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Improved methane production from brown algae under high salinity by fed-batch acclimation

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HIGHLIGHTS

- Fed-batch cultivation was conducted to improve methane production from brown algae.
- The methane production rate and salinity increased following cultivation.
- The methane production rate remained high, even at the equivalent of 5% NaCl.
- Specific bacteria and methanogens became predominant after cultivation.

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ABSTRACT

Here, a methanogenic microbial community was developed from marine sediments to have improved methane productivity from brown algae under high salinity. Fed-batch cultivation was conducted by adding dry seaweed at 1 wt% total solid (TS) based on the liquid weight of the NaCl-containing sediment per round of cultivation. The methane production rate and level of salinity increased 8-fold and 1.6-fold, respectively, at the 10th round of cultivation. Moreover, the rate of methane production remained high, even at the 10th round of cultivation, with accumulation of salts derived from 10 wt% TS of seaweed. The salinity of the 10th-round culture was equivalent to 5% NaCl. The improved methane production was attributed to enhanced acetoclastic methanogenesis because acetate became rapidly converted to methane during cultivation. The family *Fusobacteriaceae* and the genus *Methanosaeta*, the acetoclastic methanogen, predominated in bacteria and archaea, respectively, after the cultivation.
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1. Introduction

Marine macroalgae can be used as a feedstock for biomethane production because they do not compete with food crops cultivated on agricultural land (Wei et al., 2013). However, marine macroalgae contain salts (Roesijadi et al., 2010), leading to inhibition of microbial activity in macroalgae-based methane production (Oren, 1999). Indeed, methane can be produced from the feedstock by diluting salinity (Costa et al., 2012; Hinks et al., 2013; Jard et al., 2013; Vergara-Fernández et al., 2008). However, methane can also be produced without dilution of salinity by using salt-adapted microbial sources, such as marine sediments (Miura et al., 2014; Schramm and Lehnberg, 1984). Methane production under nondiluted conditions would be advantageous because of the requirement for less water and the resulting improvement in methane productivity.

To make methane production feasible, the methane production rate should be improved during methane production without dilution of salinity. Methane production consists of three steps: hydrolysis/acidogenesis, acetogenesis, and methanogenesis (McCarty and Smith, 1986). Acetogenesis and acetoclastic methanogenesis are rate-limiting steps unless the feedstocks are stable and difficult to degrade (Ito et al., 2012; Jeihanipour et al., 2011; Noike et al., 1985; Ras et al., 2011). A previous study found that acetogenesis of propionate and acetoclastic methanogenesis can become the rate-limiting steps in methane production from a brown alga at seawater salinity (Miura et al., 2014), suggesting that seaweeds are easy-to-degrade feedstocks, possibly because of the

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low content of lignin and cellulose (Wei et al., 2013). Thus, improvement of these rate-limiting steps is needed in order to enhance the rate of methane production.

Methane is produced in either a single-stage or two-stage digester (Nizami and Murphy, 2010). In a single-stage process, all reactions can occur in one reactor that is easy to operate compared to a two-stage digester with different microbial phases, namely acidogenic and methanogenic phases. Thus, a single-stage process is widely used. In this study, therefore, the rate-limiting steps were improved in one-stage methanization in order to increase the rate of methane production from *Saccharina japonica*, a brown alga. For this purpose, fed-batch cultivation was conducted to prepare a culture with maximal methane production for subsequent one-stage continuous operation. The microbial community was also analyzed during fed-batch cultivation.

2. Methods

2.1. Experimental design

Fed-batch cultivation was conducted to increase methane productivity by intermittently adding substrate to the marine sediment used as a microbial source. An aliquot was taken at the beginning of each cultivation to measure conductivity. The amount of methane was measured at the end of each cultivation. Volatile fatty acids (VFAs) were measured from an aliquot taken after the measurement of methane. Cultivation was conducted in triplicate. The values measured were expressed as mean ± standard deviation. The microbial community was analyzed from samples of the initial sediment and those of the culture after 10 rounds of cultivation.

2.2. Materials

Dried *S. japonica* was used as a substrate (Miura et al., 2014). The marine sediment used in this study was originally sampled from the Ariake Sea in Japan. The marine sediment was centrifuged to prepare concentrated sediment by removing the supernatant. The concentrated sediment contained 44.0 wt% total solid (TS) and 4.96 wt% volatile solid (VS).

2.3. Fed-batch cultivation

The concentrated marine sediment, a dilution medium, and the dry brown alga were added in amounts of 141 g, 71 mL, and 1.5 g TS, respectively, to a 700-mL vial. The dilution medium, using the brown alga as a substrate, was prepared as previously reported (Miura et al., 2014). The cultivation was conducted in triplicate at 37 °C under static conditions, but was shaken following the addition of substrate. The prepared medium had 150 g liquid and 62 g TS of the sediment for a total weight of 212 g. The TS of the substrate (1.5 g TS) occupied 1 wt% of the liquid weight, corresponding to about one-tenth of the TS content of the raw substrate without drying. The TS of the sediment occupied 29 wt% of the total weight. The substrate to inoculum ratio was 1:6 on the basis of the VS. When methane production almost ceased, dry brown alga was added again.

2.4. Chemical analysis

The content of TS and VS, chemical oxygen demand (COD), VFAs, and gas composition were analyzed as described previously (Miura et al., 2014).

The methane production rate was calculated by the modified Gompertz equation (Gurung et al., 2012):

$$M(t) = P\exp\{-\exp[R_{max}e/P(\lambda - 1) + 1]\} \quad (1)$$

where $M(t)$ is the cumulative methane production (mL CH_4/g VS substrate) at the time t (days), P is the maximum methane potential (mL CH_4/g VS substrate), R_{max} is the methane production rate (mL CH_4/g VS substrate/day), e is 2.71828, and λ is the lag phase (days). P, R_{max}, and λ were analyzed using Solver in Excel such that the sum of the square of subtraction between $M(t)$ and experimental data was the minimum possible value.

Conductivity was measured using a conductivity meter (LAQUAtwin B-771; Horiba, Kyoto, Japan). The sample was centrifuged to measure the conductivity of the supernatant. Conductivity was converted to NaCl concentration using a standard curve prepared by plotting NaCl concentrations and conductivity.

2.5. Pyrosequencing of 16S rRNA gene amplicons

Genomic DNA was extracted from about 250 mg of sample using a NucleoSpin Soil kit (Macherey-Nagel, Düren, Germany) and a FastPrep-24 instrument (MP Biomedicals, Solon, OH, USA) for mechanical lysis of the sample at 5 m/s for 30 s. The extraction was conducted in triplicate. The concentration of the genomic DNA solution was quantified using a spectrophotometer (Evolution 260 Bio; Thermo Fisher Scientific, Waltham, MA, USA). The DNA solution was used as a template for polymerase chain reaction (PCR). PCR was conducted in a 100-μL reaction mixture containing PCR buffer for KOD FX Neo (Toyobo, Osaka, Japan), 0.4 mM dNTPs, 0.3 μM primers, 0.5 ng/μL template, and 0.02 U/μL KOD FX Neo DNA polymerase (Toyobo). The following forward (A-27F) and reverse (B-519R) primers were used for amplification of a region of the bacterial 16S rRNA gene: A-27F, 5′-adaptor A-barcode-AGAGTTTGATCMTGGCTCAG-3′ and B-519R, 5′-adaptor B-GWATTACCGCGGCKGCTG-3′. The following primer set was used for amplification of a region of the archaeal 16S rRNA gene (Gantner et al., 2011): A-340F, 5′-adaptor A-barcode-CCCTAYGGGGYGCASCAG-3′ and B-1000R, 5′-adaptor B-GGCCATGCACYWCYTCTC-3′. The adaptor sequences were CCATCTCATCCCTGCGTGTCTCCGACTCAG for adaptor A and CCTATCCCCTGTGTGCCTTGGCAGTCTCAG for adaptor B. The barcode sequences in the forward primers were as follows: ACTACTATGT for bacteria and TGCTAGTCAG for archaea in the original sediment and AGACTATACT for bacteria and TGCCAGTCAG for archaea in the 10th-round culture. The bacterial 16S rRNA gene was amplified in the reaction mixture by 22 cycles of 98 °C for 10 s, 57 °C for 30 s, and 68 °C for 15 s, following initial denaturation at 94 °C for 2 min. Archaeal amplicons were prepared under the above conditions except for the following parameters: cycle number of 30, annealing at 55 °C, and extension for 22 s. Amplification was confirmed by agarose gel electrophoresis on 2% gels. PCR products obtained from triplicate templates were combined and purified by using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel).

Amplicons were sequenced using the Roche GS FLX+ system and analyzed by a commercial company (Hokkaido System Science, Hokkaido, Japan). Quality was checked by Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). Amplicons were further purified by excision and extraction of bands from an agarose gel. The purified amplicons were quantified to make equimolar pools of bacterial amplicons and archaeal amplicons. Each pool was titrated such that 1 bead contained 1 molecule of DNA, and the pools were then subjected to emulsion PCR using GS FLX Titanium SV emPCR Kit (Lib-L) (Roche, Mannheim, Germany). Pyrosequencing was conducted on a region of the GS FLX Titanium PicoTiterPlate 70 × 75 (Roche) using a GS FLX Titanium Sequencing Kit XLR70 (Roche). Bacterial and archaeal amplicons were sequenced separately using different regions of

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