**Semi-continuous methane production from undiluted brown algae using a
halophilic marine microbial community**

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Abstract

Acclimated marine sediment-derived culture was used for semi-continuous methane production from materials equivalent to raw brown algae, without dilution of salinity and without nutrient supply, under 3 consecutive conditions of varying organic loading rates (OLRs) and hydraulic retention time (HRT). Methane production was stable at 2.0 g VS/kg/day (39-day HRT); however, it became unstable at 2.9 g VS/kg/day (28-day HRT) due to acetate and propionate accumulation. OLR subsequently decreased to 1.7 g VS/kg/day (46-day HRT), stabilizing methane production beyond steady state. Methane yield was above 300 mL/g VS at all OLRs. These results indicated that the acclimated marine sediment culture was able to produce methane semi-continuously from raw brown algae without dilution and nutrient supply under steady state. Microbial community analysis suggested that hydrogenotrophic methanogens predominated among archaea during unstable methane production, implying a partial shift of the methanogenic pathway from acetoclastic methanogenesis to acetate oxidation.

Keywords: Semi-continuous methane production; Brown algae; Undiluted conditions; Microbial community

1. Introduction

Marine macroalgae are receiving increasing attention as a feedstock for biomethane production because they do not compete with food for farm land, have a higher growth rate than terrestrial energy crops, and are less resistant to degradation than lignocellulosic feedstocks (Montingelli et al., 2015). Methane can be produced by either thermal or biological gasification of biomass (Chynoweth et al., 2001). Marine macroalgae are suitable for biological gasification by anaerobic microbes because marine macroalgae have a high water content of around 90% (Roesijadi et al., 2010), which is not suitable for the thermal process requiring feedstocks with a low water content of less than 50% to achieve the high temperature needed for the process (Chynoweth et al., 2001). However, marine macroalgae contain high levels of salts (Roesijadi et al., 2010), which inhibit methane production (Feijoo et al., 1995; Liu and Boone, 1991; Miura et al., 2014; Mottet et al., 2014; Rinzema et al., 1988). Therefore, the use of diluted feedstocks or halophilic microbes is needed to allow methane production from these feedstocks.

Methane has been produced from marine macroalgae under semi-continuous conditions using diluted feedstocks (Briand and Morand, 1997; Hanssen et al., 1987; Hinks et al., 2013). In these conditions, the salinity of the feedstock decreases; however, the organic matter in the feedstocks concomitantly decreases because of increased water content, resulting in decreased organic loading rate (OLR) at the same hydraulic retention time (HRT). To obtain the same OLR, the HRT must be kept shorter under diluted than under undiluted conditions, which can result in excessively short HRT causing washout of microbes in traditional single-stage methane production. Therefore,

methane should be produced under undiluted conditions to obtain a high OLR without excessively shortening the HRT.

Miura et al. (2015) reported the acclimation of a microbial community from marine sediments by fed-batch cultivation for high-rate, continuous methane production from undiluted raw brown algae. The acclimation of the sediment by fed-batch cultivation enabled obtaining an increased methane production rate compared to the initial marine sediment slurry despite the presence of excess salts in the raw brown algae in addition to the 3% NaCl present in the initial slurry. However, it remains to be evaluated whether the acclimated microbial community is actually applicable to continuous methane production from raw brown algae.

The aim of this study was to evaluate the ability of the acclimated culture to produce methane continuously from brown algae under undiluted conditions. For this purpose, anaerobic digestion was conducted in a semi-continuous regime, using materials equivalent to undiluted raw brown algae, without nutrient supply. The OLR was varied during the production, which was continued beyond steady state.

2. Methods

2.1. Experimental design

Semi-continuous cultivation was carried out using acclimated marine sediment-derived culture as the starter culture. The starter culture was initiated in triplicate in 700-mL vials with intermittent replacement with fresh materials (dried brown algae and anaerobic water) with a total solid (TS) content of 10 wt%, which is equivalent to that of raw brown algae, for semi-continuous methane production. The cultures removed from the triplicate cultures at replacement were combined to measure

the volatile solid (VS) content. An aliquot was taken at the beginning of cultivation with fresh materials to measure the pH and salinity. Sampling was also conducted at the end of the cultivation to measure the volatile fatty acids (VFAs) and to analyze microbial community.

2.2. Materials

Dried and milled *Saccharina japonica* was used as substrate (Miura et al., 2015). The TS, VS, and chemical oxygen demand (COD) of the substrate were 94.2 wt%, 73.3 wt%, and 1030 mg/g TS, respectively.

2.3. Semi-continuous methane production using a fed-batch acclimated culture

The starter culture for semi-continuous methane production was prepared in accordance with a previously described protocol for fed-batch acclimation (Miura et al., 2015). In brief, marine sediment and dried *S. japonica* were used as the microbial source and substrate, respectively. Microbes in the marine sediment were acclimated by fed-batch cultivation with repeated addition of dried brown algae to the sediments at 1 wt% of water amount. Thirteen cycles of cultivation were used; anaerobic water was added at the beginning of the 11th round to decrease the salinity and to examine the effect of salinity on the rate of methane production. The cultivation was conducted in triplicate. The salinity at the beginning of 13th round culture was equivalent to approximately 4% NaCl.

Batch cultivation was conducted before starting semi-continuous cultivation to examine the methane productivity. The substrate was added at 1.5 g TS to the starter culture (approximately 200 g) in 700-mL vials. Anaerobic water prepared under N₂/CO₂

(80:20) was added so that the materials added had a TS content of 10 wt%, equivalent to the TS content of raw brown algae. Cultures were incubated statically at 37 °C in triplicate.

Semi-continuous cultivation was conducted under 3 consecutive conditions of varying OLR and HRT as follows: condition 1, OLR of 2.0 ± 0.1 g VS substrate/kg culture/day and HRT of 39.2 ± 1.9 days; condition 2, OLR of 2.9 ± 0.2 g VS substrate/kg culture/day and HRT of 27.8 ± 1.6 days; and condition 3, OLR of 1.7 ± 0.02 g VS substrate/kg culture/day and HRT of 45.8 ± 0.8 days (Fig. 1a). Culture was removed semi-continuously using a tube (3-mm internal diameter) connected with a 20-mL syringe, and replaced with fresh materials (substrate and anaerobic water) with a substrate-TS content of 10 wt%. The cultures were incubated statically at 37 °C and conducted in triplicate.

The OLR was calculated from the VS of substrate added, the weight of the culture measured at the start of cultivation with fresh materials, and the period of cultivation. The OLR at each cultivation with fresh materials was the average of triplicate cultures. The total OLR during cultivation in each of the cultivation conditions was expressed as the average $\pm$ standard deviation (SD). The HRT was calculated from the ratio of the weight of fresh materials to the weight of culture measured at the start of cultivation and the period of cultivation. The HRT at each cultivation with fresh materials was the average of triplicate cultures. The total HRT during cultivation in each of the cultivation conditions was expressed as the average $\pm$ SD.

The methane yield in the semi-continuous process was calculated as the slope of the plot of the cumulative amount of methane produced against the cumulative amount of substrate added.

For measurement of acetoclastic methanogenic activity, 9.4 mL of medium containing sodium acetate used as substrate (Miura et al., 2014) and 0.6 mL of culture from the first round of semi-continuous cultivation under condition 3 were mixed to make up 10 mL of culture containing 5 g/L of sodium acetate. Triplicate cultures were incubated statically in a test tube at 37 °C. The activity was measured under different salinity levels of 0%, 1%, 2%, 3%, and 4% NaCl. The ratio of substrate to inoculum was 5 to 1 on the basis of the VS. The acetoclastic methanogenic activity was calculated from the plot of acetate concentration against cultivation day by fitting a third-polynomial trendline. The maximum slope of the trendline was calculated as the activity.

2.4. Chemical analyses

The VS, COD, VFAs, methane, the rate of methane production, and salinity were analyzed as reported previously (Miura et al., 2015). Briefly, the VS content of the substrate and culture was measured after incineration at 600 °C of the TS, which was dried at 105 °C. The VFAs were analyzed by high-performance liquid chromatography using crotonate as the internal standard. The methane content was measured using a gas chromatograph and the total gas volume was determined by substituting the gas with water in a cylinder. The rate of methane production was calculated using the modified Gompertz equation from plots of the methane yield. The salinity was calculated from the standard curve of conductivity and the concentration of NaCl. The pH of the culture supernatant was measured by using a pH meter (LAQUAtwin B-712; Horiba, Kyoto, Japan).

2.5. Microbial community analysis

Triplicate culture samples were taken from the starter culture and at the end of culture under conditions 1, 2, and 3 using an 18-gauge needle, and they were stored at -20°C . These samples (weighing about 250 mg each) were subjected to genomic DNA extraction and subsequent 16S rRNA gene amplification as reported previously (Miura et al., 2015) except for the barcode sequences used in the amplification. The appearance of the samples was different depending on the contents of marine sediment. Although the starter culture consisted of marine sediment, the amount of sediment contained in these samples decreased with progressing conditions because the starter culture was replaced with fresh substrate during the various stages of the semi-continuous operation. However, this variation in the sediment contents probably did not affect subsequent genomic DNA extraction because the sediment amount in the samples used for extraction was within the linear region of the plot of extracted DNA amount versus the sediment amount. The barcode sequences used were as follows: 5'-TAGTG TAGAT-3' for bacteria and 5'-TGCTATT CAG-3' for archaea in the starter culture, 5'-TACAGATCGT-3' for bacteria and 5'-TGCTATT CAG-3' for archaea at the end of culture under condition 1, 5'-TACGCTGTCT-3' for bacteria and 5'-TGCTAGTCAG-3' for archaea at the end of culture under condition 2, 5'-TCGATCACGT-3' for bacteria and 5'-TGCTAGTCAG-3' for archaea at the end of culture under condition 3. Triplicate amplicons were combined, and a single sample per condition was subjected to microbial community analysis at the Hokkaido System Science (Hokkaido, Japan). This involved pyrosequencing and the Quantitative Insights into Microbial Ecology (QIIME) pipeline as reported previously (Miura et al., 2015). Amplicons obtained from the starter culture and the culture under condition 3 were simultaneously sequenced using the Roche GS

FLX+ system. Amplicons obtained from the cultures under conditions 1 and 2 were sequenced in independent runs using the Roche GS Junior.

The relative abundance of taxa within each sample was calculated by dividing the number of reads for each taxon by the total number of reads per sample.

3. Results and discussion

3.1. Semi-continuous methane production

3.1.1. Stability of semi-continuous methane production

Semi-continuous cultivation was conducted using a fed-batch acclimated marine sediment starter culture and fresh materials (substrate and anaerobic water) equivalent to raw brown algae as the substrate under varying OLR and HRT (Fig. 1a). First, batch cultivation was conducted to examine the methane productivity (Fig. 1b). The same amount of substrate (1.5 g TS) as the amount used in the fed-batch acclimation was added to the starter culture. The methane yield from 1.5 g TS of substrate was 458 ± 10 mL, which corresponds to 386 ± 9 mL CH₄/g VS and $79.1 \pm 1.8\%$ COD, after 6.9 days of cultivation. After 3 days of cultivation, the methane yield was 393 ± 14 mL, corresponding to 331 ± 12 mL CH₄/g VS and $67.9 \pm 2.4\%$ COD of substrate. Because of the high performance of the methanogenic microorganisms in batch cultivation, semi-continuous cultivation was subsequently conducted.

Semi-continuous cultivation was started with intermittent replacement of part of the culture with fresh materials. Cultivation was started under condition 1 (OLR of 2.0 g VS substrate/kg culture/day and HRT of 39 days), where the amount of replaced materials (substrate and anaerobic water) contained 1.5 g TS of substrate and the cultivation period was approximately 2.9 days (Fig. 1a). VFA did not accumulate, and

the methane production was generally stable although the production seemed to be unstable in the initial phase, between the first and eighth addition of fresh materials, because of a gas leak owing to a damaged butyl rubber tube (Fig. 1b). Therefore, the methane yield was calculated between the ninth and last addition of fresh materials, and was 358 ± 1 mL CH₄/g VS.

To produce methane at a higher OLR, cultivation was continued under condition 2 (OLR of 2.9 g VS/kg culture/day and HRT of 28 days), where the amount of replaced materials was increased to 2.0 g TS of substrate, and the cultivation period was approximately 3.0 days (Fig. 1a). Methane production was stable until day 83, which corresponded to 1.3-fold HRT under condition 2 and 2.3-fold HRT under conditions 1 and 2 cumulatively (Fig. 1b). However, VFAs began to accumulate from day 86 (1.4-fold HRT under condition 2 and 2.4-fold HRT under conditions 1 and 2, cumulatively) with decreasing methane production. The salinity and VS content were nearly constant from day 86, suggesting that the accumulation of VFAs began from the initial steady-state phase under condition 2. The methane yield under condition 2 was 335 ± 3 mL CH₄/g VS, which was 93.5% of that under condition 1, owing to incomplete methanization of VFAs. This result indicated that the yield decreased under condition 2, and that methane production became unstable during the transition from the non-steady state to the steady state.

To recover the methane yield, OLR was decreased to 1.7 g VS substrate/kg culture/day by lengthening the HRT to 46 days (condition 3), where the amount of replaced materials was the same as under condition 2 (2.0 g TS of substrate with appropriate amount of anaerobic water) and the cultivation period was approximately 4.6 days (Fig. 1a). The methane yield was high at the beginning of condition 3 because

the VFAs that accumulated during cultivation under condition 2 were converted to methane (Fig. 1b). Methane production, VS content, and salinity were stable throughout the 2.1-fold-HRT period of condition 3, which corresponded to 3.5- to 5.6-fold HRT under all the conditions, indicating that the cultivation under condition 3 was stably conducted under steady state. The methane yield was 346 ± 5 mL CH₄/g VS, which was higher than that under condition 2 (335 ± 3 mL CH₄/g VS), indicating that the methane yield was indeed recovered.

These results indicated that for steady-state, semi-continuous methane production from materials equivalent to raw brown algae, the OLR was limited to between 1.7 g VS/kg culture/day under condition 3 and 2.9 g VS substrate/kg culture/day under condition 2.

3.1.2. Estimation of the cause of unstable methane production

The accumulation of VFAs during cultivation under condition 2 led to unstable methane production (Fig. 1a and 1b). Therefore, we investigated the cause of VFA accumulation.

In general, decreased pH inhibits methanogens utilizing acetate, resulting in accumulation of acetate. However, the pH did not decrease during the entire experiment (Fig. 1c) and thus, could not have affected methane production.

The salinity decreased throughout cultivation under conditions 1 and 2 from 3.8 to 1.9% NaCl equivalent (Fig. 1c). The reduction in the salinity resulted from intermittent replacement of culture, because the starter culture contained salts equivalent to approximately 4% NaCl as described in Section 2.3. Moreover, ash content of the materials added was approximately 2 wt%, which was calculated from 10 wt% TS

content of the materials and approximately 20 wt% VS content of TS. The significant decrease in salinity might have affected methanogenic activity to cause unstable semi-continuous methane production. Thus, the effect of salinity on methanogenic activity was investigated. Acetoclastic methanogenic activity was measured at various salinity levels (Table 1) because acetoclastic methanogenesis is a rate-limiting step (Ito et al., 2012; Miura et al., 2014; Noike et al., 1985; Ras et al., 2011), causing accumulation of acetate as the major intermediate product (Fig. 1b). A culture removed at the first round of cultivation under condition 3 was used to measure acetate consumption rates. As shown in Table 1, the acetate consumption rate at 2% NaCl equivalent was higher than that at 4% NaCl equivalent. This result indicated that acetoclastic methanogenesis was not inhibited by the decreased salinity. Thus, methane production was not affected by the significantly decreased salinity in the semi-continuous operation.

VS content of the culture significantly decreased from 3.4 to 1.9 wt% throughout cultivation under conditions 1 and 2 (Fig. 1c). Reduced VS resulted from washout of VS by the semi-continuous operation. Moreover, VS content reflects microbial content. Thus, the reduction in VS probably affected methane production. Indeed, the methane production rate decreased from 1386 ± 61 mL CH₄/kg culture/day during initial batch cultivation to 745 ± 37 mL CH₄/kg culture/day from day 177 to day 181 under condition 3. Furthermore, specific methane production rates (mL CH₄/g VS culture/day) were almost the same in both conditions. These results suggested that the semi-continuous operation decreased microbial content, leading to decreased methane production with accumulation of mainly acetate from day 86 under condition 2, even though acetoclastic

methanogenesis, which is a rate-limiting step during methane production, was enhanced by the decreased salinity.

3.2. Microbial community

3.2.1. Bacterial community

The numbers of bacterial chimera-filtered operational taxonomic units (OTUs) and reads in the chimera-filtered OTUs obtained for each of the cultivation conditions were as follows: 452 OTUs and 22,610 reads for the starter culture, 286 OTUs and 10,729 reads for culture under condition 1, 282 OTUs and 15,629 reads for culture under condition 2, and 216 OTUs and 18,069 reads for culture under condition 3. The results indicated that the number of OTUs decreased in continuous cultivation compared to the number of OTUs in starter culture.

The abundance at phylum and family levels for the major bacterial taxa with a relative abundance $\geq 1\%$ is shown in Fig. 2. Analysis of the bacterial phyla suggested that *Fusobacteria* was the predominant phylum throughout all culture conditions, although the abundance decreased in semi-continuous cultivation compared to the starter culture, and the *Bacteroidetes* gradually increased throughout the conditions to become another dominant phylum (Fig. 2a). The family *Fusobacteriaceae* was the only component of the phylum *Fusobacteria*, and the increase in the phylum *Bacteroidetes* could be mainly attributed to bacteria of the order *Bacteroidales* (Fig. 2b). The members of the family *Fusobacteriaceae* of the phylum *Fusobacteria* can ferment carbohydrate into acids (Nelson et al., 2013). The *Bacteroidetes* have the capacity to degrade polysaccharides (Hanreich et al., 2013). Thus, these predominant bacterial phyla may be involved in the conversion of carbohydrates.

Bacteria involved in acetogenesis were undetected. In the acetogenesis step, propionate and butyrate are oxidized to acetate by depletion of hydrogen produced from the VFAs by hydrogenotrophic methanogens. Known propionate oxidizers are *Syntrophobacter* spp., *Smithella propionica*, *Pelotomaculum* spp., and *Desulfotomaculum* spp., and known butyrate oxidizers are *S. propionica*, *Desulfotomaculum* spp., *Syntrophus aciditrophicus*, *Algorimarina butyrica*, *Syntrophomonas* spp., *Thermosyntropha* spp., and *Syntrophothermus lipocalidus* (Stams et al., 2012). All species were undetected in this study. However, the genus *Pelotomaculum* possibly involved in propionate oxidation and the genus *Syntrophomonas* possibly involved in butyrate oxidation were detected with abundances of less than 0.1% and 0.2%, respectively, throughout all culture conditions. This is in accordance with a previous study in which these species were detected in methanogenic digesters at less than 2% (Stams et al., 2012).

3.2.2. Archaeal community

The numbers of archaeal chimera-filtered OTUs and reads in the chimera-filtered OTUs obtained for each of the cultivation conditions were as follows: 48 OTUs and 19,678 reads for the starter culture, 37 OTUs and 11,723 reads for culture under condition 1, 42 OTUs and 13,225 reads for culture under condition 2, and 32 OTUs and 15,764 reads for culture under condition 3. These results indicated that the number of OTUs did not significantly change under the different culture conditions.

The relative abundance at the genus level for the major archaeal taxa with a relative abundance $\geq 1\%$ is shown in Fig. 3. Analysis of the archaeal taxa suggested that *Methanosaeta*, the acetoclastic methanogen, was predominant in the starter culture and

under condition 1, indicating that methane was directly produced from acetate, the major intermediate product, by *Methanosaeta*. However, subsequent operation at the highest OLR under condition 2 changed the archaeal community; the abundance of *Methanosaeta* decreased and *Methanoculleus*, a hydrogenotrophic methanogen, became predominant. An increase in hydrogenotrophic methanogens under increased OLR has been observed in other studies (Blume et al., 2010; Lerm et al., 2012; Razaviarani and Buchanan, 2014). Further operation under condition 3 did not recover the relative abundance of *Methanosaeta* despite recovery of methane production, but further enhanced the predominance of *Methanoculleus*. *Methanosarcina*, another acetoclastic methanogen, was a minor genus throughout all the conditions. These results implied that the methanogenic pathway partially shifted from direct conversion of acetate to methane by acetoclastic methanogens to an indirect pathway involved in hydrogenotrophic methanogens.

In general, hydrogenotrophic methanogens can grow syntrophically with acetate oxidizers (Hattori, 2008). Oxidation of acetate to carbon dioxide and hydrogen is thermodynamically unfavorable, but becomes feasible by depleting hydrogen. Hydrogenotrophic methanogens enable acetate oxidation by acetate oxidizers by converting hydrogen to methane. Therefore, the predominance of the hydrogenotrophic methanogens indicated that methane might have been produced via acetate oxidation and hydrogenotrophic methanogenesis during cultivation under conditions 2 and 3 in addition to via acetoclastic methanogenesis. This hypothesis is supported by a study that showed that possible acetate oxidizers and hydrogenotrophic methanogens increased with increasing OLR during anaerobic digestion of food wastewater (Jang et al., 2014).

The bacterial phyla *Firmicutes*, *Spirochaetes*, *Synergistetes*, and *Thermotogae*, which are involved in the oxidation of acetate under thermophilic and/or mesophilic conditions, increased under the highest OLR in condition 2 (Fig. 2a). However, no known acetate oxidizers were detected in the culture under the mesophilic condition at high salinity levels.

Bacteria within the family *Lachnospiraceae* were mainly responsible for the increase in the phylum *Firmicutes* under the mesophilic condition (Fig. 2b). The phylum *Firmicutes* contained known acetate oxidizers: a strain from *Clostridium ultunense* (Schnürer et al., 1996) belonging to the family *Clostridiaceae*, a strain from *Thermacetogenium phaeum* (Hattori et al., 2000) and strains from *Tepidanaerobacter acetatoxydans* (Westerholm et al., 2011) of the family *Thermoanaerobacteraceae*, and a strain from *Syntrophaceticus schinkii* (Westerholm et al., 2010) of the family *Thermoanaerobacterales* (Family III). Among these strains, *C. ultunense* is the only known acetate oxidizer under mesophilic conditions among these strains. Thus, bacteria of the family *Lachnospiraceae* may not be the acetate oxidizers in this study.

Bacteria classified into the families *Sphaerochaetaceae* and *Spirochaetaceae* were responsible for the increase in the phylum *Spirochaetes* (Fig. 2b). The *Spirochaetes* are known to include acetate oxidizers, both in mesophilic and thermophilic conditions, but are not further classified (Lee et al., 2015). Thus, bacteria of the phylum *Spirochaetes* might be unknown acetate oxidizers in this study.

Bacteria of the family *Aminiphilaceae* were mainly responsible for the increase in *Synergistetes*, and the family *Thermotogaceae* for the increase in *Thermotoga* (Fig. 2b). The phylum *Synergistetes* contains known acetate oxidizers, which form the *Synergistes* group 4 (Ito et al., 2011) of the family *Synergistaceae*. The phylum *Thermotoga*

contains a known acetate oxidizer, *Thermotoga lettingae* (Balk et al., 2002), which belongs to the family *Thermotogaceae*. Those strains were obtained from non-mesophilic conditions (Balk et al., 2002; Ito et al., 2011). However, resolution to the genus level showed that bacteria of the genus *Kosmotoga* were mainly responsible for the increase in the family *Thermotogaceae*. Therefore, bacteria of the families *Aminiphilaceae* and *Thermotogaceae* may not be the acetate oxidizers in this study.

The results indicated that unknown acetate oxidizers of the *Spirochaetes*, which increased under the highest OLR in condition 2 leading to unstable methane production, might have caused a partial shift of methanogenic pathway from acetoclastic methanogenesis to hydrogenotrophic methanogenesis via acetate oxidation.

3.3. Comparison of the ability to produce methane from brown algae

The performance of methane production under the conditions described in this study was compared to that in other studies that also used semi-continuous methane production from brown algae under mesophilic conditions (Table 2). In this study, methane was produced from materials equivalent to raw brown algae by sediment microbes without nutrient supply at 3 consecutive different OLRs as follows: stable methane production without accumulation of VFA at OLR of 2.0 g VS/kg culture/day under non-steady state condition, unstable methane production with accumulation of VFAs at subsequent OLR of 2.9 g VS/kg culture/day during the transition from non-steady to steady state, and stable methane production without accumulation of VFAs at subsequent OLR of 1.7 g VS/kg culture/day under steady state.

In previous studies, raw algal feedstocks were generally diluted with water (Hanssen et al., 1987; Hinks et al., 2013) (Table 2). The OLR for stable methane production under

undiluted conditions in this study (1.7–2.0 g VS/kg culture/day) was comparable to or higher than that under diluted conditions with or without nutrient supply in the other studies.

Jard et al. (2012) performed methane production under undiluted conditions (Table 2). However, the methane yield in the study rapidly decreased during the non-steady state, between 1.3- and 1.9-fold HRT, and the highest OLR was 2.2 g VS/L culture/day, despite the nutrient supply (Jard et al., 2012). In contrast, in our study, the methane yield was stable during the non-steady state, but gradually decreased from the beginning of the steady state during the highest OLR of 2.9 g VS/kg culture/day. These findings indicate that the performance of methane production from raw brown algae without dilution in our study was higher than that in the previous study.

The achievable methane production rate was estimated by multiplying the OLR by the methane yield (Table 2). Comparison of the methane production rates showed that the ability to produce methane from brown algae in the current study was the highest among the studies, which may be attributable to differences in the starter culture used. In this study, acclimated culture derived from marine sediment was used. In contrast, in the previous studies, a culture from non-marine origin was used. The non-marine microbes are likely less resistant to the salts derived from the brown algae than those of marine origin.

4. Conclusions

Methane was produced semi-continuously from brown algae under undiluted conditions by acclimated marine sediment-derived culture beyond steady state. Microbial community analysis implied that the methanogenic pathway remained

partially shifted from acetoclastic methanogenesis to acetate oxidation from increased OLR. The ability of the acclimated culture to produce methane semi-continuously was higher than that observed in previous studies using cultures of non-marine origin. The OLR is limited by the HRT in the case of marine macroalgae because of their high water content. Therefore, future studies should aim to increase the OLR by shortening the HRT.

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Figure captions

Fig. 1. Semi-continuous methane production from brown algae under 3 different undiluted conditions. (a) OLR and HRT. (b) Methane production and VFA accumulation. (c) pH, salinity, and VS.

Fig. 2. Relative abundance of bacterial taxa at the phylum (a) and family (b) levels. Taxa with an abundance of $< 1\%$ were grouped as minor taxa.

Fig. 3. Relative abundance of archaeal taxa at the genus level. Taxa with an abundance of $< 1\%$ were grouped as minor taxa.

Figure1

Figure2

Figure3

Table 1

Acetate consumption rate at various salinity levels.

NaCl (%)	Acetate consumption rate (mmol/L/day)	Relative activity (%)
4	7.5 ± 0.9	100 ± 12
3	7.9 ± 0.3	105 ± 4
2	9.3 ± 0.6	124 ± 8
1	13.6 ± 0.6	181 ± 8

Table 2

Comparison of methane production from various species of brown algae.

Substrate	OLR (g VS/L/day) ^a	Nutrient supply	Methane yield (mL/g VS)	HRT (days)	State (-fold HRT)	Methane production rate (mL/L/day) ^b	Reference
Diluted conditions							
<i>Laminaria hyperborea</i>	1.65	–	280	24	ND ^c	462	(Hanssen et al., 1987)
<i>Laminaria saccharina</i>	1.65	–	230	24	ND	380	(Hanssen et al., 1987)
<i>Ascophyllum nodosum</i>	1.75	–	110	24	ND	193	(Hanssen et al., 1987)
<i>Laminaria hyperborea</i>	1.00	+	230	20	Steady (0–3)	230	(Hinks et al., 2013)
Undiluted conditions							
<i>Saccharina latissima</i>	1.73	+	238	38	Non-steady (0–1.3)	412	(Jard et al., 2012)
	2.17	+	137	33	Non-steady (1.3–1.9)	297	(Jard et al., 2012)

<i>Saccharina japonica</i>	2.00	–	358	39	Non-steady (0–1.0)	695	This study
	2.87	–	335	28	Steady (1.0–3.5) ^d	961	This study
	1.74	–	346	46	Steady (3.5–5.6)	602	This study

Methane was produced under mesophilic conditions.

^{a,b} The unit of OLR and methane production rate in this study were g VS/kg/day and mL/kg/day, respectively.

^b The rate was calculated by multiplying the OLR by the methane yield.

^c ND, not described.

^d VFAs began to accumulate from 2.4-fold HRT.