinated methane. With a concentration of 20 mg/kg, the inhibition efficiency of chloroform, dichloromethane, and tetrachloromethane reached 98.1%, 34.2%, and 42.7%, respectively. Chlorinated hydrocarbons are extremely toxic to methanogens (Bauchop, 1967; Van Nevel and Demeyer, 1977) and many chlorinated hydrocarbons can decrease methane

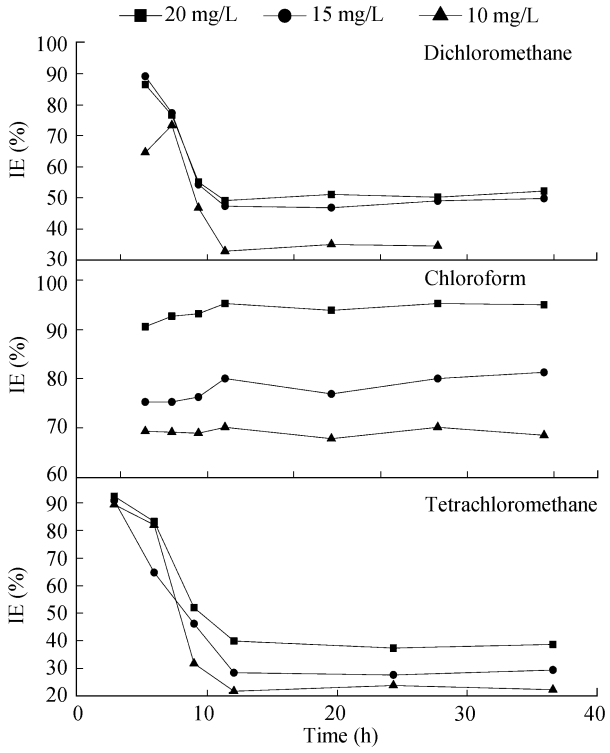

Fig. 1 Effect of inhibition time on methanogenesis.

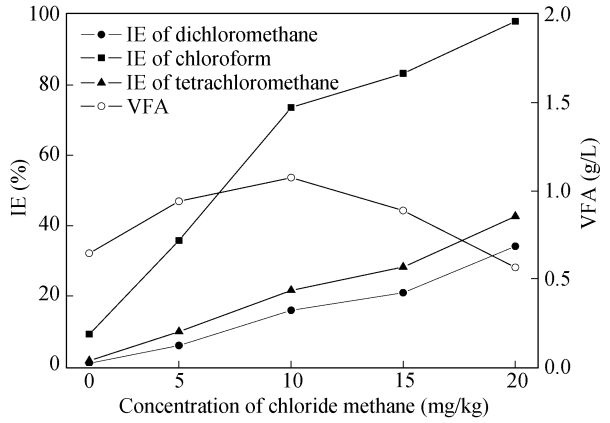

Fig. 2 Effect of chloride methane on methanogenic bacteria inhibition.

production *in vivo* and *in vitro* (Cole and McCroskey, 1975). The inhibition effect of chloroform was higher than that of dichloromethane and tetrachloromethane. The last steps of metabolic pathway of methanogens all required unusual coenzymes and cofactors with novel structures, which could be found only in the methanogens (DiMarco *et al*., 1990). The methyl was transferred to the cofactor tetrahydrosarcinapterin (THSPt) firstly, and then from CH_3-THSPt to coenzyme M (HS-CoM). The CH_3-S-CoM was reductively demethylated to methane by methyl-CoM reductase (Ermler *et al*., 1997; Ferry, 1997). Chloroform, with a lively carbon-hydrogen bond (bond energy is 392.5 ± 2.5 kJ/mol), was likely to serve as competitive inhibitor of methyl (Luo, 2002), which could participate in enzyme reaction with THSPt or HS-CoM. Moreover, similar stereoscopic structure of chloromethyl and methyl could promote the combination of chloroform and coenzyme.

VFA was characterized to test the activity of acid-producing bacteria in anaerobic digestion system. The effect of chloroform on stabilization process of food origin wastes anaerobic digestion was investigated (Fig. 2). When low concentrations of chloroform were added, the concentration of VFA increased with chloroform addition because chloroform inhibited the conversion of VFA into methane. The highest concentration of VFA was achieved chloroform concentration of 10 mg/kg. However, with further increase of the chloroform concentration, acid-producing bacteria were inhibited and then led to a decline of VFA concentration. Although chloroform with concentration of 20 mg/kg could inhibit methanogens completely, it was adverse to the stabilization of food origin wastes anaerobic acid-producing process illustrated by the low VFA concentration.

The inhibition efficiency and VFA production of acetylene on food origin wastes anaerobic digestion are shown in Fig. 3. The complete inhibition of methanogenesis was observed as the concentration of acetylene was in the range of 12×10^{-3}–20×10^{-3} (*V/V*) in the gas phase. Acetylene inhibited the growth of several bacteria normally capable of growth on lower molecular weight hydrocarbons (De Bont and Mulder, 1976). Sprott *et al*. (1982) reported that ethylene was similarly less inhibitory than acetylene for methanogenesis, and dissolved acetylene resulted in a decrease of methanogens functions which required a H^+-flux, including ATP synthesis, Ni^{2+} uptake and methanogenesis. Therefore, the inhibition mechanism of acetylene was different from that of chloroform, but both chloroform and acetylene could effectively inhibit methanogens.

Figure 3 shows that the concentration of VFA decreased significantly when acetylene concentration increased from 12×10^{-3} to 20×10^{-3} (*V/V*). This is likely because of the inhibition of acid-producing bacteria. However, at lower acetylene concentration from 1×10^{-3} to 12×10^{-3} (*V/V*), the increase of VFA was mainly attributed to the methanogenesis inhibition.

2.2 Central composite design experiments for co-inhibition system

High concentrations of chloroform and acetylene can inhibit methanogens well. However, acid-producing bacteria inhibition in high concentrations of inhibitors would

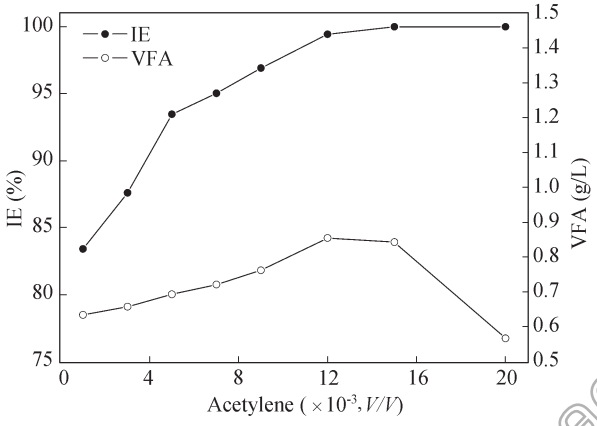

Fig. 3 Effect of acetylene on methanogenic bacteria inhibition.

affect the stabilization process of wastes in refuse landfills. Meanwhile, chloroform and acetylene have different methanogens inhibition mechanism. Therefore, second-order RSREG and multi-factor ANOVA were proposed to determine co-inhibition effect of chloroform and acetylene on food origin wastes anaerobic methanogenesis and acid-producing process.

A three-level-two-factor central composite design (CCD) was obtained using the SAS 8.1 software (SAS Institute Inc., Cary, NC, USA), which was employed to find out the interactive effects of two variables, *viz*. chloroform concentration and acetylene concentration. The levels of factors used for optimization are presented in Table 1. The trial was essentially a full factorial design augmented by four axial points coded ± α and four replications of center points (all factors at level zero), resulting in a total number of 12 experiments. The center runs provided a means for estimating experimental errors, and the axial points were added to the factorial design to provide for estimation of curvature of the model. The distance from the centre point was given by $\alpha = 2^{n/4}$ (for two factors $n = 2$, $\alpha = 1.414$). Chloroform concentration (X_1) and acetylene concentration (X_2) were identified by single factor experiment and calculated with Eq. (2):

$$x_1 = (X_1 - 5)/3, \quad x_2 = (X_2 - 4)/2 \tag{2}$$

Chloroform concentration and acetylene concentration were chosen as two independent variables in this experiment design; methane inhibition efficiency (IE), VFA and the molar ratio of propionic acid to acetic acid (P/A) were dependent variables. The matrix corresponding to the CCD is shown in Table 2. Three dependent variables were fitted using a predictive polynomial quadratic equation to correlate the response variable to the independent variables. The general form of the predictive polynomial quadratic equation is Eq. (3):

$$Y = A_0 + \sum_{i=1}^{k} A_i x_i + \sum_{i=1}^{k} A_{ii} x_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} A_{ij} x_i x_j \tag{3}$$

where, Y is the predicated response, x_i are the input variables, which affect the response variable Y, A_0 is the ith linear coefficient, A_{ii} is the quadratic coefficient and A_{ij} is ijth interaction coefficient. The quality of fit of the model equation was expressed by the coefficient of determination of R^2, and its statistical significance was determined by F-test. The significance of the regression coefficients was tested by t-test.

The design matrix in actual terms and the experimental results are presented in Table 2. The obtained values were subjected to the analysis. Summary of RSREG and ANOVA procedure is shown in Table 3. Applying multiple regression analysis, it was found that the results fitted to a second-order polynomial equation well. Thus the mathematical regression model for two dependent variables fitted in terms of uncoded factors was obtained as follows:

$$Y_{IE} = 73.4966 + 0.9203X_1 + 6.6149X_2 - 0.0801X_1^2 + 0.05658X_2X_1 - 0.5988X_2^2 \quad (R^2 = 0.9687;\ p < 0.01) \tag{4}$$

$$Y_{VFA} = 0.3685 + 0.1210X_1 + 0.09976X_2 - 0.006543 - 0.000125X_2X_1 - 0.008407 \quad (R^2 = 0.9719;\ p < 0.01) \tag{5}$$

$$Y_{P/A} = 0.2654 + 0.1757X_1 + 0.08261X_2 - 0.009133 - 0.002917X_2X_1 - 0.007857 \quad (R^2 = 0.9587;\ p < 0.01) \tag{6}$$

Table 1 Levels of factors used for optimization

Variable	Label	Level				
		–1.414 (–α)	–1	0	1	1.414 (α)
x_1	Chloroform conc. (mg/kg)	0.758	2	5	8	9.242
x_2	Acetylene conc. (10^{-4}, *V/V*)	1.172	2	4	6	6.828

2.3 Effect of chloroform and acetylene on co-inhibition efficiency

Three-dimensional plot (response surface) and contour lines for co-inhibition efficiency obtained from Eq. (4) are shown in Figs. 4 and 5. The curvatures of the graphs imply that there was a relatively weak interaction between chloroform and acetylene, which was also confirmed by RSREG analysis of cross product ($F = 0.46$; $p > 0.05$).

Table 2 IE, VFA, and P/A at various chloroform and acetylene concentrations

Run	Code value		Real value		IE (%)	VFA (g/L)	P/A
	x_1	x_2	X_1	X_2			
1	–1	–1	2	2	86.670	0.754	0.752
2	1	–1	8	2	88.108	0.909	1.120
3	–1	1	2	6	92.752	0.751	0.880
4	1	1	8	6	95.548	0.983	1.217
5	–1.414	0	0.758	4	91.451	0.698	0.542
6	1.414	0	9.242	4	94.314	1.181	1.415
7	0	–1.414	5	1.172	82.395	0.866	0.939
8	0	1.414	5	6.828	96.677	0.834	0.871
9	0	0	5	4	94.154	0.839	1.102
10	0	0	5	4	94.276	0.896	1.036
11	0	0	5	4	93.830	0.843	1.037
12	0	0	5	4	94.161	0.803	1.052

IE: inhibition efficiency; VFA: volatile fatty acids; P/A: the molar ratio of propionic acid to acetic acid.

Table 3 Analysis of variance of the model

Variable		RSREG procedure				ANOVA procedure	
	Regression	Linear	Quadratic	Cross-product	Total Model	X_1	X_2
IE	d*f*	2	2	1	5	4	4
	R^2	0.7755	0.1909	0.0024	0.9687	0.9909	
	F	74.42	18.32	0.46	37.19	9.72	99.15
	p	< 0.0001	0.0028	0.5249	0.0002	0.1976	0.0437
VFA	d*f*	2	2	1	5	4	4
	R^2	0.8827	0.0892	0	0.9719	0.9948	
	F	94.31	9.53	0	41.54	121.3	23.41
	p	< 0.0001	0.0137	0.9685	0.0001	0.0012	0.0134
P/A	d*f*	2	2	1	5	4	4
	R^2	0.8557	0.1003	0.0027	0.9587	0.9682	
	F	62.19	7.29	0.4	27.87	22.46	0.41
	p	< 0.0001	0.0248	0.5507	0.0004	0.0142	0.7973

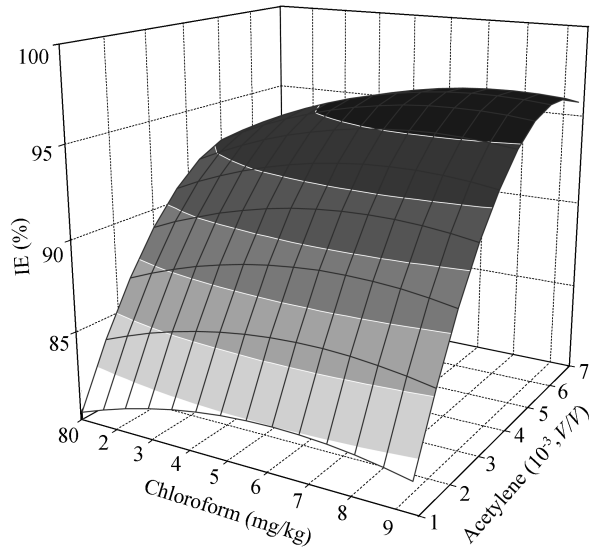

Fig. 4 Response surface for the optimization of inhibition efficiency.

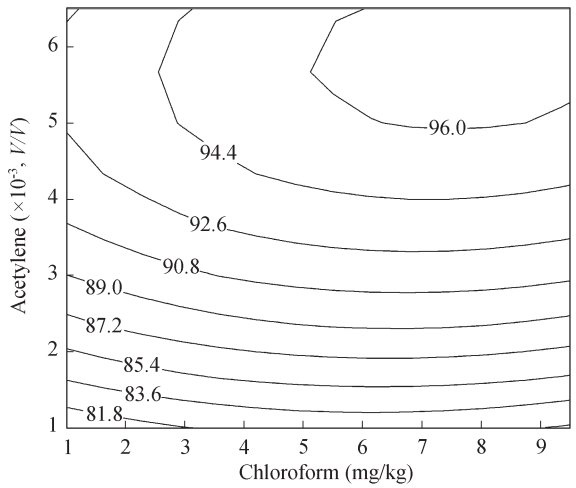

Fig. 5 Contour lines for the optimization of inhibition efficiency.

As shown in Fig. 5, acetylene had more significant effect on inhibition efficiency than chloroform (F = 99.15; p < 0.05). Compared with single factor experiments (Figs. 2 and 3), as the concentrations of chloroform and acetylene were 5 mg/kg and 3×10^{-3} (*V/V*), the inhibition efficiencies were 35.82% and 87.60%, respectively. However, the co-inhibition efficiency was calculated by Eq. (4) to be 92.40%. The results indicated that higher inhibition efficiency can be achieved at lower concentrations of chloroform and acetylene. It was meaningful for methanogens inhibition in refuse landfills, because diffusion loss of inhibitors could be reduced with a lower effective inhibition concentration in an open system. In addition, acetylene inhibited the activity of methanogens, while chloroform inhibited metabolic process of methanogens. Therefore, due to different inhibition mechanisms, some methanogens were inhibited by acetylene as well as chloroform.

2.4 Effect of chloroform and acetylene on the concentration of VFA

As shown in Table 3, chloroform (F = 121.3; p < 0.01) had more significant effect on the concentration of VFA than acetylene (F = 23.41; p < 0.05) according to ANOVA. No interactions between chloroform and acetylene were found, which is confirmed by RSREG analysis of cross-product (F = 0). Figure 6 represents contour plots, which generated with one inhibitor kept at its specific concentration, and varying another within the experimental range. Figure 6a shows the effect of acetylene on the concentration of VFA with different concentration of chloroform. Low concentration acetylene (less than 6×10^{-3}, *V/V*) resulted in the increase of VFA concentration because of the methanogenesis inhibition. However, inhibition of acid-producing bacteria occurred at the higher concentration acetylene. As shown in Fig. 6b, chloroform, within the experimental range, had no inhibition on VFA production. It appeared that chloroform had a positive effect for the increasing of VFA concentration, since chlorinated hydrocarbons could change the metabolic pathway of acid-producing bacteria (Russell and Martin, 1984).

2.5 Effect of chloroform and acetylene on the molar ratio of propionic acid to acetic acid

The response surface and contour lines (Figs. 7 and 8) for the molar ratio of propionic acid to acetic acid showed that a maximum value of 1.208 can be achieved at chloroform and acetylene concentrations of 9.05 mg/kg and 3.6×10^{-3} (*V/V*), respectively. Increasing acetylene concentration resulted in the increase of P/A, and the P/A

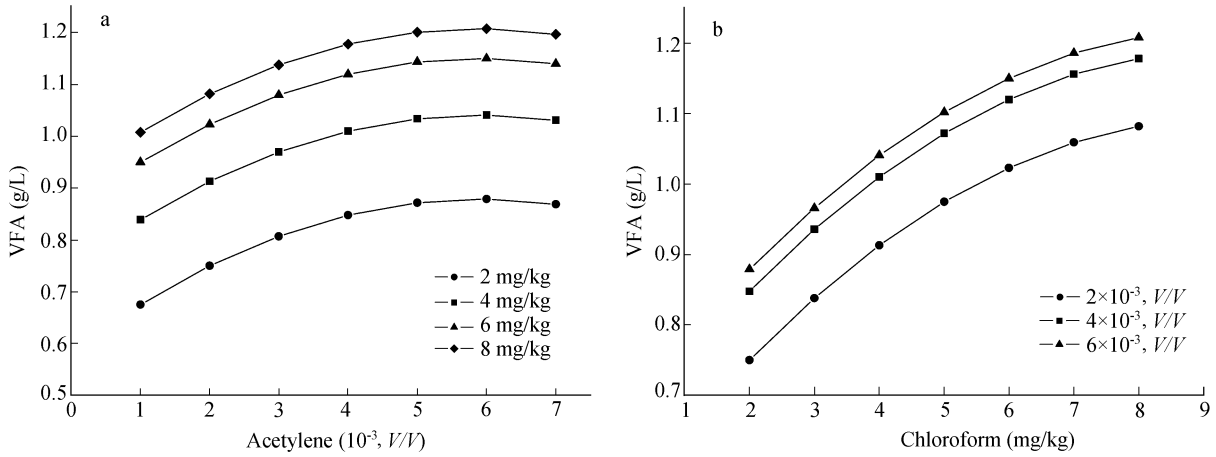

Fig. 6 Effect of acetylene (a) and chloroform (b) on VFA production with different concentration of chloroform (a) and acetylene (b), respectively.

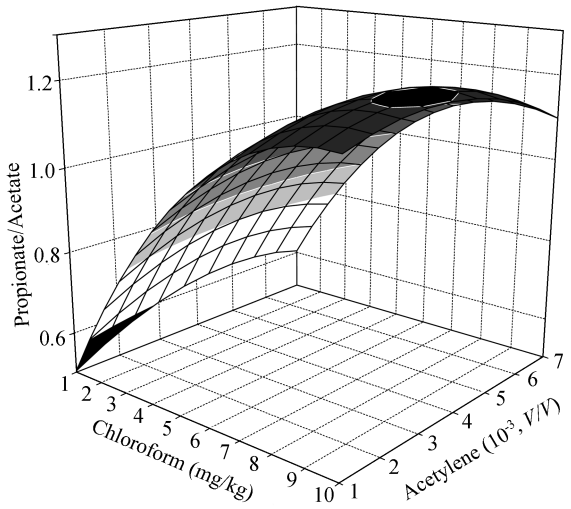

Fig. 7 Response surface for the optimization of the molar ratio of propionic acid to acetic acid.

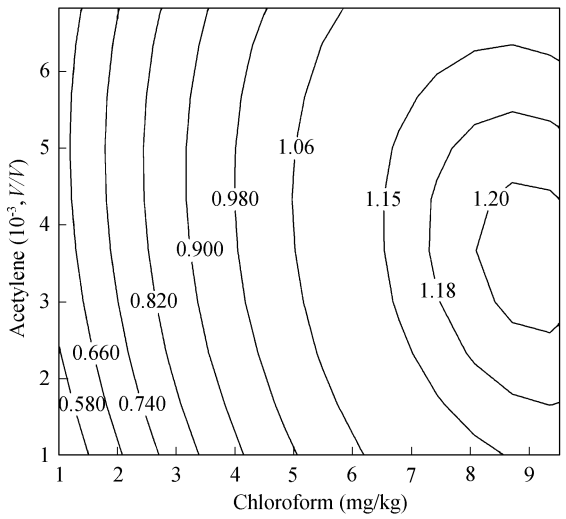

Fig. 8 Contour lines for the optimization of the molar ratio of propionic acid to acetic acid.

decreased rapidly as the concentration of acetylene exceeded 3.6×10^{-3} (*V/V*). As shown in Fig. 8, the concentration of acetylene had no significant effect on P/A, which is also confirmed by ANOVA ($F = 0.41$; $p > 0.05$). Chloroform contributed most to increase the P/A ($F = 22.46$; $p < 0.05$), which confirmed the conclusions that chlorinated hydrocarbons could change the metabolic pathway of acid-producing bacteria.

Co-inhibition, which had blocked reductive steps in methanogenesis, resulted in a decrease of acetic acid and an increase of propionic acid. Russell and Martin (1984) reported that Monensin, lasalocid, chloroform, and carbon monoxide decreased acetate production from trypticase. It is significant for methanogens inhibition and stabilization process of refuse landfills, since propionic acid could hardly be converted to acetic acid by acetogens and utilized by methanogens (Gallert and Winter, 2008; Li *et al*., 2008).

2.6 Prospect on co-inhibition technology practice at landfills

Higher inhibition efficiency at lower concentrations of chloroform and acetylene was achieved under co-inhibition. Due to much lower concentration of chloroform, it can be dissolved in leachate completely and reused by leachate recirculation without volatilization. Additionally, acetylene inhibition practice at landfills can be achieved by slow release of CaC_2, which has been studied at the scale-up experiment (300 kg substrate) by our research team. The controlled group began to generate methane (1%–5%, *V/V*) after 20 days and the concentration of methane reached 20%–30% after 3 months, but the experimental group hardly generated methane (< 1%, *V/V*) within 3 months. Moreover, chloroform can be dechlorinated by some uninhibited methanogenic strains after a long period acclimation (Fathepure and Boyd, 1988). According to the analysis and experimental results mentioned above, co-inhibition technology practice at landfills was feasible and the environmental damage was negligible.

3 Conclusions

This work investigated the inhibition effects and mechanisms of chloroform and acetylene on methanogenesis during the food origin wastes anaerobic digestion. Both of them had a specific inhibition effects on methanogenesis. RSREG was employed for experiments design. The results

showed that chloroform had significant effect on VFA production (F = 121.3; p < 0.01) and acetylene had significant effect on inhibition efficiency (F = 99.15; p < 0.05). A maximum molar ratio of propionic acid to acetic acid of 1.208 was estimated under the optimum conditions of chloroform concentration of 9.05 mg/kg and acetylene concentration of 3.6×10^{-3} (*V/V*). Finally, co-inhibitor technology can resolve the problem methane mitigation of landfills effectively before the final cover operation and have no environmental damage.

Acknowledgments

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