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Shifts in bacterial and archaeal community structures during the batch biomethanation of *Ulva* biomass under mesophilic conditions

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HIGHLIGHTS

- Mesophilic biomethanation of *Ulva* biomass was demonstrated with a high CH_4 yield.
- Changes in microbial community structure during the biomethanation were monitored.
- Bacterial community had dynamic structural shifts while archaeal community did not.
- Hydrogenotrophic *Methanolinea*-like population was likely the dominant methanogen.

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ABSTRACT

Mesophilic biomethanation of *Ulva* biomass was performed in a batch bioreactor, and a high organic removal of 77% was obtained on the basis of chemical oxygen demand (COD) after a month of operation. The estimated methane yield was 0.43 ± 0.02 L CH_4/g $COD_{removed}$ which is close to the theoretical methane potential. Transitions of bacterial and archaeal community structures, associated with process performance data, were investigated using a combination of molecular fingerprinting and biostatistical tools. During the operation, archaeal community structure had no significant changes while bacterial community structure shifted continuously and dynamically. The reactor completely stabilized volatile fatty acids (primarily acetate and propionate) accumulated from the acidogenesis phase, with *Methanosaeta*- and *Methanolinea*-related microbes respectively being the main aceticlastic and hydrogenotrophic methanogens. *Methanolinea*- and *Syntrophobacter*-related populations were likely the key members to form a syntrophic propionate-degrading consortium. A *Methanolinea*-related population was likely the dominant methane producer in the experimental reactor.

1. Introduction

With increasing concerns about environmental pollution and resource depletion, developing alternative energy sources has become an urgent global issue. Biomass energy has received great attention as a viable option for renewable energy production, due to its carbon neutral nature (Dave et al., 2013). Algae are currently acknowledged as among the highest potential biomass feedstocks for energy production, particularly due to their fast growth rate and high biomass yield (Costa et al., 2012; Dave et al., 2013). Research efforts on algal biofuels so far have concentrated mostly on using microalgae as feedstock for various energy products such as biodiesel, bioethanol, biohydrogen, and biomethane (Hinks et al., 2013). However, despite macroalgae also often have much higher productivity and degradability than terrestrial plants (Briand and Morand, 1997; Bruhn et al., 2011), relatively little attention has been directed to their potential as a prospective energy feedstock.

Seaweeds are characterized by high carbohydrate content and low lignocellulose content (Morand and Merceron, 2005), which makes them have a high biodegradation potential. This carbohydrate-rich biomass is therefore more favored for bioconversion, e.g., fermentation and anaerobic digestion (AD), while algal biodiesel production requires mass cultivation of lipid-rich microalgae. Although the full-scale economic feasibility is yet to be addressed, methane production from seaweed through AD is a potential option for sustainable energy production. AD is a series of biological reactions whereby complex organic compounds are converted to methane and carbon dioxide. Biogas, the end-product of AD, generally has a methane content of 60–70% and can be readily used for, for example, heat/electricity generation. A number of studies

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on seaweed biomethanation have been conducted, and several algal species have been reported as good substrates for AD in terms of methane yield. However, most of those 'good' ones (e.g., *Macrocystis* and *Laminaria* spp.) have comparatively high edible and commercial values, which lower their economic feasibility as a feedstock for biomethanation (Bohutskyi and Bouwer, 2013). Recently, on this account, increasing attention has been paid to seaweeds of less commercial value, for example, *Ulva* species. This algal group is known to often cause marine green tides, which could pose a serious environmental risk, in coastal areas worldwide (Nelson et al., 2003; Sidharthan et al., 2004; Zhang et al., 2011). These make biomethanation of *Ulva* biomass economically and environmentally more appealing, although its biomethane potential has been reported to be relatively low (ca. 30% lower methane yield than *Laminaria digitata*; Vanegas and Bartlett, 2013).

Although AD of *Ulva* biomass has been investigated on a number of occasions, previous works have mostly focused on the methane potential and paid little attention to the underlying microbial ecology. AD consists of three steps, hydrolysis, acidogenesis, and methanogenesis, which are mediated by extremely diverse microbial populations of different physiological and biochemical characteristics. Therefore, a harmonized activity of the microbes coexisting in an AD ecosystem is necessary for stable biomethanation, and a good understanding of microbial behavior can help reveal the underpinnings of the process. In this context, this study aimed to identify the key microbial players and investigate the shifts in microbial community structure, associated with the changes in chemical profiles, during the AD of *Ulva* biomass. For this, an anaerobic batch reactor was operated at a mesophilic temperature using *Ulva* biomass, freshly collected from a local beach experiencing *Ulva* bloom, as the sole substrate with no additives. Both bacterial and archaeal community structures were analyzed using a culture-independent molecular fingerprinting technique, denaturing gradient gel electrophoresis (DGGE). Shifts in microbial community structure with time were described using a combination of phylogenetic and statistical analyses. Although there are limited numbers of studies that have examined the microbial communities in seaweed AD environments (Hinks et al., 2013; Pope et al., 2013), to the best of the authors' knowledge, the present study is the first to look into the underlying microbial ecology of *Ulva* biomethanation. This study provides fundamental information that can help address the knowledge gap in understanding seaweed AD.

2. Methods

2.1. Seaweed substrate

Fresh *Ulva* biomass was collected from a beach near Ulsan, Korea, experiencing *Ulva* bloom. Collected seaweeds were washed twice with a small amount of water and finely ground using a kitchen blender. The seaweed slurry was then diluted in distilled water (DW) to yield a total chemical oxygen demand (COD) concentration of 6 g/L and stored at 4 °C until use. Physical and chemical characteristics of the prepared *Ulva* substrate are shown in Table S1.

2.2. Bioreactor operation

A completely mixed tank reactor with a working volume of 2 L was anaerobically operated in batch mode for biomethanation of *Ulva* biomass. The bioreactor was initially inoculated with anaerobic sludge from a full-scale sewage sludge digester. The inoculum sludge was starved for a week before inoculation to minimize the noise from endogenous biogas generation. The seeding ratio was 10% (v/v), which was equivalent to 1.3 g volatile suspended solids (VSS)/L working volume. The dilute *Ulva* slurry prepared in Section 2.1 was used as the sole substrate with no other carbon and energy source added, in order to assess the anaerobic digestibility of *Ulva* biomass as a sole feedstock. The operating temperature was maintained at 35 ± 2 °C, and the reactor pH was kept over 7.0 with 3 N NaOH. The reactor was run with periodic sampling and analysis until the conclusion of biomethanation.

2.3. DNA extraction

Total community DNA was extracted from the inoculum sludge, *Ulva* substrate, and reactor samples using a fully automated nucleic acid extractor (ExiProgen, Bioneer, Daejon, Korea) according to the manufacturer's instructions. One milliliter of each sample was centrifuged at 12,000 g for 5 min, and the spun-down pellet was washed by repeated centrifuging (1 min at 12,000 g), decanting (900 μL supernatant), and resuspending (adding 900 μL DW) to remove impurities. A 200-μL portion of the final resuspension was loaded onto the extractor with the ExiProgen Bacteria Genomic DNA kit (Bioneer). The extracted DNA was eluted in 200 μL of elution buffer and kept at -20 °C until use.

2.4. DGGE and sequencing analysis

Bacterial and archaeal 16S rRNA genes were amplified by polymerase chain reaction (PCR) using domain specific primer pairs BAC338F/805R and ARC787F/1059R, respectively (Yu et al., 2005). A 40-bp GC-clamp was attached to the 5′ end of each forward primer for better resolution of the amplified fragments on a gel (Muyzer et al., 1993). A touch-down PCR was performed according to the following thermal cycling program: pre-denaturation at 94 °C for 10 min; 20 cycles of denaturation at 94 °C for 30 s, annealing at 65–55 °C for 30 s (decreasing temperature by 0.5 °C/cycle), and extension at 72 °C for 30 s; additional 20 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C; and further extension at 72 °C for 7 min. The resulting amplicons (20 μL) were electrophorized in 8% (w/v) polyacrylamide gels for 16 h at 80 V in 1 × Tris–acetate-EDTA (TAE) buffer. The bacterial and archaeal DGGE gels respectively had denaturant gradients of 25–60% and 35–65%, where 100% is defined as 7 M urea with 40% (v/v) formamide. Gel running was conducted at 60 °C in a D-code system (Bio-Rad, Hercules, CA). The DGGE gels were then stained with SYBR Safe dye (Molecular Probe, Eugene, OR) and scanned under blue light illumination to visualize the band patterns. Bands of interest were cut out of the gel and eluted in 40 μL of sterile DW. A 2-μL aliquot of each elution was amplified by PCR using the same primer sets as for DGGE amplicons but without GC-clamp attached. The resulting PCR products were gel-purified and cloned into the pGEM-T Easy vector (Promega, Madison, WI). The cloned 16S rRNA gene sequences were analyzed using the vector-specific T7 primer and compared against the GenBank and RDP databases. DNA sequence alignment and phylogenetic analysis were conducted using MEGA 5 software, and phylogenetic trees were constructed using the neighbor-joining algorithm in the software.

2.5. Statistical analysis of DGGE profiles

The DGGE profiles were transformed into binary matrices by scoring the presence or absence of individual bands as 1 or 0, respectively, without considering band intensity. The gel images were processed by TotalLab 1D software (TotalLab, Newcastle, UK) to score the band pattern. Non-metric multidimensional scaling (NMS) and cluster analysis with unweighted pair group method with arithmetic means (UPGMA) algorithm were conducted on the obtained matrices to explore the relationships