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Effect of enhanced biomass retention by sequencing batch operation on biomethanation of sulfur-rich macroalgal biomass: Process performance and microbial ecology

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ABSTRACT

This study investigated the anaerobic digestion of *Ulva* biomass as an approach to diversifying energy sources and managing seaweed waste in a cost-effective manner. *Ulva* species are often identified as the main culprit of serious macroalgal blooms around the world, and their sulfur-rich nature causes difficulties in handling the biomass. Two reactors in continuous (Rc) and sequencing batch (Rs) modes were operated with decreasing hydraulic retention time in a stepwise manner from 20 to 6 days. Rs allowed significantly higher methane productivity (0.19–0.22 L/g chemical oxygen demand [COD] fed) and biomass retention capacity than Rc (0–0.16 L/g COD fed) throughout the experiment. Interestingly, sulfide production was also higher in Rs than in Rc. These findings, together with microbial quantification results, suggested that Rs operation enhanced biomass retention and the activity of both methanogens and sulfate-reducing bacteria. The Rc microbial community was less diverse and more variable than the Rs community. Accordingly, the performance of Rc was more significantly affected by changes in hydraulic and thus organic loads. Aceticlastic *Methanosaetaceae* dominated the methanogen community in both reactors, with the abundance of methanogens being significantly higher in Rs than in Rc. This may explain the more efficient and stable methane production despite the greater sulfidogenic activity in Rs, particularly at high hydraulic loads. Together, the results suggest that sequencing batch operation is advantageous over conventional continuous flow operation for the biomethanation of *Ulva* biomass and potentially other sulfur-rich feedstocks.

1. Introduction

Anaerobic digestion (AD) of waste biomass is considered a practical solution to the energy and environmental challenges faced today. Organic carbons are converted to biogas, consisting mainly of methane and carbon dioxide, via a series of biochemical reactions involved in AD. This indicates that waste treatment and energy production can be achieved simultaneously. As the demand for alternative energy sources has grown rapidly, extensive efforts have been made to improve the energy and economic feasibility of AD in the past decades. A simple approach is to use locally available, inexpensive substrates, and in this context, macroalgae (i.e., seaweeds) are gaining increasing attention as a bioenergy feedstock. Macroalgal biomass contains a large fraction of readily biodegradable carbohydrates and little lignin, and it does not compete with food crops [1], indicating its promising potential for biogas production.

Macroalgal blooms, particularly green tides caused mainly by *Ulva* species, are becoming more intense and prevalent worldwide as a consequence of anthropogenic marine eutrophication and global warming [2]. Unwanted blooming of *Ulva* species can lead to serious coastal pollution and public hygiene problems. An unprecedented massive *Ulva* bloom struck the western Yellow Sea off China in 2008; approximately US$90 million was spent on the cleanup [3]. It is difficult to manage such large amounts of seaweeds because they decompose fast and release malodorous substances such as sulfide. Given that *Ulva* is nutrient-rich and biodegradable, AD can provide an environmentally as well as economically appealing option for dealing with waste *Ulva* biomass. Despite its potential, *Ulva* biomass has practical limitations for AD in terms of methane potential and process stability, particularly because of its high sulfur content. *Ulva* species contain ulvan, a highly sulfated polysaccharide, as the major cell wall polysaccharide and thus, have very low C/S ratios [4]. Sulfate released upon degradation of *Ulva* biomass can stimulate the growth of sulfate-reducing bacteria (SRBs) that compete with methanogens for common

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substrates (e.g., acetate and hydrogen) and produce H_2S, which inhibits methanogenesis [5]. Therefore, control of sulfidogenic activity is considered key to efficient and stable AD of *Ulva* biomass.

Continuously stirred tank reactors (CSTRs) are the most widely used reactors for treating continuous waste streams owing to their simplicity and easy operation. However, they are sensitive to changes in operating conditions due to continuous loss of biomass in the effluent, particularly at short hydraulic retention times (HRTs). This affects the reactor performance and stability more significantly in bioprocesses involving slow-growing microbes, such as AD. An effective way to avoid this limitation is the decoupling of the solids and hydraulic retention times by retaining biomass within the system independently of influent flow. Sequencing-batch reactors (SBRs) represent a high-biomass-retention reactor configuration that runs through repeated cycles of fill, react, settle, and decant. This operation mode enables achieving an extended solids retention time (SRT) and consequently, retaining more abundant and diverse microbial populations in the reactor [6]. Therefore, in general, an SBR is expected to show a performance comparable to that of a CSTR at a shorter HRT or with a smaller reactor volume.

Although various previous studies have reported the positive effect of enhanced biomass retention on the methanogenic performance in SBRs compared to conventional CSTRs, it is questionable whether sequencing-batch operation would have a beneficial effect on the AD of *Ulva* biomass. In a mixed-culture AD process, sequencing-batch operation will allow both methanogens and SRBs, which are both relatively slow-growing, to reside in the reactor for longer periods. Given the high sulfur content of *Ulva* biomass and the competitive relationship between SRBs and methanogens, it is difficult to predict how the enhanced biomass retention in an SBR will affect microbial abundance and dynamics, and thus, AD performance. Little research has addressed this question. Therefore, this study aimed to compare the process performance and microbial ecology during AD of sulfur-rich *Ulva* biomass in continuous versus sequencing-batch modes. For this purpose, a CSTR and an SBR were run in parallel with decreasing HRT to compare process and microbial responses to increasing hydraulic and organic loads. To provide insight into the underlying microbiology associated with process behavior, a combination of molecular and statistical techniques was applied.

2. Materials and methods

2.1. Reactor setup and operation

Ulva biomass was freshly collected from a beach near Busan, Korea, and rinsed twice with a small amount of tap water to remove impurities. The biomass was stored in a freezer and ground into a slurry using a kitchen blender prior to use. The slurry was diluted to a chemical oxygen demand (COD) concentration of 5.0 g/L to be used as the substrate for reactor operation, in line with the authors' preceding studies on the biomethanation potential of *Ulva* biomass [7–9]. Two completely mixed tank reactors with a working volume of 2 L were initially filled up with anaerobic sludge collected from a full-scale biogas plant treating sewage sludge (Busan, Korea). Each reactor was purged with pure nitrogen gas for 10 min to remove dissolved and headspace oxygen before starting the feeding. Both reactors were operated at 35 ± 2 °C, and the pH was controlled near neutral by adding 3 N NaOH solution throughout the experiment. Both reactors were fed on a daily basis, but operated in different modes: one in continuous mode (Rc) and the other in sequencing batch mode (Rs). One operating cycle of Rs consisted of feeding (< 10 min), reacting (~21 h), settling (~3 h), and decanting (< 10 min). Feeding and decanting were performed manually using a syringe. The reactors were tested at increasing organic loading rates (OLRs) by reducing the HRT from 20 to 6 days over five experimental phases (i.e., 20, 15, 12, 9, and 6 days; Phases 1 to 5; Table 1). For each phase, steady-state data were obtained after at least three turnovers of the reactor working volume. In both reactors agitation was provided by a magnetic stirrer. Physicochemical characteristics of the *Ulva* substrate and the inoculum sludge used in this study are presented in Table S1.

2.2. DNA extraction

Total DNA was extracted from the steady-state reactor sample using an automated nucleic acid extractor (Exiprogen, Bioneer) according to the manufacturer's instructions. One milliliter of a sample was pelleted at 13,000 *g* for 5 min and repeatedly washed by resuspending in distilled water (up to 1 mL), supernatant decanting (900 μL), and centrifuging (13,000*g* for 1 min). The resulting pellet was resuspended in 1 mL of distilled water, and a 200-μL aliquot of the final resuspension was loaded onto the extractor with the ExiProgen Bacteria Genomic DNA kit (Bioneer). The purified DNA was recovered in 200 μL of elution buffer and stored at − 20 °C until use.

2.3. Denaturing gradient gel electrophoresis (DGGE)

Bacterial and archaeal 16S rRNA genes were amplified from total DNA samples by touch-down polymerase chain reaction (PCR) using BAC338F/509R and ARC787F/1059R primer pairs, respectively [10], as previously described [11]. Amplicons were electrophoresed in 8% (*w/v*) polyacrylamide gels with denaturant gradients of 25–60% for bacteria and 35–65% for archaea (100% denaturant corresponds to 7 M urea and 40% (*v*/v) formamide) at 80 V for 16 h in a D-code system (Bio-Rad). After electrophoresis, the gels were stained with SYBR Safe Dye (Molecular Probes) and scanned under blue light to visualize the band patterns. Selected bands were cut from the gels and eluted in 40 μL of sterile water.

2.4. Real-time PCR

Real-time PCR was used to estimate the abundance of target microbial groups, i.e., bacteria, methanogens, and SRBs. The 16S rRNA gene concentrations of total bacteria and methanogens were determined as previously described [12]. Six primers/probe sets specific for the domain *Bacteria* and five methanogen groups at the order or family level (i.e., *Methanobacteriales*, *Methanomicrobiales*, *Methanococcales*, *Methanosarcinaceae*, and *Methanosaetaceae*) were used to detect the corresponding microbial groups [10]. For the quantification of SRBs, real-time PCR was carried out using two primer sets targeting the functional genes *aprA* and *dsrA* [13]. Oligonucleotides used in this study are listed in Table S2. Reactions (20 μL) were prepared using the THUNDERBIRD Probe qPCR Mix (TOYOBO) for the runs targeting bacteria or methanogens: 10 μL of premix, 2 μL of TaqMan probe (final concentration, 200 nM), 1 μL of each primer (final concentration, 500 nM), 4 μL of PCR-grade water, and 2 μL of template DNA. For SRBs, reactions (20 μL) were prepared using the THUNDERBIRD SYBR qPCR Mix (TOYOBO): 10 μL of premix, 1 μL of each primer (final concentration, 500 nM), 7 μL of PCR-grade water, and 1 μL of template DNA. All reactions were run on a QuantStudio 12 K Flex system (Life Technologies) in a two-step thermal cycle procedure consisting of predenaturation (10 min at 95 °C) followed by 40 cycles of amplification (15 s at 95 °C and 1 min at 60 °C).

A standard curve was prepared as previously described for each primers/probe set used for the quantification of bacteria or methanogens using an equimolar mixture of the corresponding standard plasmids [14]. A 10-fold serial dilution series was constructed for each standard plasmid mixture and analyzed by real-time PCR with its corresponding primers/probe set. The crossing point values determined from the standard dilutions were plotted versus the logarithm of their template concentrations to generate a standard curve. For the quantification of SRBs, a standard curve was constructed for each target sequence in the same manner using standard plasmids. Three standard plasmids were generated for *aprA* and *dsrA* each by cloning the real-