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Letter

Enhanced Methane Recovery from Waste-Activated Sludge by Alginate-Degrading Consortia: The Overlooked Role of Alginate in Extracellular Polymeric Substances

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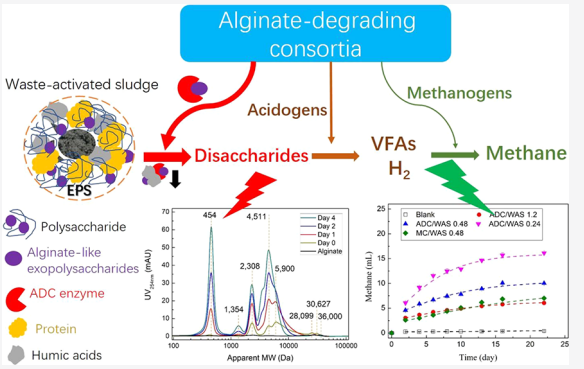
ABSTRACT: The hydrolysis of extracellular polymeric substances (EPS) in waste-activated sludge (WAS) is considered as the rate-limiting step in anaerobic digestion. Uronic acids such as alginate are one of main polysaccharide components in EPS; however, their roles on WAS conversion are overlooked until now. Previously, we described alginate-degrading consortia (ADC) that have high activity for alginate conversion. In this work, ADC was studied for polysaccharide hydrolysis and methane production from WAS for the first time, which increased the methane production by 115%−185%. Dosing ADC also increased the values of biological methane potential from 131 to 172 mL/gVSS. An alginate-like exopolysaccharide was extracted from WAS, and the content was 65 mg/g-VSS. Then, the molecular weight profiles at UV_{254nm} showed that disaccharides were the final hydrolysates of alginate by ADC enzyme. Extracted EPS could be utilized by ADC for methane production with acetate as the main intermediate. The mechanism was proposed that ADC played a key role in WAS conversion. These results indicated that alginate in EPS shall not be overlooked, which offers a new microbial method to enhance methane recovery from WAS. The microbial changes in ADC for the stability of WAS digestion should be investigated in the future.



1. INTRODUCTION

The disposal of waste-activated sludge (WAS) is an environmental problem that can be expensive, as it consumes up to 50% of the total operating cost of wastewater treatment plants.[1,2] Anaerobic digestion is an important biotechnology that can stabilize and reduce volatile solids while recovering bioenergy as methane.[3,4] Polysaccharides (including neutral sugars and uronic acids) and proteins are the main components of extracellular polymeric substances (EPS) in WAS.[5] WAS hydrolysis is considered as the rate-limiting step in anaerobic digestion, which is mainly caused by difficult-to-degrade EPS.[1,6] Various pretreatment technologies, including mechanical, thermal, and chemical methods, have been proposed to enhance the hydrolysis process,[7,8] which increases the operating cost for WAS treatment.

In previous studies, the neutral sugars such as glucose and glucan were considered as the typical components of polysaccharides in WAS.[9] However, the uronic acids (such as alginate and polygalacturonate), as important constituents of the extracellular matrix in sludge flocs and granules,[10,11] are often overlooked. For example, McSwain et al. reported that EPS of polysaccharide and protein was concentrated in a flocculent center of sludge flocs.[11] Felz et al. recently reported the percentage of neutral sugars and uronic acids were similar, 10.7% and 13.2%, respectively.[5] Lin et al. showed that the alginate-like exopolysaccharides (ALE) in aerobic granular sludge were about 10% w/w of the organic matter,[12] which provides a new substrate to enhance WAS utilization. Subsequently, alginate degradation in EPS may promote the methanogenesis of WAS, but it has not been detailed in the literature to the best of our knowledge.

Alginate degradation has been previously described for pure cultures such as *Algibacter alginolytica* HZ22^T.[13] Mixed cultures provide several advantages for the utilization of WAS over pure cultures because there is no requirement for sterilization, and it can be adapted to variation in feedstocks and process conditions.[14] Recently, we enriched alginate-degrading consortia (ADC) using alginate as a substrate, which demonstrated a high activity of alginate conversion to methane (>80%).[15] A metagenomics analysis showed that 12 species, such as

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Algibacter lectus and *Bacteroides clarus*, could excrete an oligoalginate lyase to degrade alginate.[15] The objectives of this research were to (1) investigate methane production from WAS and extracted EPS by dosing ADC, (2) analyze the contents of ALE in WAS, and (3) reveal the role of ADC saccharolytic enzymes on WAS utilization. Lastly, the underlying mechanism was proposed according to these results.

2. MATERIALS AND METHODS

2.1. Inocula and Waste-Activated Sludge. Waste-activated sludge was collected from the Jinshan wastewater treatment plant (Fuzhou City, China). The characteristics, including pH, total COD, soluble COD, suspended solids, and volatile suspended solids (VSS), are summarized in Table S1. The ADC biomass was collected from a mesophilic (35 °C) anaerobic reactor fed with alginate.[15] Another methanogenic culture (MC) was set as a control culture and was collected from a lab-scale anaerobic digester.[15]

2.2. Enhancing Methane Production and Biological Methane Potential by Dosing ADC. To evaluate methane production from WAS by enriched ADC, five tests (n = 3) were performed as shown in Table S2: a control with no addition of ADC in 55 mL WAS (as ADC/WAS 0), no addition of WAS to demonstrate methane production from only ADC (as Blank), the addition of a nonhydrolytic anaerobic culture in 55 mL of WAS (as MC/WAS 0.02), ADC culture in volumes with 5 or 15 mL added to 55 mL of WAS (as ADC/WAS 0.02 or MC/WAS 0.06).

Then, 0, 2, 5, and 10 mL of WAS was mixed with 10 mL of ADC in four groups (Blank, ADC/WAS 1.2, ADC/WAS 0.48, and ADC/WAS 0.24, n = 3) to demonstrate the biological methane potential (BMP, Table S3) of WAS. The group of MC/WAS 0.48 was used to determine the BMP value of WAS (5 mL) by dosing MC (1 mL). The serum bottles were cultured at 35 °C and pH values of 7.0 ± 0.2.

2.3. Alginate Hydrolysis and Methane Production by ADC Saccharolytic Enzyme. To demonstrate the hydrolysis mechanism of the ADC saccharolytic enzyme on alginate, the enzyme was harvested from the supernatant of 5 mL of ADC broth by centrifugation at 10,000 rpm for 10 min and 0.45 m membrane filtration.[16] Then, 55 mL of medium, 5 g/L of alginate, and 5 mL of ADC saccharolytic enzyme or water (control group) were added into a 120 mL serum (n = 3). An ADC cell was harvested from the sediment of ADC broth after removing the supernatant by centrifugation. To demonstrate the effect of the ADC saccharolytic enzyme on methane production from WAS, the ADC enzyme was harvested, and four groups (control, ADC saccharolytic enzyme, ADC cell, and ADC) were carried out (n = 3) at 35 °C and neutral pH.

2.4. Methane Production from Extracted EPS by ADC. To demonstrate the conversion of EPS layers by ADC, the three EPS layers, including S-EPS, LB-EPS, and TB-EPS, were extracted from 300 mL of WAS according to Li and Yang.[17] Then, the inorganic medium was added into three extracted EPS solutions to ensure the final volume of 300 mL. Four groups (control, S-EPS, LB-EPS, and TB-EPS) were used to demonstrate methane production from extracted EPS by dosing ADC (n = 3). A sample of 5 mL of ADC and 55 mL of extracted EPS layers were added into the serum bottle to ensure the working volume of 60 mL.

2.5. Analysis. The contents of hydrogen and methane and concentrations of ethanol and VFAs (including acetate, propionate, and butyrate) were determined with two gas chromatographs (Lunan model SP7890, CN, and Agilent 7890, CA, respectively).[15] Profiles of alginate and hydrolysate apparent molecular weight were measured at UV_{254nm} by a high-performance liquid chromatography system (Waters 1525, Waters, USA).[18] ALE was extracted from dried WAS (0.8 g)[19] and characterized by FT-IR (iS 50, Thermo Scientific Nicolet). Soluble carbohydrate was measured by the phenol-sulfuric method with glucose as the standard.[20] Soluble protein was determined by the Lowry−Folin method with bovine serum albumin as the standard.[21] Statistical analysis was carried out using SPSS (v. 20.0).

3. RESULTS AND DISCUSSION

3.1. Methane Production from WAS by Dosing ADC. Figure 1A shows that dosing ADC could notably improve

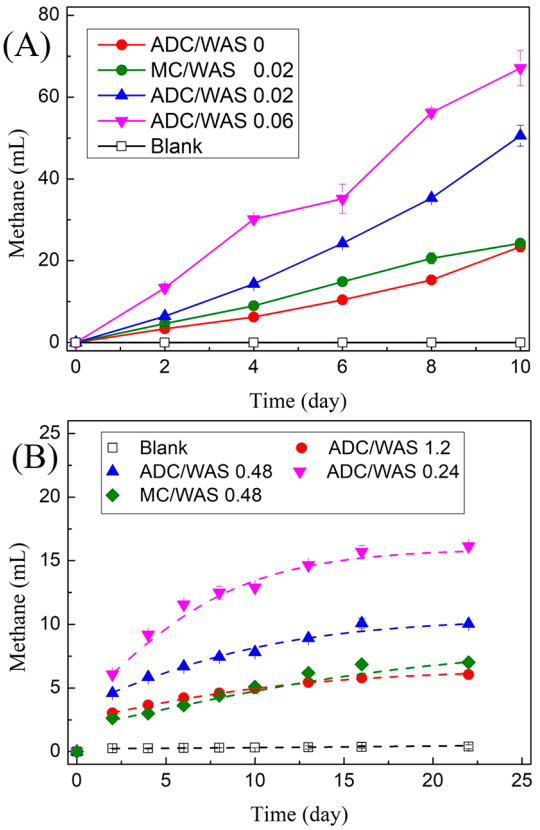

Figure 1. Profiles of methane production (A) and biological methane potential (B) of WAS by dosing ADC.

methane production from WAS, and the methane production was 50.6 mL (ADC/WAS 0.02) and 67.1 mL (ADC/WAS 0.06) on Day 10, respectively. No methane was detected without adding WAS in the blank group. The addition of MC did not increase methane formation over the control (24.3 mL in MC/WAS 0.02 vs 23.4 mL in ADC/WAS 0). Thus, dosing ADC offered an increase in methane production of 115% and 185% (p < 0.05), respectively.

The modified Gompertz model was used to analyze the BMP values as shown in Figure 1B, and the results are summarized in Table S4. The calculated maximum methane production (M_{CH4}, ADC/WAS 1.2, 6.5 mL; ADC/WAS 0.48, 10.5 mL; and ADC/WAS 0.24, 16.1 mL) was comparable to that of the experimental results (p > 0.05, no significant difference). The maximal methane production rate of ADC/

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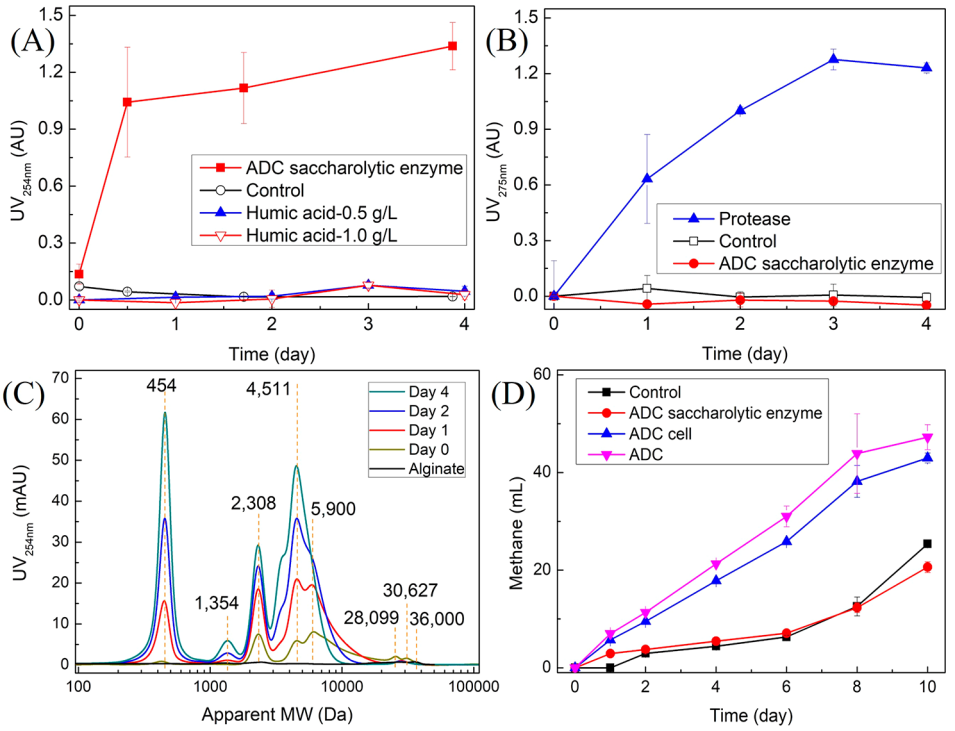

Figure 2. UV_{254nm} of alginate hydrolysis (A), UV_{275nm} of protein hydrolysis (B), apparent molecular weight changing during the alginate hydrolysis (C), and methane production from WAS (D) by the ADC saccharolytic enzyme.

WAS 0.24 was 4.8 mL/day, which was higher than that of other groups (0.1, 2.9, 3.3, and 2.1 mL/day). After considering a VSS of 12.96 g/L, the methane production was 172 mL/g of VSS, while for MC, the value was just 131 mL/g of VSS. Thus, dosing ADC increased the BMP value of WAS by over 32%. This value was also comparable to that of former works via thermal or chemical treatments ($p > 0.05$), as summarized in Table S5. Besides, another parameter, the hydrolysis rate coefficient of WAS by dosing MC was 0.099 d^{-1}, which was within the range of former reports from 0.03 to 0.3 d^{-1}.[22] After dosing ADC, the value increased to 0.17 d^{-1}. Thus, the ADC could promote both WAS hydrolysis and methanogenesis, which indicated that their role in EPS degradation should be not overlooked.

3.2. Alginate Extraction from WAS and FT-IR Spectra. Higher methane production and hydrolysis rate coefficients may be due to the presence of ALE in EPS and high enzyme activity in ADC. Thus, in this section, WAS was first collected from a local wastewater treatment plant, and the ALE content was 65 ± 22 mg ALE/g-VSS ($n = 4$). This result was similar to that of Lin (72 mg/g-VSS).[10] FT-IR spectra (Figure S1) showed that the typical peaks of alginate were identified, including a broad rounded absorption band of O−H stretching vibrations above wavenumber 3000 cm^{-1}, a weak C−H stretching peak at 2930 cm^{-1}, and a stretch of C−O−C, C−O at 1000−1200 cm^{-1}.[23] The bands at 1651 and 1532 cm^{-1} were identified as the asymmetric and symmetric stretching of carboxylate O−C−O vibrations.[19,23] The band at 1224 cm^{-1} is assigned to the presence of O-acetyl ester for bacterial alginates. These results supported that ALE was extracted from WAS.

3.3. Alginate Hydrolysis and Methane Production from WAS by the ADC Saccharolytic Enzyme. The hydrolytic activity and inhibition by humic acid of ADC saccharolytic enzyme in ADC supernatant were evaluated. After dosing the enzyme that was harvested from the supernatant of ADC, the values of UV_{254nm} increased notably from 0.14 to 1.04 after 12 h (Figure 2A), which indicates that the enriched enzyme could quickly hydrolyze alginate. In the following 4 days, the value of UV_{254nm} did not change notably and had a mean of 1.34. In the control experiment, the value of UV_{254nm} did not increase and was below 0.1. However, dosing humic acid of 0.5 and 1.0 g/L inhibited the activity of the ADC enzyme as the value of UV_{254nm} did not change, which was due to the electrostatic force of the humic acid.[22] Also, ADC saccharolytic enzyme could not degrade protein (Figure 2B). Thus, alginate was hydrolyzed by the ADC enzyme with high selectivity.

Figure 2C further depicts the molecular weight spectrum of alginate after dosing the ADC enzyme. The initial absorbance at 36,000 Da was identified as original alginate. The molecular weights of alginate gradually lowered to 454 Da after dosing the ADC enzyme, which was identified as the disaccharides of mannuronate or guluronate. The disaccharides can be further consumed by ADC after diffusing into the cytoplasm, analogous to the conversion of cellulose by anaerobic fermentation.[24] Figure 2D shows that dosing the ADC enzyme did not increase methane production from WAS, and the final methane productions were 25.4 mL (control) and 20.6 mL (ADC enzyme). Dosing ADC provided the maximum methane production of 47.2 mL, which is consistent with the methane production shown in Figure 1. Dosing an ADC cell could continuously excrete the enzyme that could hydrolyze alginate in EPS; consequently, the methane production increased to 43.0 mL after 10 days of operation. Thus, rather than the saccharolytic enzyme in ADC, dosing ADC provides a more suitable method to promote methane production from WAS.

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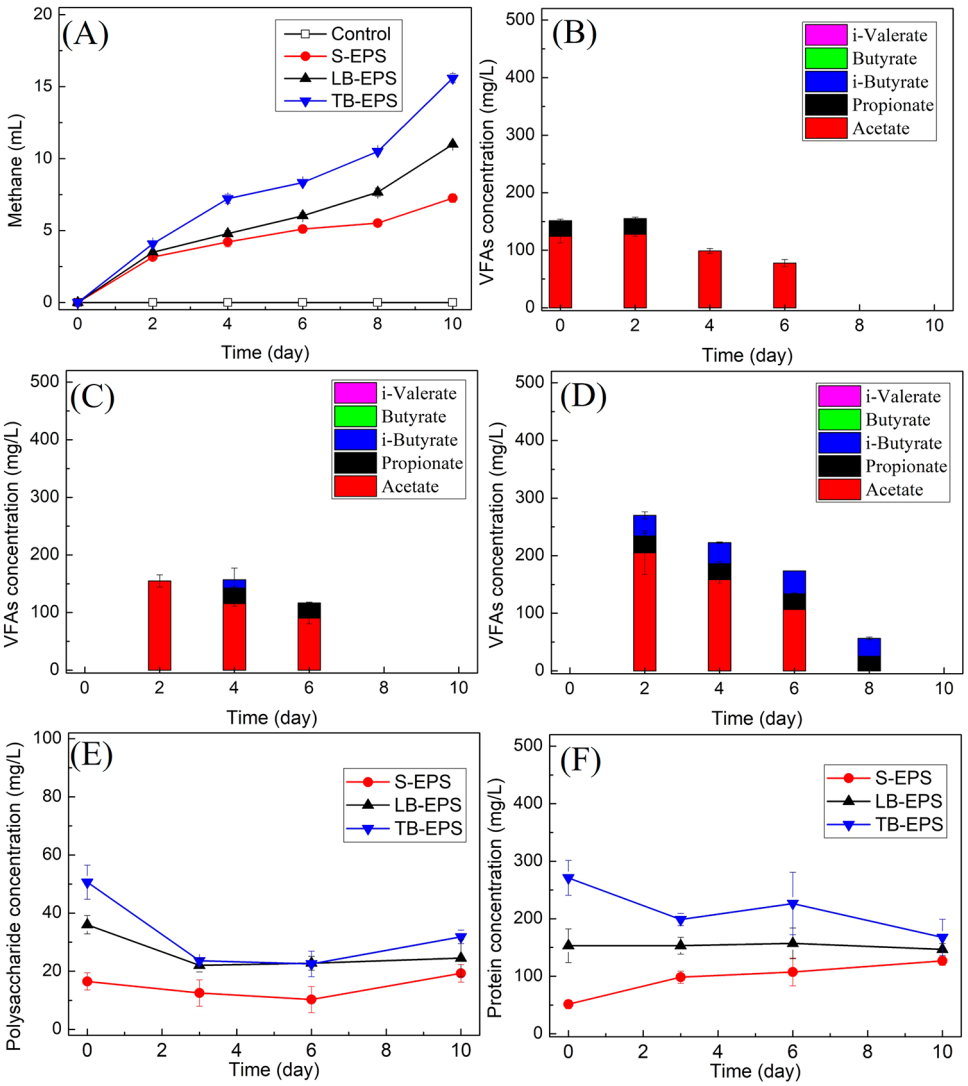

Figure 3. Methane and intermediate production from EPS compounds of WAS: (A) methane production, (B) VFAs production from S-EPS, (C) VFAs production from LB-EPS, (D) VFAs production from TB-EPS, (E) polysaccharide, and (F) protein

3.4. Methane Production from Extracted EPS of WAS by Dosing ADC. To evaluate the conversion of EPS by dosing ADC, S-EPS, LB-EPS, and TB-EPS were extracted and used as the substrates for methane production (Figure 3). In the control experiment (Figure 3A), no methane was detected within 10 days. Similarly, no methane was detected from S-EPS, LB-EPS, and TB-EPS without dosing ADC. With EPS addition, the methane production occurred in the experiments in the sequence: TB-EPS > LB-EPS > S-EPS, and the methane production after 10 days was 15.6, 11.0, and 7.3 mL, respectively. Acetate was the main intermediate during methane production from S-ESP (Figure 3B), LB-EPS (Figure 3C), and TB-EPS (Figure 3D). After 10 days, acetate and other metabolites in EPS, such as propionate and i-butyrate, were consumed.

Figure 3E and F illustrates the changes in polysaccharide and protein in extracted EPS. Polysaccharides in TB-EPS and LB-EPS were all degraded by ADC, and the concentration decreased from 50.7 to 31.8 mg/L and from 36.0 to 24.5 mg/L, respectively. The protein concentration in TB-EPS declined from 271.1 to 167.6 mg/L, while the concentration of protein in S-EPS unexpectedly increased from 51.4 to 126.8 mg/L, which might be also degraded by other bacteria in ADC because ADC was mixed culture enriched initially from anaerobe sludge.[15] Thus, our result supported the hypothesis that EPS could be degraded by enriched ADC.

3.5. Underlying Mechanism and Environmental Implications of Enhancing Methane Production by ADC. As a typical component of uronic acid in EPS, alginate plays an important role in the integration of the flocs structure,[11] but it is generally overlooked in WAS utilization. The results in this work for the first time demonstrated that ADC, a mixed culture of alginate-degrading bacteria, acidogens, and methanogens,[15] could promote methane production from WAS. As shown in Figure S2, the mechanism is proposed as follows: (1) Alginate-degrading bacteria excrete alginate saccharolytic enzyme that can convert ALE in EPS of WAS to disaccharides. (2) The disaccharides and other accessible substrates are converted to the intermediates of VFAs and H_2 by acidogens. (3) These intermediates are finally utilized by methanogens to produce CH_4 in the synergetic pathway. Since the WAS hydrolysis is considered as the rate-limiting step in anaerobic digestion, it is reasonable that the ADC bacteria that excrete the alginate saccharolytic enzyme

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play the key role in WAS conversion. Moreover, although the content of alginate is not very high, dosing ADC can notably promote methane recovery from WAS.

In this work, the alginate-degrading consortia offers a new but promising microbial method of enhancing methane production, but much research is still needed. The interaction between ADC enzyme and EPS compounds should be clarified. Besides alginate, other biomaterials including glycoproteins and polysaccharides (such as fucoidan and polygalacturonate) are also identified in WAS,[9,25] providing additional substrates for WAS utilization. For example, dosing polygalacturonase is unexpectedly effective in mobilizing polysaccharides with an increase up to 7 times in total EPS polysaccharides amount.[26] Meanwhile, the key bacteria in ADC should be also revealed by the isotopic labeling test and metagenomic and metatranscriptomic analyses,[27] which are essential to confirm the degrading pathway of EPS components. For full-scale operation, the microbial changes in ADC are important factors to demonstrate the stability of WAS digestion.[28] Labatut et al. reported that mesophilic anaerobic digestion offered the merit of stability when treating a mixture of cow manure and dog food,[28] but it should be verified for WAS digestion. Besides, dosing extra ADC periodically can improve the stability of operation. Purifying the ADC saccharolytic enzyme may also benefit the analysis of enzyme selectivity. Other factors such as divalent cations (e.g., Ca^{2+} and Mg^{2+}) may interact with alginate in EPS[23] and disturb the degradation by ADC, which should also be revealed.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.0c00784.

Detailed description of experimental design and methods, results of modified Gompertz model, summary of biological methane potential data, FT-IR spectra of alginate, and proposed mechanism of enhancing methane production (PDF)

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Notes

The authors declare no competing financial interest.

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