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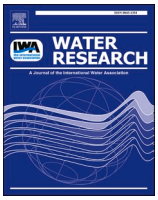

Coupling extracellular glycan composition with metagenomic data in papermill and brewery anaerobic granular sludges

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ABSTRACT

Glycans are crucial for the structure and function of anaerobic granular sludge in wastewater treatment. Yet, there is limited knowledge regarding the microorganisms and biosynthesis pathways responsible for glycan production. In this study, we analysed samples from anaerobic granular sludges treating papermill and brewery wastewater, examining glycans composition and using metagenome-assembled genomes (MAGs) to explore potential biochemical pathways associated with their production. Uronic acids were the predominant constituents of the glycans in extracellular polymeric substances (EPS) produced by the anaerobic granular sludges, comprising up to 60 % of the total polysaccharide content. MAGs affiliated with *Anaerolineacae, Methanobacteriaceae* and *Methanosaetaceae* represented the majority of the microbial community (30–50 % of total reads per MAG). Based on the analysis of MAGs, it appears that *Anaerolinea* sp. and members of the *Methanobacteria* class are involved in the production of exopolysaccharides within the analysed granular sludges. These findings shed light on the functional roles of microorganisms in glycan production in industrial anaerobic wastewater treatment systems.

1. Introduction

Anaerobic wastewater treatment is a widely applied technology to convert industrial wastewater into methane-rich biogas, a renewable energy carrier (van Lier et al., 2015a). This technology relies on the activity of microbial communities, often self-assembled into aggregates (granules) of 1–5 mm diameter. Sludge granulation is crucial for compact high-rate reactor technology, as the high density of granules permits the uncoupling of solid and hydraulic retention times (Hulshoff Pol et al., 2004; van Lier et al., 2015b).

The phenomenon of sludge granulation is linked to the production of extracellular polymeric substances (EPS) by microorganisms (Schmidt and Ahring, 2000). These EPS form a matrix that embeds the microorganisms in a gel-like structure, providing protection against external stressors. Previous studies on surface-associated biofilm-forming microorganisms revealed the diverse composition of EPS, including proteins, polysaccharides and lipids, and their combinations, like glycoproteins and lipopolysaccharides (Neu and Kuhlicke, 2017; Seviour et al., 2019). The ability of the extracellular matrix to form hydrogels is attributed mainly to the presence of functional groups in EPS that carry negative charges (Decho and Gutierrez, 2017), e.g. sialic acids, sulfated polymers and charged sugar monomers (Boleij et al., 2020; de Bruin et al., 2022; Felz et al., 2020). Yet, due to the complexity of glycans present in EPS, the composition of EPS individual glycans in the glycome (polysaccharides and glycoconjugates) remains relatively unexplored (Seviour et al., 2019). This is particularly the case in the context of mixed communities like anaerobic granular sludge. There, specific sugar monomers within glycans, such as rhamnose, mannose, glucose and galactosamine, are believed to be significant (Veiga et al., 1997). Furthermore, research also indicates that the glycoconjugate composition in anaerobic granular sludge is highly dynamic and diverse, allowing microorganisms to adapt swiftly to their environment. For example, anaerobic granules adapted to saline wastewater (20 g/L Na^+) showed an elevated content of N-acetyl-galactosamine and galactose

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glycoconjugates compared to granules grown in low-salinity (5 g/L Na^+) wastewater (Gagliano et al., 2018).

Related to the lack of knowledge on the glycome composition of anaerobic biofilms, the EPS synthesis pathways also remain poorly characterized in anaerobes. On the contrary, in aerobes several pathways have been described for EPS synthesis, including the wzy/wzx-dependent, the synthase-dependent, and the ABC transporter-dependent pathways (Limoli et al., 2015; Sun and Zhang, 2021; Whitfield et al., 2020). Genes required for EPS production encode functions related to regulation, synthesis of sugar monomers, chain-length determination, repeat-unit assembly, polymerization and export (Schmid et al., 2015). However, these genes are not always grouped in operons, and some may overlap with central metabolism, making glycoconjugates and exopolysaccharide-associated biosynthetic gene clusters challenging to detect (Jennings et al., 2015). A recent large-scale metagenomic study of aerobic sludge found that 11% of the metagenome assembled genomes (MAGs) contained known operons with all necessary genes to produce extracellular polysaccharides (Dueholm et al., 2023). This indicates that either EPS biosynthesis potential is restricted to a small subset of the microbial community, or that new EPS biosynthetic clusters remain to be discovered.

This study aimed to provide a way to qualitatively link the EPS glycome of anaerobic granular sludge with the microbial glycan-biosynthetic potential. By linking the chemical and metagenomic data it would be possible to guide the search of novel EPS biosynthetic clusters. Samples of anaerobic granular sludge were collected from industrial bioreactors with two distinct configurations (IC and UASB) and treating wastewater of varying composition (papermill or brewery wastewater). Through a combination of chemical analyses of the sludge EPS glycome and metagenomic sequencing, it was possible to study the glycan and microbial composition of the anaerobic granular sludges. Additionally, by analyzing MAGs encoding biosynthesis genes for the glycan-associated sugar monomers, it was possible to pinpoint potential microorganisms contributing to the production of the anaerobic sludge EPS glycome.

2. Materials and methods

2.1. Source of anaerobic granular sludge

Sludge samples were obtained from three full-scale anaerobic digesters, each with specific configuration and wastewater source (Table 1). Two internal circulation (IC) reactors received wastewater from papermill industry, while the upflow anaerobic sludge blanket (UASB) reactor was supplied with brewery wastewater. Industrial paper recycle/kraft wastewater can be highly contaminated with lignin, lignin-derivatives, and toxic compounds that are difficult to digest (Kumar et al., 2022). Brewery wastewater is characterized by a high organic content derived from sugars, soluble starch, and ethanol (Simate et al., 2011).

Table 1
Anaerobic granular sludge characteristics.

Sample	Papermill A	Papermill B	Brewery
Reactor type	IC	IC	UASB
Wastewater source	Paper recycle & kraft	Paper recycle & kraft	Brewery
Temperature (°C)	36	35	29
Influent COD (mg/L)	1200	1200	3900
Effluent VFA (mEq/L)	124.0	112.2	47.2
Pre-acidification (% of COD)	19 %	19 %	n.a.
Granule morphology	Round, slightly fluffy	Round, smooth	Round, grainy
Color	beige	beige	black

COD – chemical oxygen demand; VFA – volatile fatty-acids; pre-acidification – refers to the percentage of COD removed by an acidification treatment ahead of AD.

2.2. Extraction of extracellular polymeric substances

Solubilization of EPS from the extracellular matrix involved applying an alkaline extraction protocol to lyophilized granules, following the method described by Pinel et al. (Pinel et al., 2020). In brief, lyophilized samples were added to 0.1 M NaOH solution, in a 10 mg/mL concentration, and stirred vigorously for 30 min at 80 °C. The mixture was cooled to 4 °C and centrifuged at 3300 x g for 30 min. The supernatant was collected and dialyzed with a 3.5 kDa cut-off dialysis bag against demi-water overnight at room temperature and, subsequently, lyophilized.

2.3. General EPS characterization

Colorimetric carbohydrate and protein quantification in the granular EPS was performed as described by Dubois et al. (Dubois et al., 1951). In brief, lyophilized extracts obtained as described above were resolubilized in 0.01 M NaOH to a final concentration of 0.5 mg/mL. The protein content was measured in triplicate using the Pierce™ BCA protein assay kit (Thermo Scientific, Rockford, IL) according to the manufacturer's instruction. Bovine serum albumin was used as a standard. Carbohydrate content was measured in triplicate with phenol-sulfuric acid method (Dubois et al., 1951), using glucose as a standard.

Fourier-transform infrared spectroscopy (FT-IR) was used to measure the absorbance of functional groups in the lyophilized EPS extracts in triplicate with a Spectrum 100 spectrometer (PerkinElmer, Shelton, CT). The absorbance of the EPS extracts was recorded with FT-IR in attenuated total reflectance mode over a wavenumber range of 600 - 4000 cm^{-1} with 16 accumulations and a 4 cm^{-1} resolution. MATLAB was used for spectral data processing and consisted of baseline correction and feature scaling.

2.4. Hydrolysis and sugar monomer quantification

Composition of sugar monomers in the EPS was determined with high-performance anion exchange with pulsed amperometric detection (HPAE-PAD). Sugar monomers were released from polysaccharides through hydrolysis following the procedure described by Felz et al. (Felz et al., 2019). In brief, samples were hydrolyzed using 1 M hydrochloric acid at a concentration of 10 gs of sample per liter of hydrochloric acid. Hydrolysis was performed at 105 °C for 8 h in a heating block. Samples were centrifuged at 13,300 x g for 5 min and were neutralized with equal volume of 1 M sodium hydroxide. The samples were diluted 1:5 with ultrapure water and filtered through a 0.22 μm PVDF filter. Quantification of the sugar monomers was performed by calibration of sugar monomers in a range of 0.025 g/L to 0.2 g/L. The sugar monomer standards used were: galacturonic acid, glucuronic acid, rhamnose, glucose, glucosamine, mannose, galactose and fucose. The detection of sugar monomers in the standards and hydrolyzed EPS samples in triplicates was performed with a Dionex ICS-5000+, equipped with a CarboPac PA20 column and an Aminotrap pre-column as mentioned in Felz et al. (Felz et al., 2019).

2.5. Glycosyl composition analysis by TMS method

Lyophilized EPS samples were sent to the Complex Carbohydrate Research Center for an additional measurement of the sugar monomer composition in EPS (Athens, GA, USA). The method is optimized for measuring sugar monomers in lipo-polysaccharides. The analysis was performed by combined gas chromatography-mass spectrometry (GC–MS) of the *O*-trimethylsilyl (TMS) methyl glycoside derivatives produced from the sample by acidic methanolysis. These procedures were carried out as previously described (Santander et al., 2013). In brief, 300 mg of lyophillized EPS samples, and 20 mg of inositol as

internal standard, were added to separate tubes. Samples were hydrolyzed in 1 M HCl in methanol at 80 °C for 16 h. This was followed by re-N-acetylation of amino-sugars with pyridine and acetic anhydride in methanol. Subsequently, samples were per-o-trimethylsilyated with Tri-Sil reagent at 80 °C for 30 min. GC–MS analysis of the TMS methyl glycosides was performed on an AT 7890A GC interfaced to a 5975B MSD, using an EC-1 fused silica capillary column (30 $m \times 0.25$ mm ID).

2.6. DNA extraction and sequencing

Total genomic DNA was extracted from refrigerated sludge samples (+4 °C) using DNeasy PowerSoil Pro-kit (Qiagen, Germany), following the manufacturer's protocol. Library preparation and sequencing with Illumina NovaSeq 6000 PE150 was done at Novogene (Cambridge, UK), yielding on average 20Gb of raw data per sample. Raw reads were deposited to the European Nucleotide Archive (ENA) under the study accession number ERP148022. Paired-end reads were trimmed for the adapter and quality sequences and assembled with MEGAHIT v1.2.9 (Li et al., 2015). Trimmed reads were mapped to the assembled contigs with bowtie2 in BBMap suite v38.84 (Bushnell, 2015) and used for differential coverage binning with MetaBAT2 (Kang et al., 2015). The quality of the resulting metagenome assembled genomes (MAGs) were checked with CheckM (Parks et al., 2015) using the lineage-specific workflow. Taxonomic affiliation of the MAGs was done using GTDB-Tk 1.7.0 (Chaumeil et al., 2020).

2.7. Annotation of the assembled metagenomes

Annotation of MAGs was done with Prokka 1.14.6 (Seemann, 2014) with the kingdom-specific annotation mode (Bacteria or Archaea), based on the phylogenetic affiliation provided by GTDB-Tk. For the analysis of the biosynthetic potential, only the MAGs passing these quality criteria were used: presence of SSU rRNA (small subunit ribosomal ribonucleic acid gene(s)) with length of more than 1000 bp), presence of essential marker genes (Parks et al., 2015), bin completeness of more than 70 % with contamination of less or equal to 5 %. MAGs passing these criteria were called "qualified MAGs" for the correspondent samples. The relative abundance of each MAG was estimated by calculating the proportion of mapped reads to each MAG relative to total amount of reads.

Additional re-annotation of the metagenomes was done through the MG-RAST (Meyer et al., 2008) to place genes to the distinct metabolic sub-systems. Amino acid sequences from the translated sequenced genes were classified as belonging to the specific subsystems with the E-value threshold of 1e-5 and sequence identity of more than 60 %. Quality-filtered reference MAGs were additionally re-annotated in RAST (Overbeek et al., 2014). Automatic annotations were manually curated with the assistance of KEGG (Kyoto Encyclopedia of Genes and Genomes, Release 103.0, July 1, 2022 (Kanehisa et al., 2014)).

To identify carbohydrate-active enzymes that might be involved in the biosynthesis of EPS, translated protein sequences of the qualified MAGs were mapped to the CAZy database (Drula et al., 2022) with dbCAN-fam-HMMs.txt (dbCAN release 4.0) with criteria of E-value of less than 1e-5 and sequence coverage of more than 0.35 (Xia et al., 2013; Yin et al., 2012). For each sample unique CAZy instances belonging to one of the groups (glycoside hydrolases, glycosyl transferase, polysaccharide lyases, carbohydrate esterases, carbohydrate-binding modules) were calculated and averaged for all reference MAGs from that sample. Genes and proteins for the biosynthesis of the specific sugar nucleotides were selected by querying the MetaCyc database (Caspi et al., 2020) with the search terms like "rhamnose biosynthesis", "UDP-sugar biosynthesis", "alginate biosynthesis" and selecting the relevant Bacterial or Archaeal pathways. Amino acid sequences of the selected genes (Supplementary Spreadsheet 1) were then used to query the MAGs with BlastP using the E-value of less than or equal to 1e-2 as a cutoff (McGinnis and Madden, 2004).

3. Results

In this study we characterized chemical and microbiological composition of three different granular anaerobic sludges that originated from two types of organic industrial wastewater: paper recycle/kraft, and brewery. The two IC bioreactors treating paper recycle/kraft wastewater differed by the type of generated sludge: fluffy granular (Papermill A) and smooth granular (Papermill B); while the UASB reactor treating brewery wastewater had smooth granular sludge.

3.1. General characterization of the granular sludge extracellular matrix

Conventional colorimetric carbohydrate and protein quantification (see Section 2.3) was used to get a general distribution of two of the major constituents of EPS in the sludges: polysaccharides and proteins. Proteins were predominant in the EPS of all sludges, but especially in the papermill granules. The total carbohydrate content was 9.9 ± 1.5, 9.5 ± 0.3 and 6.5 ± 0.8% (glucose equivalent) of total EPS in granules from Papermill A, Papermill B and Brewery EPS, respectively. The PN/PS ratio was 9.4 ± 1.4, 9.8 ± 0.9, and 8.8 ± 1.4 g BSA-eq/g glucose-eq for EPS in granules from Papermill A, Papermill B and Brewery.

The quantification of the total carbohydrates fraction of EPS obtained with the colorimetric methods was validated by measuring the relative abundance in functional groups with FT-IR (Fig. 1). An overview of the assigned wavenumbers corresponding to the functional groups can be found in Table S1. Bands corresponding to protein absorbance regions were dominant in the spectrum (bands 1, 2, 6, 7, 9 and 11) and were most pronounced in the papermill samples. The measured absorbance height in the polysaccharide region (band 12) was 3 % higher in the spectrum of the Brewery sludge compared to both Papermill sludge samples. This showed that based on the absorbance height in the FT-IR spectra, the overall EPS composition in the granules is similar, but their polysaccharide content slightly varies.

3.2. Characterization of glycan composition in the extracellular matrix

Total sugar content in the EPS varied slightly between the sludge samples (Figures S2, S3, Table S2): 9.0 ± 0.8, 12.2 ± 1.1 and 10.1 ± 1.8 % of total EPS, for Papermill A, Papermill B and Brewery samples, respectively. The sugar monomer composition of the two Papermill samples was similar, with differences in the galactosamine, glucosamine and galacturonic acid content (Fig. 2). Compared to Papermill samples, the Brewery sample had lower glucose and mannose content, but higher content of glucuronic acid.

To get additional information on sugar monomers present in the

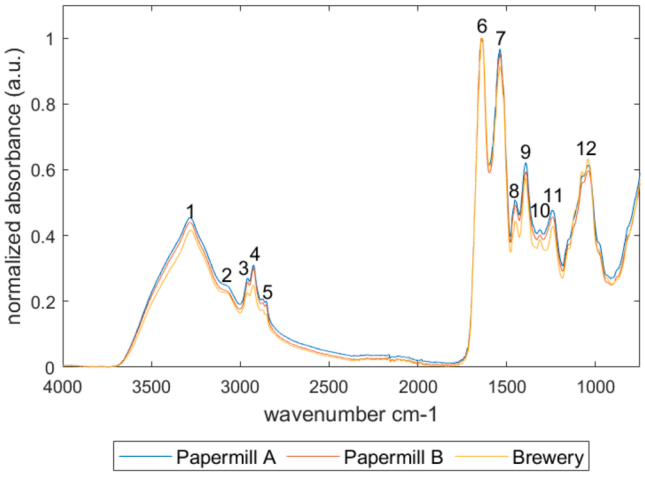

Fig. 1. Normalized absorbance FT-IR spectra of EPS extracted from Papermill A, Papermill B and Brewery granular sludge.

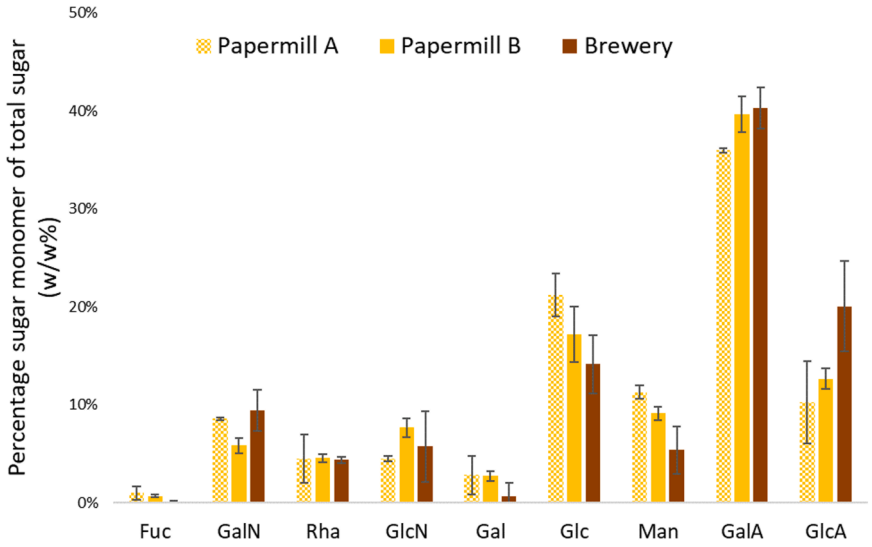

Fig. 2. Sugar monomer composition of EPS measured with HPAE-PAD chromatography. The sugar monomers are displayed as a percentage of the total sugar concentration found in the EPS. Abbreviations are according to symbol nomenclature for glycans (Varki et al., 2015). Fu stands for fucose, GalN for galactosamine, Rha for rhamnose, GlcN for glucosamine, Gal for galactose, Glc for glucose, Man for mannose, GalA for galacturonic acid and GlcA for glucuronic acid. See Supplemental Table S3 for the raw data. The error bars represent the standard deviation.

sludge EPS, TMS-derivatization analysis was performed (Supplemental Figure S4). Since this method is typically used for lipopolysaccharide analysis, differences between the sugar monomers composition obtained by TMS-derivatization and HPAE-PAD method were expected. The hydrolysis of the polysaccharides for TMS-derivatization was performed under less harsh conditions, which was reflected in the total sugar content (4.0 % and 1.9 %). Compared to the HPAE-PAD data (Fig. 2), practically no uronic acids were found with TMS-derivatization. This could indicate that the hydrolysis method used for the TMS-derivatization might not be strong enough for the hydrolysis of all glycans present in the EPS.

3.3. *Metagenome assembly and taxonomic classification*

Analysis of the sludges metagenome allowed to establish the link between EPS composition and microbial community. Extracted DNA from each granular sludge sample was sequenced to the same depth (Figure S1), generating 69.9 Gb of raw data in total, with 140–160 million raw reads per sample. Quality scores were above 91 % for each of the samples in Q30.

The highest number of MAGs were retrieved from the Papermill A sample (113), followed by Papermill B (117) and Brewery (106). MAGs from Papermill A had a higher mean completeness (78 %), while Papermill B had the lowest (42 %) (Figure S5, A). All samples shared low

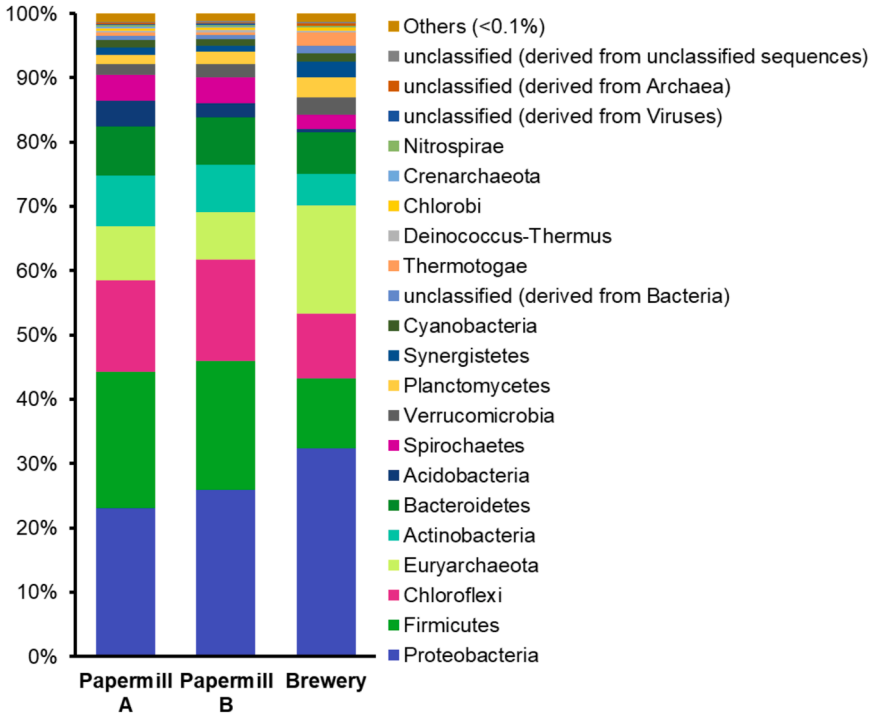

Fig. 3. Taxonomic classification of the metagenome sequences from the three anaerobic sludge samples on the phylum level.

(less than 5 %) contamination of the MAGs with the genetic material of the different phylogenetic placement (Figure S5, B).

Taxonomic affiliation of the 16S rRNA gene sequences in the metagenomes showed that samples from IC reactors (Papermill A and B) were more similar than the UASB Brewery sample (Fig. 3). Brewery-retrieved metagenomes had more 16S rRNA gene sequences belonging to the *Proteobacteria* phylum and less belonging to the *Firmicutes, Chloroflexi* and *Actinobacteria* phyla. Interestingly, the number of 16S rRNA gene sequences belonging to the *Euryarchaeota* phylum was twice as high in the Brewery granular sludge, then in either Papermill samples.

Normalized abundance and phylogenetic placement of all the retrieved MAGs from the three anaerobic sludge samples (Figure S6) demonstrates a diverse range of dominant taxa across the three samples, regardless of the shared bioreactor specification (Papermill A and B samples), or similar granulation state of the sludge (Papermill B and Brewery sample, smooth granules). The shared and distinct phylogenetic groups were visualized by constructing a Venn diagram (Figure S7). Notably, 10 MAGs belonging to the *Anaerolineaceae* family were shared among all three samples, as well as 2 MAGs from *Syntrophobacteraceae* and one belonging to the *Desulfovibrionaceae* family (Supplementary Spreadsheet 1). Apart from these, distinct MAGs were shared across the three samples belonging to the classes of *Clostridia, Clostridia*_A, *Actinomycetia, Syntrophorhabdia, Verrucomicrobiae, Syntrophia, Thermoleophilia, Spirochaetia, Syntrophobacteria, Phycisphaerae, Bacteroidia* and *Desulfovibrionia*. Among MAGs classified as Archaea, those belonging to the classes *Bathyarchaeia, Methanosarcinia* and *Methanobacteria* were found in all three samples, while Brewery sample had distinct MAGs belonging to the *Methanomicrobia* and *Thermococci* classes, and Papermill A sample had also MAGs belonging to *Thermoplasmata* class (specifically, to *Methanomassiliicoccus* genus). MAG classified as *UBA233* family within *Bathyarchaeia* class was found in all three anaerobic sludge samples.

Across all three samples, MAGs belonging to the *Anaerolineaceae* family had the highest bacterial coverage, with an average of 100–400 reads assigned per MAG and representing 9–16 % of all mapped reads. For Archaea, MAGs belonging to the genus *Methanobacterium* (400 – 900 reads per MAG) had the highest coverage.

3.4. Analysis of the pangenome across the three sludge samples

Gene-centric analysis was used to identify shared and distinct gene functions within the three metagenomes. Addressing the glycan and EPS focus of this study, we investigated the distribution of the genes associated with the metabolism of polysaccharides (Fig. 4). Four sub-systems were identified with MG-RAST and KEGG (see Methods) to harbor the relevant genes, within the overarching "capsular polysaccharides biosynthesis and assembly": (1) Exopolysaccharide biosynthesis, (2) Rhamnose containing glycans, (3) dTDP-rhamnose synthesis and (4) Alginate metabolism. Brewery MAGs had more genes classified within "capsular polysaccharides biosynthesis and assembly" (1913 genes) than either of the Papermill samples (1679 (A) and 1799 (B) genes). High number of genes with the general glycosyltransferase (GT) functions (Figure S8) explains a diversity of the sugar monomers identified in the HPAE-PAD analysis of the sludges EPS (Fig. 2). Meanwhile, biosynthesis of monosaccharides, such as rhamnose and its precursor dTDP-rhamnose, is almost evenly distributed across the three sludges (Figure S9, S10), matching the uniform distribution of rhamnose sugar monomer content in the three samples (Fig. 2).

Interestingly, the heteropolysaccharide biosynthesis potential of the MAGs revolved around alginate metabolism cluster, which was 30 % more abundant in smooth sludge (Papermill B and Brewery) compared to the floccular (Papermill A). However, only Papermill B sample had the full operon for the synthesis of alginate (Fig. 5).

3.5. Carbohydrate active enzymes across the sludge MAGs

Since the majority of the EPS-associated genes in the three sludges were glycosyltransferases of diverse functions (Figure S7), we looked closer into their distribution among MAGs to pinpoint potential EPS-producing microorganisms. GTs represent one of the classes in the carbohydrate-active enzymes (CAZymes) in CAZy database (http://www.cazy.org/, (Drula et al., 2022)), together with glycoside hydrolases (GH), polysaccharide lyases (PL), carbohydrate esterases (CE) and carbohydrate-binding modules (CBM). Enzymes within GT group catalyze transfer of sugar moieties from donor to acceptor molecules, resulting in the formation of glycosidic bonds in the polysaccharides. Gene clusters associated with the synthesis of the known EPS molecules (ex. xanthan, gellan, succinoglycan, alginate) always

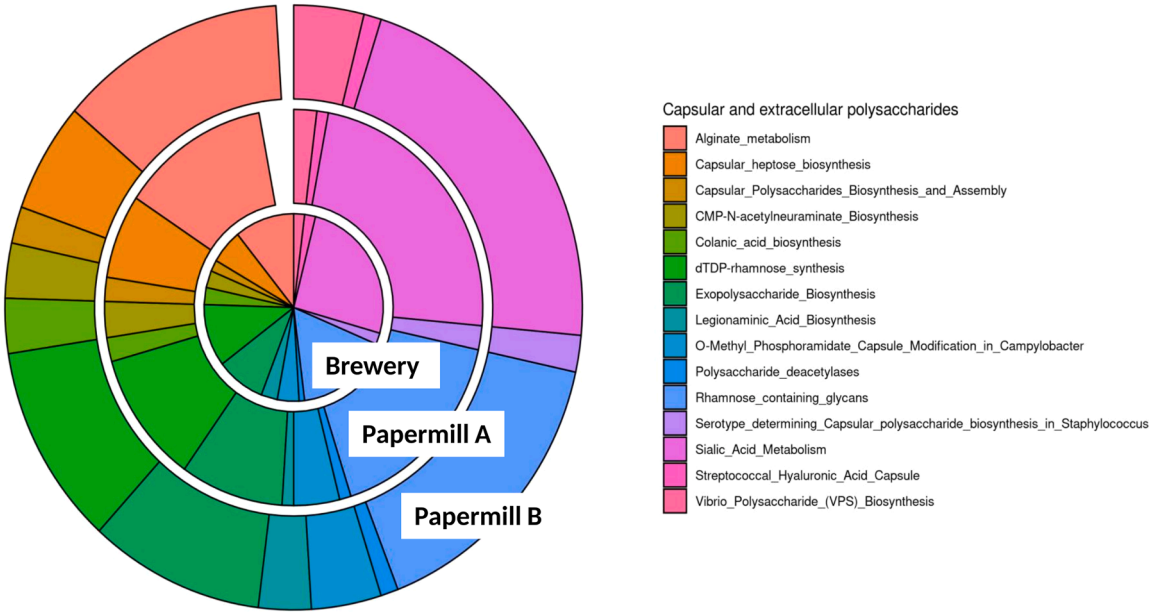

Fig. 4. Genes identified in the three sludge samples related to the metabolism of capsular and extracellular polysaccharides.

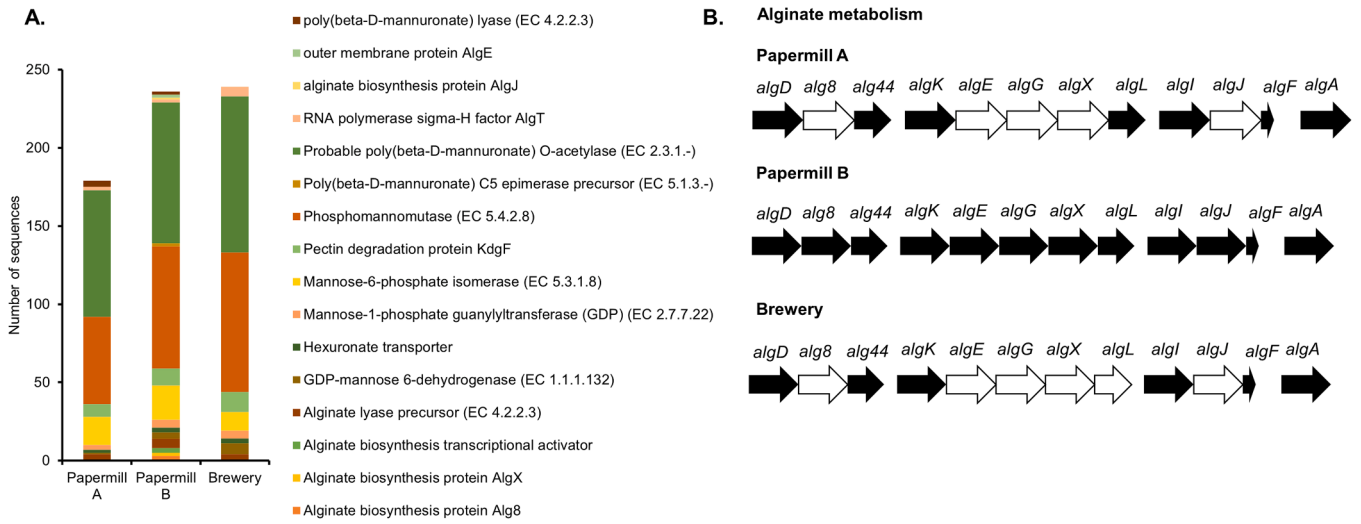

Fig. 5. Genes identified in the three sludge samples related to the alginate metabolism (A) and presence/absence of these genes within the alginate operon (B).

have one or multiple GT-encoding genes (Sun and Zhang, 2021).

Qualified MAGs, with more than 70 % completeness and less than 5 % contamination, had a diverse representation of CAZymes across all classes in all three sludges (Figure S11). Qualified MAGs in Papermill A with the greatest number of CAZymes belonged to the bacterial classes *Phycisphaerae* (MAG36), *UBA5829* (MAG122), *Bacteroidia* (MAG124) and *UB6911*(MAG125). While glycoside hydrolases were the predominant CAZymes in the floccular sample Papermill A, glycosyltransferases were the most common in Papermill B and Brewery MAGs. Among the archaeal qualified MAGs, Brewery sample had the most CAZymes encoded in MAG20 (*Methanomicrobia*), MAG116 (*Methanobacteria*) and MAG123 (*Methanomicrobia*). Interestingly, MAG116 had almost twice as many CAZymes as any other archaeal MAGs from the three samples, with most in the glycosyltransferase family.

Most abundant MAGs (with the coverage above 100 reads) in Papermill A and B samples, *Anaerolineae* MAG22, 29, and 89 had slightly lower number of CAZymes than qualified MAGs from the same samples (Fig. 6). Glycosyltransferases were represented by the GT2 and GT4 groups, that include enzymes involved in the biosynthesis of lipopolysaccharides and osmoprotectants (mannosylglucosylglycerate synthase). Presence of glycogen phosphorylase in MAG22 hints on the potential of this microorganism to synthesize an intracellular starch-like polysaccharide, glycogen. MAG22 also encoded cellobiose phosphorylase, supporting earlier claims that members of the *Anaerolineae* class can ferment carbohydrates, such as cellobiose and cellulose. Function of the CAZymes identified in the MAG29 and 89 were similar to the MAG22, but with more glycosyltransferases involved in the transport of mannose and galacturonic acid, as part of the lipid and lipopolysaccharide biosynthesis pathways (polyprenol monophosphomannose synthases and dodecaprenyl-phosphate galacturonate synthases). MAG89 also had diverse N-acetylglucosaminyltransferases, which might be used for the biosynthesis of poly-beta-1,6-N-acetyl-d-glucosamine, a polysaccharide intercellular adhesin (Arciola et al., 2015).

Most abundant MAGs in the Brewery sample also had the highest number of CAZymes: *Methanosarcinia* MAG108 and *Methanobacteria* MAG122 (Fig. 6, Figure S10). 60 % of the GTs in MAG108 and MAG122 belonged to the GT41 group, that includes β-N-acetylglucosaminyltransferases, N-β-glucosyltransferases and O-α-l-fucosyltransferases.

		GT	GH	PL	CE	CBM	Completeness
Papermill A	Methanobacteria bin 17	34	2	0	16	0	●
	Anaerolineae bin 22	38	13	4	12	10	◑
	Anaerolineae bin 29	60	32	2	18	12	◑
	Bacteroidia bin 40	23	67	3	22	11	◕
	Actinomycetia bin 62	18	16	0	20	5	●
	Methanobacteria bin 63	23	2	0	5	0	●
	Clostridia bin 102	19	68	2	23	13	●
	Methanosarcinia bin 106	41	5	2	10	3	◑
Papermill B	Bacteroidia bin 12	21	47	2	13	9	◑
	Clostridia bin 23	17	56	2	12	8	◕
	Anaerolineae bin 89	48	27	4	14	12	◑
	Anaerolineae bin 100	26	18	4	10	9	◑
	Actinomycetia bin 107	16	14	0	16	5	●
Brewery	Methanobacteria bin 55	22	5	0	8	2	◑
	Desulfuromonadia bin 64	21	3	0	0	2	◑
	Methanosarcinia bin 108	34	6	4	4	2	◕
	Methanobacteria bin 122	30	2	3	14	0	◕

Fig. 6. Number of CAZymes from the most abundant MAGs across all three anaerobic sludge samples. GT – Glycosyl Transferases, GH – Glycoside Hydrolases, PL – Polysaccharide Lyases, CE – Carbohydrate Esterases, CBM – Carbohydrate-Binding Modules.

It is important to also note that 40–50 % of the identified glycosyltransferases in all the MAGs described here did not have exact functional roles assigned (aka. putative glycosyltransferases).

3.6. Biosynthesis of sugar monomers by the abundant MAGs

To match the HPAE-PAD identified sugar monomers with the MAGs biosynthetic capabilities, we analyzed the metagenomes for the presence of genes for the biosynthesis of correspondent precursor nucleotide sugars. Nucleotide sugars, as activated monosaccharides, serve as important intermediates to the production of polysaccharides (Franklin et al., 2011). As a result, we found complete operons for the biosynthesis of UDP-α-d-galacturonate (UDP-GalA), UDP-N-acetyl-d-galactosamine (UDP-GalNAc) and dTDP-β-l-rhamnose (dTDP-l-Rha) in most of the abundant MAGs (Fig. 7).

Abundant MAGs in the Papermill A and B sludges classified as *Anaerolineae*, all had a high number of nucleotide sugar biosynthesis genes, matching the GT2 and GT4 classes of glycosyltransferases predicted with the CAZy search (Fig. 6). These MAGs also had genes for the biosynthesis of nonulosonic acids (NulO-Sia). Abundant MAGs of methanogenic archaea also had a potential to synthesize UDP-GalNAc and dTDP-l-Rha, while biosynthetic potential for GDP-Rha was more characteristic to the bacterial MAGs.

Since in the pangenome analysis we identified complete operons for the biosynthesis of alginate (in Papermill B sludge (Fig. 5), we checked whether individual abundant MAGs had the capacity to synthesize this polysaccharide. However, we only identified a few genes in the alginate biosynthesis pathway such as GDP-mannose 6-dehydrogenase (*algD*) and mannuronan C5-epimerase (*algG*).

4. Discussion

Extracellular polymeric substances produced by anaerobic microorganisms remain vastly unknown and poorly characterized. Despite a widespread industrial application of microbial biotechnologies relying on EPS-based aggregate formation, chemical and biological make-up of these micro-factories are rarely linked in a comprehensive way. In this study we investigated the chemical composition of EPS from three anaerobic granular sludges, coming from papermill and brewery wastewater treatment facilities, and used the chemical EPS composition to guide the assessment of EPS biosynthetic potential in the sludge microbiomes.

4.1. Importance of glycan analysis in protein dominated EPS

The PN/PS ratio of 8.8–9.8 (g BSA-eq/g glucose-eq) demonstrates that EPS from papermill and brewery granular sludges was protein-rich. High protein content is typical for slow-growing microbial communities and similar PN/PS ratios were found in anammox granular sludges (~10 g BSA-eq/g glucose-eq) (Lotti et al., 2019). Higher protein concentrations were linked to a higher hydrophobicity and granule formation capacity (Santschi et al., 2020). Liu and Fang suggested a positive correlation between higher Archaeal abundance and PN/PS ratio, since methanogenic sludge had 10x PN/PS ratio compared to the acidogenic one (Liu and Fang, 2002). Although proteins are often the dominant part of studied EPS, in the extracellular matrix they are often linked with polysaccharides that can alter their structural properties, offer protection from digestion and aid in osmotic regulations (Varki et al., 2022). Since polysaccharides (glycans) can constitute 40–95 % of the overall extracellular matrix (More et al., 2014), their diversity in the granular sludge may play a central role in defining the granules' activity and microbial interconnections.

4.2. Glycan composition of the granular sludge

In previously studied non-granular microbial biofilms, both bacteria and archaea produced a wide array of unique glycans in the process of microbial glycosylation that bears distinct steps from the eukaryotic ones (Dell et al., 2010). These diverse microbial glycans can be commonly found attached to lipids, proteins or loosely floating in the extracellular matrix; while the most dominant ones are associated with the microbial cell-surface and include mono- and heteropolysaccharides (Barrett and Dube, 2023; Messner et al., 2010). The complexity of the microbial communities and the added diversity of glycans produced by

		UDP-GalA	UDP-GalNAc	GDP-Man	GDP-Fuc	UDP-L-FucNAm	dTDP-l-Rha	GDP-Rha	NulO-Sia
Papermill A	Methanobacteria bin 17	5	12	6	3	4	11	3	0
	Anaerolineae bin 22	12	12	1	6	8	16	7	1
	Anaerolineae bin 29	13	17	1	6	7	15	10	5
	Bacteroidia bin 40	6	8	4	3	5	6	2	1
	Actinomycetia bin 62	2	8	4	1	3	4	3	0
	Methanobacteria bin 63	2	11	7	1	4	9	2	0
	Clostridia bin 102	8	18	6	5	1	13	6	1
	Methanosarcinia bin 106	10	7	3	6	7	12	7	0
Papermill B	Bacteroidia bin 12	3	4	3	3	3	4	3	0
	Clostridia bin 23	3	11	3	4	2	4	4	1
	Anaerolineae bin 89	12	17	3	7	9	18	13	2
	Anaerolineae bin 100	9	15	1	7	6	12	8	0
	Actinomycetia bin 107	3	8	3	2	1	3	4	0
Brewery	Methanobacteria bin 55	5	12	4	3	2	8	2	1
	Desulfuromonadia bin 64	3	5	1	2	2	7	2	0
	Methanosarcinia bin 108	3	7	2	2	2	6	5	0
	Methanobacteria bin 122	4	11	5	3	4	9	3	0

Fig. 7. Number of glycan-associated genes found across the abundant MAGs. UDP-GalA – biosynthesis of UDP-α-D-galacturonate; UDP-GalNAc – biosynthesis of UDP-N-acetyl-D-galactosamine; GDP-Man – biosynthesis of GDP-mannose; GDP-Fuc – biosynthesis of GDP-fucose; UDP-L-FucNAm – biosynthesis of UDP-N-acetyl-β-L-fucosamine; dTDP-l-Rha – biosynthesis of dTDP-β-L-rhamnose; GDP-Rha – biosynthesis of GDP-D-rhamnose; NulO-Sia – biosynthesis of NulO sialic acid.

the microbial community make isolation and identification of single glycans challenging. Therefore, to be able to characterize the full spectrum of the microbially-produced glycans in the tested here granular sludges, we hydrolyzed the total extracted granular EPS to identify the monosaccharides composition of the microbe-associated glycans. The full hydrolysis applied to the samples in this study allowed to overcome the inconsistent reports addressing EPS composition in granular sludge from the papermill and brewery wastewater treating bioreactors (Gonzalez-Gil et al., 2015; Liu and Fang, 2002). In support of the previously reported high molecular weight of the granular sludge EPS (Gonzalez-Gil et al., 2015), we indeed observed that 16.9 – 27.4 % of the total EPS from papermill and brewery granular sludge studied here had a molecular weight of over 5500 kDa (Chen et al., 2023).

Distribution of monosaccharides in papermill and brewery sludges (Fig. 2) partially matches the monosaccharide diversity reported for other anaerobic granular sludges. For example, Veiga et al. (Veiga et al., 1997) showed that the monosaccharide composition of in anaerobic granules composed of syntrophic acetogenic bacteria and methanogenic archaea contained glucose (19.3 %), rhamnose (15.0 %), mannose (12.9 %), fucose (10.7 %), galactose (10.1 %), glucosamine (8.6 %) and galactosamine (6.4%). While papermill and brewery sludges studied here were more microbiologically diverse (Fig. 3), the most dominant identified sugar monomers were negatively charged uronic acids (46–60 %), and not glucose (Fig. 2). Presence of other negatively charged glycans, was previously reported for the similar granular sludges to the ones studied here (papermill and brewery) (Gonzalez-Gil et al., 2015). Using ^{1}H NMR and MALDI-TOF analyses, authors reported presence of spectral signatures of mannuronic acid, which is closely related to alginate (Gonzalez-Gil et al., 2015). While the sugar monomer composition demonstrated here shows presence of glucuronic and galacturonic acids (Fig. 2), their molecular weight is similar to the previously found mannuronic acid (194 g/mol), making the results comparable to the previous reports. Also consistent with previous findings (Liu and Fang, 2002) is the presence of higher concentrations of uronic acids in the floccular sludge (Papermill A) compared to the smooth granular sludges (Papermill B and Brewery).

4.3. Microbial glycan biosynthetic potential

Analysis of the sugar monomers composition of the granular sludge with HPAE-PAD was then used to guide the search for the glycans biosynthetic genes in the sludges microbiomes. By sequencing the metagenomes of the three anaerobic granular sludges we were able to look closely into the glycan biosynthetic potential of the assembled high quality abundant MAGs (Fig. 6, 7). The combined analysis of the MAGs-encoded CAZymes and biosynthesis genes for the glycans precursors, nucleotide-sugars, demonstrated matching results for the two search strategies. Similarly to the earlier reported metagenome analysis of papermill and brewery anaerobic granular sludges (Gonzalez-Gil et al., 2015), we identified more alginate-associated biosynthesis genes in the Papermill metagenome than in the Brewery. However, we were not able to pinpoint the exact MAGs that contained the full alginate biosynthetic gene cluster. A number of abundant MAGs in all three sludges shared instead a common potential for the biosynthesis of galacturonate, N-acetyl-d-galactosamine and rhamnose (Fig. 7). Based on HPAE-PAD analysis, these sugars were also the most abundant monosaccharides contributing to the glycan part of the three anaerobic sludges (Fig. 2). Therefore, it is plausible to suggest that the MAGs with the identified genes for the biosynthesis of these sugar nucleotides are involved in the formation of sludge extracellular matrix.

Strikingly, the MAGs with the highest number of glycan and nucleotide sugars biosynthetic capabilities were also the most abundant across the three samples and belonged to the *Anaerolineae* and *Methanobacteria* classes. Their high abundance is supported by other studies on the microbial composition of various mesophilic anaerobic sludges, where *Methanobacteria* were the most common and abundant hydrogenotrophic methanogens (Griffin et al., 1998; McHugh et al., 2003; Song et al., 2010), while *Anaerolineae* contributed to the 45 % of the overall anaerobic microbial sludge population in the fed-batch, UASB and IC reactors treating lipid, alkane-rich or papermill wastewater, as well as waste activated sludge (Liang et al., 2015; McIlroy et al., 2017; Rivière et al., 2009). Some members of *Anaerolineae* were even found in the beneficial trophic relationships with the hydrogenotrophic methanogens from the genus *Methanospirillum* (Sun et al., 2016). Currently known representatives of *Anaerolineaceae* family are anaerobic nonmotile slow growing acetogens, with chemo-organoheterotrophic fermentative metabolism and multicellular filamentous morphology (Yamada and Sekiguchi, 2018). Although common in the dispersed anaerobic sludges, morphology of these representatives of *Chloroflexota* phylum can be contributing to the anaerobic sludge granulation (Sekiguchi et al., 2001; Yamada et al., 2005), while their ability to produce acetate may contribute to the beneficial association with the acetoclastic methanogens to further improve the strength of the anaerobic granular structures. What is more, Zhu et al. (Zhu et al., 2017) proposed that the filamentous structure of the *Anaerolineaceae* cells may be essential to maintain the granular stability during the temperature and pH fluctuations inside the UASB reactors. High abundance of the *Anaerolineaceae* (up to 30 %) and *Methanosaeta*-classified microorganisms (up to 60 %) in the sludge treating starch-rich wastewater ensured stability of the bioreactor operation even after the bioreactor pre-acidification (Wu et al., 2021). High number of CAZymes and genes for the production of nucleotide-sugars (glycans precursors) in the abundant *Anaerolineae* and *Methanobacteria* identified in this study suggest a strong link between these microorganisms and the observed glycan profile of the tested anaerobic granular sludges from IC and UASB reactors treating papermill and brewery wastewaters. However, further experimental evidence for the glycan producing activity of these microorganisms (*Anaerolineae* and *Methanobacteria*) is still needed.

5. Conclusions

In this study, by combining chemical characterization of glycans in anaerobic granular sludge with metagenome analysis, it was possible to find genes that might be involved in the biosynthesis of these glycans in anaerobic granular sludge microbial communities. The presence of these genes allowed for the identification of microorganisms that potentially take part in the production and secretion of glycans in anaerobic granular sludges from industrial wastewater treating facilities. The approach presented here shows how the chemical analysis of the sugar monomers in EPS can aid in identifying the putative EPS biosynthesis genes in the sludge MAGs. Functions of the identified carbohydrate-active enzymes encoded in the sludges MAGs were also in line with the presence of specific biosynthesis genes for the glycans precursors, nucleotide-sugars, which were identified as prevalent components of the sludge EPS. Gaining a deeper understanding of the glycan biosynthetic potential of the microorganisms in anaerobic granular sludge is essential for future endeavors to optimize biological production of specific EPS and improve resource recovery from wastewater.

CRediT authorship contribution statement

Anna Doloman: Conceptualization, Funding acquisition, Investigation, Methodology, Writing – original draft. **Stefan de Bruin:** Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Mark C.M. van Loosdrecht:** Funding acquisition, Project administration, Supervision, Writing – review & editing. **Diana Z. Sousa:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Yuemei Lin:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

Authors have no competing financial interests/personal relationships which may be considered as potential competing interests.

Data availability

DNA sequencing data is deposited in the European Nucleotide Archive (referenced in paper), and the rest of the data is attached as a supplement to this submission

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Supplementary materials

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References

Arciola, C.R., Campoccia, D., Ravaioli, S., Montanaro, L., 2015. Polysaccharide intercellular adhesin in biofilm: structural and regulatory aspects. Front. Cell. Infect. Microbiol. 5 https://doi.org/10.3389/fcimb.2015.00007.

Barrett, K., Dube, D.H., 2023. Chemical tools to study bacterial glycans: a tale from discovery of glycoproteins to disruption of their function. Isr. J. Chem. 63 https://doi.org/10.1002/ijch.202200050.

Boleij, M., Kleikamp, H., Pabst, M., Neu, T.R., van Loosdrecht, M.C.M., Lin, Y., 2020. Decorating the anammox house: sialic acids and sulfated glycosaminoglycans in the extracellular polymeric substances of anammox granular sludge. Environ. Sci. Technol. 54, 5218–5226. https://doi.org/10.1021/acs.est.9b07207.

Bushnell, B., 2015. BBMap [WWW Document]. URL sourceforge.net/projects/bbmap/.

Caspi, R., Billington, R., Keseler, I.M., Kothari, A., Krummenacker, M., Midford, P.E., Ong, W.K., Paley, S., Subhraveti, P., Karp, P.D., 2020. The MetaCyc database of metabolic pathways and enzymes - a 2019 update. Nucleic. Acids. Res. 48, D445–D453. https://doi.org/10.1093/nar/gkz862.

Chaumeil, P.A., Mussig, A.J., Hugenholtz, P., Parks, D.H., 2020. GTDB-Tk: a toolkit to classify genomes with the genome taxonomy database. Bioinformatics 36, 1925–1927. https://doi.org/10.1093/bioinformatics/btz848.

Chen, L.M., de Bruin, S., Pronk, M., Sousa, D.Z., van Loosdrecht, M.C.M., Lin, Y., 2023. Sialylation and sulfation of anionic glycoconjugates are common in the extracellular polymeric substances of both aerobic and anaerobic granular sludges. Environ. Sci. Technol. https://doi.org/10.1021/acs.est.2c09586.

de Bruin, S., Vasquez-Cardenas, D., Sarbu, S.M., Meysman, F.J.R., Sousa, D.Z., van Loosdrecht, M.C.M., Lin, Y., 2022. Sulfated glycosaminoglycan-like polymers are present in an acidophilic biofilm from a sulfidic cave. Sci. Total Environ. 829, 154472 https://doi.org/10.1016/j.scitotenv.2022.154472.

Decho, A.W., Gutierrez, T., 2017. Microbial Extracellular Polymeric Substances (EPSs) in ocean systems. Front. Microbiol. 8 https://doi.org/10.3389/fmicb.2017.00922.

Dell, A., Galadari, A., Sastre, F., Hitchen, P., 2010. Similarities and differences in the glycosylation mechanisms in prokaryotes and eukaryotes. Int. J. Microbiol. 2010, 1–14. https://doi.org/10.1155/2010/148178.

Drula, E., Garron, M.L., Dogan, S., Lombard, V., Henrissat, B., Terrapon, N., 2022. The carbohydrate-active enzyme database: functions and literature. Nucleic. Acids. Res. 50, D571–D577. https://doi.org/10.1093/nar/gkab1045.

Dubois, M., Gilles, K., Hamilton, J.K., Rebers, P.A., Smith, F., 1951. A colorimetric method for the determination of sugars. Nature 168. https://doi.org/10.1038/168167a0, 167–167.

Dueholm, M.K.D., Besteman, M., Zeuner, E.J., Riisgaard-Jensen, M., Nielsen, M.E., Vestergaard, S.Z., Heidelbach, S., Bekker, N.S., Nielsen, P.H., 2023. Genetic potential for exopolysaccharide synthesis in activated sludge bacteria uncovered by genome-resolved metagenomics. Water Res. 229, 119485 https://doi.org/10.1016/j.watres.2022.119485.

Felz, S., Neu, T.R., van Loosdrecht, M.C.M., Lin, Y., 2020. Aerobic granular sludge contains Hyaluronic acid-like and sulfated glycosaminoglycans-like polymers. Water Res. 169, 115291 https://doi.org/10.1016/j.watres.2019.115291.

Felz, S., Vermeulen, P., van Loosdrecht, M.C.M., Lin, Y.M., 2019. Chemical characterization methods for the analysis of structural extracellular polymeric substances (EPS). Water Res. 157, 201–208. https://doi.org/10.1016/j.watres.2019.03.068.

Franklin, M.J., Nivens, D.E., Weadge, J.T., Howell, P.L., 2011. Biosynthesis of the pseudomonas aeruginosa extracellular polysaccharides, Alginate, Pel, and Psl. Front. Microbiol. 2 https://doi.org/10.3389/fmicb.2011.00167.

Gagliano, M.C., Neu, T.R., Kuhlicke, U., Sudmalis, D., Temmink, H., Plugge, C.M., 2018. EPS glycoconjugate profiles shift as adaptive response in anaerobic microbial granulation at high salinity. Front. Microbiol. 9 https://doi.org/10.3389/fmicb.2018.01423.

Gonzalez-Gil, G., Thomas, L., Emwas, A.H., Lens, P.N.L., Saikaly, P.E., 2015. NMR and MALDI-TOF MS based characterization of exopolysaccharides in anaerobic microbial aggregates from full-scale reactors. Sci. Rep. 5, 14316. https://doi.org/10.1038/srep14316.

Griffin, M.E., McMahon, K.D., Mackie, R.I., Raskin, L., 1998. Methanogenic population dynamics during start-up of anaerobic digesters treating municipal solid waste and biosolids. Biotechnol. Bioeng. 57, 342–355. https://doi.org/10.1002/(SICI)1097-0290(19980205)57:3<342::AID−BIT11>3.0.CO;2-I.

Hulshoff Pol, L.W., de Castro Lopes, S.I., Lettinga, G., Lens, P.N.L., 2004. Anaerobic sludge granulation. Water Res. 38, 1376–1389. https://doi.org/10.1016/j.watres.2003.12.002.

Jennings, L.K., Storek, K.M., Ledvina, H.E., Coulon, C., Marmont, L.S., Sadovskaya, I., Secor, P.R., Tseng, B.S., Scian, M., Filloux, A., Wozniak, D.J., Howell, P.L., Parsek, M. R., 2015. Pel is a cationic exopolysaccharide that cross-links extracellular DNA in the Pseudomonas aeruginosa biofilm matrix. Proc. Natl. Acad. Sci. 112, 11353–11358. https://doi.org/10.1073/pnas.1503058112.

Kanehisa, M., Goto, S., Sato, Y., Kawashima, M., Furumichi, M., Tanabe, M., 2014. Data, information, knowledge and principle: back to metabolism in KEGG. Nucleic. Acids. Res. 42, D199–D205. https://doi.org/10.1093/nar/gkt1076.

Kang, D.D., Froula, J., Egan, R., Wang, Z., 2015. MetaBAT, an efficient tool for accurately reconstructing single genomes from complex microbial communities. PeerJ 3, e1165. https://doi.org/10.7717/peerj.1165.

Kumar, A., Singh, A.K., Bilal, M., Prasad, S., Rameshwari, K.R.T., Chandra, R., 2022. Paper and pulp mill wastewater: characterization, microbial-mediated degradation, and challenges. Nanotechnology in Paper and Wood Engineering. Elsevier, pp. 371–387. https://doi.org/10.1016/B978-0-323-85835-9.00011-8.

Li, D., Liu, C.M., Luo, R., Sadakane, K., Lam, T.W., 2015. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph. Bioinformatics 31, 1674–1676. https://doi.org/10.1093/bioinformatics/btv033.

Liang, B., Wang, L.Y., Mbadinga, S.M., Liu, J.F., Yang, S.Z., Gu, J.D., Mu, B.Z., 2015. Anaerolineaceae and Methanosaeta turned to be the dominant microorganisms in alkanes-dependent methanogenic culture after long-term of incubation. AMB Express 5, 37. https://doi.org/10.1186/s13568-015-0117-4.

Limoli, D.H., Jones, C.J., Wozniak, D.J., 2015. Bacterial extracellular polysaccharides in biofilm formation and function. Microbiol. Spectr. 3 https://doi.org/10.1128/microbiolspec.MB-0011-2014.

Liu, H., Fang, H.H.P., 2002. Extraction of extracellular polymeric substances (EPS) of sludges. J. Biotechnol. 95, 249–256. https://doi.org/10.1016/S0168-1656(02)00025-1.

Lotti, T., Carretti, E., Berti, D., Martina, M.R., Lubello, C., Malpei, F., 2019. Extraction, recovery and characterization of structural extracellular polymeric substances from anammox granular sludge. J. Environ. Manage. 236, 649–656. https://doi.org/10.1016/j.jenvman.2019.01.054.

McGinnis, S., Madden, T.L., 2004. BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic. Acids. Res. 32, W20–W25. https://doi.org/10.1093/nar/gkh435.

McHugh, S., Carton, M., Mahony, T., O'Flaherty, V., 2003. Methanogenic population structure in a variety of anaerobic bioreactors. FEMS Microbiol. Lett. 219, 297–304. https://doi.org/10.1016/S0378-1097(03)00055-7.

McIlroy, S.J., Kirkegaard, R.H., Dueholm, M.S., Fernando, E., Karst, S.M., Albertsen, M., Nielsen, P.H., 2017. Culture-Independent analyses reveal novel anaerolineaceae as abundant primary fermenters in anaerobic digesters treating waste activated sludge. Front. Microbiol. 8 https://doi.org/10.3389/fmicb.2017.01134.

Messner, P., Egelseer, E.M., Sleytr, U.B., Schäffer, C., 2010. Chapter 7 - Bacterial surface layer glycoproteins and "non-classical" secondary cell wall polymers. In: Holst, O., Brennan, P.J., Itzstein, M.von, Moran, A.P. (Eds.), Microbial Glycobiology. Academic Press, San Diego, pp. 109–128. https://doi.org/10.1016/B978-0-12-374546-0.00007-9.

Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E., Kubal, M., Paczian, T., Rodriguez, A., Stevens, R., Wilke, A., Wilkening, J., Edwards, R., 2008. The metagenomics RAST server – a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics 9, 386. https://doi.org/10.1186/1471-2105-9-386.

More, T.T., Yadav, J.S.S., Yan, S., Tyagi, R.D., Surampalli, R.Y., 2014. Extracellular polymeric substances of bacteria and their potential environmental applications. J. Environ. Manage. 144, 1–25. https://doi.org/10.1016/j.jenvman.2014.05.010.

Neu, T., Kuhlicke, U., 2017. Fluorescence lectin bar-coding of glycoconjugates in the extracellular matrix of biofilm and bioaggregate forming microorganisms. Microorganisms 5, 5. https://doi.org/10.3390/microorganisms5010005.

Overbeek, R., Olson, R., Pusch, G.D., Olsen, G.J., Davis, J.J., Disz, T., Edwards, R.A., Gerdes, S., Parrello, B., Shukla, M., Vonstein, V., Wattam, A.R., Xia, F., Stevens, R., 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic. Acids. Res. 42, D206–D214. https://doi.org/10.1093/nar/gkt1226.

Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., Tyson, G.W., 2015. CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and

metagenomes. Genome Res. 25, 1043–1055. https://doi.org/10.1101/gr.186072.114.
Pinel, I.S.M., Kleikamp, H.B.C., Pabst, M., Vrouwenvelder, J.S., van Loosdrecht, M.C.M., Lin, Y., 2020. Sialic acids: an important family of carbohydrates overlooked in environmental biofilms. Appl. Sci. 10, 7694. https://doi.org/10.3390/app10217694.
Rivière, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P., Sghir, A., 2009. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. ISME J. 3, 700–714. https://doi.org/10.1038/ismej.2009.2.
Santander, J., Martin, T., Loh, A., Pohlenz, C., Gatlin, D.M., Curtiss, R., 2013. Mechanisms of intrinsic resistance to antimicrobial peptides of Edwardsiella ictaluri and its influence on fish gut inflammation and virulence. Microbiology 159, 1471–1486. https://doi.org/10.1099/mic.0.066639-0.
Santschi, P.H., Xu, C., Schwehr, K.A., Lin, P., Sun, L., Chin, W.C., Kamalanathan, M., Bacosa, H.P., Quigg, A., 2020. Can the protein/carbohydrate (P/C) ratio of exopolymeric substances (EPS) be used as a proxy for their 'stickiness' and aggregation propensity? Mar. Chem. 218, 103734. https://doi.org/10.1016/j.marchem.2019.103734.
Schmid, J., Sieber, V., Rehm, B., 2015. Bacterial exopolysaccharides: biosynthesis pathways and engineering strategies. Front. Microbiol. 6 https://doi.org/10.3389/fmicb.2015.00496.
Schmidt, J.E., Ahring, B.K., 2000. Granular sludge formation in upflow anaerobic sludge blanket (UASB) reactors. Biotechnol. Bioeng. 49, 229–246. https://doi.org/10.1002/(SICI)1097-0290(19960205)49:3<229::AID-BIT1>3.0.CO;2-M.
Seemann, T., 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30, 2068–2069. https://doi.org/10.1093/bioinformatics/btu153.
Sekiguchi, Y., Takahashi, H., Kamagata, Y., Ohashi, A., Harada, H., 2001. In Situ detection, isolation, and physiological properties of a thin filamentous microorganism abundant in methanogenic granular sludges: a novel isolate affiliated with a clone cluster, the green non-sulfur bacteria, subdivision I. Appl. Environ. Microbiol. 67, 5740–5749. https://doi.org/10.1128/AEM.67.12.5740-5749.2001.
Seviour, T., Derlon, N., Dueholm, M.S., Flemming, H.C., Girbal-Neuhauser, E., Horn, H., Kjelleberg, S., van Loosdrecht, M.C.M., Lotti, T., Malpei, M.F., Nerenberg, R., Neu, T. R., Paul, E., Yu, H., Lin, Y., 2019. Extracellular polymeric substances of biofilms: suffering from an identity crisis. Water Res. 151, 1–7. https://doi.org/10.1016/j.watres.2018.11.020.
Simate, G.S., Cluett, J., Iyuke, S.E., Musapatika, E.T., Ndlovu, S., Walubita, L.F., Alvarez, A.E., 2011. The treatment of brewery wastewater for reuse: state of the art. Desalination 273, 235–247. https://doi.org/10.1016/j.desal.2011.02.035.
Song, M., Shin, S.G., Hwang, S., 2010. Methanogenic population dynamics assessed by real-time quantitative PCR in sludge granule in upflow anaerobic sludge blanket treating swine wastewater. Bioresour. Technol. 101, S23–S28. https://doi.org/10.1016/j.biortech.2009.03.054.
Sun, L., Toyonaga, M., Ohashi, A., Matsuura, N., Tourlousse, D.M., Meng, X.Y., Tamaki, H., Hanada, S., Cruz, R., Yamaguchi, T., Sekiguchi, Y., 2016. Isolation and characterization of Flexilinea Flocculi Gen. Nov., sp. Nov., a filamentous, anaerobic bacterium belonging to the class Anaerolineae in the phylum Chloroflexi. Int. J. Syst. Evol. Microbiol. 66, 988–996. https://doi.org/10.1099/ijsem.0.000822.
Sun, X., Zhang, J., 2021. Bacterial exopolysaccharides: chemical structures, gene clusters and genetic engineering. Int. J. Biol. Macromol. 173, 481–490. https://doi.org/10.1016/j.ijbiomac.2021.01.139.
van Lier, J.B., van der Zee, F.P., Frijters, C.T.M.J., Ersahin, M.E., 2015a. Celebrating 40 years anaerobic sludge bed reactors for industrial wastewater treatment. Rev. Environ. Sci. Biotechnol. 14, 681–702. https://doi.org/10.1007/s11157-015-9375-5.
van Lier, J.B., van der Zee, F.P., Frijters, C.T.M.J., Ersahin, M.E., 2015b. Development of Anaerobic High-Rate Reactors, Focusing on Sludge Bed Technology. https://doi.org/10.1007/10_2015_5012.
Varki, A., Cummings, R.D., Aebi, M., Packer, N.H., Seeberger, P.H., Esko, J.D., Stanley, P., Hart, G., Darvill, A., Kinoshita, T., Prestegard, J.J., Schnaar, R.L., Freeze, H.H., Marth, J.D., Bertozzi, C.R., Etzler, M.E., Frank, M., Vliegenthart, J.F., Lütteke, T., Perez, S., Bolton, E., Rudd, P., Paulson, J., Kanehisa, M., Toukach, P., Aoki-Kinoshita, K.F., Dell, A., Narimatsu, H., York, W., Taniguchi, N., Kornfeld, S., 2015. Symbol nomenclature for graphical representations of glycans. Glycobiology 25, 1323–1324. https://doi.org/10.1093/glycob/cwv091.
Varki, A., Cummings, R.D., Esko, J.D., Stanley, P., Hart, G.W., Aebi, M., Mohnen, D., Kinoshita, T., Packer, N.H., Prestegard, J.H., 2022. Essentials of glycobiology [internet].
Veiga, M.C., Jain, M.K., Wu, W., Hollingsworth, R.I., Zeikus, J.G., 1997. Composition and role of extracellular polymers in methanogenic granules. Appl. Environ. Microbiol. 63, 403–407.
Whitfield, C., Wear, S.S., Sande, C., 2020. Assembly of bacterial capsular polysaccharides and exopolysaccharides. Annu. Rev. Microbiol. 74, 521–543. https://doi.org/10.1146/annurev-micro-011420-075607.
Wu, J., Jiang, B., Kong, Z., Yang, C., Li, L., Feng, B., Luo, Z., Xu, K.Q., Kobayashi, T., Li, Y. Y., 2021. Improved stability of up-flow anaerobic sludge blanket reactor treating starch wastewater by pre-acidification: impact on microbial community and metabolic dynamics. Bioresour. Technol. 326, 124781 https://doi.org/10.1016/j.biortech.2021.124781.
Xia, Y., Ju, F., Fang, H.H.P., Zhang, T., 2013. Mining of novel thermo-stable cellulolytic genes from a thermophilic cellulose-degrading consortium by metagenomics. PLoS ONE 8, e53779. https://doi.org/10.1371/journal.pone.0053779.
Yamada, T., Sekiguchi, Y., 2018. Anaerolineaceae, in: bergey's Manual of Systematics of Archaea and Bacteria. Wiley, pp. 1–5. https://doi.org/10.1002/9781118960608.fbm00301.
Yamada, T., Sekiguchi, Y., Imachi, H., Kamagata, Y., Ohashi, A., Harada, H., 2005. Diversity, localization, and physiological properties of filamentous microbes belonging to chloroflexi subphylum I in mesophilic and thermophilic methanogenic sludge granules. Appl. Environ. Microbiol. 71, 7493–7503. https://doi.org/10.1128/AEM.71.11.7493-7503.2005.
Yin, Y., Mao, X., Yang, J., Chen, X., Mao, F., Xu, Y., 2012. dbCAN: a web resource for automated carbohydrate-active enzyme annotation. Nucleic. Acids. Res. 40, W445–W451. https://doi.org/10.1093/nar/gks479.
Zhu, X., Kougias, P.G., Treu, L., Campanaro, S., Angelidaki, I., 2017. Microbial community changes in methanogenic granules during the transition from mesophilic to thermophilic conditions. Appl. Microbiol. Biotechnol. 101, 1313–1322. https://doi.org/10.1007/s00253-016-8028-0.