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Free acetic acid as the key factor for the inhibition of hydrogenotrophic methanogenesis in mesophilic mixed culture fermentation

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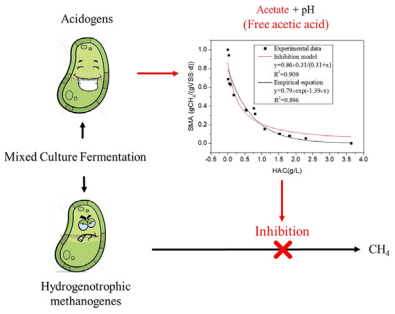
GRAPHICAL ABSTRACT



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ABSTRACT

The inhibition of acetate under acidic pH is an ideal way to reduce methanogenesis in mesophilic mixed culture fermentation (MCF). However, the effects of acetate concentration and acidic pH on methanogenesis remain unclear. Besides, although hydrogenotrophic methanogens can be suitable targets in MCF, they are generally ignored. Therefore, we intentionally enriched hydrogenotrophic methanogens and found that free acetic acid (FAA, x) concentration and specific methanogenic activity (SMA, y) were correlated according to the equation: $y = 0.86 \times 0.31/(0.31 + x)$ ($R^2 = 0.909$). The SMA was decreased by 50% and 90% at the FAA concentrations of 0.31 and 2.36 g/L, respectively. The coenzyme M concentration and relative electron transport activity agreed well with the FAA concentration. Moreover, the methanogenic activity could not be recovered when the FAA concentration exceeded 0.81 g/L. These findings indicated that neither acetate nor acidic pH, but FAA was the key factor to inhibit methanogenesis in MCF.

1. Introduction

The degradation process of organic waste in the mixed culture fermentation (MCF) can be divided into four cascading stages, namely hydrolysis, acidification, acetogenesis and homoacetogenesis, and methanogenesis (Chen et al., 2017). After methanogenesis is inhibited, volatile fatty acids (VFAs), such as acetate, propionate and butyrate, noticeably accumulate during the metabolism of acidogens. VFAs are

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impotant compounds and are also the indispensable precursors in the production of liquid biofuels (Zhang et al., 2013). It is established that methanogenesis includes acetoclastic methanogenesis and hydrogenotrophic methanogenesis. Acetoclastic methanogens, such as *Methanosaeta* species, convert acetate to methane and carbon dioxide (Dai et al., 2017). Hydrogenotrophic methanogenesis is performed by hydrogenotrophic methanogens, such as *Methanosphaera stadtmanii* and *Methanobrevibacter wolinii*, which convert hydrogen and carbon dioxide to methane (Demirel & Scherer, 2008). Accordingly, to produce more VFAs in MCF, both acetoclastic methanogenesis and hydrogenotrophic methanogenesis should be totally inhibited.

Conventional methods to inhibit the methanogenic activity include thermal treatment and nonspecific/specific inhibitors. Through thermal treatment, non-spore-forming microorganisms are killed, while some spore-forming bacteria, like *Clostridiaceae* and *Thermoanaerobacteriacea*, may survive (Fang et al., 2002). However, thermal treatment can only temporarily inhibit methanogens (Liu et al., 2016; Luo et al., 2011). Currently, as a typical nonspecific inhibitor, chloroform is widely used in anaerobic fermentation. However, besides inhibiting methanogens, chloroform also reduces the activity of homoacetogenic bacteria (Xu et al., 2010). In addition, 2-bromoethanesulfonate (BES) is another established specific inhibitor of methanogenesis (Liu et al., 2011). However, several microorganisms can consume BES in MCF and methanogens will be reactivated without enough BES (Zhang et al., 2013). Moreover, the high cost of BES also restricts its industrial application. Thus, exploring effective and cheap methods for the inhibition of methanogenesis is still necessary, and inhibitors, such as VFAs, are ideal candidates.

VanKessel et al. found that methanogenesis was pH-dependent, and no methane was detected at pH values below 6.0 (VanKessel & Russell, 1996). On the contrary, Kim et al. (2004) found that low pH reduced the methanogenic activity, but methane was still detectable at pH 4.8 and acetate concentration of 1 g/L. Meanwhile, Horn et al. (2003) contended that the acetate concentration was the key inhibitor of methanogenesis, as they found that at pH 4.5 the hydrogenotrophic methanogenesis was totally inhibited at the acetate concentration of just 0.3 g/L. Lins et al. found that in anaerobic digestor (AD), the activity of *Methanoculleus* spp. was hardly affected at the acetate concentration of 9.0 g/L and neutral pH (Lins et al., 2014). Recently, Xiao et al. found that methanogenesis occurred even at the acetate concentration of 8.2 g/L and pH of 4.7 (Xiao et al., 2016). These contradictory results indicate that neither the acetate concentration nor the pH is a plausible methanogenesis inhibitory factor. Zhou et al. (2008) found that neither pH nor nitrite, but instead free nitrous acid was the key inhibitory factor in a denitrifying-enhanced biological phosphorus removal sludge. Accordingly, it is reasonable to postulate that free acetic acid (FAA) is a key methanogenesis inhibitory factor.

Importantly, the different tolerance capacities of acetoclastic and hydrogenotrophic methanogens have been reported. For instance, hydrogenotrophic methanogens can outcompete acetoclastic methanogens under the same inhibitory conditions (Ahring et al., 2001; Gijzen et al., 2000). In contrast, acetoclastic methanogens were found to be the dominant archaea in mesophilic MCF (Junicke et al., 2016). Therefore, hydrogenotrophic methanogens are the logical targets and will be initially enriched when analyzing the inhibitory effects on methanogenesis in mesophilic MCF. In addition, when FAA is used as an inhibitor, the bacterial activity will be recovered when the concentration of FAA is below a certain threshold, and using high concentration costs too much. Therefore, it is also important to determine the most suitable FAA concentration that does not inhibit methanogens.

Methanogenesis is an enzyme-mediated reaction. Coenzyme M (CoM; 2-mercaptoethane sulphonate) in hydrogenotrophic methanogens can be converted into methyl CoM with carbon dioxide. In addition, methane is released after reduction of methyl CoM by the methyl CoM reductase enzyme (Pramanik & Kim, 2012). Thus, CoM is the key factor in the methanogenesis process that can influence the production of methane. INT (2-(p-iodophenyl)-3-(pnitrophenyl)-5-phenyltetrazolium chloride) is an oxidation-reduction dye, which can be reduced by electrons produced in the electron transport system (ETS) (Yin et al., 2005). Recently, INT was shown to be a suitable probe to assay the electron transport system activity of methanogens (Tian et al., 2017). Accordingly, the CoM concentration and ETS are suitable factors to analyze the activity of methanogens.

As it is known, H_2 and CO_2 are the suitable substrates for the selective enrichment of hydrogenotrophic methanogenesis over acetoclastic methanogenesis in MCF (Demirel & Scherer, 2008). In view of that, the main objectives of this work are based on the use of enriched sludge in hydrogenotrophic methanogenesis: 1) to evaluate whether FAA is a key factor in methane production inhibition; 2) to examine the correlation between the CoM concentration, ETS, and FAA concentration; 3) to determine the threshold of FFA that will cause inhibition of archaea.

2. Materials and methods

2.1. Inoculum and medium

The mixed culture was obtained from a local mesophilic digester treating starch wastewater in the Shandong province, China. The total solids (TS) and volatile suspended solid (VSS) of the mixed culture were 30 and 11.7 g/L, respectively. After grinding and dispersion to break granular sludge, 40 mL of the inoculum was added into a 2 L reactor containing 1.25 L anaerobic medium. The anaerobic sludge digestion medium was refreshed 3 times, and no VFAs were detected. The reactor was stirred with a magnetic stirrer at a speed of 120 rpm. A 700-mL gas containing 80% hydrogen and 20% carbon dioxide was injected into the headspace of the reactor every 12 h, and the reactor was operated for 1 month. After enrichment, the microbial community of the enriched sludge and raw sludge (without enrichment) were analyzed by high-throughput sequencing.

The mixed culture medium components were the same as those reported by Zhang et al. (2013), comprising the following: NH_4Cl 500 mg/L; KH_2PO_4 200 mg/L; Na_2SO_4 50 mg/L; KCl 50 mg/L; $CaCl_2$ 10 mg/L; $MgCl_2{\cdot}6H_2O$ 70 mg/L; $MnCl_2{\cdot}4H_2O$ 0.8 mg/L; $CoCl_2{\cdot}2H_2O$ 1.2 mg/L; $FeSO_4{\cdot}7H_2O$ 3.2 mg/L; $AlCl_3$ 0.5 mg/L; $NaMO_4{\cdot}2H_2O$ 0.1 mg/L; H_3BO_3 0.2 mg/L; $NiCl_2{\cdot}6H_2O$ 0.5 mg/L; $CuCl_2{\cdot}2H_2O$ 1.1 mg/L; $ZnSO_4{\cdot}2H_2O$ 3.2 mg/L; EDTA-2Na 3.0 mg/L; biotin 2.0 mg/L; folic acid 2.0 mg/L; vitamin B_6 10 mg/L; riboflavin 5.0 mg/L; vitamin B_1 5.0 mg/L; niacin 5.0 mg/L; pantothenic acid 5.0 mg/L; vitamin B_{12} 0.1 mg/L; 4-aminobenzoic acid 5.0 mg/L; lipoic acid 5.0 mg/L.

2.2. Free acetic acid (FAA) concentration and specific methanogenic activity

The correlation between the concentration of FAA and acetate at different pH is shown in Eq. (1).

$$FAA = \frac{C_{total} \times C_{H^+}}{Ka + C_{H^+}} \quad (1)$$

where FAA is the free acetic acid concentration (g/L), C_{total} is the total acetate concentration (g/L), C_{H^+} is the proton concentration (g/L), and Ka is the dissociation constant of acetate.

The specific methanogenic activity (SMA), expressed in gCH_4/(gVSS·d), reflects the activity of methanogens. In this study, the SMA was calculated based on Eq. (2).

$$SMA = \frac{r}{X} \quad (2)$$

where r is the production rate of methane (gCH_4/(L·d)), and X is the concentration of biomass (gVSS/L).