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Characterisation of cellulose-degrading organisms in an anaerobic digester

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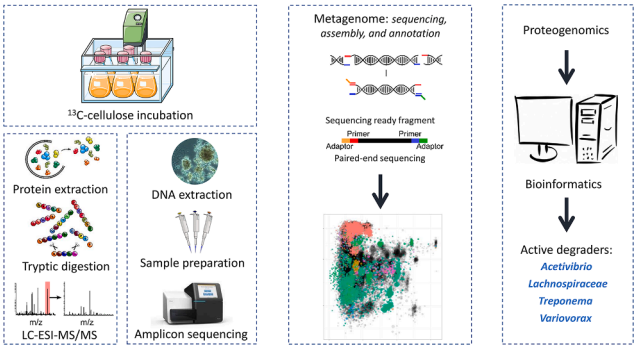
HIGHLIGHTS

- Protein stable isotope probing revealed microbial function and identity.
- *Corynebacterium* was identified through CAZyme, and metagenomic analysis.
- Proteogenomics identified six genera associated with cellulose degradation.
- Microbial ^{13}C-cellulose assimilation yielded twenty ^{13}C-labelled peptides.
- Meta-omics provides insight into microbial degradation of cellulosic biomass.

GRAPHICAL ABSTRACT



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ABSTRACT

The recalcitrant nature of lignocellulosic biomass hinders efficient exploitation of this fraction for energy production. A better understanding of the microorganisms able to convert plant-based feedstocks is needed to improve anaerobic digestion of lignocellulosic biomass. In this study, active thermophilic cellulose-degrading microorganisms were identified from a full-scale anaerobic digester fed with maize by using metagenome-resolved protein stable isotope probing (protein-SIP). ^{13}C-cellulose was converted into ^{13}C-methane with a ^{13}/^{12}C isotope ratio of 0.127 after two days of incubation. Metagenomic analysis revealed 238 different genes coding for carbohydrate-active enzymes (CAZymes), six of which were directly associated with cellulose degradation. The protein-SIP analysis identified twenty heavily labelled peptides deriving from microorganisms actively assimilating labelled carbon from the degradation of ^{13}C-cellulose, highlighting several members of the order *Clostridiales*. *Corynebacterium* was identified through CAZyme screening, amplicon analysis, and in the metagenome giving a strong identification of being a cellulose degrader.

1. Introduction

The increasing energy demand and the imminent depletion of fossil fuels necessitate more sustainable and eco-friendly energy sources. Anaerobic digestion (AD) of agro-industrial wastewaters is a feasible and versatile technology for biogas production (Weiland, 2010). In

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addition, emission of greenhouse gases from fossil fuel combustion and growing concern for climate change have led to a demand for renewable fuels and waste management with climate goals of achieving net-zero greenhouse gas emissions for EU countries by 2050 (European Union, 2020). Biogas production from currently available feedstocks has the potential to produce 10,100 to 14,000 TWh, corresponding to 6–9 % of the world's energy consumption and subsequently reduce the greenhouse gas emissions with 10 to 13% (3,290 to 4,360 Mt CO_2 eq.) (Jain et al., 2019).

Lignocellulosic biomass consists primarily of cellulose, hemicellulose and lignin, and possesses a highly resistant and recalcitrant structure (Sawatdeenarunat et al., 2015). However, it has gained interest as a substrate for biogas production due to its high energy potential. The bioconversion of lignocellulose into methane and carbon dioxide is only possible through cooperation of robust mixed microbial communities. However, due to its recalcitrant nature, the anaerobic digestion of lignocellulosic biomass may yield low methane production. Nonetheless, investigations to increase exploitation of the lignocellulosic biomass has focused on the settings and operational parameters of bioreactors, such as solid retention times and organic loading rates, in order to achieve higher degradation efficiencies (Jensen et al., 2021).

The AD process is generally well understood at a macroscopic level, however, the link between the identity and function of the microbes involved in the different stages of bioconversion are still not fully elucidated. While the organisms involved in methane production from acetate are relatively well characterised and described, it is less clear which organisms are involved in the hydrolysis of cellulosic and hemicellulosic polymers, although this is considered to be the rate-limiting factor in anaerobic digestion of lignocellulosic biomass (Jensen et al., 2021). Recent studies into anaerobic digestion have applied (multi-) omics approaches to study different microbial processes in more detail. Early proteomic studies of full-scale anaerobic digesters showed a high abundance of proteins involved in hydrolysis of monomeric sugars (Heyer et al., 2013). The taxonomic distribution of these observed proteins (primarily representatives of *Clostridiales*) was very similar to the organisms linked to the enzymatic activity of cellulose and xylanase, observed in a study focused on the degradation of straw by different anaerobic digester types (Jensen et al., 2021). However, most studies into the enzymatic processes of AD, such as hydrolysis, have so far only been able to provide information on either function or identity, without providing a direct link between both. Recently, metagenome-resolved protein stable isotope probing (protein-SIP) was applied to biogas reactors in order to extract multi-dimensional information regarding syntrophic acetate oxidation (Mosbaek et al., 2016), as well as the Wood-Ljungdahl pathway as a whole, during anaerobic digestion and biomethanation processes (de Jonge et al., 2021). These proteogenomic studies were some of the first to provide a direct link between identity and function of important microbes of anaerobic digestion systems.

In this study, cellulose-fed anaerobic digestion using a culture-independent approach, to gain insight into the active cellulose degrading populations were examined. Proteogenomics was applied to biomass from a thermophilic full-scale digester and incubated with ^{13}C-labelled cellulose. The applied approach is able to identify cellulose-degrading microorganisms based on newly synthesized proteins with ^{13}C-incorporation at high taxonomic resolution, thereby providing information regarding identity and function simultaneously.

2. Materials and methods

2.1. Batch experiment

The batch reactors were set up in 60 mL serum bottles with 30 mL of working volume and inoculated with sludge from a full-scale thermophilic anaerobic reactor (Bioenergy OL, Denmark). The inoculum was collected from a full-scale reactor operated at 50 °C treating a mixture of swine and cattle manure, maize silage, and deep litter. For DNA and protein extraction purposes, a batch reactor was fed with 1 g of ^{13}C-cellulose (IsoLife, Wageningen, The Netherlands) and a parallel batch reactor was fed with 1 g of unlabelled cellulose (Merck, Germany). The substrate was added in order to reach an optimal food to microorganism (F/M) ratio for methanogenesis of 0.3, according to the total volatile solids (TVS) of the inoculum (96 gTVS·L^{-1}) (Souto et al., 2010). Hypothetically, optimizing the methane production conditions would favour the elucidation of the microorganisms participating in the process. For biogas measurements and quantification of labelled methane ($^{13}CH_4$) production, a third batch reactor was set up with the same F/M ratio, in which the substrate composition was 90 % unlabelled cellulose and 10 % labelled cellulose. The pH of the bulk was measured to be 7.8 in all batches, and due to the nature of the substrate, and that the measured pH was close to the ideal range for methanogenesis (Parkin and Owen, 1986), the pH was not adjusted.

The batch reactors were sealed with thick butyl rubber stoppers and aluminium crimps. The headspace was purged with nitrogen gas (N_2) for 2 min to ensure anaerobic conditions. The experiment was incubated for 9 days at 50 °C and stirred at 150 rpm using a magnetic stirrer to ensure that the biomass was kept in suspension. After 9 days, the biogas production had reached the stationary phase, and the experiment was stopped. All handling and sampling were conducted using strict anaerobic techniques.

2.2. Gas production and cavity ring-down spectroscopy measurements of $^{13}CH_4$

The volume of a produced biogas was measured using a water displacement method (μFlow, Bioprocess control), preceded by a CO_2-fixing unit, at room temperature and atmospheric pressure.

The modified Gompertz model (Eq. (1)) (Zwietering et al., 1990) was adjusted to the cumulative methane production profile in order to obtain the kinetics parameters of potential methane production (P_{max}, NmL), maximum methane production rate (R_{max}, NmL·h^{-1}), and the lag phase (λ, h). P(t) and *e* are the cumulative methane production as a function of the incubation time (t) and Euler's number (2.71828), respectively. The model fitting was performed using the software IBM SPSS Statistics version 26.0 (IBM Corp., USA).

$$P(t) = P_{max} \bullet \exp\left(-\exp\left[\frac{R_{max} \bullet e}{P_{max}} (\lambda - t) + 1 \right] \right) \quad (1)$$

The production of $^{13}CH_4$ was measured over the course of the incubation period by stable isotope analysis. Samples (2 mL) were collected at 12, 24, 36, 48, 96, 144, 192, and 216 h following the beginning of the experiment from the headspace of the bottles with a gas tight syringe and stored in nitrogen-flushed 10 mL serum bottles. The $^{13/12}$C isotope ratio of CH_4 in the gas samples was analysed using a cavity ring-down spectrometer (CRDS) (G2201-*i* Isotopic Analyzer, Picarro). Initially, the CRDS was calibrated as previously described (Dalby et al., 2020). In brief, the calibration was done using 250 ± 5 ppm CH_4 isotope standards (Air Liquide, Taastrup, Denmark) with certified δ^{13}C values of − 24.2 ± 0.3 ‰, −45.5 ± 0.3 ‰, and − 68.6 ± 0.3 ‰, respectively. Zero Air (HiQ zero air generator, Linde Group) was used to dilute the gases to concentrations ranging from 3 to 30 ppm CH_4 with a total flow rate of approximately 110 or 1100 mL·min^{-1} using mass flow controllers (Bronkhorst EL-FLOW, AK Ruurlo, The Netherlands). The linearity at extreme $\delta^{13}C_{CH4}$ values has previously been validated (Dalby et al., 2020). A 1-L Tedlar bag was flushed with nitrogen twice before injection of 0.5 mL gas sample. The instrument inlet tube was attached to the Tedlar bag, and the instrument measured the content for approximately 10 min. When the recorded values stabilized, the following 2 min of data were used to calculate the mean.

The $^{13/12}$C isotope ratio was estimated from the instrument reported δ^{13}C values using Eq. (2).

$$\delta^{13}C = \frac{^{13/12}R_{sample}}{^{13/12}R_{VPDB}} - 1 \quad (2)$$

where $^{13/12}R_{VPDB}$ is the $^{13/12}C$ isotope ratio of the reference material with an $^{13/12}C$ ratio of 0.011802 (Werner and Brand, 2001).

2.3. 16S rRNA, mcrA, and FTHFS gene amplicon sequencing

Biomass samples were collected at 0, 48, 96, 144, 192, and 216 h following the beginning of the experiment, snap-frozen, and stored at −20 °C until analysis.

DNA extraction was conducted in triplicate using FastDNA Spin Kit for Soil (MP Biomedicals, Denmark), following the manufacturer's recommendations. The quality of the extracted DNA was evaluated using a TapeStation 2200 and genomic DNA ScreenTapes (Agilent, USA). DNA concentration was determined using Quant-iT HS DNA Assay kit (Thermo Fisher Scientific, USA). The V4 variable region of the 16S rRNA, methyl coenzyme-M reductase (*mcrA*), and formyltetrahydrofolate synthetase (*fhs*) gene were amplified using primer set 515F/806R (515F: 5′-GTGCCAGCMGCCGCGGTAA-3′ and 806R: 5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2011), mcrA-fw/mcrA-rev (mcrA-fw: 5′-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3′ and mcrA-rev: 5′-TTCATTGCRTAGTTWGGRTAGTT-3′) (Luton et al., 2002), and FTHFS_fwd/FTHFS_rev/3-SAO-rev (FTHFS_fwd: 5′-CCIACICCISYIGGNGARGGNAA-3′, FTHFS_rev: 5′-ATITTIGCIAAIGGNCCNSCNTG-3′, and 3-SAO-rev: 5′-ATRTTNGCRAADGGNCCNCCRTG-3′) (Singh et al., 2020), respectively. Duplicate PCR reactions of 25 μL (PCRBIO 1x Ultra Mix (PCR BIOSYSTEMS), 400 nM of each primer, 10 ng of template DNA (100 ng template DNA for amplification of the *fhs* gene), and nuclease-free water) were run under the following conditions: For 515F/806R, an initial denaturation at 95 °C for 2 min, 25 cycles of 95 °C for 15 sec, 50 °C for 15 sec, and 72 °C for 1 min, and a final elongation of 72 °C for 5 min; for mcrA-fw/mcrA-rev, an initial denaturation at 95 °C for 2 min, 35 cycles of 95 °C for 15 sec, 50 °C for 15 sec, and 72 °C for 1 min, and a final elongation of 72 °C for 5 min; and for FTHFS_fwd/FTHFS_rev/3-SAO-rev, an initial denaturation at 95 °C for 2 min, 35 cycles of 95 °C for 15 sec, 52 °C for 15 sec, and 72 °C for 1 min, and a final elongation of 72 °C for 5 min. A non-template control and a sample of known content were included to control the quality of the amplicon generation. The amplicon libraries were purified using the CleanNGS (CleanNA, The Netherlands), with sample/bead solution ratio of 5/4, and the purified DNA was eluted in 25 μL nuclease-free water. Library concentration was measured with Quant-iT HS DNA Assay (Thermo Fisher Scientific, USA) and quality checked with a TapeStation 2200, using D1000 ScreenTapes (Agilent, USA). Subsequently, all samples were barcoded for sequencing in accordance with the Nextera XT adapter protocol (Illumina, USA). DNA concentration was measured using Quant-iT HS DNA Assay kit on a plate reader (TECAN Infinite M1000). PCR products were visualized on TapeStation 2200 using D1000 ScreenTapes prior to equimolar pooling, and sequencing was subsequently performed on an Illumina MiSeq platform with a MiSeq Reagent Kit v3 (2x300PE) and 20 % Phi-X spike-in.

The raw sequencing reads obtained were quality checked and processed into amplicon sequencing variants (ASVs) using the AmpProc pipeline version 5.1 (https://github.com/eyashiro/AmpProc). The 16S rRNA and *mcrA* gene amplicons were processed as paired end reads and *fhs gene amplicons* were processed as single reads, due to the amplicon being too long to be able to be merged. The primer region was removed for *fhs* and *mcrA*. For the *mcrA* gene amplicon, the taxonomic assignment was carried out using an in-house database, consisting of ~ 500 curated *mcrA* sequences obtained from FunGene repository (Cole et al., 2014), combined with the NCBI nt database (release 234, October 15th 2019). For the V4 variable region of the 16S rRNA gene, the taxonomic assignment was carried out using MiDAS v4.8 (2020–02-28) database, and a combination of the AcetoBase database (Singh et al., 2019) and NCBI nt database (release 234, October 15th 2019) for the *fhs* amplicon. R version 4.0.3 (https://www.r-project.org/) was used for the remaining analysis through Rstudio version 1.4.1103 (https://www.rstudio.com/). The R package ampvis2 (v.2.7.17) was used for the generation of heat maps and to perform principal component analysis (PCA) on Hellinger transformed ASV counts (Andersen et al., 2018).

2.4. Metagenome and bioinformatics

A total of three metagenomes were prepared from the inoculum collected at Bioenergy OL (23–09-2020). Total DNA was extracted using FastDNA Spin Kit for Soil, following manufacturer's recommendations (MP Biomedicals, Denmark). Metagenome preparation and sequencing was performed by Novogene (United Kingdom). The raw reads were pre-processed by removing PhiX using Usearch10 (Edgar, 2010) and subsequently trimmed for adaptors and quality filtered for a minimum phred score of 20 using cutadapt (Martin, 2011). The trimmed metagenome reads were assembled using SPAdes (v.3.12.0) (Bankevich et al., 2012), applying k-mers of 21, 33, 55, and 77, and a minimum scaffold length of 1 kbp. A script downloaded from https://github.com/Kirk3gaard/misc_scripts/tree/master/prepare_data_for_mmgenome2 was used to prepare the data for being analysed in R using the mmgenome2 package (https://kasperskytte.github.io/mmgenome2/). Briefly, the script generates a prediction of open reading frames in the metagenome, identifies essential genes, and classifies the contigs taxonomically. Labelled bins were extracted using the locator feature in mmgenome2. Furthermore, the metagenome was analysed for CAZymes using Hotpep (Busk et al., 2017).

2.5. Protein extraction and protein-SIP analysis

Protein extraction was performed as previously described (Heyer et al., 2013) using TEAB (0.05 M TEAB buffer stock, 1.0 mg/L NaDOC, pH ≤ 8) as resuspension buffer. In-gel digestion of extracted proteins was conducted as previously described (Mosbaek et al., 2016). The following desalting and analysis of tryptic peptides by automated liquid chromatograph-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) were performed as previously described (Poulsen et al., 2021).

The proteomic data was analysed using the MetaProSIP tool (Sachsenberg et al., 2015), in an OpenMS pipeline (https://www.openms.de), using standard settings, as described previously (de Jonge et al., 2021; Mosbaek et al., 2016). The metaproteome, generated from the metagenome and annotated using Prokka (v.1.14) (Seemann, 2014), was used as the search database.

3. Results and discussion

3.1. Biogas production

The overall performance of the batch incubations was stable throughout the 9 day experimental period. No signs of methane production inhibition by e.g. VFA accumulation or acidification were observed.

Fig. 1 presents the cumulative methane production for both experimental data and fitted model (a) and the $^{13/12}CH_4$ isotope ratio profile (b) during the experimental period. The $^{13/12}CH_4$ isotope ratio was measured during the 9 days of incubation, reaching a maximum of 0.127. The reactor reached stationary phase and a stable $^{13/12}CH_4$ isotope ratio (~8 %) within the fourth day of incubation. This ratio is a little lower than expected (isotopic ratio of the substrate was 10 %), but this can be explained by the presence of other residual carbon sources present in the inoculum. The modified Gompertz model was applied to elucidate the kinetics parameters of the biogas production such as the maximum methane production potential (P_{max}), the maximum methane production rate (R_{max}), and the lag-phase period (λ) which were 59.8

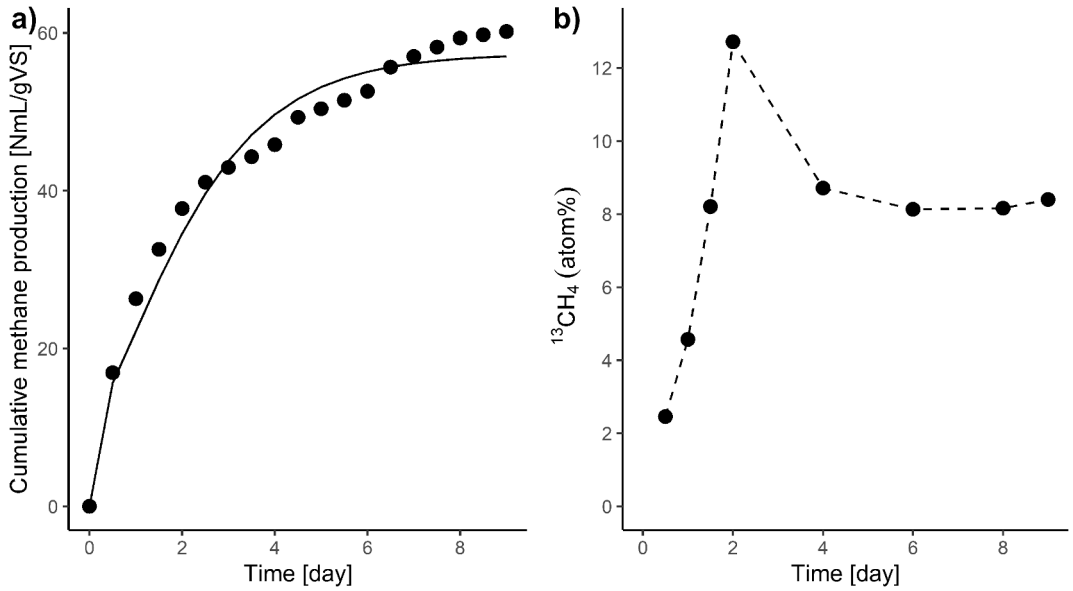

Fig. 1. a) Methane production during incubation for the U-^{13}C-cellulose reactor, measured using a gas flow meter (BPC Instruments). Methane production was measured real-time, temperature compensated and with increased frequency to ensure measurement accuracy (<5% variance) in the protein-SIP experimental setup. The data was modelled using a modified Gompertz model (Eq. (1)) and visualised by the continuous line ($R^2 = 0.953$). b) Temporal change of the atom percent of $^{13}CH_4$ in the reactors fed with 10 % U-^{13}C-cellulose (●), measured using cavity ring-down spectroscopy. The points represent mean values of the past 2 min.

NmL·gVS^{-1}, 13.3 NmL·d^{-1}·gVS^{-1}, and 0 h, respectively. The high correlation efficiency (R^2 of 0.953) shows that the mathematical model fit well to the experimental data, hereby validating the presented experimental set-up. The maximum CH_4 production rate (R_{max}) was greater than previous reported R_{max} values (10.1 NmL·d^{-1}·gVS^{-1}) for a similar experimental setting applying a F/M ratio of 3.5 (Wang et al., 2020), but less than R_{max} from systems focusing on optimizing methane production from cellulolytic substrates using different amendments with R_{max} ranging from 20.3 and 43.6 mL·d^{-1}·gVS^{-1} (Bohutskyi et al., 2018; He et al., 2022).

From one gram of cellulose, the theoretical methane production would yield 145.8 NmL·gVS^{-1} (Parkin and Owen, 1986). According to the experimental chemical oxygen demand (COD) removal of 41 %, the theoretical cumulative CH_4 production is 58.3 NmL·gVS^{-1}. This result confirms the experimental cumulative methane production (60.1 NmL·gVS^{-1}) after 9 days of incubation, which was further reproduced in the mathematical modelling (59.8 NmL·gVS^{-1}) of the CH_4 profile using the modified Gompterz model. Greater COD removal efficiency has typically been reported in the literature relative to this study, which is likely due to longer incubation periods (24 to 60 days) and higher F/M ratios (1 to 3.5). However, the obtained results confirm the suitability of energetically exploiting cellulose-source residues in anaerobic digestion technologies.

3.2. Microbial community composition

The microbial community of the cellulose-fed reactors (labelled and unlabelled) were analysed by amplicon sequencing of the 16S rRNA (microbiome), *mcrA* (methanogens) and *fhs* (acetogens) gene. The three most abundantly detected bacterial families (based on 16S rRNA gene amplicons) were *Ruminiclostridium*, *Clostridiaceae* (*Clostridium sensu stricto 1*) and *Caldicoprobacteraceae* (*Caldicoprobacter*) (Fig. 2). The family *Caldicoprobacteraceae*, which are obligate anaerobic and thermophilic bacteria and have been previously described as being able to degrade xylose, glucose, galactose, cellobiose, raffinose, and xylan and cellulosic metabolites, participating in the hydrolytic breakdown to monosaccharides, acetate, CO_2, and H_2 (Yokohama et al., 2010). Some populations were observed to either decrease or increase in relative abundance from day 6 to day 9 (Fig. 2). The most noticeable change in the microbial community seems to occur among the phyla of *Firmicutes* (increase) and *Bacteroidota* (decrease), where the genera *Clostridium* sensu stricto 1 and *Defluviitalea* were observed to increase in relative abundance, while *Lentimicrobium* was almost not detected at day 9. This is likely explained by the well-documented cellulolytic activity of members of the bacterial order *Clostridiales*, which has previously been highlighted as the primary cellulose-degrading guild in anaerobic digesters (Sun et al., 2016). The added cellulose would likely have stimulated the activity of these microorganisms.

Among the methanogens (based on *mcrA* gene amplicons), the two most abundant genera, from day 2 and forward, were *Methanothermobacter* and *Methanobacterium* (Fig. 3). *Methanosarcina* and *Methanomassiliicoccus* showed a decrease in relative abundance starting from day 2. The methanogenic families *Methanobacteriaceae*, *Methanosarcinaceae*, and *Methanomicrobiaceae*, representing hydrogenotrophic and acetoclastic methanogens, were present as the three most abundant methanogenic populations detected in the reactors, which is in accordance with previous studies from similarly operated AD which also observed a diverse representation of methanogens and metabolic preferences (Tuan et al., 2014). *Methanobacteriaceae* are able to use H_2/CO_2 (hydrogenotrophic methanogenesis) as a substrate for methanogenesis, and the *Methanobacteriaceae* family is the most commonly observed family of methanogens in anaerobic digesters, regardless of operational setup or substrate composition (Kirkegaard et al., 2017). *Methanosarcina* is the only currently known methanogenic species able to utilize all three known pathways for methanogenesis and a minimum of nine different substrates for methanogenesis, therefore able to utilize all three products from acetogenesis for methanogenesis. Furthermore, this genus has been reported as exceptionally tolerant of high ammonium concentrations, high salt concentrations, change in pH, and change in temperature (De Vrieze et al., 2012). A combination of methanogens is generally accepted to be present in a stable anaerobic digester as a means of handling stress and environmental changes, allowing this sensitive population to adapt robustly and alter the preferred pathway of methanogenesis if needed. The most well-described example of this is a shift from acetoclastic to hydrogenotrophic methanogenesis, aided by syntrophic acetate oxidation, under stressful conditions (Westerholm et al., 2015). A shift in the abundant methanogens without loss of performance during the

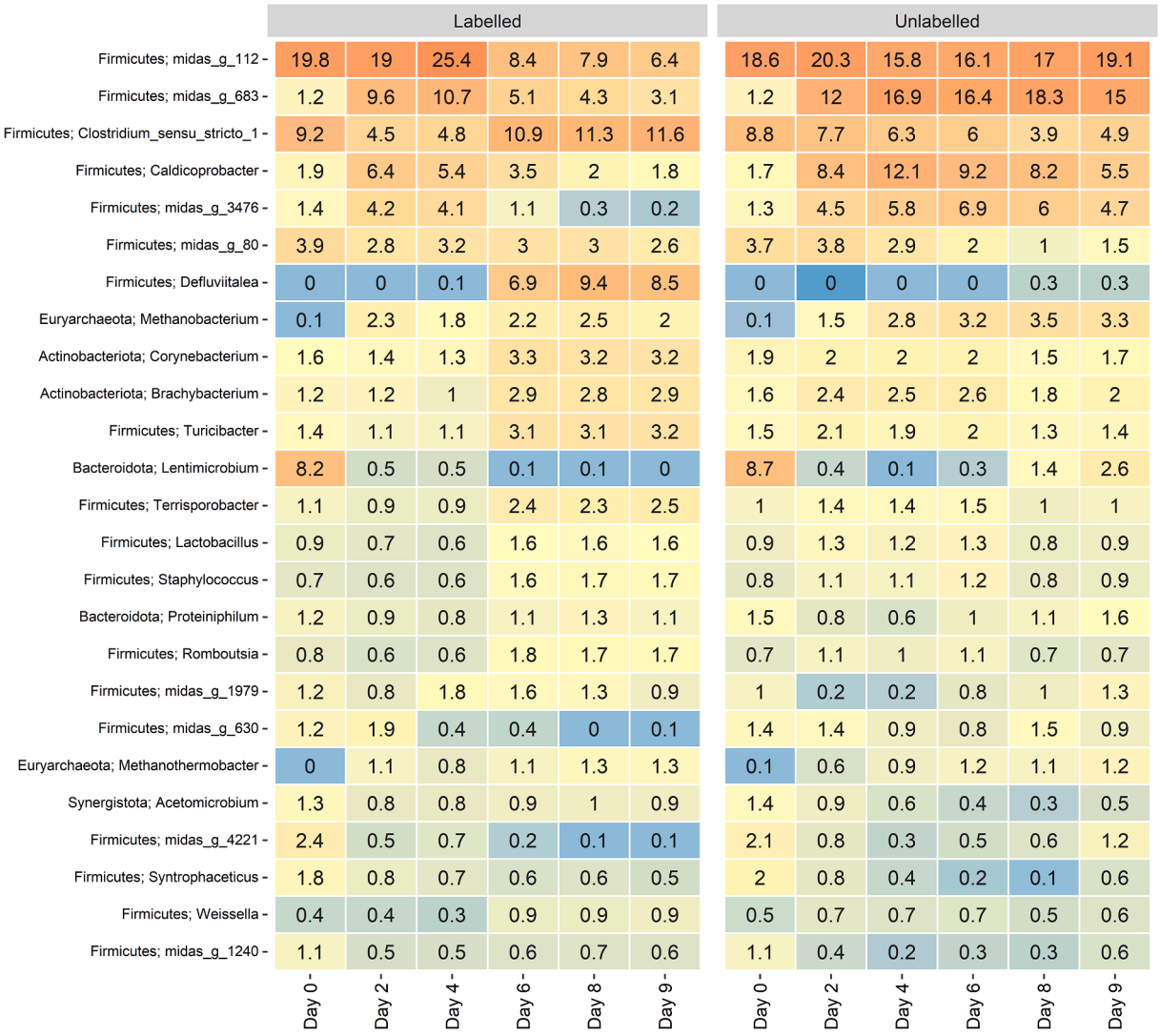

Fig. 2. Heatmap of the 25 most abundantly observed microbial populations, displayed at genus level or otherwise best possible taxonomic classification.

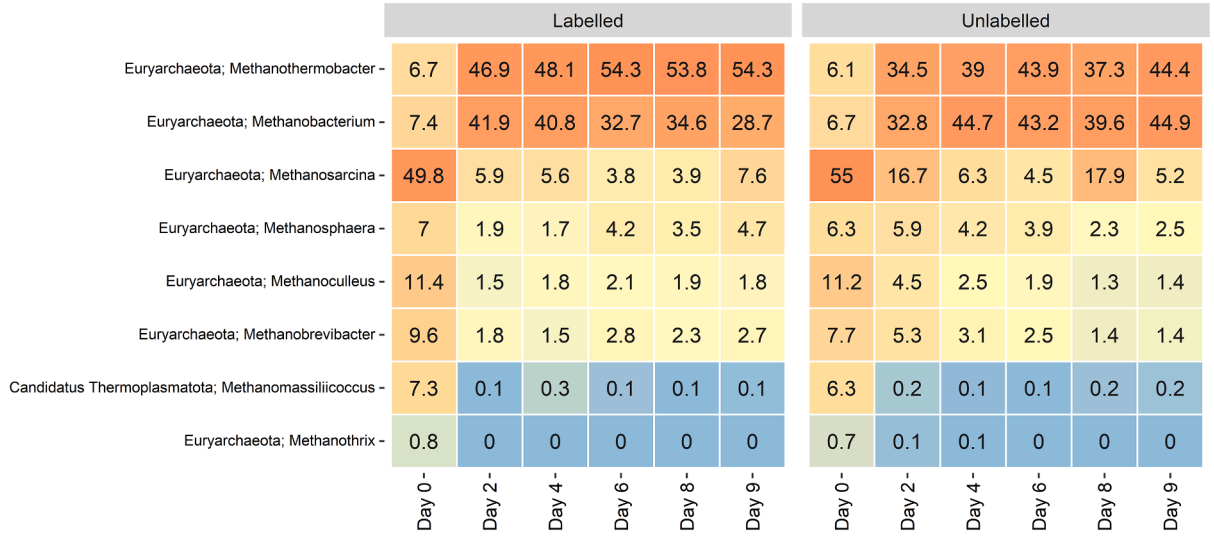

Fig. 3. Heatmap of the 8 most abundantly observed methanogenic populations, displayed at genus level or otherwise best possible taxonomic classification.

incubation time is indicative of a robust microbial response to the supplemented extra cellulose, and thereby a stable well-functioning inoculum sludge.

Among the acetogenic populations (analysed by *fhs* gene amplicons), the three most abundantly observed genera were *Acetomicrobium, Pontibaca,* and *Moorella* (Fig. 4), including a high abundance of uncharacterised sequences. *Acetomicrobium mobile* has previously been shown unable to grow on cellulose (Menes and Muxí, 2002), however, the amplicon results presented here show that *Acetomicrobium* was observed in high abundance in the cellulose-fed reactors, suggesting that it may be a secondary user of the cellulose. On the other hand, *Moorella* spp. has previously been shown to have the ability to convert cellulosic biomass directly to acetate (Karita et al., 2003), and is considered a model organism of the acetogens. A small, but noticeable difference in abundant organisms within the acetogenic populations was observed from day 0 to day 8. Acetogenic populations (and by proxy the syntrophic acetate oxidisers) produce substrate for methanogenic microorganisms, and many close syntrophic relationships have previously been described between members of these two functionally important microbial guilds (Westerholm et al., 2015). The observed evolution of both microbial populations during the incubations (Fig. 3 and S3) was therefore as expected due to their close relationship, and stimulation of certain acetogens would subsequently also very likely lead to a response from their respective syntrophic partners.

The evolution of the microbial communities in the labelled (U-^{13}C) and unlabelled (U-^{12}C) cellulose reactors, as well as the differences between the three biological replicates of each condition, were illustrated by principal component analysis (PCA) (See supplementary material). Overall, the results revealed high reproducibility of the replicates and changes over time between the labelled and unlabeled incubations, with only minor differences in microbial community change. The shift in the microbiome (16S rRNA gene amplicons) in the labelled incubations is like due to an increase in the abundance of *Defluviitalea* in the labelled reactor after day 4, as the two reactors had highly comparable microbial compositions overall (Fig. 2 and supplementary material). The methanogenic communities developed very similarly over time, with minor differences near the end of the experimental period. *Methanosarcina* was most abundant at the start of the incubation and on day 2, but hereafter, *Methanobacterium* and *Methanothermobacter* became the two most abundant genera in both the labelled and unlabelled incubations (Fig. 3 and supplementary material). For the acetogenic populations, the development of the community from day 0 to day 8 can be ascribed to a large number of populations with a shift in relative abundance, as well as the increasing abundance of *Pontibaca* in both incubation types (Fig. 4 and supplementary material). These populations underwent a large change in microbial community composition compared to the overall community structure and the methanogens, and it can be suggested at the acetogenic populations showed a strong response to the addition of cellulose. Representatives of the acetogenic community have previously been shown to be able to use cellulose as a substrate (Xu et al., 2003),

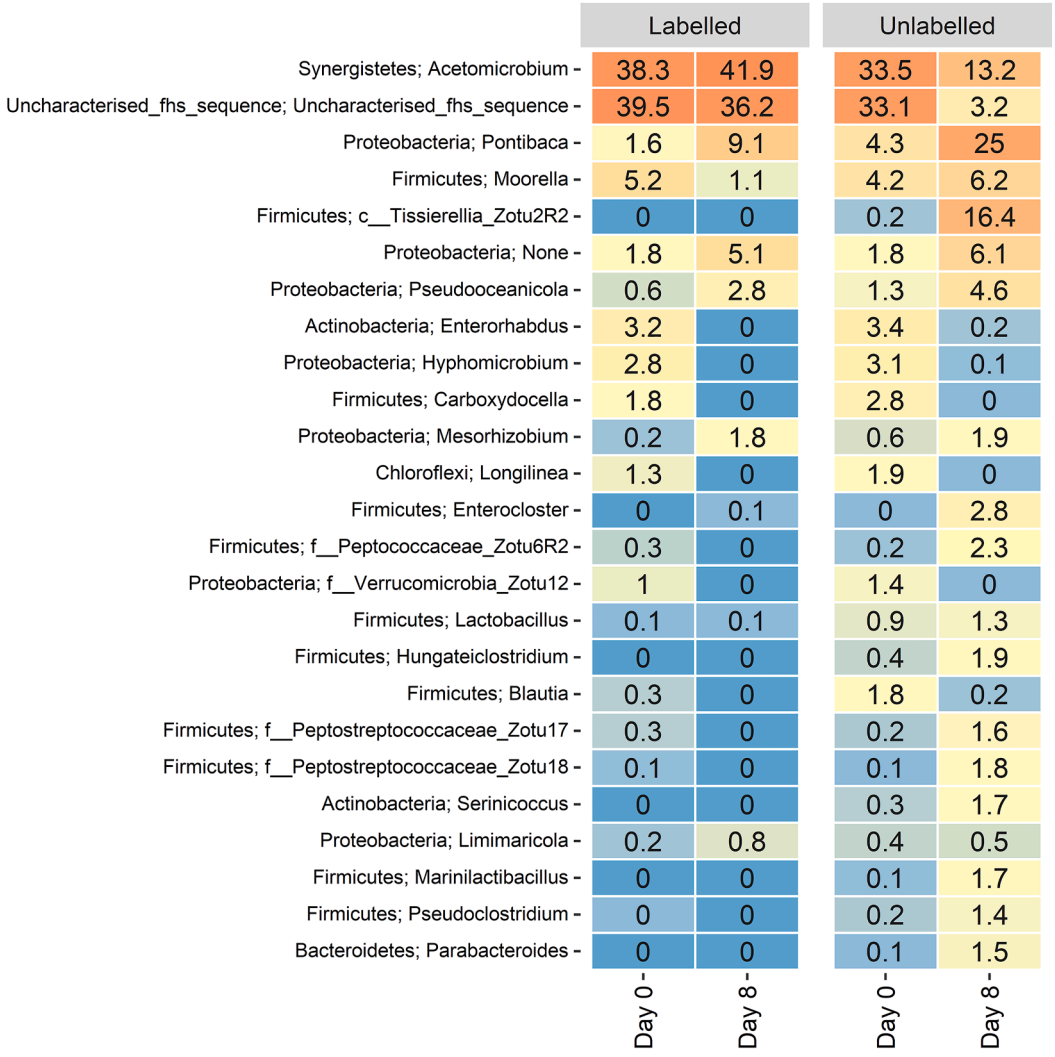

Fig. 4. Heatmap of the 25 most abundantly observed acetogenic populations, displayed at genus level or otherwise best possible taxonomic classification.

and these results are also in line with the findings of the protein-SIP analysis, which also detected several known acetogens as labelled (supplementary material).

3.3. Metagenome analysis

Three biological replicate metagenomes were generated from the inoculum, which yielded a combined assembly of 773,486,919 bp. The metagenome consisted of 28 different phyla, with *Actinobacteria* and *Firmicutes* as the most abundant, representing 40.2 % and 34.9 % of all identified scaffolds, respectively. The five most abundant families were the *Muribaculaceae* (0.35 %), *Methylococcaceae* (0.25 %), *Clostridiales* (0.27 %), *Methanomassiliicoccaceae* (0.20 %), and *Synthrophaceae* (0.13 %). The taxonomic diversity of the metagenome analysis was consistent with the diversity identified by amplicon sequencing analysis, with the observation of *Firmicutes* and *Actinobacteria* as the most abundant phyla. *Firmicutes* has previously been shown to comprise the most abundant group of microbial communities in biogas reactors, and contains a large number of well-described microorganisms with diverse functions that were first observed and/or isolated from anaerobic digester systems (Koeck et al., 2016).

Analysis of the carbohydrate-active enzyme (CAZyme) potential of the metagenome resulted in a total of 17,155 hits, corresponding to 238 different CAZymes, 6 of which are directly linked to cellulose degradation and were seen 756 times in the metagenome. These CAZymes included cellulase (EC 3.2.1.4), cellobiose phosphorylase (EC 2.4.1.20), cellodextrin phosphorylase (EC 2.4.1.49), cellulose 1,4-β-cellobiosidase (non-reducing end) (EC 3.2.1.91), cellulose 1,4-β-cellobiosidase (reducing end) (EC 3.2.1.176), and cellobiose dehydrogenase (EC 1.1.99.18), seen 250, 58, 32, 7, 5, and 26 times, respectively. The CAZymes were affiliated to 13 different genera (abundance in metagenome), *Bifidobacterium* (0.09 %), *Brachybacterium* (0.47 %), *Cellulomonas* (0.17 %), *Corynebacterium* (1.71 %), *Herbinix* (0.08 %), *Hungateiclostridium* (0.22 %), *Methanoculleus* (0.17 %), *Methanosarcina* (0.43 %), *Mycolicibacterium* (0.52 %), *Neorhizobium* (0.01 %), *Thermobifida* (0.30 %), *Thermoclostridium* (0.35 %), and *Turicibacter* (0.05 %), and are affiliated with four different phyla, *Actinobacteria*, *Euryachaeota*, *Firmicutes*, and *Proteobacteria* (Fig. 5). The genera *Brachybacterium*, *Corynebacterium*, and *Turicibacter*, have been directly linked to cellulose degradation and were abundantly observed throughout the whole experimental period by the amplicon sequencing analysis (Fig. 2). *Brachybacterium* is able to decompose cellulose into cellobiose and glucose (Zhang et al., 2007), and *Corynebacterium* has been reported to be able to simultaneously metabolize glucose and cellobiose (Sakai et al., 2007). Furthermore, another of the identified genera through its CAZymes was *Cellulomonas*, which is of special interest due to its known ability to degrade cellulose, xylan, and starch (Abt et al., 2010). Molecular work has previously identified and characterised cellulases and xylanases of *Cellulomonas*, and the genomic data revealed that 9.6 % of encoded proteins are classified into carbohydrate transport and metabolism (Abt et al., 2010).

3.4. Taxonomic diversity and proteins identified in the extracted and/or labelled bins

A total of 2,075 peptides were identified across all samples, and 20 peptides (0.1 %) were found to have incorporated labelled carbon (supplementary material). Filtering with the conditions that peptides could not be labelled at time 0 and had the relative isotope abundance (RIA) $\geq$ 10 after 216 h (9 days), left a total of 19 labelled peptides of interest. Seventeen peptides showed a high RIA ($\geq$20). Of the 19 peptides, the majority belonged to organisms representing the phylum *Firmicutes,* and four peptides were associated to the phylum *Candidatus* Bipolaricaulota. The identified peptides were mapped to 11 different scaffolds on the metagenome and identified as being part of either an ABC transporter-binding protein or a hypothetical protein, and both were identified multiple times. Metagenome-assembled genomes (MAGs) containing labelled scaffolds were extracted and annotated. The MAGs were found to be dominantly affiliated with the following phyla: *Firmicutes*, *Proteobacteria*, and *Actinobacteria* (supplementary material). The taxonomic assignment of the 11 extracted MAGs (bins) containing the genes coding for the isotopically labelled peptides revealed four different organisms with previously described cellulolytic activity: *Acetivibrio*, *Lachnospiraceae*, *Treponema*, and *Variovorax*. The genus *Acetivibrio* has previously been reported as containing species which are efficient cellulose degraders (Xu et al., 2003), and has been observed in both wastewater treatment plants and anaerobic digesters. Another study found that *Treponema* (*Spirochaetes*) was important for the hydrolysis of cellulose (Jensen et al., 2021). Furthermore, bacteria belonging to the family *Lachnospiraceae* have also been reported as having cellulolytic activity (Flint et al., 2012), and *Variovorax* has previously been suggested to possess endocellulase capacity (Ghio et al., 2012), but no xylan-degrading capacity was observed.

The peptides which had assimilated labelled carbon were found to be part of ABC transporter-binding proteins or hypothetical proteins. The ABC transporter superfamily has been shown to have emerged prior to

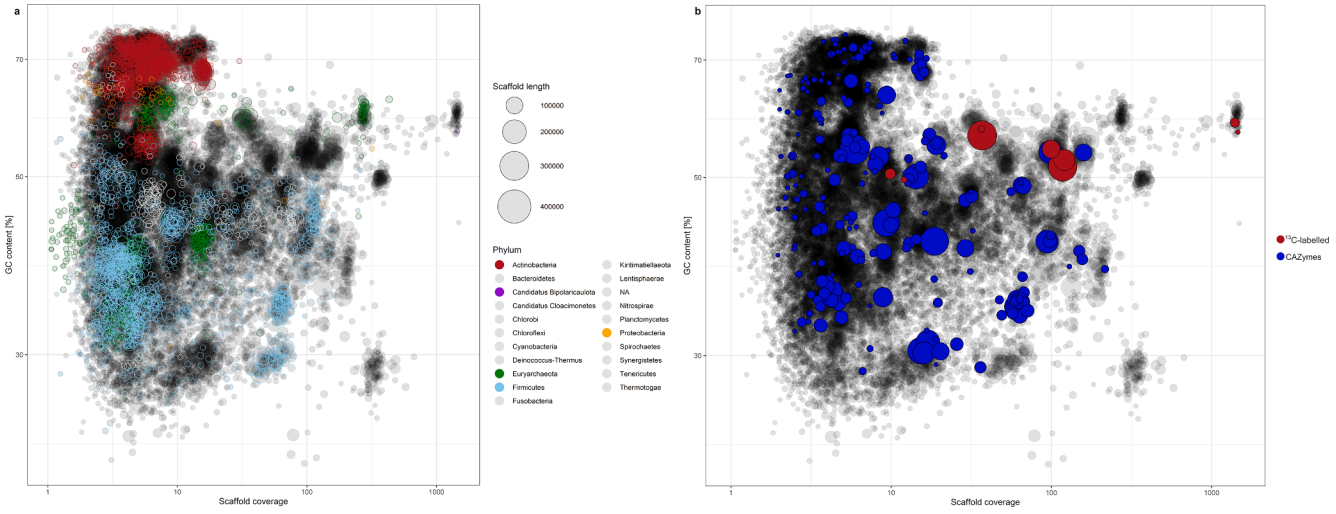

Fig. 5. The scaffold coverage and GC content from generated metagenome are plotted. The dot sizes indicate the scaffold length, and with a minimum scaffold length of 6000 bp. a) Colouring is according to phylogeny (phylum) and only the phyla holding CAZymes or ^{13}C-labelled proteins are coloured. b) Scaffolds where CAZymes or ^{13}C-labelled proteins were found is coloured.

the evolutionary divergence of prokarya, archaea, and eukarya, and evolution has adopted these transporters to such a degree that they are now present in all domains of life. Furthermore, detection of multiple ABC transporter-binding proteins is in line with the results of a previous study where protein-SIP was used to detect syntrophic acetate-oxidizing bacteria (SOAB) in anaerobic digesters using ^{13}C-labelled acetate (Mosbaek et al., 2016), as well as a protein-SIP study focused on the differences in metabolic activity between anaerobic digestion and *in-situ* H_2 biomethanation (de Jonge et al., 2021) , using ^{13}C-labelled bicarbonate.

Based on the obtained results, it can be proposed that the most abundant organisms involved in cellulose degradation by hydrolytic enzymes in the presented work originated from the genera *Acetivibrio*, *Brachybacterium*, and *Cellulomonas*. Enzymes from these organisms are able to degrade the cellulose into poly- and monosaccharides such as glucose and cellobiose (Abt et al., 2010; Xu et al., 2003; Zhang et al., 2007). Representatives of *Acetivibrio* and *Corynebacterium* potentially acted as secondary users of the cellulose during acidogenesis, during which they are able to degrade glucose and cellobiose simultaneously (Sakai et al., 2007). The poly- and monosaccharides were subsequently converted further into volatile fatty acids, and ultimately degraded into acetate, hydrogen, and carbon dioxide. *Moorella* was abundantly detected as a representative of downstream bioconversion, this organism is considered a model organism among acetogens. Furthermore, an additional number of organisms with the ability to use the Wood-Ljungdahl pathway were identified in the reactor by both the amplicon sequencing analysis and the proteogenomics. These microorganisms are capable of performing the bi-directional conversion between acetate, and carbon dioxide and hydrogen. These organisms included the genera *Acetomicrobium* and *Pontibaca*. Both acetoclastic and hydrogenotrophic methanogens were detected using amplicon sequencing and metagenomic analysis. The genera *Methanosarcina*, *Methanobacterium* and *Methanothermobacter* were observed abundantly during the incubations, and all have previously described syntrophic partnerships with acetogenic organisms (either through syntrophic acetate or propionate oxidation) (Kato et al., 2009; Plugge et al., 2010; Sousa et al., 2007). It is likely that these methanogenic organisms shared a syntrophic relationship with some of the abundantly observed acetogens, which is supported by the observed stimulation and change in both populations during the experimental period. Taken together, it could be hypothesized that all post-acetogenesis products (acetate, CO_2, and H_2) could have served as substrates for a combination of acetoclastic and hydrogenotrophic methanogenesis.

4. Conclusion

Elucidating which microorganisms are involved in the primary degradation of cellulose in AD systems contributes to better treatment of lignocellulosic biomass. Protein-SIP analysis of a full-scale thermophilic anaerobic digester showed that representatives of the phyla *Firmicutes* and *Candidatus* Bipolaricaulota were active cellulose degraders. The CAZyme screening highlighted cellulose-degrading organisms such as *Cellulomonas* and *Brachybacterium*. In addition, *Corynebacterium* was identified through both CAZyme screening and metagenomic analysis. The present work demonstrates that simultaneous identification and functional analysis of digester microbes was able to provide important insight into the degradation processes relating to lignocellulosic biomass.

5. Availability of data and materials

All amplicon and metagenome data are available at the European Nucleotide Archive (ENA) under project accession number PRJEB46131. The mass spectrometry proteomics data have been deposited at ProteomeXchange Consortium (Vizcaíno et al., 2014) via the PRIDE partner repository with the data set identifier PXD027303.

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CRediT authorship contribution statement

Jan Struckmann Poulsen: Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Nadieh de Jonge:** Methodology, Software, Writing – original draft. **Williane Vieira Macèdo:** Data curation, Writing – original draft. **Frederik Rask Dalby:** Formal analysis, Methodology, Validation. **Anders Feilberg:** Resources, Validation. **Jeppe Lund Nielsen:** Conceptualization, Methodology, Validation, Resources, Writing – original draft, Supervision, Funding acquisition.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.126933.

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