[Regular Paper]

High-rate Fermentation of Acetate to Methane under Saline Condition by Aceticlastic Methanogens Immobilized in Marine Sediment

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High-rate production of methane from acetate using a fixed-bed reactor filled with marine sediment containing halotolerant aceticlastic methanogens and 3 % NaCl was investigated. In continuous culture, the methane production rate increased with increasing dilution rate. The maximum methane production rate of 750 mM d⁻¹ was observed at a dilution rate of 16.2 d⁻¹, which is impossible without immobilization of the cells in the sediment. Using scanning electron microscopy, we observed *Methanosaeta*-like filamentous microorganisms and *Methanosarcina*-like coccoid microorganisms in granule-like structures. The results demonstrated that marine sediment is not only a promising microbial resource of halophilic aceticlastic methanogens, but also a supporting material to fix aceticlastic methanogens in a fixed-bed reactor.

Keywords

Methane, Acetate, Aceticlastic methanogen, Fixed-bed reactor, Saline condition

1. Introduction

Methane fermentation is employed worldwide for organic waste treatment and energy recovery from various kinds of organic matter including manure, food, and municipal wastes. However, methane fermentation using common anaerobic technologies in saline condition (>3 %) is often difficult because of inhibitory effect by salinity^{1)~7)}.

Methane fermentation occurs in complex microbial ecosystems, in which organic matter is converted to methane in three steps: hydrolysis/acidogenesis, acetogenesis, and methanogenesis⁸⁾. In the first step, organic matter is converted to volatile fatty acids (VFAs) by various bacteria. Then, VFAs such as propionate and butyrate are converted to acetate and hydrogen by propionate- and butyrate-oxidizing bacteria, respectively. The final step of methane fermentation, formation of methane from CO₂ with H₂ is promoted by the activity of hydrogenotrophic methanogens, whereas acetate degradation can proceed through two different path-

ways: direct cleavage of acetate by aceticlastic methanogens or syntrophic acetate oxidation (SAO)⁹⁾. The aceticlastic methanogens cleavage methyl group of acetate into methane, while the carboxyl group is converted into CO₂¹⁰⁾. On the other hand, SAO is a two-step reaction in which acetate is primarily oxidized to H₂ and CO₂ by SAO bacteria, followed by the subsequent reduction of CO₂ with H₂ to methane by hydrogenotrophic methanogens (SAO-HM pathway)⁹⁾.

It has been reported that most of the methane (approximately 65–80 %) generated from fermentation is derived from direct cleavage of acetate by aceticlastic methanogens^{9), 11)~13)}. However, recent studies have reported a significant contribution by SAO-HM pathway to the production of methane under inhibitory conditions such as the level of ammonium–nitrogen, acetate concentration, or the synergetic stress of acids and ammonium, dilution rate, temperature and prevailing methanogenic population structure^{9), 10), 14)}. Nevertheless, it is generally assumed that direct cleavage of acetate is important and predominant pathway though depending on the environmental conditions. Hence, aceticlastic methanogens play an important role in methane fermentation. Only two genera, *Methanosarcina* and *Methanosaeta*, are known to be aceticlastic methano-

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gens¹⁵). Unlike *Methanosarcina*, which prefers methylated compounds such as methanol and methylamines to acetate, *Methanosaeta* is a specialist that uses only acetate¹⁵.

Optimal design of an anaerobic digester for high-rate methane fermentation is important to achieve high organic loading rate, reduced hydraulic retention time (HRT), and high methane yields¹⁶. In this context, one- or two-stage digesters have been employed. In one-stage digestion, all microbiological phases of anaerobic digestion occur in one tank. However, conditions that are favorable to the growth of acid-forming bacteria such as short HRT and low pH, may be inhibitory to methane-forming bacteria¹⁷. On the other hand, in a two-stage system, different microbial phases are separated. In such systems, the hydrolytic and acidification phases may occur in the first reactor, while acetogenesis and methanogenesis occur in the second reactor. The concept of two-stage digestion is driven by separate optimization of each step, in which different types of microorganisms with different physiology participate. Therefore, two-stage system has several advantages over conventional one-phase processes, such as increased stability of the process by controlling the acidification-phase in order to prevent overloading and the build-up of toxic material, and higher organic loading rates and shorter HRT, resulting in potentially higher yields of biogas in smaller digesters than in one-stage reactors^{16,17}.

Application of marine aceticlastic methanogens for conventional processes in a second reactor in two-stage digestion would enable treatment of organic wastes with high salinity^{6,18,19}. We recently reported that the aceticlastic methanogen *Methanosaeta* sp. strain H_A, highly enriched from marine sediment collected from Hiroshima Bay, exhibited growth at 2.06 M (corresponding to 12 %) NaCl²⁰. Therefore, we investigated continuous methane production from acetate by enriched *Methanosaeta* sp. strain H_A in a glass column reactor packed with a polypropylene-ceramic mixture as support material for fixation of *Methanosaeta*²⁰. In this set-up, methane production rate under 3 % NaCl was 70 mM d⁻¹. However, we had previously reported that microbial consortia in coastal mud sediment produced methane from acetate originating from organic matter in the sediment at the higher rate of 96 mM d⁻¹ under 3 % NaCl²¹. This suggested that inorganic, small silt in the sediment is a promising candidate for fixing aceticlastic methanogens, allowing higher methane production from acetate than artificial support carriers. However, since the acetate concentration was limited to the amount of organic matter in the marine sediment, the potential of the sediment as support material was not fully characterized.

Therefore, in this study, we attempted high-rate of methane from acetate with acclimated aceticlastic methan-

ogens derived from marine sediment under 3 % NaCl.

2. Materials and Methods

2.1. Source of Inoculum and Culture Media

Marine sediment was collected from the coastal seabed of Hiroshima Bay as reported previously²¹. The sediment samples were refrigerated at 4 °C until use. Artificial seawater containing 30 g NaCl, 3.0 g MgSO₄·7H₂O, and 0.053 g CaCl₂·5H₂O per L of deionized water was used for acclimatization of the anaerobic marine sediment microbiota. The pH of the artificial seawater was adjusted to 8.0 with 1 M NaOH. Basal medium was employed for all experiments except for the acclimatization. The composition of the basal medium was as follows: 0.3 g KH₂PO₄, 1.0 g NH₄Cl, 0.1 g MgCl₂·6H₂O, 0.08 g CaCl₂·2H₂O, 4 g KHCO₃, 30 g NaCl, 5.0 g sodium acetate, 1.0 mg resazurin, 10 mL vitamin solution, and 10 mL trace element solution. The vitamin solution contained 2.0 mg biotin, 2.0 mg folic acid, 10.0 mg pyridoxine HCl, 5.0 mg thiamine HCl·2H₂O, 5.0 mg riboflavin, 5.0 mg nicotinic acid, 5.0 mg calcium-D-pantothenate, 5.0 mg *p*-aminobenzoic acid, 5.0 mg lipoic acid, and 0.1 mg vitamin B₁₂. The trace element solution contained 12.8 g nitrilotriacetic acid, 1.35 g FeCl₃·6H₂O, 0.10 g MnCl₂·4H₂O, 0.024 g CoCl₂·6H₂O, 0.10 g CaCl₂·2H₂O, 0.10 g ZnCl₂, 0.025 g CuCl₂·2H₂O, 0.01 g H₃BO₃, 0.024 g Na₂MoO₄·2H₂O, 1.0 g NaCl, 0.12 g NiCl₂·6H₂O, and 0.026 g Na₂SeO₃·5H₂O.

2.2. Continuous Culture in a Fixed-bed Reactor

The marine sediment was acclimated to stimulate methane production. Hereto, batch culture was performed in 700-mL vials using artificial seawater at 37 °C for approximately six months. After acclimation, the sediment was transferred [30 % (v/v) inoculum] into a column reactor with a gas-liquid-solid separator and with a volume of 640 mL (**Fig. 1**). Continuous culture was performed in basal medium with 3 % NaCl, with an initial dilution rate of 2.0 d⁻¹. The dilution rate was gradually increased during culture.

2.3. Batch Culture

After continuous culture of the marine sediment in the reactor, the sediment was harvested and inoculated [30 % (v/v) inoculum] in 120-mL serum vials containing basal medium without acetate, and incubated at 37 °C. After the acetate was completely consumed, the sediment was used as inoculum.

To measure the specific growth rate of aceticlastic methanogens in the sludge, triplicate cultures [10 % (v/v) inoculum] in 120-mL serum vials containing 50 mL of basal medium with 5 g L⁻¹ of sodium acetate were employed, and the amount of methane produced in the logarithmic growth phase (within 7 days) was measured. To test the effect of the acetate concentration on the

Fig. 1 Fixed-bed Reactor System for Methane Fermentation of Acetate under High Salinity (A), and the Picture of Sediments during the Culture in the Reactor (B)

specific growth rate, the acetate concentration in the basal medium was varied from 0.5 to 200 mM and batch culture was performed as described above. Mixed liquor volatile suspended solid (MLVSS) was determined as described previously²²⁾. Specific growth rates (μ_{CH_4}) were calculated during the exponential phase, as described previously^{22),23)}.

2. 4. Scanning Electron Microscopy (SEM)

For SEM, acclimated sludge was sampled from the reactor after completion of continuous culture. The solid fraction including cells was harvested by centrifugation at $10,000 \times g$ for 10 min, washed three times with 0.1 M phosphate buffer (pH 7.2), and fixed with 2.5 % glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 2 h at 4 °C. The sample was again washed three times with 0.1 M phosphate buffer (pH 7.2), fixed with a 2 % solution of osmium tetroxide for 2 h at 4 °C, and dehydrated by successive transfer to 50, 70, 80, 90, 95, and 99.5 % solutions of ethanol and *t*-butyl alcohol. Subsequently, the sample

was freeze-dried, mounted on stubs of a scanning microscope with patina paint, and sputter-coated with gold. The sections were examined and photographed using a JSM-5900 SEM (JEOL Ltd., Tokyo, Japan).

2. 5. Chemical Analysis

The volume of CH_4 generated during culture of methanogens was measured by displacing the gas with a saturated solution of NaCl. The concentration of methane was analyzed by gas chromatography (GC-8A; Shimadzu Corp., Kyoto, Japan) equipped with a thermal conductivity detector and a stainless steel column packed with activated carbon, at 70 °C. Argon was used as the carrier gas⁴⁾. The concentration of acetate was quantified by high-performance liquid chromatography (LC-2000 Plus HPLC; JASCO Corp., Tokyo, Japan) equipped with a refractive index detector (RI-2031 Plus; JASCO Corp.), Shodex RSpak KC-811 column (Showa Denko K.K., Kanagawa, Japan), and a guard column (Shodex RSpak KC-G; Showa Denko K.K.) at 60 °C. Ultrapure water containing 0.1 % (v/v) phosphoric acid was used as the mobile phase at a flow rate of 0.7 mL/min⁴⁾.

3. Results and Discussion

3. 1. Effect of the Dilution Rate on the Methane Production Rate

Continuous culture in the fixed-bed reactor packed with acclimated marine sediment containing 3 % NaCl was performed with basal medium containing acetate as the sole carbon source at 37 °C at a low dilution rate (**Fig. 2(A)**). The initial dilution rate was 2.0 d^{-1} . When the dilution rate was increased stepwise to 4.9 d^{-1} , the acetate removal efficiency was maintained at *ca.* 97 % (**Fig. 3(A)**), and the methane production rate increased in proportion to the increasing dilution rate (**Fig. 3(B)**). The methane production rate reached 303 mM d^{-1} at 4.9 d^{-1} dilution rate. In aceticlastic cleavage of acetate by aceticlastic methanogens, the methyl group of acetate is converted into methane, while the carboxyl group is converted into CO_2 ¹⁰⁾, *i.e.*, 1 mol of acetate is converted into 1 mol of methane and 1 mol of CO_2 theoretically. In this study, the ratio of methane production to acetate consumption was approximately equal (**Fig. 3(C)**) at this dilution rate, indicating that consumed acetate was completely converted to methane by the acclimated methanogenic sediment. To investigate the potential of the acclimated methanogenic sediment at high dilution rate, the dilution rate was gradually increased to 20 d^{-1} after treatment at 5 d^{-1} for 20 days (**Fig. 2(B)**). As the dilution rate increased, the acetate removal rate decreased (**Fig. 3(A)**). In contrast, the methane production rate increased step-wise until the dilution rate reached 16.2 d^{-1} , but decreased subsequently (**Fig. 3(B)**). Similar to the low dilution rate conditions, the ratios of

Symbols: closed diamond, acetate in the influent; open diamond, acetate in the effluent; closed circle, methane production rate; open circle, carbon dioxide production rate; closed triangle, dilution rate; closed square, pH in the influent; open square, pH in the effluent.

Fig. 2 Continuous Methane Production from Acetate under 3 % NaCl in the Fixed-bed Reactor at 2-5 d⁻¹ (A) and 4-20 d⁻¹ Dilution Rates after Acclimation of the Sediment for 60 Days (B)

methane production and acetate consumption were approximately equal at the high dilution rate (**Fig. 3(C)**).

In this study, a maximum methane production rate of 750 mM d⁻¹ with 77 % of acetate removal rate in 3 % NaCl was observed at a dilution rate of 16.2 d⁻¹. In our previous study, a maximum methane production rate of 96 mM d⁻¹ was achieved in a UASB reactor using acclimated methanogenic sludge from marine sediment under 3 % NaCl, with a dilution rate of 2.8 d⁻¹ and an acetate removal rate of 55 %²¹⁾. Therefore, these results suggested that the performance of methane production by acclimated methanogenic sediment in 3 % NaCl has been dramatically improved in this study. Furthermore, methane production rate when using moving bed biofilm reactor (MBBR) and thermophilic down-flow anaerobic packed-bed reactor (TDPR) under condition without NaCl addition have been reported to be 183 mM d⁻¹ and 598 mM d⁻¹, respectively (**Table 1**). Hence, the maximum methane production rate of

750 mM d⁻¹ in this study was significantly higher than that of other reported conditions, demonstrating that the fixed bed reactor process with marine sediment as the support carrier was useful for efficient methane production from acetate.

3. 2. Characteristics of Acclimated Aceticlastic Methanogens

In the batch culture of the acclimated marine sediment to investigate the specific growth rates (μ_{CH_4}) of acclimated aceticlastic methanogens under 3.0 % NaCl, a maximum specific growth rate of 0.152 h⁻¹ was obtained. The specific growth rate of the acclimated methanogen was higher than that of other known methanogens (**Table 2**).

To examine the acetate consumption under 3 % NaCl, the initial acetate concentration was varied from 0.5 to 200 mM. In the batch culture, the acetate was completely converted to methane in all conditions. Furthermore, when the initial acetate concentration was

Closed and open squares represent data from the experiments in Figs. 2(A) and 2(B), respectively.

Fig. 3 Efficiency of Acetate Removal (A) and Methane Production Rate (B) at Various Dilution Rates, and Methane Production Rate at Various Acetate Consumption Rates (C)

150 mM, the specific acetate-consumption activity was 3.2 mmol g of MLVSS⁻¹ h⁻¹ (Fig. 4). It has been reported that *Methanosarcina barkeri* shows 0.7 mmol CH₄ g of MLVSS⁻¹ h⁻¹ at 30 mM of initial acetate concentration²². Therefore, these results indicated that

this acclimated methanogenic sediment possessed high acetate-consumption activity.

3.3. SEM of Acclimated Marine Sediment

SEM photographs of acclimated marine sediment derived from the fixed-bed reactor with acetate as a sole carbon source and with 3 % NaCl are shown in Fig. 5. Granule-like-structures were observed in the sediment (Fig. 5(A)). In the granule-like structures, *Methanosaeta*-like filamentous microorganisms (Fig. 5(B)) and *Methanosarcina*-like coccoid microorganisms (Fig. 5(C)) were observed.

Few species of halophilic *Methanosarcina* have been reported so far. The optimal NaCl range for growth of the halophilic aceticlastic methanogen *Methanosarcina acetivorans* was reported to be 0.1–0.6 M (corresponding to 0.6–3.5 %), and no growth occurred without NaCl²⁴. *Methanosarcina mazei* strain Göl was able to grow at a concentration of 18,000 mg Na⁺ L⁻¹ (4.6 % of NaCl equivalent)^{25,26}. In contrast, only two species of halophilic and halotolerant *Methanosaeta* have been reported^{20,27}. Aceticlastic halophilic *Methanosaeta pelagica* 03d30qT, isolated from tidal flat sediment, were able to grow at Na⁺ concentrations of 0.20 to 0.80 M (1.2 to 4.7 % of NaCl equivalent), with optimum growth at 0.28 M (1.6 % of NaCl equivalent)²⁷.

We previously enriched and identified aceticlastic halotolerant *Methanosaeta* sp. strain H_A from the same marine sediment as that used in this study²⁰. The morphology of this strain was quite similar to the *Methanosaeta*-like microorganisms observed by SEM in this study; however, the growth rate was lower than that of acclimated methanogens. Hence, we speculated that the collaboration between *Methanosarcina* and *Methanosaeta* resulted in the high-performance methane fermentation under saline condition.

4. Conclusion

High-rate production of methane from acetate under 3 % NaCl was successfully achieved using continuous culture in a fixed-bed reactor with marine sediment as the microbial resource of halophilic aceticlastic methanogens and as support material. In continuous culture, a maximum methane production rate of 750 mM d⁻¹ with 77 % acetate removal rate was observed at a dilution rate of 16.2 d⁻¹. Both *Methanosaeta*- and *Methanosarcina*-like microorganisms were observed in granule-like structures in the sediment. Our results indicated that acclimated methanogenic marine sediment might be applicable to high-rate methane production from acetate under saline condition.

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Table 1 Methane Production Rate of Different Conditions

Temp. ^{a)}	Reactor	Concentration of NaCl [%]	Methane production rate [mM d ⁻¹]	Acetate removal [%]	Reference
M	UASB	3.5	19 ^{b),c)}	ND ^{d)}	1)
M	UASB	2.5	63 ^{b),c)}	ND ^{d)}	1)
M	Fixed-bed reactor	3.0	96	55	21)
M	MBBR	0	183 ^{b)}	97	10)
T	TDAPR	0	598 ^{b)}	65 ^{b)}	28)
M	Fixed-bed reactor	3.0	750	77	this study

MBBR: moving bed biofilm reactor; TDAPR: thermophilic down-flow anaerobic packed-bed reactor.

a) Temp. M: mesophilic temperature, Temp. T: thermophilic temperature.

b) Values calculated from the data reported.

c) Methane production rate when it is assumed that 100 % of acetate was converted to methane.

d) No data available

Table 2 Specific Growth Rates of Aceticlastic Methanogens

Name of methanogens	Temp. [°C]	Specific growth rate [h ⁻¹]	Reference
Thermophilic acetate-utilizing methanogen (TAM)	60	0.032	29)
<i>Methanotherx</i> sp.	58	0.022	30)
<i>Methanosaeta soehngenii</i>	37	0.008	23)
<i>Methanosarcina</i> sp. strain 227	35	0.01-0.02	31)
<i>Methanosarcina</i> sp. strain TM-1	58	0.058	32)
<i>Methanosarcina mazei</i>	30	0.059-0.096	33)
Highly enriched <i>Methanosaeta</i> sp. strain H _A (NaCl 3.0 %)	37	0.036	20)
Methanogens from marine sludge (NaCl 3.0 %)	37	0.152	this study

Fig. 4 Acetate Utilization by Acclimated Sludge in the Fixed-bed Reactor at Various Initial Acetate Concentrations

Agency.

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Fig. 5 SEM Photographs of a Granule-like Structure (A), Filamentous *Methanosaeta*-like (B), and Coccoid *Methanosarcina*-like Microorganisms (C) in the Sediment from the Fixed-batch Reactor

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要 旨

海洋底泥に固定化された酢酸資化メタノジェンを用いた塩環境下での酢酸からの高効率メタン発酵

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海洋底泥を固定化担体および耐塩性酢酸資化性メタン生成菌源として, 3%の塩濃度条件下での固定床型リアクターを用いた酢酸からのメタンの超高速生産を試みた。連続培養試験において, 16.2 d⁻¹の希釈率および3%の塩濃度下で750 mM d⁻¹の最大メタン生産効率が得られた。その際の酢酸除去率は77%であった。走査型電子顕微鏡による観察の結果, グラニュール

の形成および *Methanosaeta* 様の糸状微生物, *Methanosarcina* 様の球菌が確認された。これらの結果から, 海洋底泥は耐塩性メタン発酵の微生物源としてだけでなく, 酢酸からの超高速メタン生産を可能とする固定化担体としても優れていることが示唆された。