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DOI
10.1016/j.watres.2023.119800

Publication date
2023

Document Version
Final published version

Published in
Water Research

Water Research 233 (2023) 119800

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

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ARTICLE INFO

Keywords:
Waste activated sludge
Anaerobic digestion
Structural extracellular polymeric substances
Polygalacturonate-degrading consortium
Clostridium
Pectate lyases

ABSTRACT

By maintaining the cell integrity of waste activated sludge (WAS), structural extracellular polymeric substances (St-EPS) resist WAS anaerobic fermentation. This study investigates the occurrence of polygalacturonate in WAS St-EPS by combining chemical and metagenomic analyses that identify ~22% of the bacteria, including *Ferruginibacter* and *Zoogloea*, that are associated with polygalacturonate production using the key enzyme EC 5.1.3.6. A highly active polygalacturonate-degrading consortium (GDC) was enriched and the potential of this GDC for degrading St-EPS and promoting methane production from WAS was investigated. The percentage of St-EPS degradation increased from 47.6% to 85.2% after inoculation with the GDC. Methane production was also increased by up to 2.3 times over a control group, with WAS destruction increasing from 11.5% to 28.4%. Zeta potential and rheological behavior confirmed the positive effect which GDC has on WAS fermentation. The major genus in the GDC was identified as *Clostridium* (17.1%). Extracellular pectate lyases (EC 4.2.2.2 and 4.2.2.9), excluding polygalacturonase (EC 3.2.1.15), were observed in the metagenome of the GDC and most likely play a core role in St-EPS hydrolysis. Dosing with GDC provides a good biological method for St-EPS degradation and thereby enhances the conversion of WAS to methane.

1. Introduction

Waste activated sludge (WAS) represents a huge amount of unwanted product from wastewater treatment plants (WWTPs). For example, the amount of produced WAS with a water content of ~80% exceeded 60 million tons in China in 2019 (Wu et al., 2020). Anaerobic digestion (AD) or fermentation is an effective method to produce high-value products in the form of methane and volatile fatty acids (VFAs, including acetate and propionate) from organic wastes (Liu et al., 2021a; Toutian et al., 2020b). However, it suffers from low methane production and poor COD conversion from WAS. In many models of WAS structure, the extracellular polymeric substances (EPS), especially gel-forming polysaccharides, are used to act as a gel matrix to maintain the structural integrity of microbial aggregates and thereby resist microbial degradation (Ding et al., 2015; Wilén et al., 2003; Yu, 2020). These gel-forming polysaccharides have been subsequently named structural EPS (St-EPS) (Felz et al., 2016). To improve methane and VFA production, pretreatments using thermal, mechanical, chemical, and biological methods have been proposed which release organics from the dispersed WAS (Toutian et al., 2020a), but these methods have not been purposefully developed to convert St-EPS.

Lin et al. initially isolated a typical gel-forming polysaccharide of alginate-like exopolysaccharides (ALE, a typical uronic acid) from WAS flocs (72 ± 6 mg/gVSS) (Lin et al., 2013). Zhang et al. enriched a freshwater alginate-degrading consortium (ADC) that demonstrated methane enhancing performance (up to 185%) and VFA production (~

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https://doi.org/10.1016/j.watres.2023.119800
Received 5 December 2022; Received in revised form 22 February 2023; Accepted 22 February 2023
Available online 24 February 2023

35%) from WAS (Hu et al., 2022; Zhang et al., 2021). Guo et al. demonstrate that after 44 days of operation in mesophilic anaerobic batch reactors, the amount of St-EPS was reduced by 26% in granular sludge and by 41% in WAS (Guo et al., 2020). Thus, degrading St-EPS is seen as essential to break the structure of WAS and to promote methane production. Felz et al. recently detected other typical uronic acid monomers of galacturonate and glucuronate in hydrolyzed St-EPS and reported that the contents of uronic acids in St-EPS (7.2% using galacturonate as standard) were comparable to neutral sugars (13.8% using glucose as standard) (Felz et al., 2019). Hence, a hypothesis is proposed in this study that the polygalacturonate degrading consortium (GDC) can degrade St-EPS and thereby promote methane production from WAS. The occurrence of polygalacturonate in WAS is first identified as it is often ignored in similar studies.

When considering the degradation of complex organics such as polygalacturonate and St-EPS in WAS, hydrolysis can be the limiting step for anaerobic fermentation (Geng et al., 2021; Teo and Wong, 2014). Galacturonate, the monomer of polygalacturonate, has been studied as a substrate for anaerobic fermentation. For example, Valk, et al. enriched a *Lachnospiraceae*-species dominated consortium in mesophilic anaerobic chemostats using galacturonate as the substrate, and the main metabolites were acetate (0.53 mol/mol-galacturonate) and formate (0.16 mol/mol-galacturonate) (Valk et al., 2018). However, the hydrolysis of polygalacturonate is seldom reported, especially in WAS fermentation. For polygalacturonate hydrolysis, extracellular enzymes can be divided into polygalacturonase (EC 3.2.1.15) and pectate lyases (EC 4.2.2.2, EC 4.2.2.9, and EC 4.2.2.10) (Richard and Hilditch, 2009). Interestingly, compared with conventional cellulase (EC 3.2.1.4), α-amylase (EC 3.2.1.1), and protease (EC 3.4.22.2), Lü et al. found dosing polygalacturonase (EC 3.2.1.15) unexpectedly increased the release of total EPS polysaccharides by 7 times (Lü et al., 2016). Moreover, alginate lyase (EC 4.2.2.3) was identified as the core extracellular enzyme for alginate degradation (Geng et al., 2021), while the impact of pectate lyase on WAS hydrolysis remains unclear.

Finally, besides enriching GDC, the mechanisms whereby GDC influences St-EPS degradation and WAS fermentation are not well documented. These mechanisms may include the action of extracellular enzymes, intracellular metabolic pathways, and the change of St-EPS and gel strength. The combination of high-throughput sequencing and metagenomic analyses is often used to reveal the diversity and metabolic functions of enriched microbial consortia in anaerobic batch reactors, especially for the minor and ignored microbes. For example, the genus *Bacteroides* was identified as the core bacteria for the degradation of alginate using extracellular alginate lyase (EC 4.2.2.3), though its percentage was lower than 0.1% in an enriched mixed culture (Zhang et al., 2019). These methods may also provide solid evidence of polygalacturonate occurrence in WAS. Besides methane production, St-EPS degradation may change the gel strength of WAS, which can be revealed using rheological analysis (Li et al., 2020).

Thus, in this study, the occurrence of polygalacturonate in WAS St-EPS is first identified by combining chemical and metagenomic analysis. In order to evaluate biological methods for better WAS conversion, we then develop an enrichment culture for GDC in batch reactors using polygalacturonate as the substrate. The role of St-EPS on methane production and change of gel strength in WAS by this enriched GDC is then investigated. The metabolic functioning of GDC is also analysed by combining high-throughput sequencing and metagenomic analysis. Finally, we investigate the effectiveness of GDC for enhanced WAS fermentation. It is hoped that the outcomes of this study may improve the understanding of the effects of St-EPS degradation on WAS fermentation and subsequently help develop a microbial method to enhance WAS fermentation.

2. Materials and methods

2.1. Inocula and enrichment of GDC in mesophilic fed-batch reactors

WAS was collected from the Jinshan wastewater treatment plant (Fuzhou City, China). The typical characteristics of this WAS are summarized in Table S1. Two inocula were collected from a mixture of WAS (10 mL, VSS 10.7 g/L) and the effluent of a chemostat fed with alginate (30 mL, VSS 1.2 g/L) (Hu et al., 2022; Zhang et al., 2019). The mixture was diluted with 20 mL of inorganic medium to reach 60 mL. The composition of the inorganic medium was the same as that described in a previous work (Hu et al., 2022).

The enrichment process can be divided into three stages. In stage 1 (days 1 - 50), the inoculum (60 mL) and inorganic medium (120 mL) were added to 3 serum bottles (120 mL, working volume of 60 mL). Each bottle was sparged with N_2 (> 99.99%) for 20 min and was then cultured in a shaker at 35 °C. 5 g/L of polygalacturonate was added on days 0 10, 18, 26, 34, and 42. During each cycle, the polygalacturonate concentration and metabolites were measured. In stage 2 (days 51 - 90), the enriched GDC in three serum bottles was combined in a 1 L reactor with a working volume of 500 mL, and 5 g/L polygalacturonate was added on days 51, 61, and 76 days. In the last stage (days 90 - 96), the growth profile was investigated by adding 1 mL of enriched GDC, 59 mL of inorganic medium, and 5 g/L of polygalacturonate in three serum bottles (120 mL).

2.2. St-EPS extraction and degradation by dosing with enriched GDC

To demonstrate the role of enriched GDC on St-EPS degradation, St-EPS was extracted from 200 mL of WAS via the heated Na_2CO_3 method (Felz et al., 2016). 150 mL of the extracted St-EPS and 30 mL of enriched GDC, obtained from a 1 L reactor, were then mixed and added into three 120 mL serum bottles (working volume 60 mL, $n = 3$). 10 mM of 2-bromoethanesulfonic sodium (BES) was added to prevent CH_4 production. Three serum bottles were also sparged with N_2 and cultured for 13 days in a shaker at 120 rpm and 35 °C. The change of typical compounds in the St-EPS (i.e., uronic acid, polysaccharide, protein, and humic acid) and metabolites were all determined. Meanwhile, FT-IR spectra and synchronous correlation spectra in 2D-IR were obtained.

2.3. WAS fermentation and changing parameters after dosing with enriched GDC

To demonstrate the role of enriched GDC on WAS fermentation, three groups, GDC-0, GDC-10, and GDC-20 ($n = 3$ for each group), were prepared in 120 mL (working volume of 60 mL) serum bottles with both WAS (50 mL) and GDC of 0, 10, and 20 mL, respectively. The supernatant of the GDC was centrifuged at 10,000 rpm and added to 10 mL of inorganic medium before using. Consequently, the ratio of the GDC (1.4 ± 0.2 gVSS/L) to WAS (10.7 ± 1.6 gVSS/L) was 0, 0.03 and 0.06 gVSS/gVSS, respectively. These serum bottles were also sparged with N_2 and cultured for 30 days. The accumulation of metabolites, changes to the St-EPS, WAS destruction (i.e., $VSS_{destruction}$), zeta potential, and rheological behavior were then determined.

2.4. Activities of extracellular enzymes excreted by enriched GDC

To demonstrate the activities of the extracellular enzymes excreted by enriched GDC, the enzymes in the supernatant from the serum bottles were harvested on day 96 by centrifugation at 10,000 rpm for 5 min. Inorganic medium (50 mL), polygalacturonate (5 g/L), and enzymatic solution (10 mL) were added into a 120 mL serum bottle ($n = 3$) under a pH of 7.0 ± 0.2 and a temperature of 35 °C. The activity of lyase and hydrolase, and the change in molecular weight (MW) of the hydrolysate were all determined.

2.5. Analysis

St-EPS was extracted from dried WAS following the method of Felz et al. (Felz et al., 2016), and was characterized using a FT-IR (iS 50, Thermo Scientific Nicolet), and synchronous correlation spectra from 2D-IR were then analyzed. Monomers in the St-EPS were analysed using a HPAEC-PAD in a Dionex ICS5000 system (Dionex, USA). The absorbance change of polygalacturonate at 254 nm and 540 nm were all determined using a UV–visible spectrophotometer (A560, AOE Instruments, CN). The hydrolysates of polygalacturonate after dosing with enzymes were measured using a high-performance liquid chromatography system (Waters 1525, Waters, USA) at 254 nm (Geng et al., 2021).

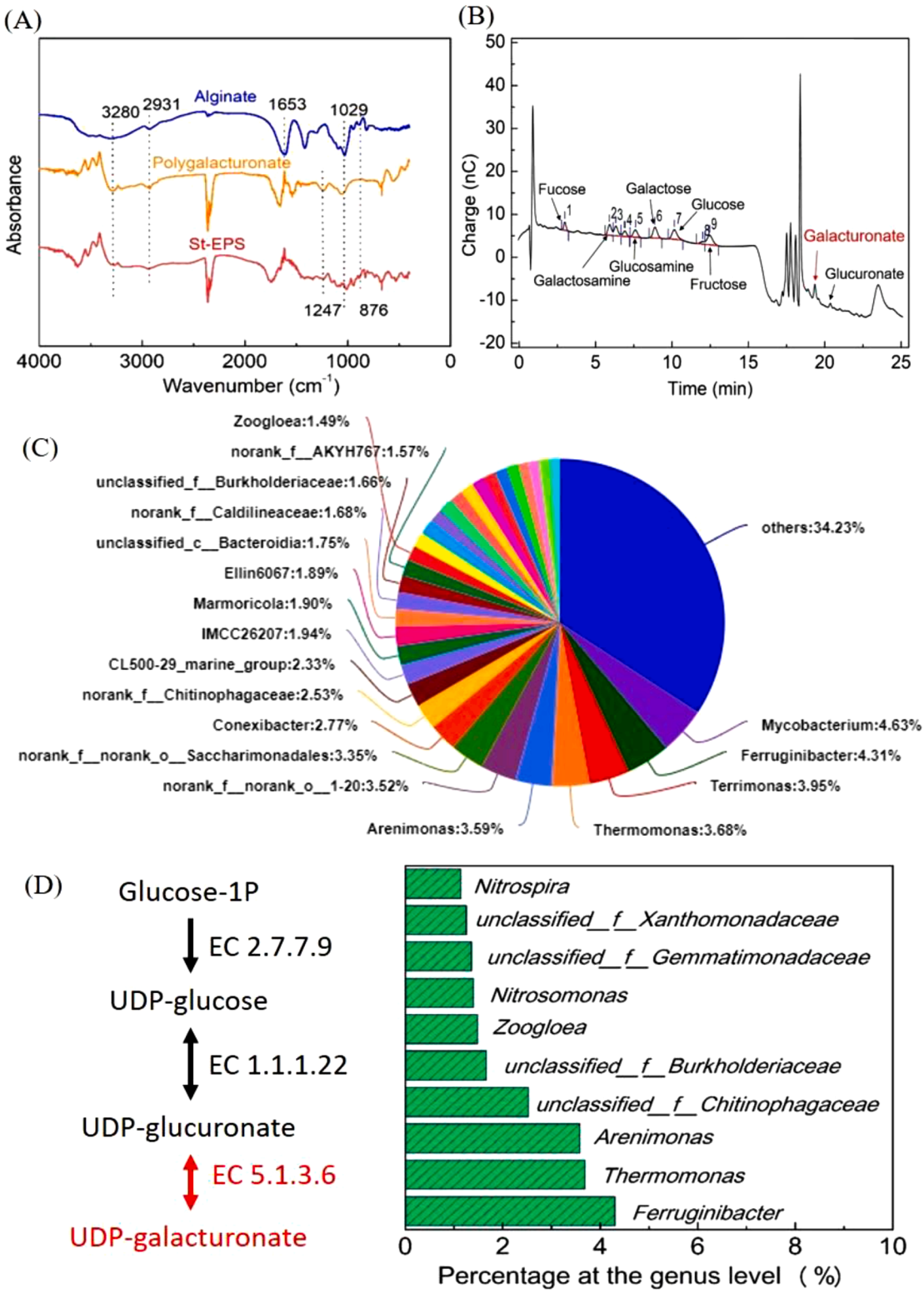

Fig. 1. Occurrence of polygalacturonate in St-EPS extracted from WAS. (A) FT-IR spectra. (B) Monomers in hydrolysed St-EPS. (C) Bacterial diversity at the genus level in the WAS sample. (D) Identification of key bacteria for polygalacturonate.

The concentrations of polygalacturonate and uronic acids in the St-EPS were measured using the carbazole-sulfuric acid method (Felz et al., 2019). The protein and humic acids concentrations were determined using the Lowry method (Felz et al., 2019). The polysaccharide concentration was determined using the anthrone-sulfuric acid method (Felz et al., 2019).

The CH_4 and H_2 contents in the headspace of the reactors were determined using a gas chromatograph (SP7890, Lunan, CN). Concentrations of ethanol and VFAs were measured using another gas chromatograph (7890, Agilent CA). $VSS_{destruction}$ was calculated according to the change of VSS during WAS fermentation (Liu et al., 2021b). The final COD balance calculation was based on the COD of each metabolite concentration (Zhang et al., 2019). The zeta potential of the WAS supernatant was analysed using a Nanosizer ZS instrument (Malvern Co., UK). The rheological behavior of the WAS was characterized using a Physica rheometer (MCR 301, Anton Par, Austria) under both steady-shear tests and dynamic measurements to obtain measurements of structural properties, and viscous and viscoplastic properties (Wang et al., 2017).

2.6. Illumina Miseq high-throughput sequencing and metagenomic analysis

The bacterial diversities associated with the WAS and enriched GDC were analysed using high-throughput sequencing (Majorbio Corporation, Shanghai, China). Six DNA samples were extracted from two inocula (activated sludge and chemostat sludge) and four enriched GDC on days 26, 50, 90, and 96. These samples were termed as the WAS, GDC1, GDC2, GDC3, GDC4, and GDC5, respectively. Amplification (primers of 341F-806R (Zhang et al., 2019)) was carried out using ABI GeneAmp® 9700. Sequencing was then performed using an Illumina Miseq PE 300 sequencer. Raw sequencing reads were quality-filtered and operational taxonomical units (OTU, > 97%) were picked for further analysis of the microbial diversities using Silva v132 database (a confidence threshold of 0.7) following the standard protocols of the Majorbio Cloud Platform (Ren et al., 2022).

Metabolic pathways in the WAS sample and the enriched GDC5 sample were further identified using metagenomic analysis in a Novaseq 6000 platform (Majorbio, China). Pair-end sequence reads were then merged and metagenomics data were assembled using MEGAHIT (version 1.1.2). KEGG annotation was finally conducted using Diamond software (version 0.8.35) with an e-value of 1e-5. The metabolic pathway and encoded enzymes were finally identified following the standard protocols of the Majorbio Cloud Platform (Ren et al., 2022).

3. Results and discussion

3.1. Identifying the occurrence of polygalacturonate in ST-EPS

The content of St-EPS in the WAS sample was 114.5 ± 13.9 mg/gVSS, which was comparable to the results of Lin (72 mg/g-VSS) and Qian (101 mg/gVSS) (Lin et al., 2013; Qian et al., 2021). A comparison of FT-IR spectra from the St-EPS, polygalacturonate, and alginate (Fig. 1A) identified several typical absorption peaks, including wide absorption peaks near 3280 cm^{-1} associated with O—H stretching vibrations, an antisymmetric stretching vibration absorption peak of methylene at 2931 cm^{-1}, a vibration peak of C = O near 1652 cm^{-1}, and a vibration peak of the C—O group near 1246 cm^{-1} (Zhang et al., 2021). The absorption peak at 1029 cm^{-1} reflects the bending vibration of the C—O-C group in the polysaccharide (Lin et al., 2013).

Synchronous correlation spectra obtained using two-dimensional correlation infrared spectroscopy (2D-IR, Figure S1) were further used to identify the similarity between St-EPS and polygalacturonate within the range of 1000~1800 cm^{-1} (Huang et al., 2021). The positive results of cross-related peaks, including 1029 cm^{-1}, 1247 cm^{-1}, and 1652 cm^{-1}, also showed that their band intensities were changed in the same direction, which verified the similarity of identified functional groups. Interestingly, uronic acid components of galacturonate and glucuronate were also identified in the St-EPS using HPAEC-PAD. This observation is similar to that of Felz et al. who noted that both galacturonate and glucuronate were detected in EPS from aerobic granular sludge (Fig. 1B) (Felz et al., 2019). These results suggest good similarity between the functional groups of St-EPS, polygalacturonate, and alginate.

Genera of *Mycobacterium* (4.6%), *Ferruginibacter* (4.3%), *Terrimonas* (4.0%), and *Thermomonas* (3.9%) were the main bacteria in the WAS sample identified using high-throughput sequencing (Fig. 1C). The genus *Zoogloea* (1.5%) was especially illustrated as a key contributor to the formation of activated sludge flocs by producing excessive amounts of viscous EPS (An et al., 2016). UDP-galacturonate is known as the precursor of polygalacturonate in anabolism, which is converted from UDP-glucuronate by UDP-glucuronate 4-epimerase (EC 5.1.3.6) (Zhao et al., 2018). UDP-glucuronate is initially produced from Glucose-1P via UDP-glucose pyrophosphorylase (EC 2.7.7.9) and Uridine-5-diphosphoglucose Dehydrogenase (EC 1.1.1.22) (Zhao et al., 2018). These three enzymes were all encoded by sequenced bacteria in the WAS sample via the metagenomic analysis (Figures S2 and S3). Approximately 22% of the bacteria, including *Ferruginibacter* and *Zoogloea*, were identified according to the key enzyme of EC 5.1.3.6 using metagenomic analysis (Fig. 1D). These results demonstrate that polygalacturonate did exist in the St-EPS and was produced by the dominant bacteria in the WAS.

3.2. GDC enrichment and degradation of ST-EPS

GDC was enriched in three serum bottles for 50 days (Stage 1) by adding 5 g/L polygalacturonate on days 0, 10, 18, 26, 34, and 42. The final concentrations of polygalacturonate at the end of each cycle were below 0.05 g/L (Figure S4A). ADC in the inocula was observed to utilize polygalacturonate using metagenomic analysis (Geng et al., 2021), which may initially consume polygalacturonate in this study. Methane was detected after dosing with polygalacturonate, and the final volume was between 113 and 142 mL (Fig. 2A). The H_2 content was always below 0.01% (the detection limit of GC). Intermediates of acetate (0.9 ± 0.2 g/L) and propionate (0.2 ± 0.05 g/L) were detected, and were completely consumed by methanogens (Figure S4B). The average COD balance was 110.1 ± 10.6%.

The enriched GDC was then transferred into a 1-L bottle on day 51 (Stage 2), and 5 g/L polygalacturonate was added on days 51, 61, and 76. The average CH_4 production was 373.7 ± 6.9 mL (Fig. 2A) and COD balance was 101.3 ± 11.9% ($n = 3$). The OD600 in the growth curve (Figure S4C) also increased from 0.05 ± 0.01 (day 90) to 0.96 ± 0.05 (day 95). These results all supported a good activity of enriched GDC using polygalacturonate as the sole carbon source.

Extracted St-EPS could not be degraded under abiotic conditions. After adding GDC to the St-EPS extracted from the WAS sample, uronic acid (polygalacturonate equivalent, Fig. 2B), polysaccharide (glucose equivalent, Fig. 2C), and protein (BSA equivalent, Fig. 2D) were all degraded within 13 days. The final concentration of these three compounds on day 13 was 0, 0, and 42.5 ± 7.5 mg/L. respectively. The concentration of humic acid did not change much and was around 150 mg/L (Figure S4D). The main metabolites of the St-EPS were acetate and propionate with final concentrations of 144.5 ± 54.7 and 48.7 ± 6.5 mg/L, respectively (Fig. 2E). Since BES was added in these tests, there was no CH_4 accumulation in the headspace, and H_2 may have been utilized by homoacetogenesis to produce acetate (Figure S4E). Guo et al. recently demonstrated that St-EPS could decrease the anaerobic biodegradability and structural decomposition of WAS (Guo et al., 2020). Lü et al. showed that dosing with polygalacturonase (EC 3.2.1.15) increased the release of total EPS polysaccharides by 7 times(Lü et al., 2016), indicating that enriched GDC may promote St-EPS degradation and methane production from WAS.

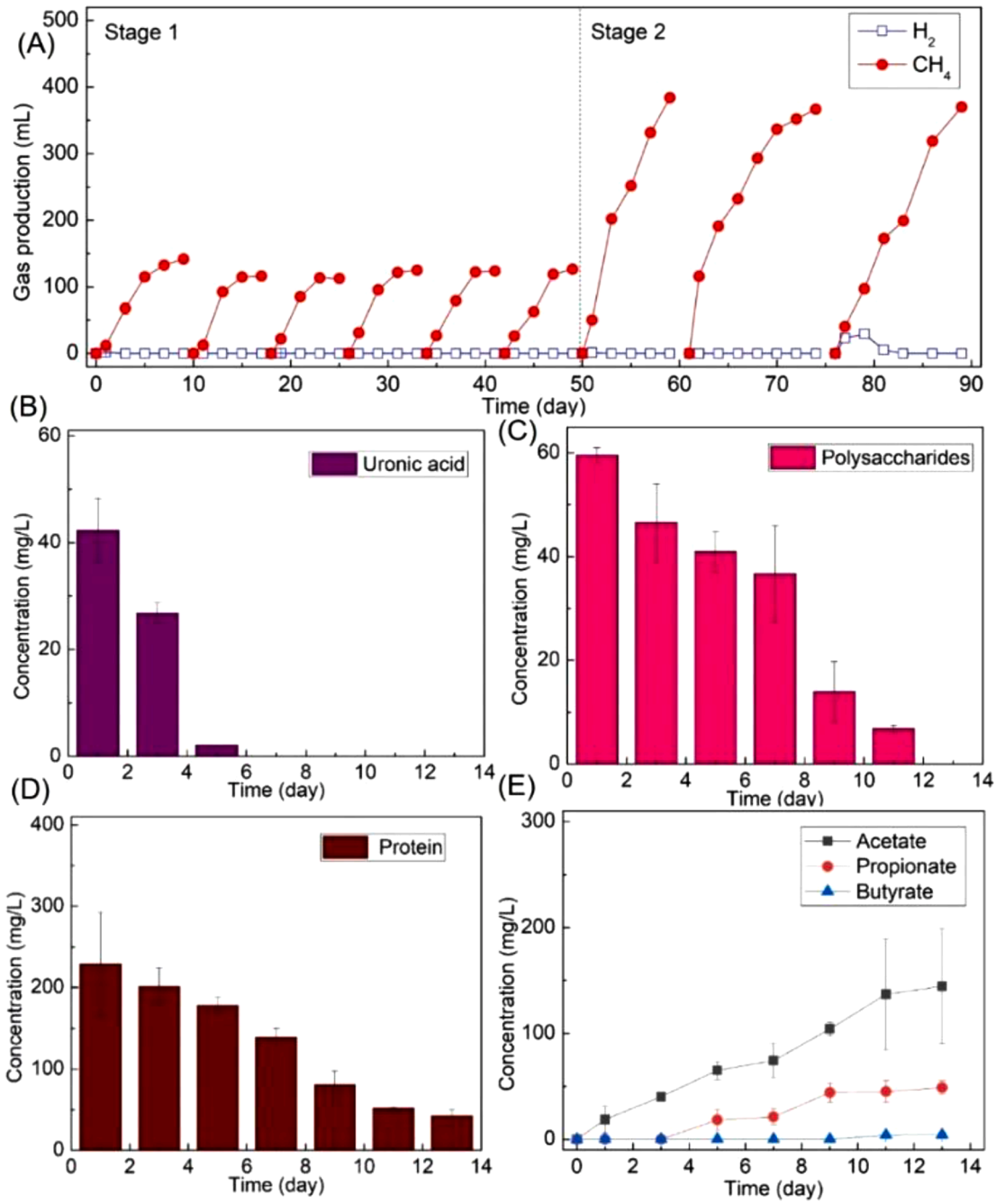

Fig. 2. GDC enrichment and St-EPS degradation. Gas production (A) and change of uronic acid (B), polysaccharides (C), protein (D), and VFAs (E) in the St-EPS.

3.3. CH_4 production from WAS by dosing with enriched GDC

As expected, dosing the WAS digestion batch reactor with enriched GDC promoted methane production over 30 days of operation. Fig. 3A shows that the final CH_4 production in the GDC-20 group (129.7 ± 2.1 mL) was much higher than that in the control group (39.5 ± 1.7 mL) and in the GDC-10 group (85.8 ± 0.9 mL). Thus, methane production was increased by up to 2.3 times. No hydrogen was accumulated (Figure S5A) in the headspace and the acetate and propionate VFAs were all consumed within 15 days (Figures S5B, S5C, and S5D).

Though St-EPS can be degraded by native bacteria in WAS, dosing with GDC further promoted St-EPS conversion (Fig. 3B). For example, the final St-EPS content was 60.0 ± 6.6 mg/gVSS in the control group and was just 17.0 ± 0.8 mg/gVSS in the GDC-20 group, indicating a higher degradation efficiency (47.6% vs 85.2%). This was much higher than that found by Guo et al. (41%) in mesophilic anaerobic batch reactors without enriching St-EPS degrading bacteria (Guo et al., 2020). Fig. 3B further shows that the $VSS_{Destruction}$ of the WAS sample after 31 days of fermentation notably increased from 11.5% ± 1.7% in GDC-0, to 17.8%±2.0% in GDC-10 and 28.4% ± 0.9% in GDC-20. WAS fermentation also changed the zeta potential of the WAS sample (Fig. 3C), which decreased from −35.2 ± 4 to −73.5 ± 1.4 mV. This is consistent with previous research results that have shown that zeta potential decreases after anaerobic fermentation (Liu et al., 2021c).

Furthermore, both the original sludge and the sludge after anaerobic fermentation showed a shear thinning behavior, in which dosing with GDC lowered both apparent viscosity and shear stress, indicating a degradation of polymers (Fig. 3D). Results of the creep test (Fig. 3E) show that creep compliance was positively correlated with the GDC dosing level, indicating that the addition of GDC promoted the

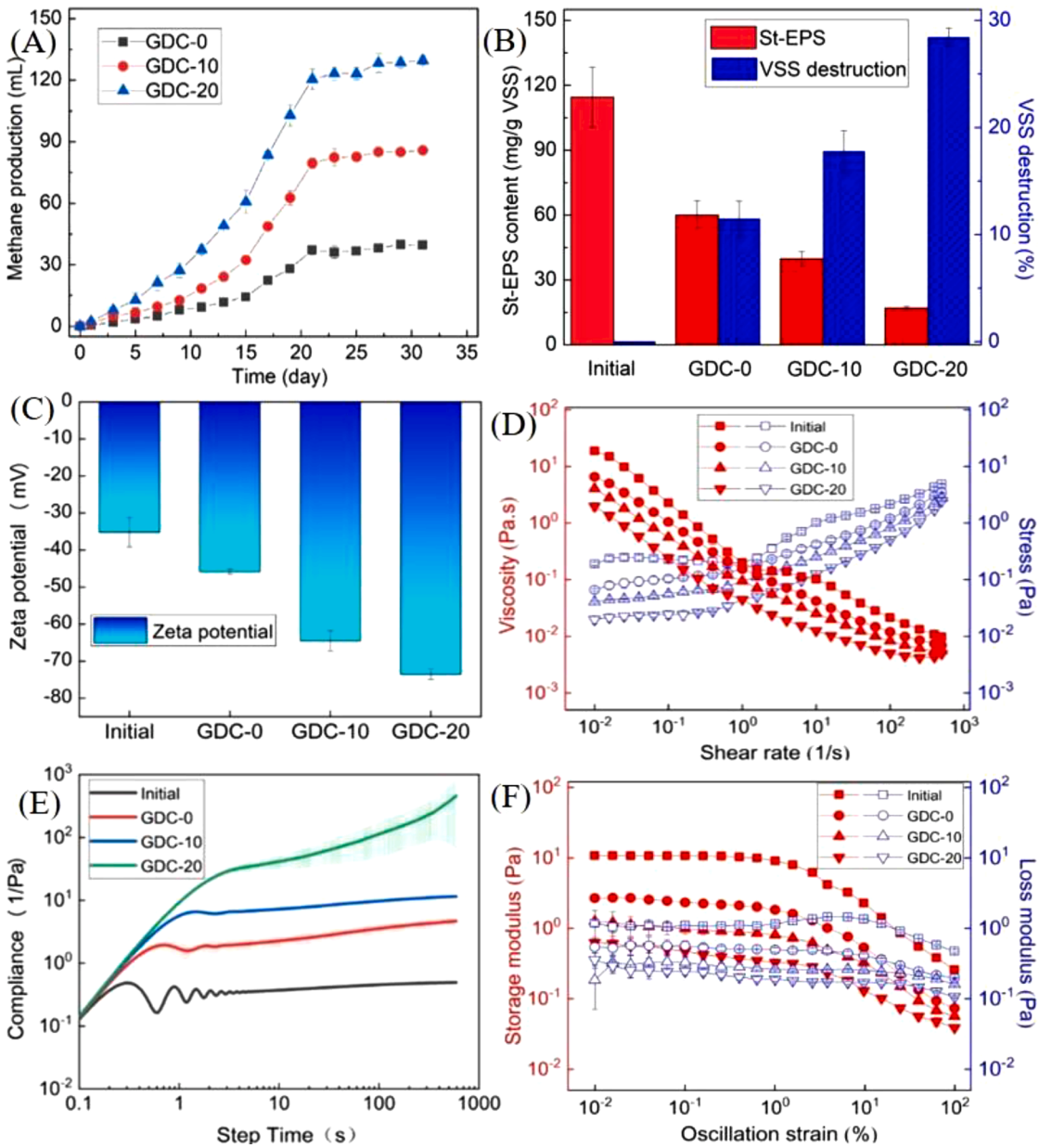

Fig. 3. WAS fermentation by dosing with GDC. (A) CH_4 production, (B) Change of St-EPS and WAS destruction. (C) Zeta potential. (D) Flow curves. (E) Creep tests. (F) Dynamic strain sweep.

degradation of St-EPS and led to the destruction of WAS, which further made the sludge easier to deform. Wang et al. have demonstrated that the ratio of loss modulus to storage modulus can reflect the proportion of sludge viscosity and elasticity (Wang et al., 2017). The dynamic strain sweep curve (Fig. 3F) shows that the maximum value of the angle tangent in the WAS sample was 7.4, which decreased to 1.76 in the GDC-20 sample, indicating that the network strength and cohesive force of WAS flocs decreased with the increasing amount of inoculant. These results support our hypothesis that St-EPS degradation can promote WAS destruction by dosing with enriched GDC, providing a promising microbial way to enhance WAS fermentation.

3.4. Microbial diversity of enriched GDC

To reveal the core bacteria in the enriched GDC, the microbial diversity of two inocula (the WAS and GDC1 samples in Section 3.1), the four enriched consortia of GDC2, GDC3, GDC4, and GDC5 were analyzed using 16S rRNA gene Illumina high-throughput sequencing. The Shannon index curve (Figure S6A), rarefaction curve (Figure S6B), and main sequencing indices (Table S2) all indicated that the sequencing results covered the whole enriched consortia. At the domain level (Figure S6C), the bacterial percentage in the final GDC5 was 99.5%.

Fig. 4A shows that *Bacteroidota* (60.3%) and *Firmicutes* (33.8%) were the main phyla in GDC1. In GDC5, the main phyla changed to *Proteobacteria* (64.5%), *Firmicutes* (25.0%), and *Bacteroidota* (9.8%). The percentages of the top enriched bacteria (>1%) at the genus level are summarized in Fig. 4B according to the Rank-Abundance curves (Figure S6D) and heatmap (Figure S6E). *Bacteroides* (47.7%) and *Oscillibacter* (20.9%) were the main genera in GDC1, while these changed to genera of *Clostridium_sensu_stricto_8* (37.5%), *Methanobacterium* (15.4%), and *Alistipes* (14.3%) in GDC4 and *unclassified_f_Enterobacteriaceae* (46.5%) and *Clostridium_sensu_stricto_8* (15.4%) in GDC5. The identified polygalacturonate producers of *Ferruginibacter* and *Zoogloea* in WAS were not detected in GDC5.

It should be noted that *Bacteroides* was enriched in a previous study as the novel genus which degrades uronic acids of alginate and

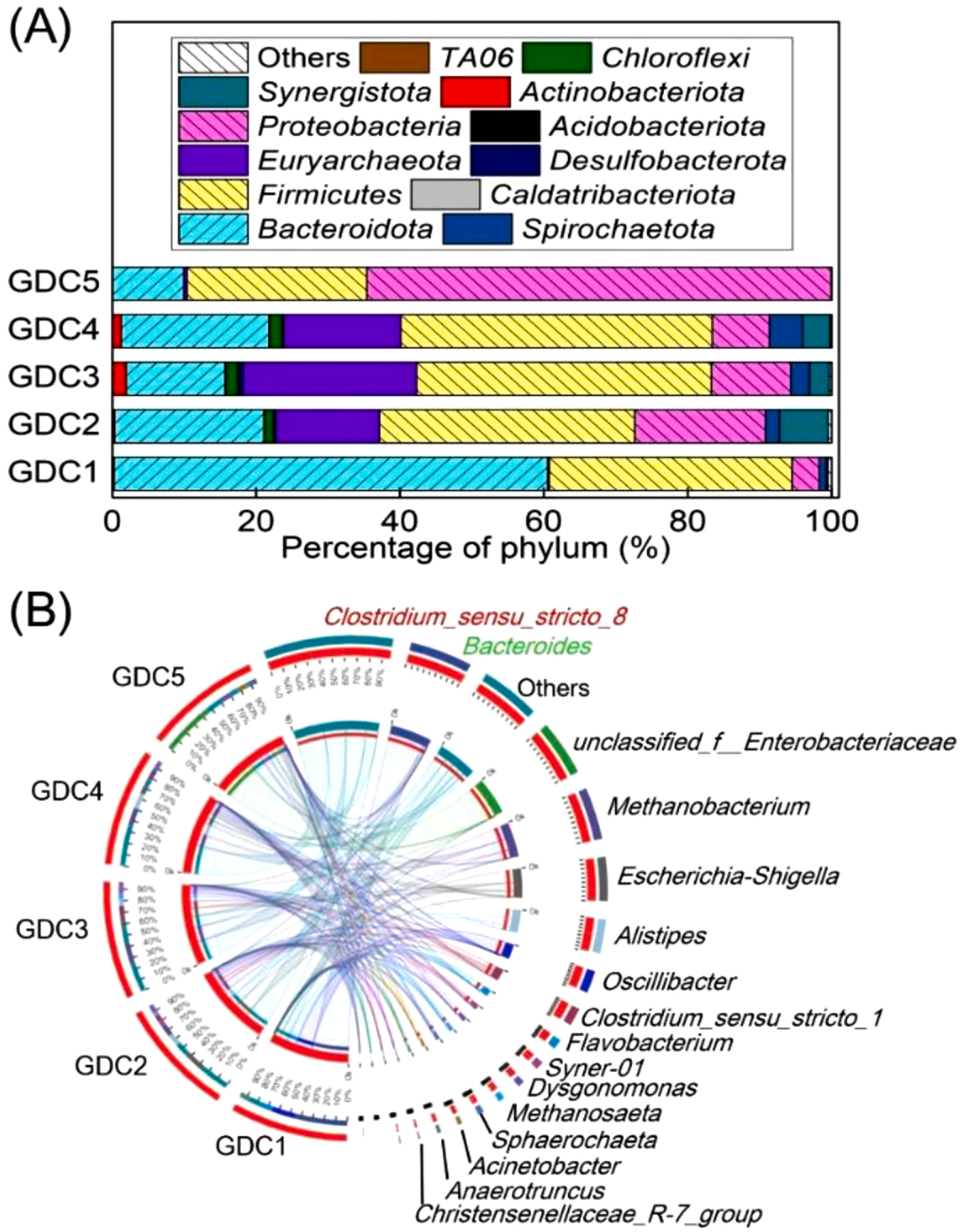

Fig. 4. Microbial diversity at the levels of phylum (A) and genus (B) based on the high-throughput sequencing.

polygalacturonate in a mesophilic chemostat (Geng et al., 2021). In the current study, after long-term operation, the percentage of the genus *Bacteroides* decreased notably from 47.7% (GDC1) to 0.5% (GDC5), which indicates that a different, potentially novel, genus (maybe two genera of *unclassified_f_Enterobacteriaceae* and *Clostridium_sensu_stricto_8*) performed polygalacturonate degradation. This might be better revealed via a metagenomic analysis. Lastly, two genera of *Methanobacterium* (hydrogenotrophic methanogens, 15.4% in GDC5) and *Methanosaeta* (acetoclastic methanogens, 0.8% in GDC5) (Zabranska and Pokorna, 2018) were identified to convert the intermediates of H_2 and acetate to methane, which is consistent with the results presented in Fig. 2.

3.5. *Extracellular enzymes and intracellular metabolic pathway of enriched GDC*

The extracellular enzymes for polygalacturonate hydrolysis include lyases of pectate lyases (EC 4.2.2.2), exo-pectate lyase (4.2.2.9), and pectin lyase (4.2.2.10), and hydrolyase of polygalacturonase (EC 3.2.1.15) (Garron and Cygler, 2014). Enzymatic activity tests for lyases in the GDC5 sample (Fig. 5A) showed an obvious activity over the control sample indicating good activity of polygalacturonate lyase and hydrolyase. The apparent MW of initial polygalacturonate was above 100,000, and after dosing with enzymatic solution, the MW profiles of final hydrolysate decreased to a MW of 266 Da (Fig. 5B). However, due to the complexity of these enzymes, it was hard to analyze the specific intermediates (such as MW ~ 45,000), which would be revealed in the future. With respect to hydrolyase, the absorbance at 540 nm (Fig. 5A) did not increase by much. Thus, the extracellular lyases, rather than

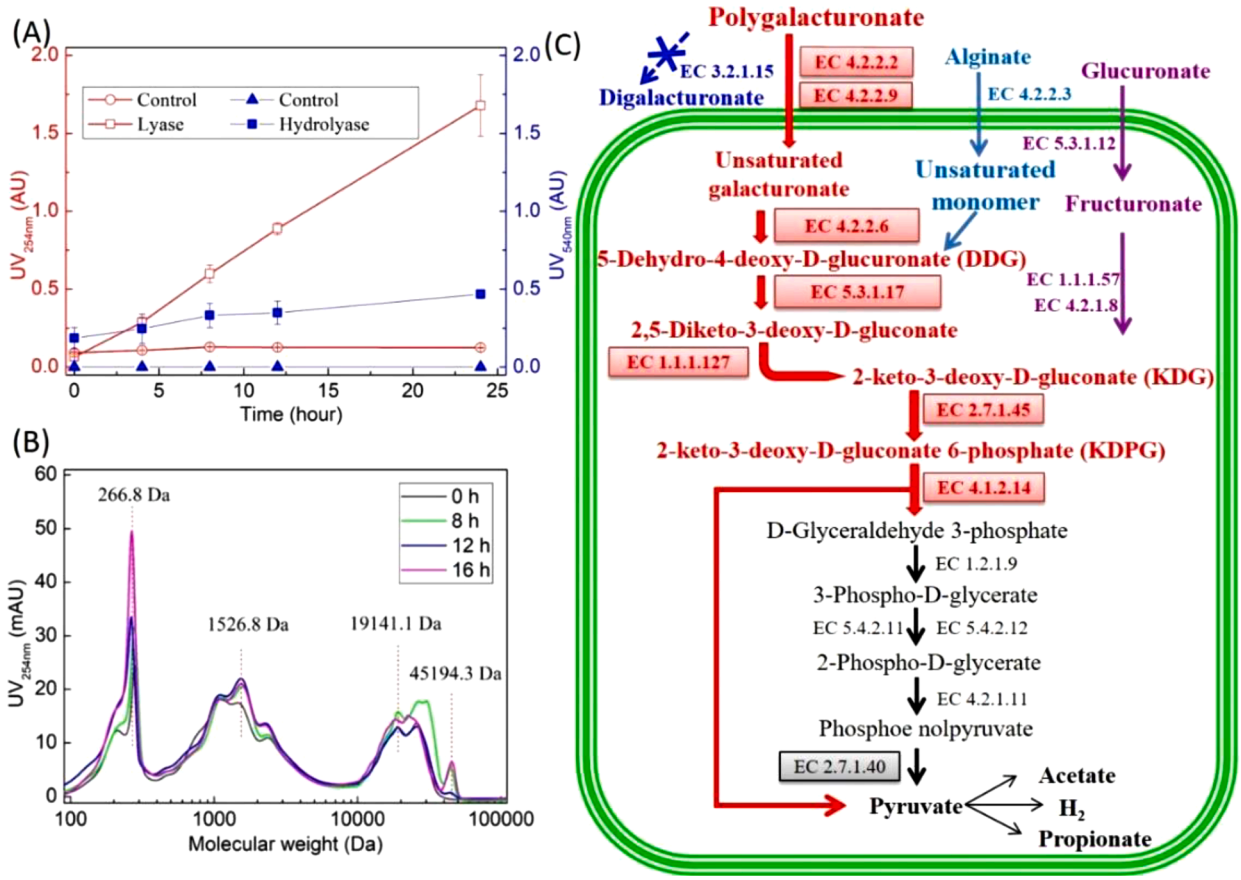

Fig. 5. Enzymatic activities (A), MW profiles (B), and proposed intracellular metabolic pathway via metagenomic analysis (C) in the GDC5 sample.

polygalacturonase (EC 3.2.1.15), might work on polygalacturonate degradation in enriched GDC.

The majority of identified sequenced reads in enriched GDC was in the metabolism section according to metagenomic analysis (Figure S7). The full metabolic pathway of carbon flow to methane was constructed by metagenomic analysis (Figure S8), in which the well-known glycolysis of Entner-Doudoroff (ED) pathway (Figure S9) and Embden-Meyerhof-Parnas (EMP) pathway (Figure S10) were encoded. Fig. 5C demonstrates the identified pathways for polygalacturonate degradation by enriched GDC. The extracellular lyases of pectate lyases (EC 4.2.2.2) and exo-pectate lyase (4.2.2.9) were identified (Figure S11), and other lyases of pectin lyases (4.2.2.10) and extracellular hydrolyase of polygalacturonase (EC 3.2.1.15) (Figure S11) were missing. This meant that polygalacturonate was first converted to unsaturated digalacturonates via pectate lyases of EC 4.2.2.2 and EC 4.2.2.9, which is consistent with the extracellular enzymatic activities seen in Figs. 5A and 5B.

The intracellular unsaturated digalacturonate was further degraded into 5-Dehydro-4-deoxy-D-glucuronate (DDG) by EC 4.2.2.6, which subsequently entered into the ED pathway via cascading reactions by intracellular enzymes of EC 5.3.17, EC 1.1.1.127 (Figure S11), EC 2.7.1.45 (Figure S11). The enzymes for other uronic acids of glucuronate (EC 5.3.1.12, EC 1.1.1.57, and EC 4.2.1.8 in Figure S11) and alginate (EC 4.2.2.3 in Figure S12) were also encoded to enter into the polygalacturonate degrading pathway. Additionally, the produced KDPG was converted to D-Glyceraldehyde 3-phosphate (GAP) and pyruvate by a KDPG aldolase (EC 4.1.2.14 in Figure S9) and finally converted into the metabolites detected in Fig. 2.

The typical enzymes of pectate lyases (EC 4.2.2.2 and EC 4.2.2.9) and intracellular enzymes of EC 4.2.2.6, EC 5.3.17, and EC 1.1.1.127 are summarized in Table S3, and the identified bacteria are summarized in Table S4. The genus *Clostridium* (17.1%), including closed genera of *Clostridium_sensu_stricto_8* (15.4%) and *Clostridium_sensu_stricto_12* (1.2%), was the main identified bacterium that can excrete extracellular lyase of both EC 4.2.2.2 and EC 4.2.2.9. Another genus of *Bacteroides*, a former enriched bacteria for alginate conversion (Geng et al., 2021), may also excrete these two enzymes, but the percentage of this genus was just 0.5%, indicating that the genus *Clostridium* outcompeted *Bacteroides* for polygalacturonate degradation during the 100 days enrichment.

It should be noted that for the intracellular metabolic pathway, EC 5.3.17 and EC 1.1.1.127 were identified in the *Clostridium* genus, but EC 4.2.2.6 was not. It is known that for alginate degradation, the conversion of unsaturated monomers is generally considered as a non-enzymatic reaction (Geng et al., 2021). Thus, the conversion of unsaturated galacutronate to DDG may also occur in a similar way. Moreover, the genus *Clostridium* was also the main bacterium for glucuronate degradation according to the enzyme of EC 5.3.1.12. However, the role of *unclassified_f_Enterobacteriaceae* on polygalacturonate degradation was still unclear due to the poor identification of enzymes summarized in Table S3. Thus, the genus *Clostridium* could be considered a core bacterium for the degradation of polygalacturonate and other uronic acids, which is consistent with the microbial diversity seen in Fig. 4B.

3.6. Implication and perspective of ST-EPS degradation and WAS fermentation via dosing with enriched GDC

Hydrolysis via extracellular enzymes is known as the first step in WAS utilization (Teo and Wong, 2014; Zhang et al., 2021). Lü et al. found that dosing polygalacturonase (EC 3.2.1.15) in WAS increased the release of total EPS polysaccharides by up to 7 times (Lü et al., 2016), but the associated mechanism was not well documented. In this work, the occurrence of polygalacturonate was revealed by combining

chemical and metagenomic analyses (Fig. 1), supporting the positive role of polygalacturonase on polysaccharide release. However, two extracellular lyases, pectate lyases (EC 4.2.2.2) and Exo-pectate lyase (4.2.2.9), were unexpectedly identified in this work when using polygalacturonate as the substrate and unsaturated galacturonate was subsequently produced (Fig. 5). Thus, the pectate lyases extracted by the *Clostridium* genus (17.1%) in the enriched GDC were the likely key extracellular enzymes to promote St-EPS destruction, as shown in Fig. 6.

This was analogous to alginate degradation, where the alginate lyase (EC 4.2.2.3) was the key extracellular enzyme in enriched genus *Bacteroides* (Zhang et al., 2019). Thus, the mechanism is proposed in Fig. 6 that the hydrolysate of WAS was utilized by the genus *Clostridium* and other acidogens to produce fatty acids such as acetate, propionate, and butyrate, which were finally converted to methane by methanogens represented by the genera *Methanobacterium* and *Methanosaeta*. This work demonstrates that integrating high-throughput sequencing and metagenomic analyses can help clarify the core bacterium and metabolic pathways of enriched microbial cultures.

Till now, physical, chemical, and mechanical treatments have been proposed to promote WAS fermentation, but toxic and refractory by-products are also unwantedly released by these methods (Toutian et al., 2020a; 2021; Wang et al., 2022). For example, Cao et al. reported that excess nitrate and nitrite remained in the effluent of WWTPs due to the complex composition and toxicity of released COD from alkaline fermentation broth (Cao et al., 2019). Thus, biological methods are deemed to offer the benefits of low operating cost and high selectivity (Zhang et al., 2021). St-EPS with typical components of polygalacturonate and ALE can strengthen the stability of the structure of WAS (Felz et al., 2019; Guo et al., 2020). The enriched GDC improved the methane production by up to 2.3 times in this work, which was supported by the observed St-EPS reduction, WAS destruction, and the rheological behaviors of both apparent viscosity and shear stress (Fig. 3).

As excreted from bacteria in WAS, EPS is generally considered to be a complex organic composed of proteins, polysaccharides, and humic acids (Yu, 2020). Besides polygalacturonate degradation, *Clostridium* is a well-known gram-positive genus which can produce hydrogen and fatty acids from carbohydrates such as carbohydrates and protein as substrates (Batstone et al., 2019; Dai et al., 2020). In our recent work, a gram-negative alginate-degrading consortium of genus *Bacteroides* was enriched and demonstrated enhancement of methane production from WAS (115% - 185%) (Zhang et al., 2021, 2019). These results demonstrate that St-EPS plays a significant role in WAS fermentation. Destruction of the EPS network by combining these enriched bacteria may further enhance WAS fermentation by degrading St-EPS. A better focus on the mechanisms behind sludge hydrolysis will also help in maximizing the methane yield from WAS.

As a polymer polysaccharide commonly found in the cell wall, polygalacturonate is also abundant in fruits and vegetables and frequently applied as a gelling agent in the food industry (Bui et al., 2019; Perpelea et al., 2022; Valk et al., 2018). Approximately 35 million tons of citrus peel, 28 million tons of sugar beet pulp, and 2 million tons of apple pomace were produced in 2016, containing ~15% polygalacturonate (Valk et al., 2018). Thus, the co-fermentation of organic wastes, including WAS, fruits, and vegetables, may further increase the activity of extracellular enzymes and promote methane production, which will be investigated in future studies. Hereinafter, the St-EPS degradation via enrichment with GDC can provide a good paradigm for WAS fermentation via biological methods.

4. Conclusions

The occurrence of polygalacturonate in the St-EPS of WAS was investigated in this work by combining chemical and metagenomic analyses for the first time. The positive role of St-EPS in WAS fermentation was subsequently documented using enriched GDC. Consequently, this work provided an elegant method for promoting WAS fermentation by identifying the key component in EPS. The main findings were as follows:

- About 22% of bacteria in collected WAS, including *Ferruginibacter* and *Zoogloea*, were identified as being associated with polygalacturonate production according to the key enzyme EC 5.1.3.6.
- 10 g/L of polygalacturonate was wholly consumed by enriched GDC with a COD recovery balance around 100%.
- The percentage of St-EPS degradation increased from 47.6% to 85.2% via dosing with GDC, and the methane production subsequently increased up to 2.3 times over a control group.
- The percentage of WAS destruction increased from 11.5% to 28.4% after dosing GDC, which was supported by the change of rheological behaviors of the sludge.
- The genus *Clostridium* (17.1%) was finally identified as the key bacterium in the GDC using high-throughput sequencing and metagenomic analyses.
- Extracellular pectate lyases (EC 4.2.2.2 and 4.2.2.9), not typical polygalacturonase (EC 3.2.1.15), were encoded to play a core role in St-EPS hydrolysis.

Appendix A. Supplementary data

The Supporting Information is available free of charge. Additional

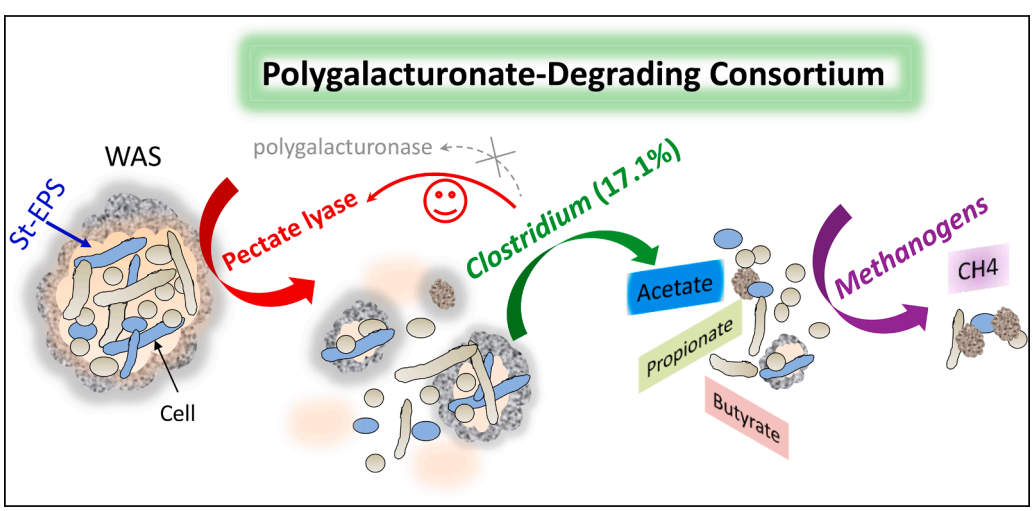

Fig. 6. Proposed mechanism of WAS destruction and fermentation via a *Clostridium* dominated GDC.

tables (Tables S1−S4) and figures (Figure S1−S12) about the growth curve, microbial diversity by Illumina high-throughput sequencing and metagenomic analysis of WAS and enriched GDC.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

Acknowledgements

The authors would like to acknowledge the financial support from the National Natural Science Foundation of China (Grant no: 52170127, 51878175, and 51808120), the Science Foundation of the Fujian Province (2020J01567), and the Program for Innovative Research Team in Science and Technology in Fujian Province University (IRTSTFJ).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2023.119800.

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