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iTRAQ quantitative proteomic analysis reveals the pathways for methanation of propionate facilitated by magnetite

Yuhang Jing [a], Jingjing Wan [a], Irini Angelidaki [b], Shicheng Zhang [a], Gang Luo [a, *]

[a] Shanghai Key Laboratory of Atmospheric Particle Pollution and Prevention (LAP^3), Department of Environmental Science and Engineering, Fudan University, 200433, Shanghai, China

[b] Department of Environmental Engineering, Technical University of Denmark, DK-2800, Kgs Lyngby, Denmark

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ABSTRACT

Methanation of propionate requires syntrophic interaction of propionate-oxidizing bacteria and hydrogenotrophic methanogens, which is referred to as interspecies electron transfer. The present study showed that 10 mg/L conductive magnetite enhanced the methane production rate from propionate by around 44% in batch experiments, and both direct interspecies electron transfer and interspecies H_2 transfer were thermodynamically feasible with the addition of magnetite. The methanation of propionate facilitated by magnetite was also demonstrated in a long-term operated continuous reactor. The methane production rate from acetate by the enriched mixed culture with magnetite was higher than that without magnetite, while similar methane production rates were found from H_2/CO_2 by the enriched mixed culture with and without magnetite. The ability to utilize molecular H_2 indicated interspecies H_2 transfer played a role in the enriched culture with magnetite, and propionate-oxidizing bacteria relating with interspecies H_2 transfer were also detected by metagenomic sequencing. Metagenomic sequencing analysis also showed that *Thauera*, possibly relating with direct interspecies electron transfer, were enriched with the addition of magnetite. iTRAQ quantitative proteomic analysis, which was used in mixed culture for the first time, showed that magnetite induced the changes of protein expression levels involved in various pathways during the methanation of propionate. The up-regulation of proteins involved in propionate metabolism were found, and they were mainly originated from propionate-oxidizing bacteria which were not reported to be capable of direct interspecies electron transfer until now. Cytochrome *c* oxidase was also revealed as the possible protein relating with direct interspecies electron transfer considering its up-regulation with the addition of magnetite and origination from *Thauera*. Most of the up-regulated proteins in methane metabolism were originated from *Methanosaeta*, while most of the enzymes with down-regulated proteins were originated from *Methanosarcina*. However, the up-regulated proteins relating with hydrogenotrophic methanogenesis were originated from neither *Methanosaeta* nor *Methanosarcina*, indicating they were not involved in direct interspecies electron transfer. The hydrogenotrophic methanogens, e.g. *Methanospirillum*, *Methanosphaerula* et al., might be involved in direct interspecies electron transfer. Overall, the present study showed that both direct interspecies electron transfer and interspecies H_2 transfer were present during methanation of propionate facilitated by magnetite.

1. Introduction

Anaerobic digestion of organic wastes for methane production is one of the few proven, economical, large-scale bioenergy strategies, and it is already a widespread practice. New approaches are expected to improve the efficiency and expand the application (Madsen et al., 2011; McCarty et al., 2011). For instance, the technology requires considerable monitoring and optimization due to the frequent process instabilities, which often result in the accumulation of propionate (Verstraete et al., 2005; Wang et al., 2006).

Propionate is a key metabolite in the anaerobic degradation of organic wastes (de Bok et al., 2004; Dong et al., 1994). The anaerobic propionate oxidation performed by propionate-oxidizing bacteria (POB) is highly endergonic under the standard conditions and is

* Corresponding author.
E-mail address: gangl@fudan.edu.cn (G. Luo).

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feasible only under conditions with low H_2 concentrations. Therefore, H_2-scavenging reaction is essential to make the propionate oxidation energetically favorable (Aulenta et al., 2013; Kang et al., 1996). Methanogenic degradation of propionate requires syntrophic interaction of POB and hydrogenotrophic methanogens, which is specifically referred to as interspecies electron transfer (Aulenta et al., 2013; McInerney et al., 2009; Stams and Plugge, 2009). Effective interspecies electron transfer is essential in methanogenic ecosystems (Bryant et al., 1967; McInerney et al. 2008, 2009). The microbial community that metabolizes multicarbon organics other than acetate requires an electron sink in order for complex organics to be converted to CH_4 and CO_2. Hydrogenotrophic methanogens can function as this sink, consuming electrons in the reduction of CO_2 to CH_4.

The first mechanism described for electron transfer in methanogenic systems was interspecies hydrogen transfer (IHT), in which microorganisms that require an electron sink reduce protons to produce H_2 and the methanogens utilize H_2 as an electron donor (Boone et al., 1989; McInerney et al. 2008, 2009; Stams et al., 2006). Formate may also act as an electron carrier (Boone et al., 1989; Lovley, 2011a; McInerney et al., 2009; Thiele and Zeikus, 1988). Recent findings, however, have pinpointed the possibility that other interspecies electron transfer mechanisms, not relying on the exchange of diffusible molecules or energy carriers among partners, may also play a role in syntrophic communities of anaerobic microorganisms (Lovley, 2011b, 2012; Malvankar and Lovley, 2012). Direct interspecies electron transfer (DIET) may be a more effective mechanism for interspecies electron exchange under anaerobic conditions than interspecies electron transfer via reduced molecules such as H_2 and formate (Liu et al., 2012; Morita et al., 2011; Summers et al., 2010).

DIET has been studied most intensively in adaptively evolved co-cultures of *Geobacter metallireducens* and *Geobacter sulfurreducens* (Kato et al., 2012a), however, it also appeared that DIET was an important process for interspecies electron exchange in multispecies aggregates from a methanogenic digester in which *Geobacter* and *Methanosaeta* species predominated (Kato et al., 2012b). Recent studies showed that the anaerobic degradation rate of organics (e.g. acetate, ethanol) could be enhanced with the addition of electrically conductive materials (e.g. magnetite) and DIET was suggested to be the main reason (Aulenta et al., 2013; Jiang et al., 2013; Kato et al., 2010). However, only batch experiments were carried out in the previous studies, and the long-term effects of electrically conductive materials on the enhancement of methane production was not investigated. Besides, only microbial community and theoretical thermodynamic analysis were used to support the presence of DIET with the addition of electrically conductive materials (Cruz Viggi et al., 2014; Kato et al., 2012a; Yamada et al., 2015; Yang et al., 2015), which were still not convincible.

Metaproteomics can identify actually translated genes revealing important metabolic information of the microbial communities (Lu et al., 2014), which provides direct proof on the changes of metabolic pathways. iTRAQ labeling is an efficient quantitative metaproteomic technology, which uses a family of isobaric isotope tags to label tryptic peptides from differential samples (Mertins et al., 2012). iTRAQ labeling can identify and quantify the proteins from multiple differential samples associated with various exposures simultaneously. Therefore, it has been used to reveal the dose-effect relationship between expression levels of the differential proteins and exposure levels of the pollutants (Gunnigle et al., 2013; Tan et al., 2012; Xie et al., 2015). However, iTRAQ quantitative proteomic analysis is currently only used for analysis of pure cultures under different exposures, and it is still not used in mixed cultures such as microbiome for biogas production.

Based on the above considerations, the present study investigated the effects of conductive magnetite on the anaerobic propionate degradation based on both batch and continuous experiments. Furthermore, iTRAQ quantitative proteomic analysis was employed to analyze the changes of metabolic pathways induced by the addition of magnetite.

2. Material and methods

2.1. Batch experiments

All the batch experiments were conducted in 118 mL serum bottles. 40 mL BA medium containing inoculum (5 gVSS/L) supplemented with sodium propionate (30 mM) and nano magnetite (0, 10 mg/L, 50 mg/L, 100 mg/L or 1000 mg/L). The composition of BA medium was described in a previous study (Angelidaki and Sanders, 2004). The pH of all the bottles were adjusted to 7. All the bottles were flushed with pure N_2 for 5 min, sealed with butyl rubber stoppers, and then incubated at temperature 37 °C in a shaker with 200 rpm. H_2 in the gas phase could be considered to be equalized with that in the liquid phase since relatively high intensity of mixing was applied. The inoculum used in this study was obtained from an anaerobic reactor treating cassava stillage in an ethanol plant (Taicang, Suzhou, China). Bottles with BA medium and inoculum only, but without propionate and magnetite, were used as blanks. All the tests were prepared in duplicate. A modified Gompertz model (equation (1)) was used to quantitatively analyze the production of methane under various conditions, which was widely used previously (Costa et al., 2012; Lü et al., 2013). $M(t)$ (mL) is the cumulative methane production at time t, P (mL CH_4) is the maximum CH_4 potential, R_m (mL/d) is the maximum CH_4 production rate, λ (d) is the lag-phase time, and e is 2.71 828. The three parameters, P, R_m, and λ were estimated through global curve-fitting using OriginPro 8.0 with a minimum residual sum of squared errors between the experimental data and model curves.

$$M(t) = P \times \exp\left\{ -\exp\left[\frac{R_m \times \mathrm{e}}{P} \times (\lambda - t) + 1\right]\right\} \quad (1)$$

2.2. Fed-batch continuous experiments

Fed-batch continuous experiments were further conducted in two 600 mL serum bottles with working volume 320 mL. The influent of one reactor (control) was BA medium containing only 30 mM propionate, while the influent of the other one was BA medium containing 30 mM propionate and 10 mg/L nano magnetite. Each reactor was inoculated with source culture from the batch experiments. The reactors were fed every 5 days and the hydraulic retention time was controlled at 20 days. All the reactors were placed in a shaker with the speed 300 rpm and the temperature 37 °C.

2.3. Methanation of acetate and H_2/CO_2 by the enriched cultures

Batch experiments for estimation of the methanogenic activity of enriched cultures from the continuous reactors on acetate and H_2/CO_2 were carried out when steady-states were achieved in continuous reactors. Similar procedure was adopted as described in 2.1. The differences were: (1) propionate were replaced by either acetate (20 mM) or H_2/CO_2 (80/20) under 1 atm, (2) the inocula

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