

# Effect of Surface Characteristics of Microorganisms in Anaerobic Sludge on Immobilization to Support Materials

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## Introduction

From the viewpoint of establishing a recycling society, developing new energy sources, and preventing global warming, anaerobic treatment has received renewed attention as a technology for recovering energy from organic waste with a high water content. The anaerobic digestion of complex organic materials to produce methane consists of a cascade of biochemical conversions catalyzed by different physiological groups of interacting microbes. Complex organic materials are first hydrolyzed to simpler organics before being fermented to volatile acids by acidogens. Those volatile acids longer than two carbons are then converted to acetate and hydrogen gas by hydrogen-producing acetogens. Finally, the acetate and hydrogen gas is converted to methane by methanogens<sup>1</sup>. Acetate is the precursor for about 70% of the methane produced during the anaerobic digestion of complex organic materials<sup>2</sup>. The decarboxylation of acetate is the rate-limiting step in methane fermentation<sup>3</sup>. Therefore, to achieve highly efficient anaerobic treatment, it is necessary to immobilize the acetate-utilizing methanogens in the fermenter. However, the basic mechanisms of immobilization are poorly understood. Adhesion is strongly dependent on the surface characteristics of the microbial cells. Surface physico-chemical properties can be treated as an indicator of the adhesion properties of microbial cells.

In this study, the effect of surface characteristics of microbes living in anaerobic sludge on the immobilization of support materials was examined experimentally. The anaerobic sludge collected from an anaerobic digester was used for the experiment. *Methanosaeta* and *Methanosarcina* species are the only known methanogens that are capable of acetate catabolism. *Methanosaeta* species can use acetate as sole growth substrate. In contrast, *Methanosarcina* species can use acetate, H<sub>2</sub>/CO<sub>2</sub>, methanol, and methylamines as growth substrates<sup>4</sup>. Both acetate and methanol were used as substrate in the current experiments to investigate the immobilization of *Methanosaeta* and *Methanosarcina* species.

## Materials and methods

### *Microbial cells*

Five typical microbes were selected to investigate the surface characteristics of microbes living in anaerobic digester. Three different acidogens (proteolytic bacteria, amyolytic bacteria, lipolytic bacteria) were selectively cultivated in a specific medium supplemented with a specific substrate per 1,000 ml of PGY medium. A specific substrate for proteolytic bacteria was skim milk (10 g), for amyolytic bacteria it was amylogen (2 g), and for lipolytic bacteria it was tributyrin (5 g). Selective cultivation was performed in a 1-L serum bottle. The supernatant liquid (0.1%), fractionated anaerobic sludge by centrifugation at 3,000 rpm for 10 min, was inoculated to 500 ml of specific medium in each bottle. The anaerobic sludge was collected from the anaerobic treatment plant operated at Yagi bio-ecology center, Yagi-cho, Kyoto, Japan. *Methanosarcina barkeri* (JCM 10043) was purchased from the Japan Collection of Microorganisms. *Methanosaeta concilii* (DSM 3671) was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen. These acetate-utilizing methanogens were

grown anaerobically without shaking at 37 °C and neutral pH in pressure culture bottles sealed with a butyl rubber stopper and aluminum crimp seal<sup>5</sup>. Cells were harvested by centrifugation at 10,000 rpm at 4 °C for 10 min and washed in triplicate using 0.9w/v% NaCl aqueous solution. The washed cells were resuspended in the sterile solution to evaluate the physico-chemical properties of the microbial cells.

### ***Support materials***

Two different support materials were used: bamboo charcoal is a hydrophobic and negatively-charged particle, and alumina is a hydrophilic and positively-charged particle. The size of these support materials was about 5 mm in diameter.

### ***Measurements of electrophoretic mobility***

The electrophoretic mobility (EPM) of microbial cells was measured using an electrophoretic light-scattering spectrophotometer (ELS-800; Otsuka Electronics, Osaka, Japan). In this procedure, the migration velocity of microbial cells within the electric field of an electrophoresis cell is determined by measuring the Doppler effect using a laser beam. The washed microbial cells were resuspended in the sterile NaCl aqueous solution at the desired concentration and vortexed for 3 min. All measurements were carried out in triplicate.

### ***Measurements of hydrophobicity***

The surface hydrophobicity of microbial cells was determined by microbial adhesion to hydrocarbon (MATH) assay<sup>6</sup>. 0.4 ml of hydrocarbon (n-hexadecane) was added to a test tube containing 2.4 ml of the washed cell suspension resuspended in a PUM buffer. The mixtures were vortexed uniformly for 2 min. The solution was then allowed to stand for 15 min to ensure complete separation of the two phases. The percentage of cells adhering to hydrocarbon  $F$ , which was used as a measure of cell-surface hydrophobicity, was calculated using the following equation:  $F = (1 - A_t/A_0) \times 100$ , where  $A_0$  is the initial absorbance of the microbial suspension before mixing, and  $A_t$  is the absorbance after mixing.

### ***Immobilization test of microbes***

The immobilization test of microbes is composed of three steps: incubation and immobilization of microbes, washing of support materials, and incubation using immobilized methanogens. 4.0 ml of DSM 120 medium inoculated with 5% of anaerobic sludge and 1.0 g of support material were placed into each of the 21-ml serum bottles. The bottles were then capped with butyl rubber stoppers and crimped with aluminum seals. After sealing, the headspaces of the bottles were purged with an oxygen-free 80% N<sub>2</sub>-20% CO<sub>2</sub> gas mixture to ensure anaerobic conditions. The serum bottles were then incubated at 37 °C. Methane production was monitored by TCD gas chromatograph (GC-8APT; Shimazu, Kyoto, Japan). Sodium acetate and methanol were used as substrate at initial concentrations of 20 mol/m<sup>3</sup>. Control cultures were prepared in the same way without dissolving substrates in the content of the bottle. After saturation of biogas production, the aluminum caps of the bottles were removed, and the support materials were taken out from the bottles. The support materials were washed with the 0.9w/v% sterile NaCl aqueous solution to remove the residues on the support materials. The washed support materials were put into new 21-ml serum bottles filled with 4.0 ml of DSM 120 medium dissolved in 20 mol/m<sup>3</sup> of the substrate. The substrates used were the same at both the first and final steps. Subsequently, the bottles were sealed with butyl-rubber and aluminum caps. Cultures were grown at 37 °C under N<sub>2</sub>/CO<sub>2</sub> atmosphere.

## Results and discussion

### *Electrostatic properties of microbes*

Table 1 shows the EPM of five typical microbes living in an anaerobic digester. Zeta potentials were calculated using the Smoluchowski equation. The EPM measurement was conducted in phosphate buffer (pH 7.0, ionic strength 100 mol/m<sup>3</sup>). The EPM of three acidogens (proteolytic bacteria, amylolytic bacteria, lipolytic bacteria) selectively cultivated from the anaerobic sludge were  $-2.19 \times 10^{-8}$ ,  $-1.35 \times 10^{-8}$ , and  $-1.32 \times 10^{-8}$  m<sup>2</sup>/V/s, respectively. It was found that these acidogens were negatively charged. The EPM of *M. barkeri* was  $-1.60 \times 10^{-8}$  m<sup>2</sup>/V/s, and *M. barkeri* was also negatively charged. The majority of microbial cells are generally negatively charged because the carboxyl group and phosphoric acid group at the microbial surface are dissociated at neutral pH. In contrast, the EPM of *M. concilii* was  $0.09 \times 10^{-8}$  m<sup>2</sup>/V/s. This value was significantly smaller than that of the three acidogens, as well as *M. barkeri*. Therefore, it was shown that *M. concilii* was hardly charged, which was remarkable, since it is unlike general microbes.

Table 1 Electrophoretic mobility of five typical microbes living in an anaerobic digester.

	Microbial cell	Electrophoretic mobility (10 <sup>-8</sup> m <sup>2</sup> /V/s)	Zeta-potential (mV)
Acidogen	<i>Proteolytic bacteria</i>	-2.19	-28.3
	<i>Amylolytic bacteria</i>	-1.35	-17.5
	<i>Lipolytic bacteria</i>	-1.32	-17.2
Acetate-utilizing	<i>Methanosaeta concilii</i>	0.09	1.2
Methanogen	<i>Methanosarcina barkeri</i>	-1.60	-20.7
Control	<i>Escherichia coli</i>	-1.45	-18.8

### *Hydrophobic properties of microbes*

Fig. 1 shows the microbial adhesion to hydrocarbon as a function of the initial absorbance of the cell suspension. Three acidogens showed an affinity to n-hexadecane. The percentage of proteolytic bacteria, amylolytic bacteria, and lipolytic bacteria adhering to hydrocarbon was 54.4, 64.3, and 72.3, respectively, when the initial absorbance of the cell suspension was about 0.1. Two acetate-utilizing methanogens also showed an affinity to n-hexadecane. The percentage of *M. concilii* and *M. barkeri* adhering to hydrocarbon was 66.7 and 66.1, respectively, when the initial absorbance of the cell suspension was about 0.1. The degree of adhesion decreased with increasing initial absorbance because microbial cells were unable to sufficiently adhere to the hydrocarbon. In contrast, *E. coli* did not show any affinity to n-hexadecane. Since the adhesion of five typical microbes was greater than 50%, we speculated that the microbes living in anaerobic sludge are relatively hydrophobic. The adherence of bamboo charcoal to n-hexadecane was 86.6%, and to alumina it was 7.3%. It was confirmed that bamboo charcoal was hydrophobic, and alumina was hydrophilic.

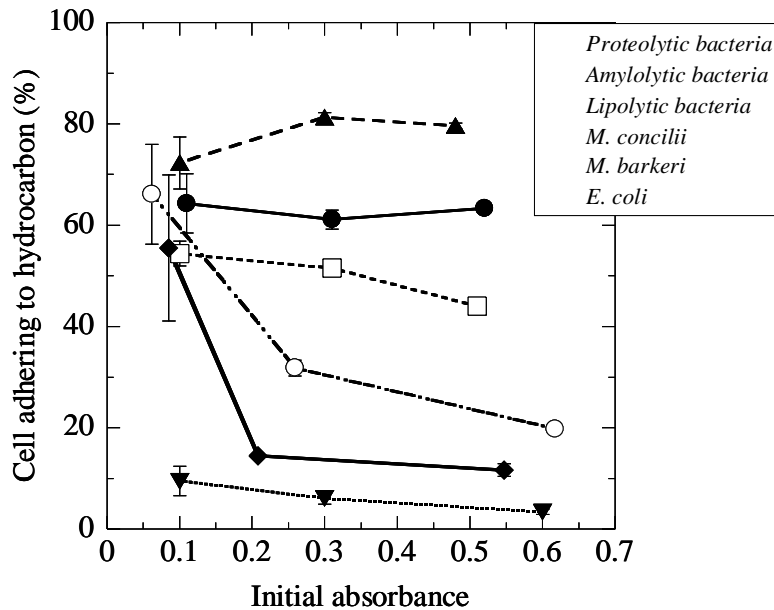


Fig. 1 Microbial adhesion to n-hexadecane as a function of the initial absorbance of the cell suspension.

### Immobilizing of acetate-utilizing methanogens

Fig. 2 shows the methane production using immobilized methanogens on support material. When sodium acetate was used as a substrate, *Methanosaeta* species were a dominant species in the acetate-utilizing methanogens. In contrast, *Methanosarcina* species were a dominant species when using methanol as substrate. When the microbes were cultivated by sodium acetate, the methane production increased gradually over time using the methanogens immobilized on bamboo charcoal and could not be observed using the methanogens immobilized on alumina. When the microbes were cultivated by methanol, the methane production increased rapidly using the methanogens immobilized on bamboo charcoal and was observed 3 days later using the methanogens immobilized on alumina. These results indicated that *Methanosarcina* species adhered to both surfaces of bamboo charcoal and alumina, while *Methanosaeta* species adhered to bamboo charcoal, and not adhered to alumina.

*M. concilii* are non-charged and hydrophobic cells. This indicates that the Coulomb force acts only weakly between *M. concilii* and support materials, while the van der Waals force universally acts between materials. *M. concilii* approaches the support materials by the van der Waals attraction which acts between *M. concilii* and support materials. When the support material is bamboo charcoal, *M. concilii* could be adhered to the surface by hydrophobic attraction between *M. concilii* and bamboo charcoal. In contrast, since alumina is hydrophilic, *M. concilii* would not adhere to the surface of alumina. On the other hand, *M. barkeri* and acidogens are negatively charged and hydrophobic cells. The electrostatic repulsive force and hydrophobic attractive force act between the microbes (except for *Methanosaeta* species) and the bamboo charcoal. Since *Methanosarcina* species more easily adhered to the hydrophobic surface of bamboo charcoal than the hydrophilic surface of alumina, it was assumed that the hydrophobic attractive force was more dominant than the electrostatic repulsive force in this experimental condition. Therefore, after the microbes approach the solid surface, it is important that the microbial cells have an affinity to the support material to immobilize the microbe. These results of our experiment indicated that the hydrophobic and negatively charged support material, which could suppress the immobilization of microbes except for *Methanosaeta* species, was suitable for the selective immobilization of the *Methanosaeta* species that are the most important microbes in anaerobic digestion.

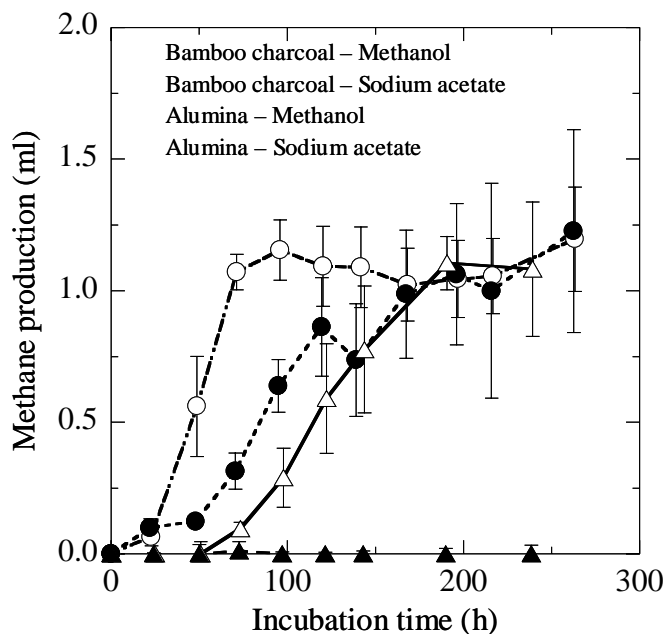


Fig. 2 Methane production using immobilized methanogens on support material.

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