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Article

Identification of Extracellular Key Enzyme and Intracellular Metabolic Pathway in Alginate-Degrading Consortia via an Integrated Metaproteomic/Metagenomic Analysis

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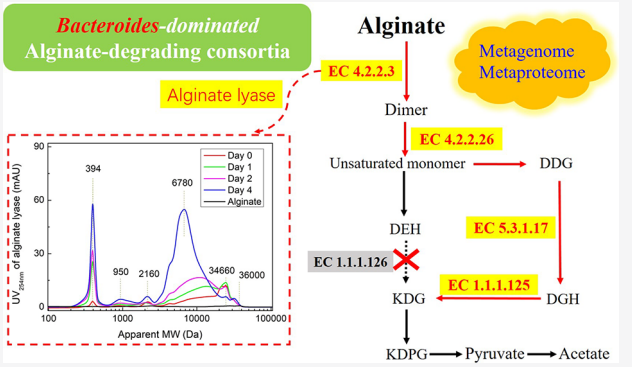
ABSTRACT: Uronic acid in extracellular polymeric substances is a primary but often ignored factor related to the difficult hydrolysis of waste-activated sludge (WAS), with alginate as a typical polymer. Previously, we enriched alginate-degrading consortia (ADC) in batch reactors that can enhance methane production from WAS, but the enzymes and metabolic pathway are not well documented. In this work, two chemostats in series were operated to enrich ADC, in which 10 g/L alginate was wholly consumed. Based on it, the extracellular alginate lyase (~130 kD, EC 4.2.2.3) in the cultures was identified by metaproteomic analysis. This enzyme offers a high specificity to convert alginate to disaccharides over other mentioned hydrolases. Genus *Bacteroides* (>60%) was revealed as the key bacterium for alginate conversion. A new Entner−Doudoroff pathway of alginate via 5-dehydro-4-deoxy-D-glucuronate (DDG) and 3-deoxy-D-glycerol-2,5-hexdiulosonate (DGH) as the intermediates to 2-keto-3-deoxy-gluconate (KDG) was constructed based on the metagenomic and metaproteomic analysis. In summary, this work documented the core enzymes and metabolic pathway for alginate degradation, which provides a good paradigm when analyzing the degrading mechanism of unacquainted substrates. The outcome will further contribute to the application of *Bacteroides*-dominated ADC on WAS methanogenesis in the future.



KEYWORDS: *alginate-degrading consortia, two chemostats in series, extracellular alginate lyase (EC 4.2.2.3), Bacteroides, new Entner−Doudoroff pathway, DDG and DGH*

1. INTRODUCTION

Anaerobic digestion offers the merits of reducing waste-activated sludge (WAS) production and recovering methane as bioenergy.[1,2] Extracellular polymeric substances (EPS) in WAS contain functional groups (e.g., carboxyl and hydroxyl groups) and can bind with common bivalent cations (i.e., Ca^{2+}) in wastewater to form aggregation and flocculation structures.[3,4] This stable EPS structure causes the slow hydrolysis of WAS and the low efficiency of anaerobic digestion.[5,6] The alginate-like exopolysaccharides, i.e., a linear polysaccharide of α-L-guluronate (G) and β-D-mannuronate (M), are ~7% w/w of the organic matter in WAS.[7] Moreover, GG blocks (i.e., the dimers of G) in alginate can act as ionic bridges of divalent cations (especially Ca^{2+}) and other uronic acids.[8] Recently, structural extracellular polymeric substances (St-EPS) are extracted and identified with typical alginate isomers of glucuronic acid and polygalacturonic acid that can form gel-like materials in WAS.[9,10] Felz *et al.* reported that the content of uronic acid (13.2%, galacturonic acid equivalent) was comparable to that of sugar (10.7%, xylose equivalent) in WAS.[10] Till now, most reports on alginate degradation focus on marine pure culture bacteria and alginate lyases for brown macroalgae utilization, such as *Algibacter alginolytica* HZ22^T.[11−13] For WAS methanogenesis, the mixed culture is known to offer much more benefits than the pure culture, including no requirement for sterilization, robust adaptation to variation in feedstocks and process conditions, etc.[14,15] Thus, promoting the conversion of alginate and the isomers in St-EPS via a mixed culture may improve methane recovery from WAS, but it is seldom reported as far as we know.

Previously, we enriched a freshwater alginate-degrading consortium (ADC) from anaerobic sludge using alginate as the substrate for the first time.[16] The enriched ADC demonstrates

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the expected performance of enhancing the methane production from WAS, which could increase the biological methane potential from 131 to 172 mL/gVSS.[17] This value is comparable to that of thermal or mechanical pretreatment technologies for enhancing methane production from WAS. However, the core enzymes and metabolic pathway for alginate conversion in ADC are not well documented, which is mainly due to two reasons: (1) a low percentage of 12 putative bacteria (<0.1%, such as *A. lectus* and *Bacteroides* sp. D2) that can excrete M-specific oligo-alginate lyase (EC 4.2.2.3)[16] and (2) the phylogenic distinguishing from the well-known marine alginate-degrading bacteria. The chemostat is known as a process configuration to enrich highly active bacteria.[15] This benefits the study of the core enzymes and metabolic pathway for alginate conversion, which are the prerequisite to promote the application on WAS methanogenesis.

The alginate degradation by a marine bacterium of *A. alginolytica* is reported via the coupling of several extracellular alginate lyases and the intracellular modified Entner−Doudoroff (ED)−Embden−Meyerhof−Parnas (EMP) pathways in bacteria enriched from seaweed.[12] Generally, alginate lyase can be divided into four types according to the substrate specificity, that is, extracellular M-specific, G-specific alginate lyases (EC 4.2.2.11), bifunctional lyases (EC 4.2.2.-), and intracellular exo-alginate lyase (EC 4.2.2.26).[18] However, the alginate lyase and metabolic pathway in ADC enriched from the freshwater niche are not clear. To clarify the core enzymes and/or metabolic pathway in an enriched mixed culture, a metaproteomic/metagenomic analysis is suited.[19−21] For example, Zhao *et al.* reported the components of biocake proteins in a submerged membrane bioreactor by metaproteome analysis to understand the mechanism of membrane fouling.[20] Park *et al.* constructed a N-involved metabolic pathway in enriched anammox bacteria in a full-scale glycerol-fed nitritation−denitritation process.[22] Thus, integrating metaproteomic and metagenomic analysis is necessary to reveal the alginate degradation by enriched freshwater ADC.

Consequently, the objectives of this research were to (1) establish a mesophilic two chemostats in series process to highly enrich alginate-hydrolysing bacteria in ADC, (2) identify the extracellular alginate lyase via metaproteomic analysis, (3) reveal the core bacterial evolution in ADC by Illumina Miseq high-throughput sequencing, and (4) uncover the intracellular metabolic pathway in ADC by metagenomic and metaproteomic analysis. The outcomes can improve the understanding of alginate degradation and contribute to promote methane recovery from WAS by ADC.

2. MATERIALS AND METHODS

2.1. Inocula and Setup of Mesophilic Two Chemostats in Series. The ADC biomass was collected from a mesophilic (35 °C) anaerobic reactor for methane production using alginate as the substrate.[16] Two chemostats in series were operated under anaerobic conditions to demonstrate hydrolysis and acidogenesis in R1 and methanogenesis in R2, in which the effluent of R1 was utilized as the influent of R2. The total volume of R1 and R2 was the same as 3.2 L, with working volumes of 1.1 and 2.5 L, respectively. The stirring velocity in the two reactors was all set at 200 rpm. The inocula were sparged with N_2 (>99.99%) for 20 min. The initial volatile suspended solid (VSS) was 2.0 g/L. The influent concentration of alginate was 10 g/L. The detailed compositions of the inorganic medium were the same as the former work.[16] The hydraulic retention time (HRT) in R1 was stepwise reduced to 1.8 days within 15 days. Then, the whole experiment included two stages: in stage 1 (1−30 days), the HRTs of R1 and R2 were set at 1.8 and 4.1 days, respectively; and in stage 2 (30−60 days), the HRTs were shortened to 1.0 and 2.3 days in R1 and R2, respectively.

2.2. Alginate Hydrolysis by Hydrolytic Enzymes. Hydrolysis by extracellular enzymes of enriched ADC was the first step to utilize alginate. Thus, to demonstrate the activity of alginate lyase, the supernatant of R1 broth was harvested by centrifugation at 10,000 rpm for 10 min. Another three enzymes including α-amylase (EC 3.2.1.1), protease (EC 3.4.22.2), and α-glucosidase (EC 3.2.1.20) were purchased from Aladdin (Aladdin Bio-Chem Technology). Then, 55 mL of medium, 5 g/L alginate, and 5 mL of enzymes were added into a 120 mL serum (n = 3). The absorbance change of alginate at $UV_{254\ nm}$ was determined by a UV−visible spectrophotometer (A560, AOE Instruments, CN). Profiles of alginate and hydrolysate at $UV_{254\ nm}$ were measured by a high-performance liquid chromatography system (Agilent 1260, USA).[23] The m/z values of alginate hydrolysate after dosing alginate lyase on day 4 were analyzed by MALDI-TOF-MS (Ultraflextreme, Bruker, GER).

2.3. Metabolite Analysis. The hydrogen and methane contents were determined with a gas chromatograph of SP7890 (Lunan, CN).[16] The concentrations of volatile fatty acids (VFAs, including acetate, propionate, and butyrate) and ethanol were measured by another gas chromatograph of GC-2030 (Shimadzu, JP).[16] Alginate was measured by the carbazole-sulfuric acid method.[10] The biomass composition of ADC in two chemostats was considered as $CH_{1.8}O_{0.5}N_{0.2}$. The COD balance was calculated according to COD of the above detected metabolites, i.e., alginate, 0.74 gCOD/g; acetate, 1.07 gCOD/g; propionate, 1.53 gCOD/g; butyrate, 1.82 gCOD/g; CH_4, 4 gCOD/g; biomass, 1.37 gCOD/g.

2.4. Characterization of ADC Enzyme and Microbial Communities. *2.4.1. Alginate Lyase Identification by the Metaproteomic Analysis.* The enzyme solution was first harvested from the supernatant of a 5 mL ADC broth in R1 by centrifugation at 10,000 rpm for 10 min. The proteins in ADC broth was first visualized by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Then, the proteins were hydrolyzed and analyzed by liquid chromatography coupled with tandem mass spectrometry (LC−MS/MS, Thermo Fisher, Germany) in Majorbio (Shanghai, China). Lastly, the raw data were processed and blasted in the UniProt database according to the protocol of Majorbio.

2.4.2. DNA Extraction and Illumina Miseq High-Throughput Sequencing. The five microbial samples were each collected in R1 on days 0, 15, 30, 45, and 60 and were named R1-0, R1-1, R1-2, R1-3, and R1-4, respectively. DNA samples were extracted and amplified via the primers of 341F-806R.[24] Sequencing was then performed by a Miseq PE 300 sequencer (Majorbio, China). The bacterial diversities were analyzed via the picked operational taxonomical units (>97%). The sequencing data of the five samples were deposited in the NCBI (SRR12876902−SRR12876906).

2.4.3. 16S rRNA Gene Clone Library Sequencing. The DNA sample of R1-4 for 16S rRNA gene clone library sequencing was amplified using primers of 27F and 1492R. The 28 positive clones were then sequenced by the ABI DNA Analyzer (PRISMTM 3730XL, Majorbio, China). A neighbor-

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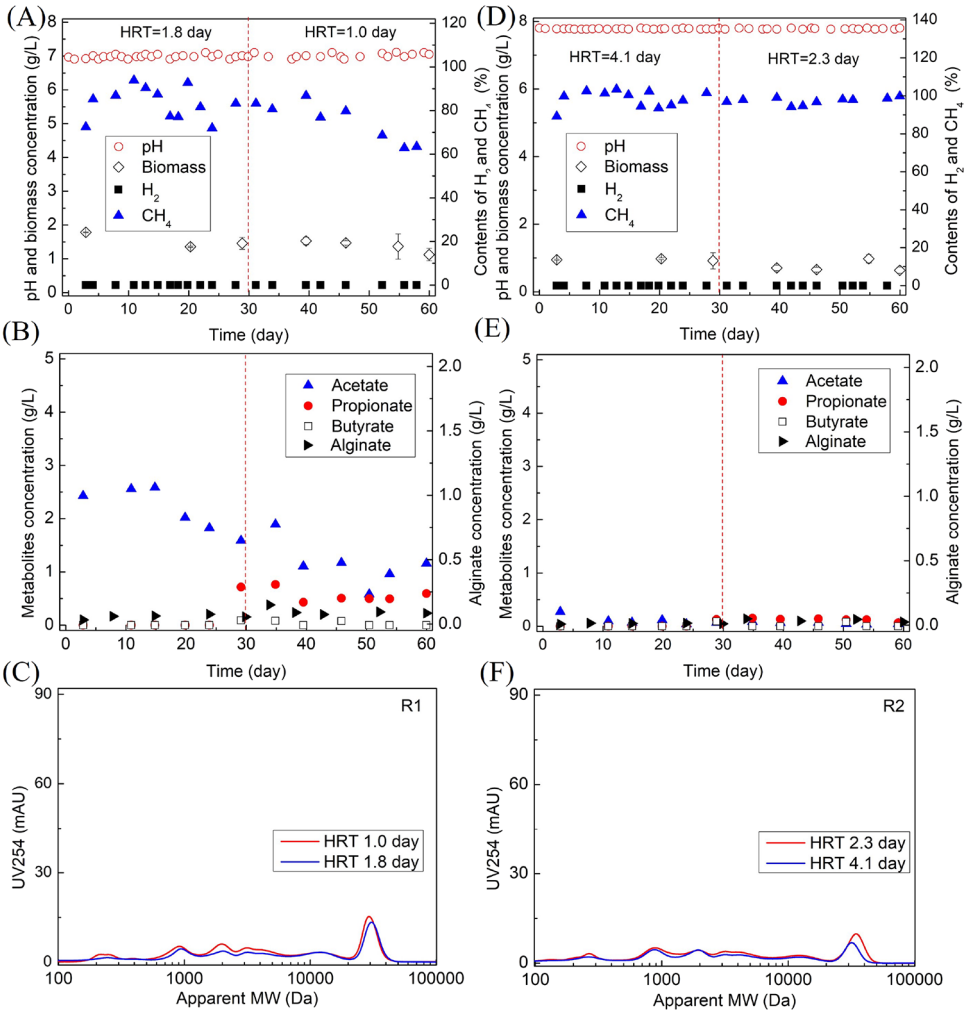

Figure 1. Performance of alginate conversion by ADC in two chemostats in series. Gaseous (A), metabolites in broth (B), and molecular profiles of effluents (C) in R1; gaseous (D), metabolites in broth (E), and molecular profiles of effluents (F) in R2.

joining tree was constructed by the MEGA (version 6.06). Twenty-eight clone data were deposited in the NCBI (MW133006−MW133033).

2.4.4. ADC Metabolic Pathway by Metagenomic and Metaproteomic Analysis. The genomic sample of R1-4 was sequenced by a Hiseq 2000 platform (Majorbio, China). The pair-end sequence reads were then merged and analyzed according to the protocol of Majorbio. The sequencing data were deposited in the NCBI (SAMN1651645). Meanwhile, the intracellular proteins were also analyzed by iTRAQ-based metaproteomic methods (Majorbio, China) and the data were analyzed according to the protocol of Majorbio. The identified enzymes in ADC were finally classified according to the metabolic pathway in the KEGG database.

3. RESULTS AND DISCUSSION

3.1. Alginate Conversion by ADC in Mesophilic Two Chemostats in Series. Figure 1 represents the performance in the two reactors using alginate as the sole carbon source. During the whole operation, pH values in R1 and R2 did not change much and remained at 7.0 and 7.8, respectively. Biomass concentrations in R1 and R2 were 1.2−1.5 gVSS/L and 0.6−0.9 gVSS/L, respectively. The alginate concentrations in the effluent were always below 0.2 g/L even in R1 with HRTs of 1.8 and 1.0 days, which meant that over 98% of alginate was consumed in R1.

The primary gaseous metabolite was methane, and the content was around 80% in R1. In R2, CO_2 was totally adsorbed as pH values increased to 7.8, and thus the content of methane was close to 100%. The methane production rates in R1 were 529 ± 125 mL/(L day) at an HRT of 1.8 days (n = 6) and 685 ± 50 mL/(L day) for an HRT of 1.0 days (n = 6). In R2, these values were 293 ± 49 mL/(L day) for an HRT of 4.1 days (n = 6) and 524 ± 4 mL/(L day) for an HRT of 2.3 days (n = 6). The contents of H_2 in the gas from each reactor were always below 0.01% (the detecting limit of GC). For metabolites in the liquor of R1 (Figure 1B), acetate (2.2 ± 0.4 g/L, n = 6) was the main soluble metabolite at an HRT of 1.8 days. As the HRT was lowered to 1.0 day, acetate decreased to 1.1 ± 0.5 g/L (n = 6), while propionate increased to 0.55 ± 0.1 g/L (n = 6). The butyrate concentrations were rather low, below 0.1 g/L. In the R2 reactor (Figure 1E), the accumulated VFAs were almost fully consumed; the concentrations of the volatile fatty acids were below 0.1 g/L and even close to 0 g/L. The molecular profiles of effluent in R1 (Figure 1C) and R2 (Figure 1F) showed that the low molecular-weight metabolites of alginate were nearly all consumed even in R1 under both tested HRTs, and the peak values in R2 were also lower than those in R1. The COD balance is summarized in

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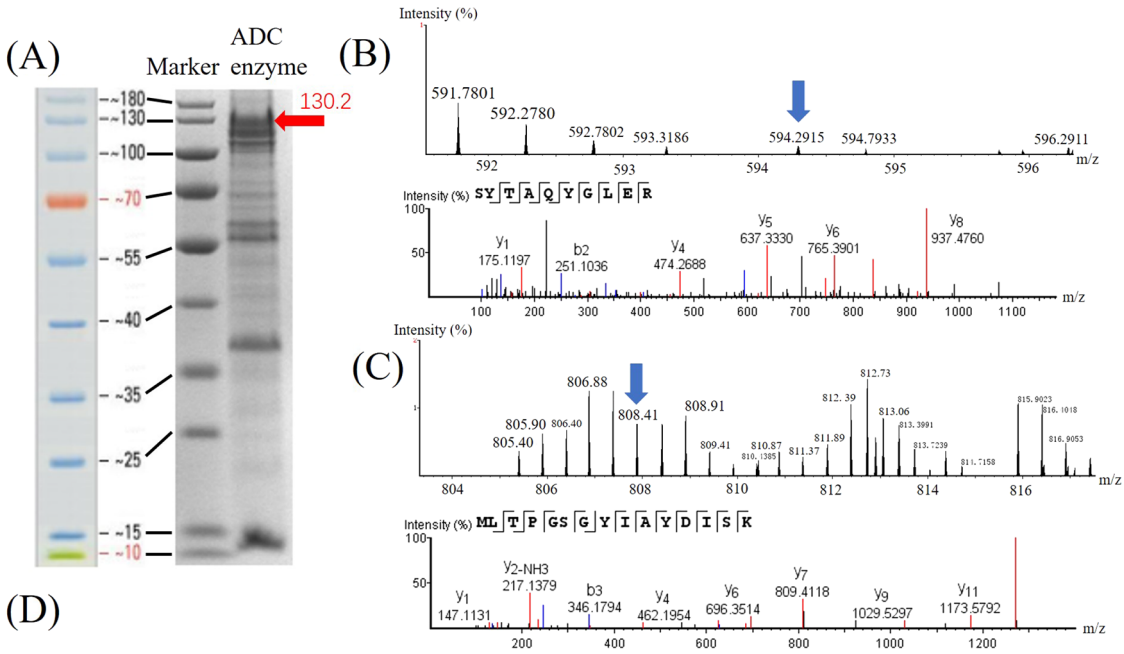

Peptide Name	m/z	Identity (%)	Q-Begin	Q-End	MW (kDa)	Description
R. SYTAQYGLER. F	594.29	35.4	1109	1191	130.2	Alginate lyase (EC. 4.2.2.3) of *Marinoscillum sp.*
R. MLTPGSGYIAYDISK. D	808.41	25.5	550	652	130.2	Alginate lyase (EC. 4.2.2.3) of *Marinoscillum sp.*

Figure 2. ADC enzyme identification by metaproteomics analysis. SDS-PAGE bands (A). MS1 and MS2 spectra of R.SYTAQYGLER.F and R.MLTPGSGYIAYDISK.D by LC−MS/MS (B, C). Blasting results of two identified peptides (D).

Table S1, and the recovery percentage was between 91 and 102%, which supported the results that the main metabolites were detected. The COD percentage of alginate converted to methane was lower than 40% in R1 but increased to almost 80% in R2. Meanwhile, in our previous work, it took 10 days to consume 8.0 g/L alginate with a percentage of genus *Bacteroides* below 0.1%.[16] Pham *et al.* reported that the maximum yield of VFAs was only 37.1% within 5 days by an unknown mixed culture.[25] Also, Ji *et al.* found that the remaining percentage of alginate was about 23% after 2 days of operation by a thermophilic *Defluviitalea phaphyphila*.[11] It is known that the chemostat could remove low-activity bacteria under low HRTs.[24] Thus, ADC with a high activity of alginate conversion was obtained in R1 with a low HRT of 1.0 day, which may be due to a higher percentage of enriched genus *Bacteroides* in R1.

3.2. Extracellular Alginate Lyase Identification by Metaproteomic Analysis and the Enzymatic Activity. Degrading alginate into oligosaccharides by extracellular alginate lyases is the first step in the bioconversion process. Therefore, the metaproteome of the ADC supernatant in R1 was evaluated by LC−MS/MS to reveal the extracellular alginate lyases. Figure 2A shows several protein bands with molecular weights ranging from 10 to 180 kDa in the R1 supernatant by SDS-PAGE. Two peptides of R.SYTAQYGLER.F (m/z 594.29 in MS1 and MS2 spectra in Figure 2B) and R.MLTPGSGYIAYDISK.D (m/z 808.41 MS1 and MS2 spectra in Figure 2C) were identified to be related to a modular protein of alginate lyase (EC 4.2.2.3) with an MW of 130.2 kDa (marked in Figure 2A). These two peptides were blasted with the identified percentages of 35.4% (Figure S2) and 25.5% (Figure S3) to this modular alginate lyase (Figure 2D). This low similarity percentage may be due to the fact that most reported alginate lyases are enriched from marine brown macroalgae fermentation,[11,18] while ADC is enriched from WAS fermentation, a freshwater system. For example, Sun *et al.* reported that a famous alginate-degrading genus of *Algibacter*, including 12 species, had been isolated from seawater, green algae, intertidal sediments, marine sediments, sea urchins, and brown alga reservoirs.[26] Ji *et al.* isolated a thermophilic alginate-degrading bacterium from the marine sediment of a coastal region of the Yellow Sea in China.[11] Moreover, Boleij *et al.* recently proposed a pathway for the legionaminic acid synthesis by anammox, while the percentage of enzymes were also not high enough (32.7−50.1%), which was attributed to the difference between "*Ca. Brocadiales*" and a reference of *Halorubrum* sp. PV6.[27] Thus, more works such as alginate lyase purification shall be carried out to reveal the amino sequence and structural characterization in the future.

Generally, alginate lyase can be divided into four types according to the substrate specificity such as extracellular M-specific and G-specific alginate lyases.[18] Several enzymes also show multi-functionality, for example, the alginate lyase from *Sphingomonas* sp. is active against M, G, and MG blocks, with a

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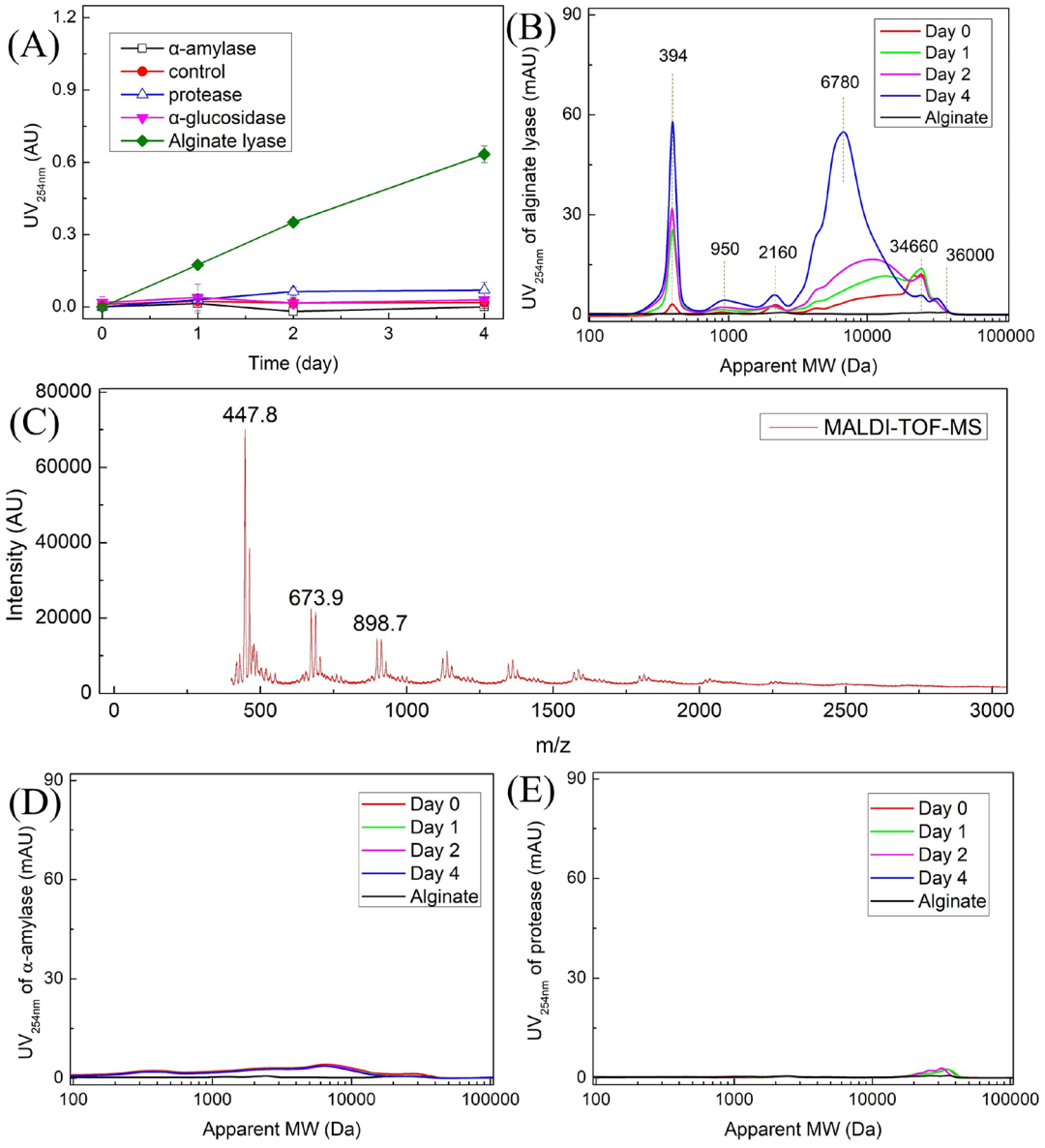

Figure 3. Impact of hydrolytic enzymes on alginate degradation. Change in $UV_{254\ nm}$ (A). MW profiles (B) and MALDI-TOF-MS (C) of alginate hydrolysate after dosing ADC enzymes. MW profiles of alginate after dosing α-amylase (D) and protease (E).

preference for the M block.[28] Thus, the identified alginate lyase might also convert G and MG blocks in alginate, which is a prerequisite for alginate utilization. The hydrolytic enzymes α-amylase, protease, and α-glucosidase are the most commonly mentioned hydrolytic enzymes to accelerate EPS hydrolysis.[2,29] However, as a linear polysaccharide composed of G and M blocks with a linkage of the β-1,4-glycosidic band, alginate is resistant to these enzymes. As expected, the absorbance at $UV_{254\ nm}$ increased only in the test of alginate lyase in the ADC supernatant and finally to 0.63 ± 0.03 after 4 days (Figure 3A), while the absorbance in the other three hydrolytic tests did not increase much (below 0.1). Moreover, the apparent MW profiles (Figure 3B) of alginate further demonstrated that alginate gradually changed from an initial 36,000 Da to a lower MW of 394 Da. Similarly, the m/z value of alginate hydrolysate by using MALDI-TOF-MS was 447.8 (Figure 3C). These data were similar to that of our recent work (454 Da).[17] Since the monomer MW is 192 Da, disaccharides shall be the final hydrolysate of alginate, which is analogous to the conversion of cellulose to cellobiose by cellulase.[30] Meanwhile, dosing α-amylase (Figure 3D), protease (Figure 3E), and α-glucosidase (Figure S4) did not result in hydrolysis of alginate to a lower MW, which is consistent with the observed $UV_{254\ nm}$ values (Figure 3B). Thus, the identified alginate lyases had high selectivity on alginate conversion.

3.3. Illumina High-Throughput and 16S rRNA Gene Clone Library Sequencing of Alginate-Degrading Consortia. Illumina Miseq high-throughput sequencing was carried out to evaluate the bacterial community of the enriched ADC in R1 operated at HRTs of 1.8 and 1.0 days, and the main sequencing indices are summarized in Table S2. According to the coverage index (>0.999, Table S2), Shannon diversity (Figure S5A), and rank−abundance (Figure S5B) curves, the sequencing depth in this work was enough to analyze the whole community of five samples. Figure S5C shows the sequencing results of enriched ADC at the phylum level. The percentages of archaea (phyla of Euryarchaeota) in five samples were rather low (<1%). Bacteroidota and

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Spirochaetota were the two main phyla in ADC (~90%), and they remained stable during the operational period. For example, in R1-0, the percentages were 70.2 and 16.8%, respectively.

Figure 4A shows the top 40 genera in five samples by Illumina high-throughput sequencing, and the whole results of

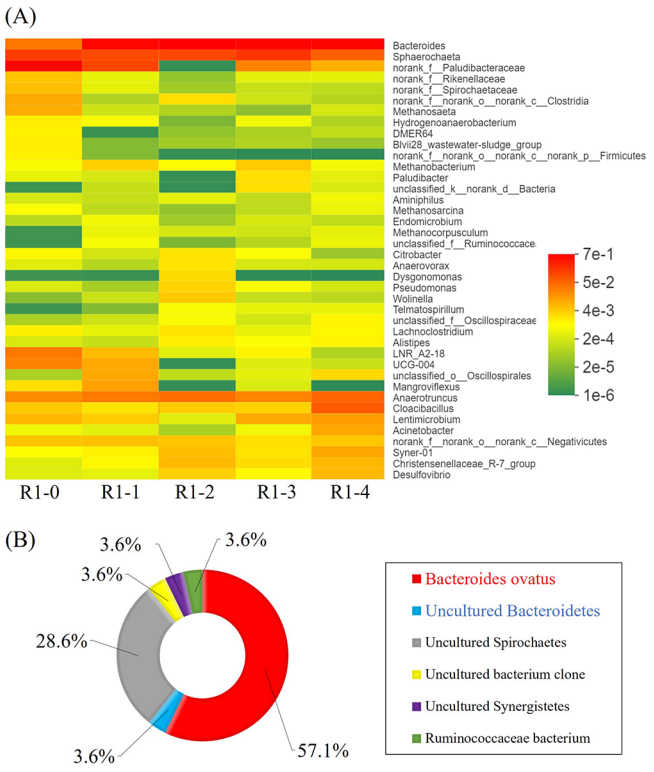

Figure 4. Top 40 bacteria at the genus level in R1 by Illumina high-throughput sequencing (A) and percentages of enriched bacteria in R1-4 at the species level (B).

enriched ADC are summarized in Figure S5D, in which the main bacteria of *Bacteroides* and *norank_f__Paludibacteraceae*, all belonging to the Bacteroidota phylum, were changed notably. The percentage of *Bacteroides* increased from 3.45% (R1-0) to 72.5% (R1-4), while the percentage of *norank_f__Paludibacteraceae* decreased from 64.5% (R1-0) to 0.8% (R1-4). Another bacterium of *Sphaerochaeta* was around 10% in five samples. Genera of *Methanobacterium* (hydrogenotrophic methanogen) and *Methanosaeta* (acetoclastic methanogen)[31] were the two main archaea, but the percentages were all below 1% in five samples and were even 0.07 and 0.03% in R1-4, respectively. These results were much different from the former work, in which genera *Thauera* (16.7%), *Clostridium-sensu-stricto 1* (14.6%), *vadinBC27-wastewater-sludge group* (13.2%), and *Lentimicrobium* (11.8%) were the main bacteria in a batch reactor,[16] while the genus *Bacteroides* was rather low (<0.1%). A high percentage of enriched genus *Bacteroides* in R1 (>60%) resulted in a high activity of alginate conversion (Figure 1). In addition, the percentage of genus *Cloacibacillus* in R1 was below 0.4% in samples of R1-0, R1-1, R1-2, and R1-3, while, it increased to 7.6% in R1-4. Ganesan *et al.* isolated *Cloacibacillus evryensis* from a mesophilic anaerobic sludge digester that could utilize proteins to produce acetate, propionate, and butyrate.[32] However, the function of degrading alginate by this genus was still unclear, which would be clarified in the future via pure culturing of enriched genus *Cloacibacillus*.

To reveal the bacterial compositions of R1-4 at the species level, the 16s rRNA gene clone library sequencing was further analyzed (Figure S6 and Table S3). Figure 4B clearly shows that *Bacteroides ovatus* (60.7%, belonging to genus *Bacteroides*) and *Uncultured Spirochaetes* (28.6%, belonging to genus *Sphaerochaeta*) were the main detected bacteria in R1-4, which was consistent with the results from Illumina high-throughput sequencing. In a previous work, the genus *Bacteroides* (such as *Algibacter lectus* and *B. clarus*), which could excrete alginate lyase (EC 4.2.2.3), was identified by metagenomic analysis,[16] but the percentage (<1%) was much lower than that in this work. Thus, compared to batch mode, the chemostat could remove low-activity bacteria and enrich high-activity ADC to a high percentage. Enrichment in a continuous system has therefore preference over batch enrichment.

Till now, the genus *Bacteroides*, including *B. ovatus* and *B. intestinalis*, has been mainly described in the context of a healthy human intestine,[33,34] but its alginate-degrading function is seldom reported. The clone ELU0066 is also enriched as an uncultured bacterium in human ileum.[35] Other clones grouped close to the bacteria of uncultured bacterium clone NBBPI0209_44 (3.6%), Ruminococcaceae bacterium Marseille-P3646 (3.6%), and uncultured Synergistetes bacterium clone 3CP(-)_78 (3.6%) were rather low. Thus, these results verified that in the chemostat operation, a more specialized community was enriched and secondary species occurring in the batch-enriched ADC community were effectively washed out. This resulted in a higher enrichment of *Bacteroides* in the ADC. Therewith, a metagenome analysis was carried out to evaluate the role of genus *Bacteroides* in alginate degradation.

3.4. Bacteria and Intracellular Enzymes for Alginate Degradation in *Bacteroides*-Dominated ADC by Metagenomic and Metaproteomic Analysis. In *Bacteroides*-dominated ADC, the majority of sequenced reads were identified in the metabolism section by metagenomic analysis (Figure S7), in which the carbohydrate metabolism (~6,000,000 reads) was the most abundant one. Moreover, the full metabolic pathway of carbon flow to methane and CO_2 was constructed by metagenomic analysis (Figure S8), in which the well-known glycolysis pathways of ED (Figure S9) and EMP (Figure S10) were identified. For alginate conversion, the specific metabolic pathway is initially proposed in Figure 5A according to four typical enzymes of alginate lyases (EC 4.2.2.3, 4.2.2.11, 4.2.2.-, and 4.2.2.26) and 4-deoxy-L-erythro-5-hexoseulose (DEH) reductase (EC 1.1.1.126).[12] The subsequently produced 2-keto-3-deoxy-gluconate (KDG) enters into the ED pathway of 2-keto-3-deoxy-phosphogluconate (KDPG) via KDG kinase (EC 2.7.1.45). Meanwhile, the metaproteomic analysis in Figures S12−S16 also supported these results that the same extracellular alginate lyase (EC 4.2.2.3 in Figure S12), intracellular full carbon flux (Figure S13), and glycolysis pathways of ED (Figure S14) and EMP (Figure S15) were all identified. Thus, the alginate lyases and DEH reductase were chosen as the markers to identify the core bacteria in *Bacteroides*-dominated ADC.

For alginate lyases, though two enzymes (EC 4.2.2.11 and 4.2.2.-) were not detected, the specific enzymes of M-specific alginate lyase (EC 4.2.2.3) and exo-type alginate lyase (EC 4.2.2.26) were identified, which is consistent with the

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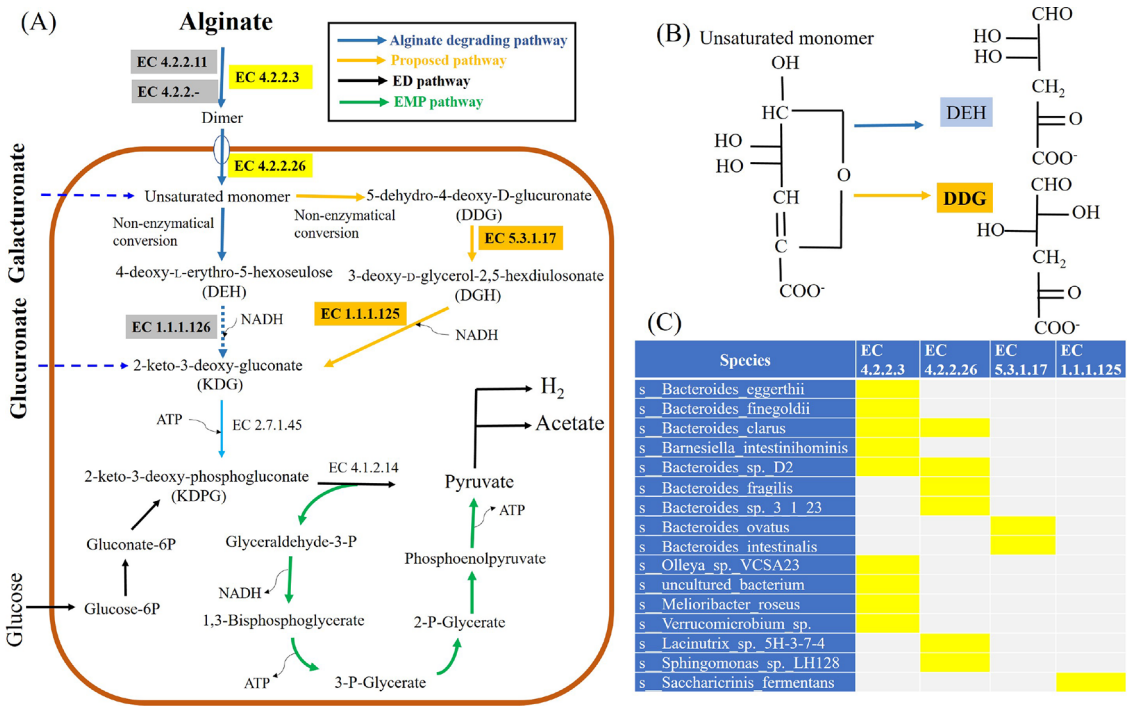

Figure 5. Constructed metabolic pathway of alginate conversion in ADC bacteria by metagenomic analysis. Constructed metabolic pathway (A). Identified core bacteria for alginate conversion (B). Non-enzymatic conversion of unsaturated monomer to DEH and DDG (C).

extracellular metaproteomic analysis (Figure 2). Hereafter, based on the presence of the two characteristic lyases, nine (Table S4) and six (Table S5) species were identified, in which six and five species belonged to the genus *Bacteroides*. Two species of *B. clarus* and *Bacteroides* sp. *D2* were identified having both lyases (Figure 5B). However, the DEH reductase (EC 1.1.1.126) was not detected in the constructed metagenomic library, which indicates that other pathways may occur for intracellular unsaturated monomer conversion.

Recently, Hobbs *et al.* reported that a novel enzyme (KdgF) in *Yersinia enterocolitica* can catalyze the conversion of polygalacturonate- and alginate-derived 4,5-unsaturated mono-uronates to linear ketonized forms.[36] Sun *et al.* also reported a KdgF-like protein in *A. alginolytica* (an aerobe belonging to the Flavobacteriaceae family) in the alginate degradation pathway via the complete genome sequencing.[26] Therefore, a novel pathway for alginate conversion is proposed in which alginate-derived 4,5-unsaturated mono-uronates are converted to 5-dehydro-4-deoxy-D-glucuronate (DDG) and not to DEH (Figure 5C). Interestingly, the enzymes that could convert DDG and 3-deoxy-D-glycerol-2,5-hexdiulosonate (DGH) to KDG were identified via metagenomic and metaproteomic analysis, including 4-deoxy-L-threo-5-hexulose uronate isomerase (EC 5.3.1.17, Figures S11 and S16) and 2-deoxygluconate dehydrogenase (EC 1.1.1.125, Figure S11). Additionally, the bacteria belonging to the genus *Bacteroides* (Figure 5B, including *B. ovatus*, *B. intestinalis*, and *B. uniformisis*) were also annotated to encode these two enzymes (Tables S6 and S7). Consequently, alginate could be converted to pyruvate by genus *Bacteroides* via a new pathway and following ED and EMP pathways. This result is congruent with the experimental results in Figure 1 and the bacterial diversity in Figure 4.

Interestingly, besides the metabolic pathway of alginate, the isomers of intracellular galacturonate (including enzymes of EC 5.3.1.17, EC 1.1.1.125, EC 2.7.1.45, and EC 1.2.14) and glucuronate (including enzymes of EC 5.3.1.2, EC 1.1.1.57, EC 4.2.1.8, EC 2.7.1.45, and EC 1.2.14.) degradation pathways can also be identified to produce pyruvate in Figure S11 and lumped in Figure 5. Since galacturonate and glucuronate are identified in St-EPS,[10] ADC may also utilize these components to produce methane from WAS, which supports the better performance of dosing ADC to promote methane production in our recent work.[17]

3.5. Environmental Implication of Applying ADC for WAS Utilization. This work demonstrates that integrating metaproteomic/metagenomic analysis is a good tool to clarify the core enzymes and/or metabolic pathway in enriched microbial cultures.[37] After removing the low activity bacteria via chemostat, we obtained the *Bacteroides*-dominated ADC in this study. It shall be noted that two phyla *of* Bacteroidetes and Firmicutes are generally considered as the main hydrolytic bacteria in the mesophilic reactors.[38,39] However, the role of genus *Bacteroides*, belonging to phylum Bacteroidetes, on anaerobic digestion is seldom reported. For example, *B. cellulosolvens* and *B. xylanolyticus* are commonly deemed to degrade cellulose and xylan in biogas plants, respectively.[40] The function of alginate degradation was not identified to the best of our knowledge. Moreover, the metaproteomic analysis identified the key extracellular alginate lyase (EC 4.2.2.3) in the broth of *Bacteroides*-dominated ADC. Combined metagenomics and metaproteomic analysis indicated a new pathway for alginate conversion, i.e., via DDG and DGH as the intermediates to KDG and not via the well-known DEH, broadening the understanding of alginate degradation pathways. Thus, genus *Bacteroides* shall be the key bacterium for the alginate conversion process. More works will be needed to verify the pathway by enzyme activity measurements or metabolomic techniques.[41] Other technologies, such as whole-genome sequencing of isolating bacterium in ADC

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and metatranscriptome, are not only useful to verify this novel alginate pathway of genus *Bacteroides* but also for better understanding the hydrolytic process in general. Thus, this work provides a good paradigm when analyzing the degradation mechanism of unacquainted substrates.

By the end of 2019, it is estimated that the production of sludge (80% water content) in China is up to 39 million tons.[42] There is a strong drive to minimize this amount, and anaerobic digestion of WAS is greatly desired.[2,17,43] Felz *et al.* recently reported that the contents of neutral sugars and uronic acids in EPS were comparable (10.7 and 13.2%).[10] Several researchers reported that uronic acids containing polymers in EPS are resistant to anaerobic degradation.[8,29] In this work, we demonstrate that *Bacteroides*-dominated ADC with the trait to excrete alginate lyases (EC 4.2.2.3) can enhance EPS conversion in WAS. Combination with other well-known hydrolytic enzymes of α-amylase, protease, α-glucosidase, and lysozyme may further promote the hydrolysis process. Thus, ADC can help increase the WAS digestion rate, which should be further evaluated at a large-scale digestion installation.

Besides alginate, other uronic acids such as polygalacturonate are also detected in St-EPS.[10] Interestingly, DDG is a typical intermediate of polygalacturonate via a novel enzyme of KdgF.[36] Thus, the *Bacteroides*-dominated ADC in this work may also offer the merit to convert galacturonate and glucuronate (Figure 5). It shall also be noted that other components including glycoproteins and sulfated polysaccharides (such as carrageenan and heparin) are also identified in WAS,[44,45] providing additional substrates for WAS utilization. Meanwhile, the toxicity of humic acids on the activity of alginate lyase shall also be considered. As known, the mixed culture provides several advantages for the utilization of WAS because there is no requirement for sterilization and it can be adapted to variation in feedstocks and process conditions.[46] Thus, exploiting EPS-degrading mixed cultures including *Bacteroides*-dominated ADC offers a promising biotechnology for WAS utilization.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.1c05289.

Additional tables (Tables S1−S7) and figures (Figure S1−S16) about the performance of mesophilic two chemostats in series, clustx results of protein sequences of extracellular ADC enzyme, α-glucosidase activity, microbial diversity of enriched ADC by Illumina high-throughput sequencing and 16s rRNA gene clone library sequencing, and metagenomic/metaproteomic analysis of enriched ADC (PDF)

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Notes

The authors declare no competing financial interest.

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