Study on the cellulose-degrading bacterial community in biogas systems

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Abstract

Renewable energy sources, such as biogas, play an important role in the transition to a climate neutral society. Despite the great abundance of lignocellulosic biomass, its use for biogas production is limited by its recalcitrant structure. The microbial degradability of lignocellulosic biomass in the biogas system can be enhanced by making use of the system's native cellulose-degrading bacterial community. The development of such operational strategies for enhanced degradability of lignocellulosic biomass would, however, benefit from more knowledge on (i) the taxonomy of this community and (ii) its response to process parameters.

This study aimed to contribute to a better understanding of these two areas by (i) characterising a putative novel cellulose-degrading bacterial species (strain Dc1) isolated from an industrial biogas reactor and (ii) investigating how the abundance of cellulose-degrading bacteria correlates with process parameters. Physiological features of strain Dc1 were characterised according to current standards for classification of novel species. Furthermore, the abundance of a functional gene-marker for cellulose degradation, *cel48*, was quantified by quantitative PCR and correlated to a set of process parameters in Swedish farm-based biogas plants.

The results indicate that Dc1 is mainly cellulolytic and saccharolytic, with some ability to degrade amino acids and fumarate. Dc1 exhibited close similarity to a known cellulose-degrading bacterium, *Acetivibrio cellulolyticus* (98.5%), based on 16S rRNA gene sequence analysis, but more experiments are needed to validate whether it belongs to the same species or represents a novel one. The relative abundance of *cel48* (copies/ng total DNA) correlated positively with hydraulic retention time (HRT) in the range of 16-49 days ($p<0.001$). There was also an indication of negative correlation of the relative amount of *cel48* with the total ammonium-nitrogen (TAN) concentration in the digestate, in the ranges 1.7 – 3.9 g/L.

The results from both parts of the study must, however, be interpreted with care, as they were subject to shortcomings that reduced the reliability of the results. Nevertheless, the results still offer new insights into the substrate utilisation and taxonomy of Dc1, as well as an indication of a positive effect of increased HRT and a negative effect of TAN on the cellulose-degrading bacterial community in agricultural biogas processes. The absence of correlations with other process parameters suggests that the abundance of cellulose-degrading bacteria may depend on the overall environment created by several process parameters rather than on individual ones.

Keywords: anaerobic digestion, anaerobic microbiology, biogas, *cel48*, cellulose-degrading bacteria, characterisation, farm biogas, glycoside hydrolase family 48, lignocellulosic biomass, quantitative PCR.

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Abbreviations

AD	Anaerobic digestion
cel48	Collective name for glycoside hydrolase family 48 genes
HPLC	High-performance liquid chromatography
HRT	Hydraulic retention time
GH48	Glycoside hydrolase family 48
TAN	Total ammonium-nitrogen
VFA	Volatile fatty acids

1. Introduction

1.1 Background

1.1.1 Biogas – a source of renewable energy

Today's climate crisis has galvanised the search for and development of alternatives to fossil fuels. The European Green Deal within the European Union (EU) has set the goal of reaching climate neutrality by 2050 (The European Green Deal 2019). Renewable energy plays an important role in the subgoal of clean energy transition, and the member states of the EU have committed to ensuring that 42.5% of the total union's energy consumption comes from renewable energy sources by 2030 under the Renewable Energy Directive (Directive EU/2023/2413 2023).

Biogas is principally composed of methane and carbon dioxide. It is produced through anaerobic digestion (AD) of organic material by a complex and interdependent anaerobic microbial community (Schnürer & Jarvis 2018). Food waste, manure, sewage sludge and crop residues are examples of organic material (substrates) that can be used to produce biogas. Biogas can be used for heat and electricity. After increasing the proportion of methane through the removal of carbon dioxide, it can also be used as a vehicle fuel. Combustion of methane emits carbon dioxide and water. However, the net emission remains zero, given that the emissions come from carbon in substrates already in circulation. Therefore, biogas is a renewable energy source (Schnürer & Jarvis 2018).

The biogas system is also a model for circularity and sustainability as it enables the flow and recycling of biological resources and energy between cities and farms and provides a system for waste management (Figure 1) (Schnürer & Jarvis 2018). The residual digestate produced in the process can be used as a biofertiliser. This enables the replacement of mineral fertilisers, which is positive from an environmental point of view, as their production is energy-intensive and leads to the emission of greenhouse gases due to the consumption of fossil fuels. Furthermore, utilisation of the biofertiliser closes nutrient cycles when the nutrients are brought back to the soil. Additionally, the unique biological system in the anaerobic digester is a potential source of biochemicals (Schnürer & Jarvis 2018).

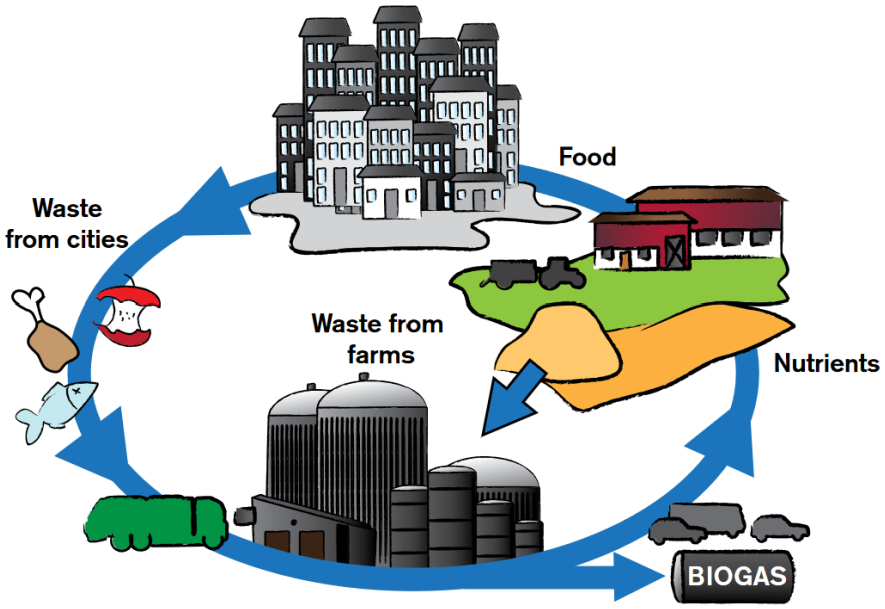

Figure 1. In addition to renewable energy (biogas), the unique biogas system provides a method for the valorisation and upgrading of biological compounds, production of biofertiliser and a system for waste management (Schnürer & Jarvis 2018). Illustrated by Cajsa Lithell, RedCap Design.

1.1.2 Biogas production from lignocellulosic biomass

Cellulose is the most abundant organic polymer on Earth (Schwarz 2001). The fact that lignocellulosic waste streams are also highly abundant and available has made them an interesting and important substrate for the production of bioenergy (Himmel et al. 2010; Olatunji et al. 2021). As much as 76% of the biogas potential from Swedish raw material is estimated to come from agricultural activity (agricultural residues and manure) if forestry raw material is excluded (Avfall Sverige 2008). Unfortunately, biogas from lignocellulosic biomass cannot be produced to its full potential as its complex structure is challenging for the microbial community in the biogas system to degrade, as further described below.

Recalcitrance of breakdown of lignocellulosic materials

Lignocellulosic biomass mainly consists of cellulose, hemicellulose, and lignin (Bajpai 2016). These are all components of the plant cell wall, together forming a complex structure (Figure 2). Cellulose is the most abundant of the three and has structural importance for the plant cell wall. The basic components of cellulose are chains of glucose, linked together by β-1,4 glycosidic bonds (Bajpai 2016). When these glucose chains bind to each other, they form a crystalline structure of microfibrils that, in turn, form cellulose fibres (Schwarz 2001; Bajpai 2016). Cellulose is insoluble due to this structure, rendering it recalcitrant to breakdown

(Schnürer & Jarvis 2018). The recalcitrance is further enhanced by hemicelluloses and lignin interacting with the cellulosic microfibrils (Sawatdeenarunat et al. 2015).

Hemicellulose is a branched, heterogeneous compound, comprising different monosaccharides (uronic acids, pentoses and hexoses) (Bajpai 2016). Lignin is a large and complex aromatic molecule (Bajpai 2016) that is difficult to degrade under anaerobic conditions (Benner et al. 1984; Dinsdale et al. 1996). In the plant cell wall, lignin protects cellulose and hemicellulose against interference with microbial enzymes (Himmel et al. 2010; Bajpai 2016).

The hydrolytic step, where biomass is broken down into soluble monomers and oligomers, is the rate-limiting step during biogas production from lignocellulosic biomass (Noike et al. 1985; Tsavkelova & Netrusov 2012). The degradation of lignocellulosic biomass in the biogas system can be facilitated with biological pretreatments with enzymes, fungi or microbial consortia as previously reviewed (Monlau et al. 2013; Zheng et al. 2014). Studies have also indicated priming, *i.e.* the addition of easily degradable carbons to the system, to increase both the degradation of lignocellulosic biomass and the abundance of cellulolytic species in biogas processes (Eliasson et al. 2023; Axelsson Bjerg et al. 2025). Furthermore, the degradability of lignocellulosic biomass can be increased by strengthening the system's native cellulose-degrading microbial community. However, more knowledge on the cellulose-degrading community in the biogas system is needed to further explore and develop such methods.

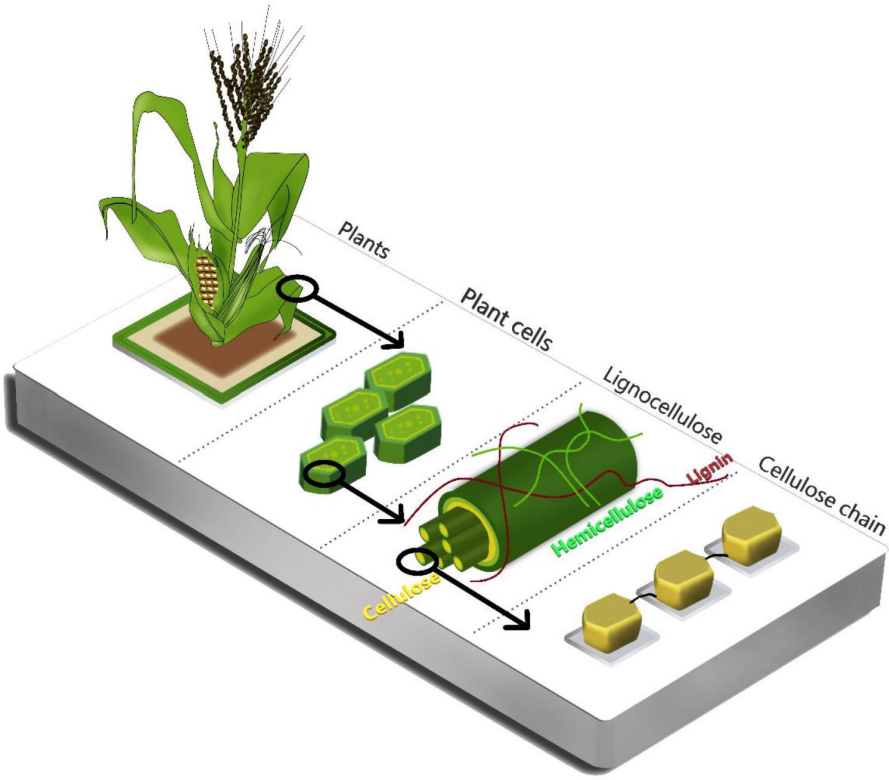

Figure 2. Cellulose consists of chains of glucose moieties, assembled into chains and fibres. The cellulose fibres, hemicellulose and lignin together form lignocellulose, a complex and rigid structure in the plant cell wall (Liu 2019).

1.1.3 Cellulose-degrading bacterial community in the biogas system

The microbial community in biogas systems consists of anaerobic microorganisms from all domains (Bacteria, Archaea and Eukarya) at various proportions (Schnürer 2016). Bacteria are most abundant in terms of the total number of species and are responsible for the degradation of various substrates. Interestingly, although the methane-producing Archaea (methanogens) play an important role in the system, they only constitute a small part of the microbial community (Schnürer 2016).

Taxonomy of the cellulose-degrading bacterial community in biogas systems

The cellulose-degrading bacterial community in biogas systems is typically dominated by members within the phyla Bacillota and Bacteroidetes, and the orders Clostridiales (Bacillota) and Bacteroidales (Bacteroidetes) (Azman et al. 2015; Sun et al. 2016; Westerholm et al. 2019). Examples of other phyla containing cellulose-degraders in biogas systems include Fibrobacterota, Spirochaetota, Thermotogota and Chlorobiota as reviewed by Azman et al (2015).

The composition and abundance of cellulose-degrading taxa can, however, vary greatly between different biogas processes. On the one hand, several studies have

shown clostridia (phylum Bacillota) to be particularly abundant in the cellulose-degrading bacterial community in the biogas system and to play a key role in cellulose degradation (Schlüter et al. 2008; Izquierdo et al. 2010; Zakrzewski et al. 2012; Azman et al. 2015). The 16S rRNA gene analysis of a set of Swedish farm-based biogas plants operating on manure (included in the present study) also revealed the highest relative abundance of Clostridium sensu stricto 1 (unpublished data). On the other hand, another study found *Defluvitoga* (phylum Thermotogota), a known cellulose-degrading bacterium, to be particularly abundant in the community (Perman et al. 2024).

More efforts are needed to gain a better understanding of the cellulose-degrading bacteria in the biogas system (Azman et al. 2015). Consequently, more knowledge on the functional and taxonomic properties of this community remains to be characterised.

Influence of process parameters on the cellulose-degrading community in biogas systems

Studies have shown that the cellulose-degrading bacterial community and/or the rate of cellulose degradation in the biogas system can be influenced by the alteration of process parameters. However, there is no unified understanding of the underlying mechanisms and reported effects can be contradictory between studies, as discussed below.

A specific process parameter of interest is the ammonia levels in the process. To compensate for the high C/N-ratio in lignocellulosic biomass (Li et al. 2013), it is often co-digested with nitrogen-rich, proteinaceous substrates such as manures and slaughterhouse wastes (Wang et al. 2012; Schnürer & Jarvis 2018). Ammonia (NH_3) and ammonium (NH_4^+) are released during protein degradation. These substances are in equilibrium with each other, and the total amount of both substances in a process is measured in the total ammonium nitrogen (TAN) (Schnürer & Jarvis 2018). Consequently, these types of processes can have high levels of ammonia, a component that can inhibit microorganisms (Schnürer & Jarvis 2018). This means that the cellulose-degrading bacterial community in the biogas system is often exposed to high ammonia levels. The ammonia tolerance of this community is, however, not yet fully understood (Azman et al. 2015). A better understanding of this aspect would help to develop methods for increasing the efficiency of microbial lignocellulose degradation in biogas systems at high levels of ammonia.

Other process parameters with possible influence on the cellulose degradation and the cellulose-degrading bacterial community are hydraulic retention time (HRT), temperature, pH, substrate composition, and the presence of inhibitors such as volatile fatty acids (VFA), as reviewed by Azman et al (2015).

Mechanism of anaerobic cellulose degradation

The rigid structure of native, crystalline cellulose is degraded by a consortium of enzymes employing different breakdown strategies (Schwarz 2001), including glycoside hydrolases (GHs) such as cellulases and hemicellulases (Himmel et al. 2010). GHs from anaerobes mostly belong to GH families 5, 9 and 48 (Pereyra et al. 2010).

Instead of secreting individual cellulose-degrading, free enzymes, some anaerobes possess a membrane-bound complex of enzymes that break down cellulose, named cellulosome (Bayer et al. 1983; Schwarz 2001). The cellulosomes are constructed of both non-catalytic and catalytic domains (Bayer et al. 2004). During cellulose degradation, the cellulosome physically attaches to cellulose fibres, thus ensuring that the cellulose-degrading enzyme complex is in close proximity to the cellulose (Himmel et al. 2010). This is mediated by the carbohydrate-binding domains (CBMs), attached to scaffoldin domains. The scaffoldin also contains cohesion domains (cohesins) that bind to the dockerin domains of the catalytic domains.

1.1.4 Culture-dependent and culture-independent approaches to the study of microorganisms and microbial consortia

Microorganisms can be studied using both culture-dependent and culture-independent approaches. Both approaches, with their respective advantages and disadvantages, are of equal relevance. Although the modern era of microbiology and biotechnology is largely characterised by studies based on molecular techniques, there is a remaining importance of the cumbersome practice of isolation and characterisation of novel microorganisms (Clavel et al. 2025).

Culture-independent techniques have enabled the study of microbial communities in complex environments without the need for cultivation, opening up a new world of possibilities and insights in microbiology (Zarraonaindia et al. 2013; Hugerth & Andersson 2017). However, these techniques also have limitations. An important limitation is their dependence on databases (Hugerth & Andersson 2017); the interpretation and significance of studies based on culture-independent techniques will ultimately depend on the quality and availability of information in the used database. This information can only be gained by the isolation and characterisation of novel species, which is a culture-dependent technique. Important information is still missing in such databases. For example, it is still challenging for molecular techniques to assign sequences to taxa of various levels (Zakrzewski et al. 2012; Li et al. 2014). When applying Illumina sequencing of a functional gene-marker for cellulose-degradation, *cel48* encoding GH48 genes (an enzymatic group containing cellulose-degrading enzymes (see section 1.1.3)), Rettenmaier et al (2020) found that, at mesophilic conditions, most sequences had an unknown taxonomic identity. This further emphasises the need for more

knowledge on enzymes involved in cellulose degradation in biogas processes and suggests that they may be assigned to species yet to be discovered. Other examples of limitations with culture-independent techniques include the biases introduced by *e.g.* the DNA-extraction and the PCR reaction (Brooks et al. 2015; Hugerth & Andersson 2017; Douglas et al. 2020).

Isolation and characterisation are of special value and interest in anaerobic microbiology, a generally less discovered field than aerobic microbiology (Hanišáková et al. 2022). Although culture-dependent techniques are important for the generation of knowledge on individual microbes, they also have limitations. For example, they are limited to the study of microbes that can be cultivated under current existing laboratory techniques (Hugerth & Andersson 2017). Furthermore, culture-dependent techniques have limited capacity for elucidating characteristics of microbial communities as they typically study microbes isolated from both other microorganisms as well as from their natural environmental conditions (Vanwonterghem et al. 2014). Therefore, high-quality and robust research is ultimately dependent on both approaches (Al-Awadhi et al. 2013).

The cellulose-degrading bacterial community has previously been studied with different techniques. In addition to the isolation and characterisation of novel species from this community, several culture-independent techniques have been applied. One example is the detection and quantification of functional genetic markers for cellulose degradation from glycoside hydrolase families 5 and 48 (GH5 and GH48) designed by Pereyra et al (2010). Rettenmaier et al (2020) further improved the primers for the GH48 genes from Pereyra et al (2010) and additionally designed a mix of primers for improved detection of the same genes. The cellulose-degrading bacterial community can also be studied with 16S rRNA gene–based studies. This has been studied using universal primers, targeting the whole prokaryotic community (Eliasson et al. 2023), or with specific primers, targeting a species/genus/family, etc of interest (Denman & McSweeney 2006; McDonald et al. 2012; Zeng et al. 2015). The primers can also target functional genes in the bacterium or bacterial group of interest, such as *CipA* (gene encoding a cellulosomal scaffolding protein) in *Acetivibrio thermocellum* (Tang et al. 2015).

Characterisations of novel organisms are validated and published by the International Journal of Systematic and Evolutionary Biology (IJSEM). A valid characterisation of a novel microbial species should contain information on the isolates' DNA (full genome, 16S rRNA sequence), similarity to other species, as well as physiological data including growth characteristics, substrate utilisation, morphology, etc (Microbiology Society).

1.1.5 The novel cellulose-degrading bacterium Dc1

The cellulose-degrading bacterium Dc1 investigated in the present study originated from an industrial biogas plant operating on thin stillage (Moestedt et al. 2013) and was isolated by Li Sun in 2015 (Sun 2015).

Dc1 was thereafter partly characterised in a previous Master's thesis (Sjöberg 2023), which suggested that it could represent a novel species. Its substrate utilisation was investigated for a range of carbohydrates, and the strain showed fast growth on cellulose and cellobiose, and slower growth on xylose, straw, glucose and ribose. The ability of Dc1 to degrade other types of substrates has not been investigated. Additionally, some other tests on Dc1 have been performed, including its growth characteristics. Growth occurred in 25-51 ºC and within the pH range 6 - 9, but optima are yet to be determined (Sun 2015; Sjöberg 2023).

Initial taxonomic analysis of the 16S rRNA gene (NCBI BLAST, core_nt database) revealed close similarity to *Acetivibrio cellulolyticus* strain HPc (99.11%) (Patel et al. 1980). Although only 97% identity of 16S rRNA gene sequences is required for strains to be assigned to the same species (Tindall et al. 2010), physiological data on Dc1 is still required for the investigation of its uniqueness given the fact that they were isolated from distinct environments: *A. cellulolyticus* was isolated from the ammonia-low environment of sewage sludge, whereas Dc1 comes from an ammonia-rich biogas-reactor. Should Dc1 be physiologically different from *A. cellulolyticus*, it could represent a novel species. If not, the results will confirm the occurrence of *A. cellulolyticus* in both low and high ammonia environments.

1.2 Aim of the study

The study aimed to gain knowledge of the cellulose-degrading bacterial community in biogas systems by

(i) characterising a putative novel cellulose-degrading bacterial species isolated from an industrial biogas reactor according to the prevailing requirements of the IJSEM

(ii) correlating the abundance of a functional gene marker for cellulose degradation, *cel48,* in Swedish farm-based biogas plants to a set of process parameters.

2. Materials and Methods

2.1 Characterisation of the isolate Dc1

2.1.1 Source of bacterium

The first isolation of Dc1 by Li Sun was achieved by first enriching cellulose-degrading bacteria from the inoculum of the industrial biogas plant, followed by picking single colonies using the agar shake tube dilution method with cellulose as the sole carbon source throughout the process (Sun 2015).

2.1.2 Growth medium and culturing conditions

The growth medium, used for all experiments unless otherwise stated, was prepared as previously described (Westerholm et al. 2010). In short, and for the preparation of 1 L growth medium, a phosphate buffered basal medium (BM) was prepared by mixing a phosphate buffer (final concentrations) (KH_2PO_4 and Na_2HPO_4, both 3mM), redox indicator (Resazurin) (1.99 µM), yeast extract (0.2 g/L) and Selenium/Wolfram solution (0.1 µM of each) with distilled water to a final volume of more than one litre. The mixture was boiled for 20 minutes to a final volume of 900 mL and cooled on ice while flushing with N_2. Subsequently, the medium was aliquoted to serum bottles (18 mL BM added for a 118 mL serum bottle) while flushing with N_2. The vials were sealed with butyl rubber stoppers (while still flushing with N_2) and aluminium caps. The gas phase was exchanged to N_2/CO_2 (80/20 v/v, 0.2 bar) with vacuum-gas cycling. Following autoclaving (20 min, 121 ºC) and cooling to room temperature, the serum bottles were supplemented with pre-sterilised (filter 0.2 µm) solutions Cold 1 (C1) and Cold 2 (C2) (5% v/v of final volume) containing trace elements, vitamins and salts (C1), and reducing agents and a bicarbonate buffer (C2) (Westerholm et al. 2010). Following the addition of C2, the redox indicator Resazurin turned colourless, which illustrated a suitable Oxygen-Reduction Potential (ORP). The pH of the final solution was 6.9 -7.2.

The serum bottles were inoculated with 1 mL of inoculum of Dc1, which was grown on either cellobiose (10 mM) or cellulose (5 g/L). Unless otherwise stated, inoculated bottles were incubated in the dark without shaking at 37 ºC.

2.1.3 Isolation

From the first isolation of Dc1 (see section 2.1.1), the bacterium was maintained through regular inoculation in growth medium supplemented with cellulose or cellobiose until the start of the present study. From this culture, Dc1 was again re-isolated to ensure the purity of the culture. This was achieved with the agar shake dilution method (Heller 1921; Hanišáková et al. 2022). This method is based on a serial dilution of the culture in agar shake tubes as follows: Balch tubes were filled with BM supplemented with 2 % agarose (w/v) (4.5 mL) under the flushing of N_2. The tubes were sealed, followed by gas exchange as previously described (see section 2.1.2). Following autoclaving (20 min, 121 ºC), the tubes were supplemented with C1 and C2 (0.25 mL each) and cellobiose (to a final concentration of 4.1 mM). The inoculum of Dc1 (0.1 mL) was added to the first Balch tube, and subsequently, 0.5 mL was transferred from one tube to the next. This was repeated for a series of 12 tubes in total. Subsequently, the tubes were stored upside down at 37ºC and in the dark until colonies appeared.

Individual colonies were picked with syringes from the agar shake tubes at the highest possible dilution with colonies, inoculated in separate serum bottles (118 mL) in liquid growth medium supplemented with cellobiose (10 mM) and incubated. The purity of the cultures was verified with 16S rRNA gene sequencing as described below (see section 2.1.5). Upon confirmation of its purity, one of the cultures was chosen as the source of inoculum for all subsequent experiments.

2.1.4 Substrate utilisation screening

The isolate was grown in growth medium in serum bottles (118 mL) supplemented with the following substrates (final concentration 10 mM unless otherwise stated): glycerol, malate, 1,2-propandiol, glucose, cysteine, serine, ethanol, citrate, lactate, methanol, 2-propanol, 1-butanol, 2,3-butandiol, benzoic acid, proline, dimethylamine, formate, pyruvate, betaine, acetoin, methionine, fumarate, tryptophan, leucine, isoleucine, histidine, asparagine, acetate (25 mM), vanillate (3 mM), syringate (2 mM), ethylene glycol (5 mM), cellobiose, xylose, cellulose (5 g/L), casaminoacids (3 g/L) and tryptone (3 g/L). Except for cellulose that was autoclaved with the BM in the serum bottles, all substrates were prepared anaerobically and sterile filtered into the sterile and anaerobic serum bottles. The anaerobic preparation of the substrates was achieved by dissolving the substrates in oxygen-free water while flushing with N_2. Duplicate cultures were prepared with the respective substrates, and when necessary, negative controls without inoculum were also prepared. Additionally, negative controls containing only growth medium

and inoculum were prepared to determine the background growth of Dc1 on the yeast extract included in the BM.

The serum bottles were incubated for at least one month. Growth was assessed by visual examination of turbidity of the culture and by measuring the volatile fatty acid (VFA) production of Dc1 as a degradation product of the substrates with High-performance Liquid Chromatography (HPLC). The VFA production of Dc1 from the yeast extract in the growth medium was subtracted from the VFAs produced from the substrates when the growth of Dc1 on different substrates was assessed.

For the HPLC analysis, the samples (1 mL culture) were stored in the freezer. Subsequently, the frozen samples were centrifuged (11 000 rpm, 15 min), and, in case of pellet formation, the supernatant was transferred to a new Eppendorf tube. Following vortexing (700 µL of the sample), the samples were transferred to a new Eppendorf tube to which sulphuric acid (70 µL, 5 M) was added. The solution was sterile filtered (0.2 µM) into HPLC vials and stored at 4 ºC until the HPLC run. The HPLC run was performed with a Shimadzu 2050 Series instrument, based on an ion exclusion column (Rezex ROA – Organic Acid H+, 300x7.80 mm, Phenomenex) and detected by a UV detector at a wavelength of 210 nm. The mobile phase was 5 mM sulfuric acid (0.6 mL/min).

2.1.5 16S rRNA gene analysis

The following workflow was used to verify the purity of different cultures throughout the project.

First, DNA was extracted from the serum bottles with the DNeasy Blood and Tissue kit for gram-positive bacteria (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was quantified using Qubit fluorometer and AccuGreen Broad Range dsDNA Quantitation kit (Biotium, Fremon, USA) according to the manufacturer's instructions. Samples were stored in the freezer.

Next, the 16S rRNA gene was PCR-amplified using universal primers 8F and 1522R (Edwards et al. 1989). The PCR mixture was prepared using the protocol for *Taq* DNA polymerase or DreamTaqGreen PCR Master Mix (2X) (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The PCR settings were as follows: 95 ºC (10 min), 30 x [95 ºC (45 s), 55 ºC (30 s), 72 ºC (1 min 30 s)], 72 ºC (10 min). Following PCR, the samples were frozen or stored at 4ºC. Next, the PCR product was analysed with gel electrophoresis. The PCR product was cleaned with magnetic beads (HighPrep PCR, MagBio Genomics, Kraichtal, Germany) with some modifications from the manufacturer's instructions. The volume of the magnetic beads corresponded to 0.8x of the sample volume, and pipette-mixing of the sample with magnetic beads was increased to 10 times. The magnetic beads were washed with 180 µL of 70% ethanol. After the addition of elution buffer (EB) (modified to 20 µL), an extra incubation step of 2

minutes at room temperature (RT) was added. Throughout the whole process, the separation time for the magnetic beads was increased to 5 minutes. Following cleaning of the PCR product, DNA was again quantified using Qubit Fluorometer as described above.

The samples were sequenced with Sanger sequencing (Macrogen Europe sequencing service). The sequences were quality trimmed (UNIPRO Ugene 50, (Okonechnikov et al. 2012) and a consensus sequence was created for each pair of forward and reverse reads from the same sample (BioEdit version 7.7.1). The resulting consensus sequences were analysed in the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST) with the Core Nucleotide Database (core_nt) and 16S ribosomal RNA sequences (Bacteria and Archaea) from the rRNA/ITS databases, with uncultured/environmental samples excluded. The investigated cultures were considered pure if no background signal was visible in the chromatograms.

2.2 Abundance of *cel48* in a set of Swedish farm-based biogas plants

2.2.1 Source of samples

The samples used in the present study were collected from 22 different Swedish farm biogas plants during 2020 and 2021. These biogas plants operated with swine and cow manure as the main substrate (>90%) with different process parameters (Table A2.1, Appendix 2). Before the start of the present study, DNA had been extracted from these samples in duplicates using the FastDNA Spin Kit for Soil (MP Biomedicals Europe) as previously described (Danielsson et al. 2017). The samples were subsequently stored at -20 ºC.

2.2.2 Abundance of *cel48* in a set of Swedish farm-based biogas samples

In the present study, a functional gene-marker for cellulose degradation, *cel48*, was detected and quantified in the above-mentioned farm biogas plants with a qPCR using a standard curve prepared from one culture of Dc1. The chosen primers were designed by Rettenmaier et al (2020).

Choice of primers

The choice of primers in the present study was preceded by initial tests. Both types of primers designed by Rettenmaier et al (2020), *i.e.*, cel48_490F_I and cel48_920R_I and the primer mix (cel48-Mix2F and cel48-Mix2R) were evaluated by endpoint PCR on both a Dc1 culture (with some contamination) and a farm-

based biogas plant sample. While both primer types gave a PCR product of the expected size on the Dc1-culture, only the primer pair cel48_490F_I and cel48_920R_I gave a result for the environmental sample. Therefore, these primers were chosen for the subsequent qPCR.

Construction of the standard curve

Quantification of *cel48* was conducted with a standard curve. The standard curve was constructed with plasmids containing *cel48* extracted from a Dc1 culture (contaminated), PCR-amplified with primers cel48_490F_I and cel48_920R_I (Rettenmaier et al. 2020) and Platinum *Taq* polymerase. The product was stored in the freezer.

Following gel-electrophoresis of an aliquot of the PCR product, it was purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with a modified DNA elution step. DNA was eluted once with 15 µL EB, incubated (1 min, RT) and centrifuged (1 min). Subsequently, the product was stored in the freezer.

The PCR product was ligated into plasmids and transformed into *Escherichia coli* with the pGEM-T Easy Vector system (Promega Corporation, Madison, USA) according to the manufacturer's instructions, with some modifications for the transformation. After the incubation of the ligation reaction, the ligation products were stored in the freezer until the start of the transformation. The following modifications from the manufacturer's instructions were applied to the transformation: Top10 competent cells (Thermo Fischer Scientific, Waltham, MA, USA) were used and mixed with the ligation products in 2 mL microcentrifuge tubes. After incubation, the plates were subsequently stored at 4 ºC. The presence of inserts in individual colonies was confirmed with colony PCR and gel electrophoresis. Colony PCR was performed with the DreamTaq Green Master Mix (2X) with the following reaction mixture: DreamTaq Green PCR Master Mix (2X) (12.5 µL), primers cel48_490F_I and cel48_920R_I (0.5 µL each, 100 pmol/µL), 1 colony and PCR-H_2O to a final volume of 25 µL. The PCR programme was the same as previously described (Rettenmaier et al. 2020).

Individual colonies with inserts were cultivated in liquid LB medium supplemented with Ampicillin (100 µg/mL) (6 mL) in Falcon tubes (15 mL) (37 ºC, 17 h, 200 rpm). Plasmids were purified from these cultures with the GeneJET Plasmid Miniprep kit (Thermo Scientific, Vilnius, Lithuania) according to the manufacturer's instructions, with a modified DNA elution step with 40 µL EB. DNA concentration was measured (Nanodrop), and an aliquot of each culture was sequenced with the universal primer pair M13-pUC (forward and reverse) (Sanger sequencing, Macrogen Europe sequencing service). Samples were stored in the freezer.

The insert sequences were analysed regarding the quality of sequencing (chromatograms) and the similarity of the sequenced gene to the target (BLAST). Three different plasmids were evaluated in a qPCR run. The standard curves were prepared through a dilution series to obtain $10^1 – 10^8$ copies of plasmid/µL. qPCR was performed on QuantStudio 5 Real-Time PCR system (Applied Biosystems, Thermo Fischer Scientific, Waltham, MA, USA). The qPCR reactions contained: 10 µL ORA SEE qPCR Green ROX L Mix 2x (highQu, GmbH, Kraichtal, Germany), 0.4 µL of each primer cel48_490F_I and cel48_920R_I (100 pmol/µL) (2 µM), 1 µL plasmid DNA ($10^1 – 10^8$ copies/µL) and PCR-H_2O (8.2 µL) to a final volume of 20 µL. Each reaction was performed in triplicate. The PCR programme followed the instructions from the ORA SEE qPCR Green ROX L Mix 2x protocol with an additional extension step at 65 ºC (20 s): initial denaturation (95 ºC, 3 min), 40x [95 ºC (5 s), 60 ºC (20 s), 65 ºC (20 s)] and melting curve analysis: 95 ºC (15 s), 60 ºC (1 min), 95 ºC (1 s). The melting curve settings were determined by the machine. Based on the qPCR results of the three plasmids, one plasmid was chosen for the standard curve in the qPCR.

Quantitative PCR on a set of Swedish farm biogas samples

Quantitative PCR was performed on samples from 22 different Swedish farm-based biogas plants to investigate their abundance of *cel48*. Most samples were a pool of duplicate DNA extractions with equal DNA amounts. In some cases, however, only one of the duplicates was available for analysis.

Quantitative PCR was performed on a CFX Connect Real-Time system (BioRad, Hercules, California, USA). The qPCR reactions contained: 10 µL ORA SEE qPCR Green ROX L Mix 2x (highQu GmbH, Kraichtal, Germany), 0.4 µL of each primer cel48_490F_I and cel48_920R_I (100 pmol/µL) (2 µM), DNA (1 µL plasmid DNA ($10^1 – 10^8$ copies/µL) for the standard curve, 2 µL sample DNA (0.5 ng/µL) and PCR-H_2O to a final volume of 20 µL. Each sample was analysed in triplicate. The added amount of DNA from the farm-based biogas samples corresponded to a 1:9 – 1:89 dilution of the samples. This dilution factor was deemed suitable based on initial optimisation tests, where different dilutions of the samples were investigated in a PCR reaction. The chosen dilution factor ensured a sufficient initial amount of DNA in the qPCR reaction to obtain the product of interest while diluting the samples as much as possible to avoid inhibition of the PCR reaction from humic acid. The PCR programme followed the instructions from the ORA SEE qPCR Green ROX L Mix (2X) protocol: initial denaturation (95 ºC, 3 min), 40x [95 ºC (5 s), 60 ºC (30 s)] and melting curve analysis: 60 ºC (1 min), 95 ºC (time unspecified). The melting curve settings were determined by the machine.

Correlation of cel48 to process parameters in Swedish farm-based biogas plants

The resulting abundances of *cel48* in the farm-based biogas plants were correlated to the following process parameters in the samples: temperature, HRT, TAN, ammonium, carbon reduction and substrate carbon content with the Pearson correlation test.

3. Results

3.1 Characterisation of the isolate Dc1

3.1.1 Isolation

Within the current work, Dc1 was re-isolated to verify that the culture was pure. This was achieved with the agar shake dilution method. This method is based on serial dilution of the original culture (from which Dc1 was to be isolated) in Balch tubes containing growth medium supplemented with agarose. Single colonies of Dc1 appeared in the Balch tubes at higher dilutions. Dc1 formed round, flat and white colonies, approximately 1-2 mm in diameter (Figure 3).

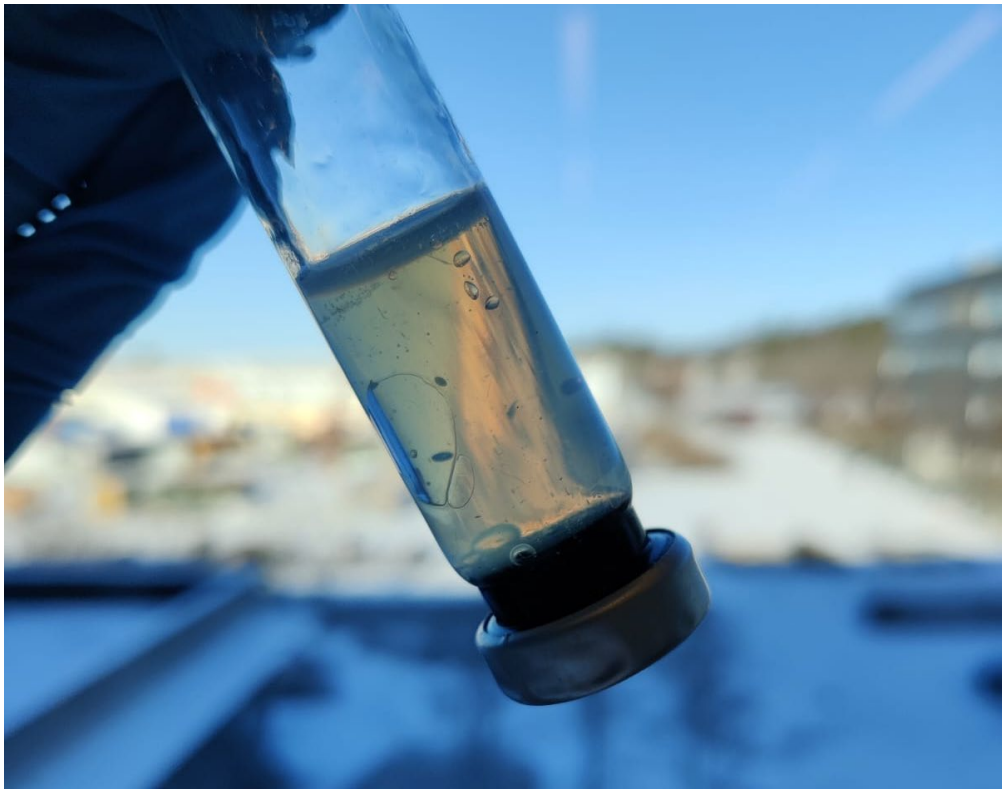

Figure 3. Individual colonies of Dc1 in an agar shake dilution tube.

3.1.2 Substrate utilisation screening

Growth of Dc1 was examined on a range of different substrates (alcohols, carbohydrates, amino acids, carboxylic acids, methoxybenzoates, and one amine) and assessed by visual inspection of turbidity and by measuring the substrate degradation products (VFAs) with HPLC.

Based on visual inspection, fast growth occurred with glucose, xylose, cellobiose and cellulose (Figure 4). Growth on cellulose (not with other substrates) produced a yellow colour in the culture. Based on results from HPLC, weak growth occurred with fumarate, methionine and tryptone (Table 1; Table A1, Appendix 1). During growth with these three substrates, the culture produced acetate, butyrate and isovalerate (Figure 5). No growth was observed with any of the other substrates, i.e. with alcohols, most amino acids and carboxylic acids, the methoxybenzoates and the amine.

However, the culture was contaminated during the substrate utilisation screening (see section 3.1.3). Therefore, it cannot be concluded whether the observed growth with fumarate, methionine and tryptone was due to growth Dc1 or a contaminant species. Since growth of the culture occurred on cellulose and the carbohydrates, these cultures were never analysed with HPLC. Additionally, the results for serine, glycerol and betaine were unclear due to inconsistencies in VFA production between the duplicates, *e.g.* some cultures indicated growth and some did not (Table A1, Appendix 1).

Figure 4. Growth of the culture in cellobiose (left) and cellulose (right) in serum bottles. Growth on cellulose produced a yellow colour in the culture.

Table 1. Growth of Dc1 on different substrates. Growth, assessed by both visual inspection of turbidity and HPLC analysis, was scaled as follows: no growth (-), weak growth (+), moderate growth (++), rapid growth (+++) and not determined (ND).

Substrate	**Growth**	***Substrate***	**Growth**
Glycerol¤	ND	*Betaine*¤	ND
1,2-propanediol	-	Tryptone*	+
Ethanol	-	*Glucose**	ND
Methanol	-	*Xylose**	ND
2-propanol	-	*Cellobiose**	ND within this work
1-butanol	-	*Cellulose**	ND within this work
2,3-butandiol	-	*Syringate*	
Ethylene glycol	-	*Vanillate*	-
Acetoin	-	*Acetate*	-
Asparagine	-	*Fumarate**	-
Histidine		*Pyruvate*	+
Cysteine	-	*Citrate*	-
Serine¤	ND	*Lactate*	-
Proline	-	*Benzoic acid*	
*Methionine**	-	*Formate*	-
Tryptophan	-	*Malate*	-
Leucine	-	*Dimethylamine*	
Isoleucine	-		
Casaminoacids	-		

* Need to be confirmed with a pure culture of Dc1 due to contamination of the inoculum.

¤ Growth could not be determined due to inconsistencies between the duplicates in HPLC.

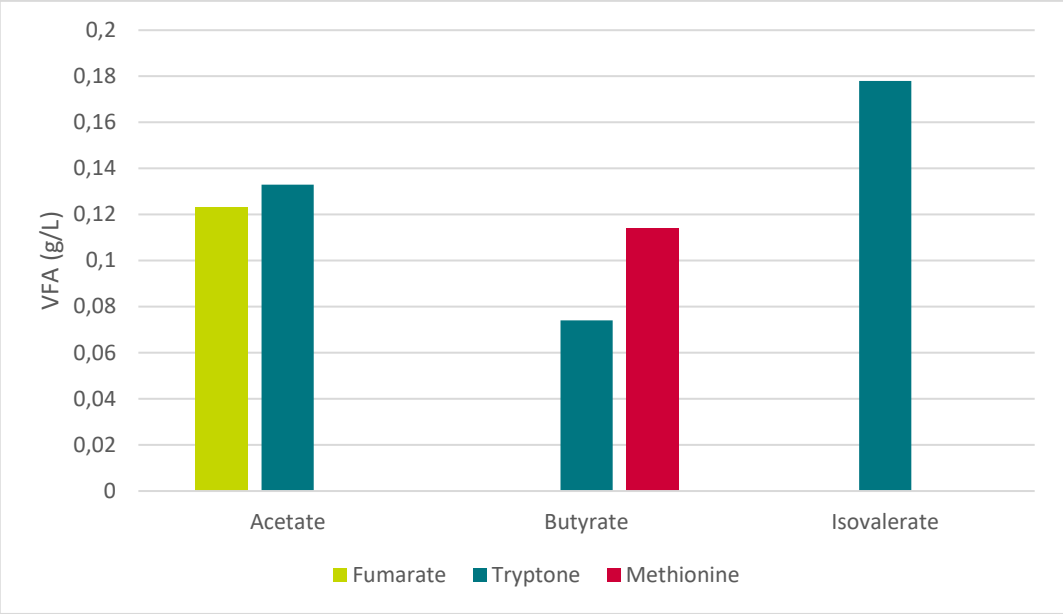

Figure 5. VFA production of the culture in the substrate utilisation screening. Values presented correspond to VFA production in addition to what was produced from the background growth of the culture on yeast extract in the growth medium.

3.1.3 16S rRNA gene analyses

16S rRNA gene sequencing analyses were performed to confirm the taxonomic identity and purity of the cultures. The 16S rRNA gene of Dc1 exhibited at most 98.54% similarity to *A. cellulolyticus* strain HPc. In the 16S rRNA gene database (BLAST), Dc1 also exhibited close similarity to *A. cellulolyticus* strain CD2 (96.27%) and other species from the genus *Acetivibrio* (family Oscillospiraceae, class Clostridia, phylum Bacillota) such as *A. aldrichii*, *A. straminisolvens*, *A. clariflavus* and *A. thermocellus*.

Throughout the characterisation of Dc1, contaminations occurred multiple times in both inocula and sample bottles from the substrate utilisation screening. The contaminations were recognised through growth in negative controls, contaminated 16S rRNA gene sequences, as well as background signal in the sequencing chromatograms. Interestingly, all contaminated bottles analysed with 16S rRNA gene analysis appeared to contain the same contaminant species, exhibiting up to 95.5% similarity to *Thermicanus aegyptius* strain ET-5b.

Due to the constant problems with contamination, two separate isolations of Dc1 were made from the same set of agar shake dilution tubes. Interestingly, the cultures from both sets of isolations initially contained *A. cellulolyticus* (90.41 – 98.54% similarity), but, after incubation of at least one month, the cultures instead contained *T. aegyptius* (89.36-95.53% similarity) (Table 2). This suggests that the contaminant species took over the cultures first at a later stage. There was, however, an exception to this pattern with a Dc1-culture that still exhibited the highest

similarity to *A. cellulolyticus* after 6 weeks of incubation. The contaminant was also discovered in a cellobiose stock solution and another bottle containing the same cellobiose (neither was inoculated with the culture) (Table 2).

Table 2. Results of the 16S rRNA gene analyses of various Dc1-cultures in the characterisation.

Culture	**Time of incubation**	**BLASTn (core_nt) (% similarity)**
Isolation nr 1	2 days	*A. cellulolyticus* strain HPc (98.54)
	2 months	*T. aegyptius* strain ET-5b (93.06)
Isolation nr 2	2 days	*A. cellulolyticus* strain HPc (92.1)
	1 month	*T. Aegyptius* strain ET-5b (95.53)
Cellobiose stock solution	Not inoculated	*T. Aegyptius* strain ET-5b (93.73)
Cellobiose	Not inoculated	*T. Aegyptius* strain ET-5b (93.43)
Dc1 culture	6 weeks	*A. cellulolyticus* strain HPc (93.7)
Cellulose (substrate utilisation test)	5 weeks	*T. aegyptius* strain ET-5b (89.36)

3.2 Abundance of *cel48* in a set of Swedish farm-based biogas plants

The abundance of a functional gene marker for cellulose degradation, *cel48,* was analysed in Swedish farm-based biogas plants. Quantification of *cel48* was achieved with a qPCR using a standard curve constructed with plasmids containing inserts with the gene of interest.

3.2.1 Performance of the standard curve

Analysis of the plasmids used for the standard curve

Before the construction of the standard curve for qPCR, plasmids cloned in *E. coli* were sequenced to verify that they had an insert containing the gene of interest. The selected plasmid contained an insert with the highest similarity to glycosyl hydrolase family 48 proteins (up to 100% identity), as well as an endoglucanase. Taxonomically, the sequence was most similar to a sequence from *A. clariflavus* but also resembled sequences from e.g. *A. cellulolyticus, Anaeromicropila populeti* and uncultured species.

Performance of the standard curve

The standard curve was based on a dilution series of the plasmid with known absolute quantities of the gene (10^1 – 10^8 copies/µL). The efficiency of the standard curve was 101.4% (after removal sample with 10^1 gene copies and one replicate from the dilution with 10^7 gene copies), and the R^2-value of the linear regression was 0.994 (Figure 6). The standard curve had two melt peaks; one at 80.5 – 81.5 ºC and another at 87 – 88.5 ºC (Figure 7). The first peak was visible for all concentrations, while the second peak was only visible for 10^3-10^8 gene copies. The variance between the replicates increased with decreasing DNA concentration (Figure 7).

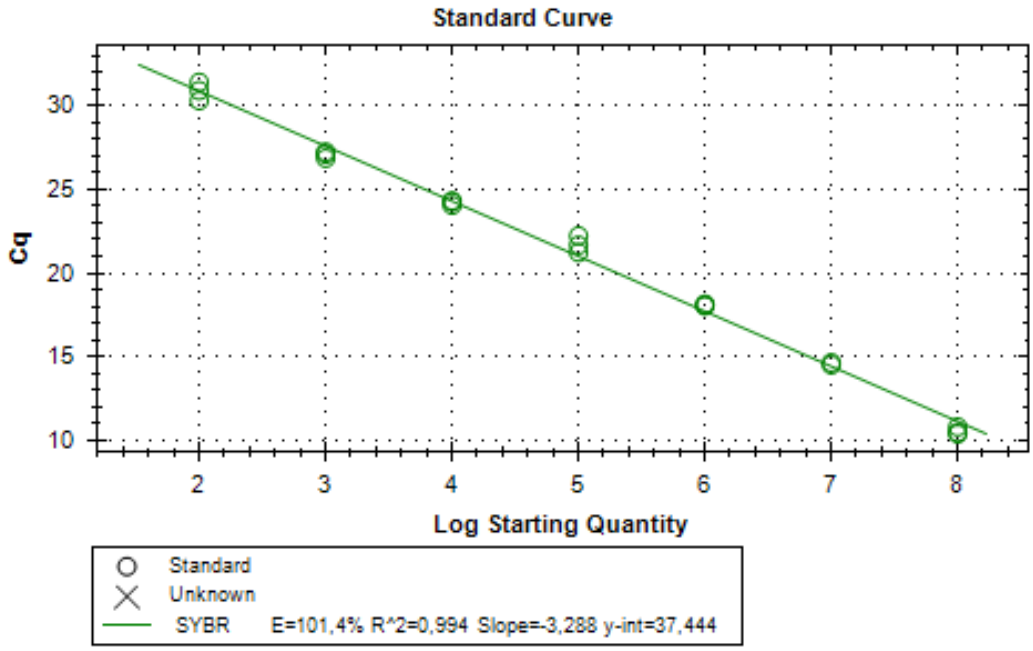

Figure 6. Standard curve for the qPCR with 101.4% efficiency, R^2 0.994 and slope -3.288 after removal of all values from 10^1 copies and one of the 10^7 copies.

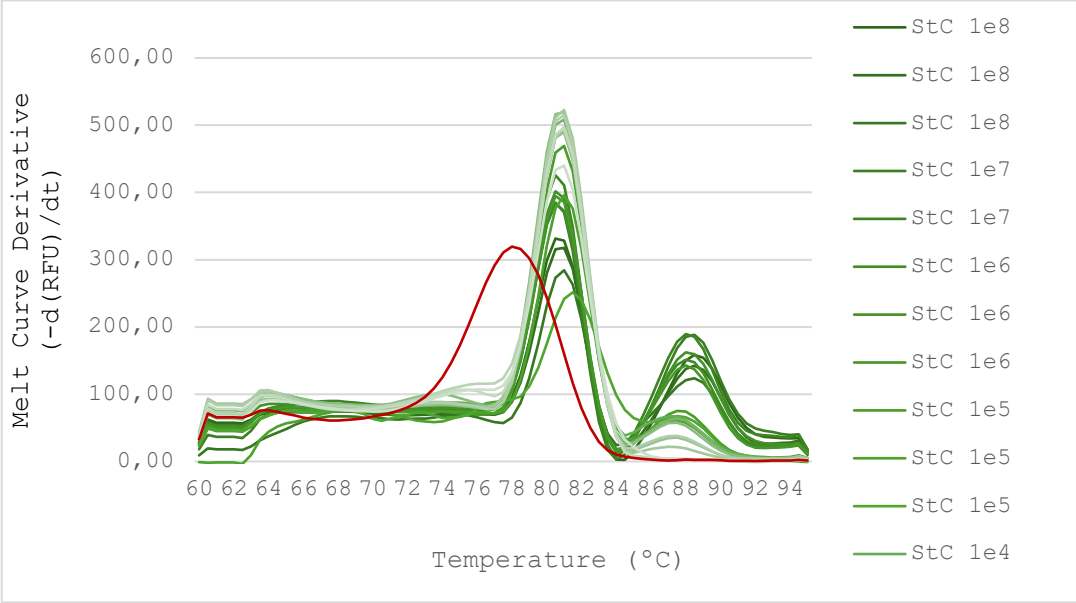

Figure 7. Melt peak for the standard curve. Two peaks are visible: one at 80.5 – 81.5 ºC and another at 87-88.5 ºC. The peak for the negative control (red) appeared at around 78.5 ºC.

3.2.2 Abundance of *cel48* in the farm-based biogas samples

The absolute abundance of the functional marker for cellulose degradation, *cel48*, was measured in a set of DNA samples extracted from different Swedish farm-based biogas plants.

The DNA samples had a variety of different melt peaks (Figure A2.1, Appendix 2). The most abundant melt peak occurred at 84-85.5 ºC (Figure 8). For most of these samples, this peak was clear and the only one. Instead of a single peak, some samples had several smaller peaks within the temperature range of around 72-95 °C Figure 9). These smaller peaks coincided with the first and second peaks from the standard curve and the major peak from the samples in different variations. Some of these samples also had a distinct peak at 89 °C.

The negative control and a few sample replicates gave peaks at 77.5 – 79.5 ºC (Figure 8; Figure A2.1, Appendix 2). The melt peaks from the biogas plants I and K varied significantly between the replicates. One of the replicates from K was removed from the analysis as it coincided with the negative control.

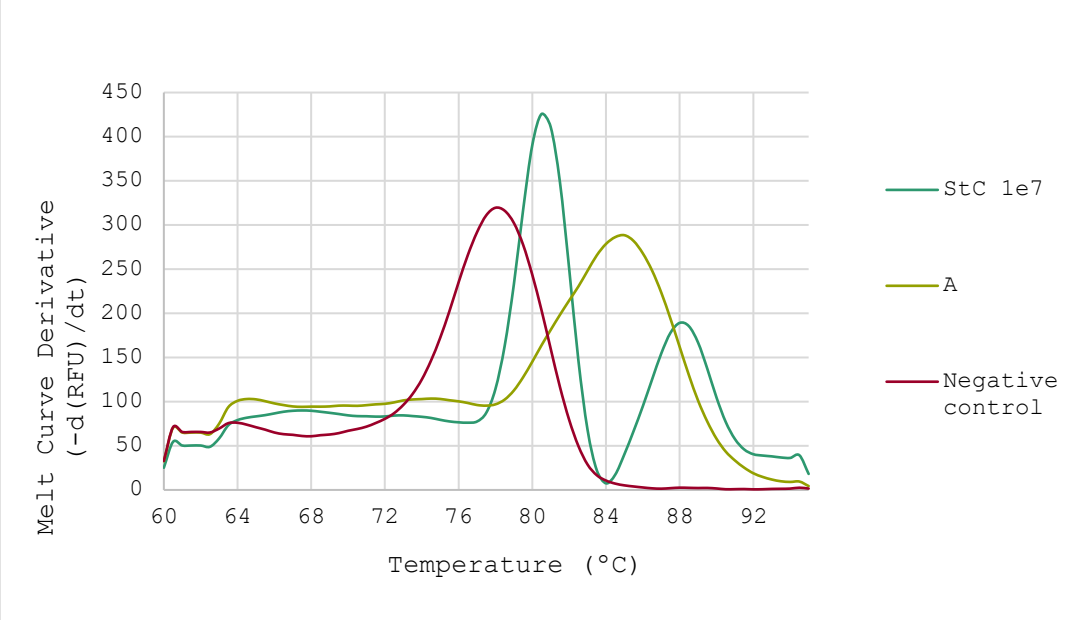

Figure 8. Representative melt peaks from the qPCR. Negative control (78 ºC), Standard curve (80.5 ºC and 88.5 ºC) and farm-based biogas sample (represented by A) (85 ºC).

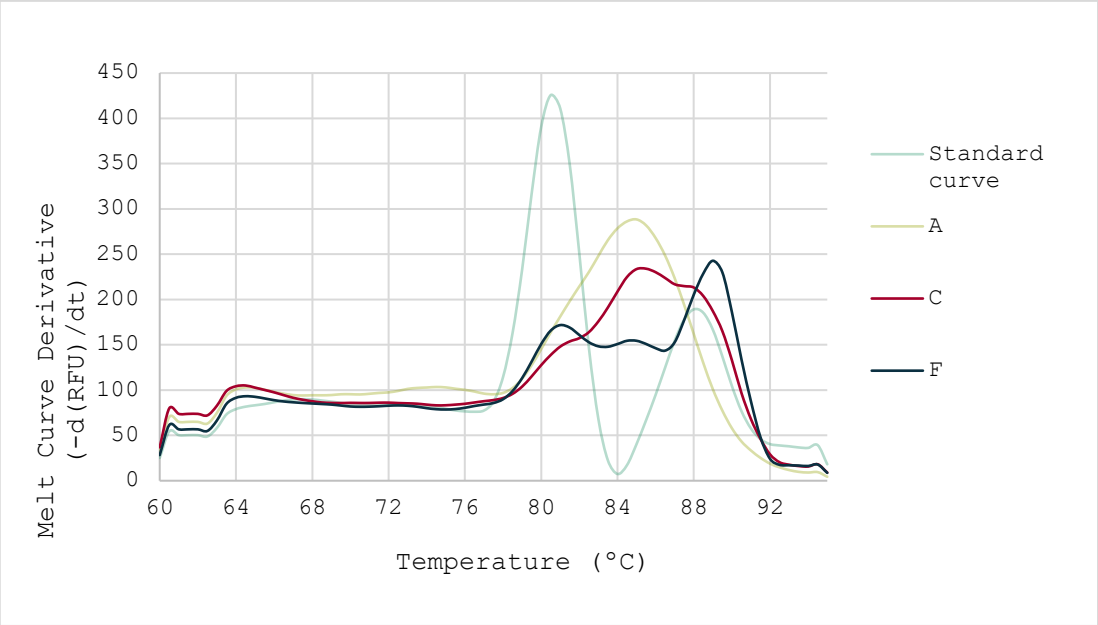

Figure 9. The melting curves from C and F are examples of alternative melt peaks from the farm-based biogas plant samples, compared to the standard curve and the most common peak from the samples (represented by A).

Gel electrophoresis was performed on all PCR products from the qPCR to verify that all samples contained the right product. The gel electrophoresis showed that all samples from the standard curve and the farm-based biogas samples had a distinct band of the desired length (367 bp) (Figure 10). Unspecific amplification occurred in samples K (shorter than the main band) and L (longer than the main band). The presence of these extra bands was disregarded in the analysis of the qPCR.

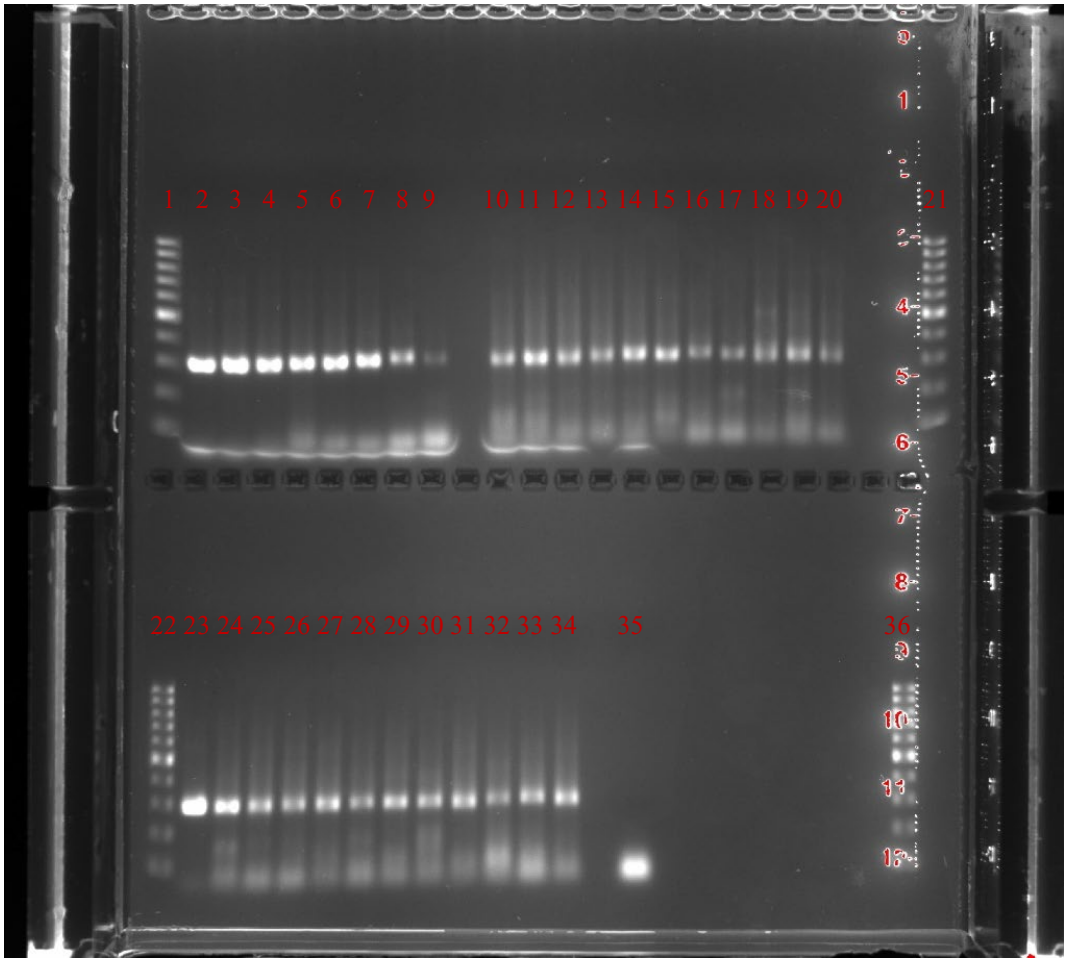

Figure 10. Gel electrophoresis of the qPCR products after amplification of cel48 in different farm-based biogas plants. The gel was loaded with ladder (100 bp) (1, 21, 22, 36), standard curve in decreasing DNA amount from 10^8 – 10^1 copies (2-9), farm-based biogas samples in the following order: A, C, D, E, F, G, I, K, L, M, N, O, P, S, U, V, X, Y, Z, Å, BB, Ö (10 -20; 24 – 34), standard curve 10^8 copies for reference (23) and negative control (35). Unspecific amplification occurred in samples K (17) and L (18).

According to the qPCR, the farm-based biogas plants contained $7 \cdot 10^2 – 1 \cdot 10^4$ copies *cel48*/ng DNA (relative amount) and $7 \cdot 10^5 – 1.5 \cdot 10^7$ copies *cel48*/mL digestate (absolute amount) (Figure 11; Table A2,2, Appendix 2). The standard deviation was <16% for all biogas samples (relative and absolute values) except for G (±23%, ±27%) and I (±135%, ±165%) (relative and absolute values, respectively). No standard deviation could be determined for K, as one of the triplicates was removed as an outlier.

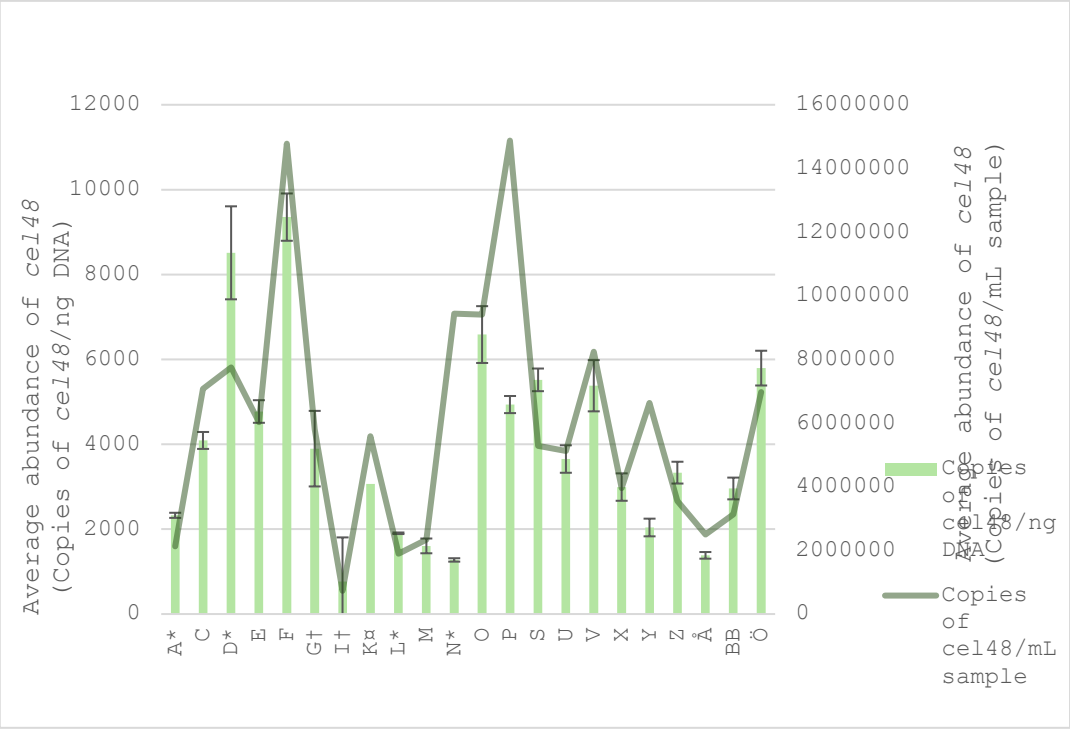

*Figure 11. Abundance of cel48 in a set of Swedish farm-based biogas plants. Abundance is presented in relative (copies cel48/ng total DNA) and absolute (copies/mL digestate) terms. * Only one of the two duplicate DNA extractions was analysed. † Standard deviation ≥23% (relative and absolute amount). ¤ No standard deviation is available for this sample (only duplicates from the qPCR were included).*

The abundance of *cel48* in the farm-based biogas samples was correlated to a set of process parameters. This was achieved by both plotting the values as well as investigating the statistical significance with the Pearson correlation test.

There was a significant correlation between the relative and absolute amounts of *cel48* in the samples ($p < 0.001$) (Figure A2.3, Appendix 2); however, some samples did not follow this trend. The sample from the farm-based biogas plant D had a comparatively high relative abundance of the gene, while its absolute amount was comparatively low. The inverse trend was identified in the samples from the farm-based biogas plants N, P and Y, which had a comparatively low relative amount of the gene, while the absolute amount was comparatively high.

The relative abundance of *cel48* was positively correlated with HRT ($p < 0.001$) after the removal of plant E, which was regarded as an outlier (Figure 12). There was also a negative trend with *cel48* and increasing total ammonium-nitrogen (TAN) (NH_4^+-N) in the digestate (more pronounced in the relative values than the absolute) (not statistically significant) (Figures A2.4 and A2.5, Appendix 2). No trends or statistical significance could be observed between the abundance of *cel48* and ammonia levels in the digestate, carbon reduction (%), substrate carbon content or the process temperature.

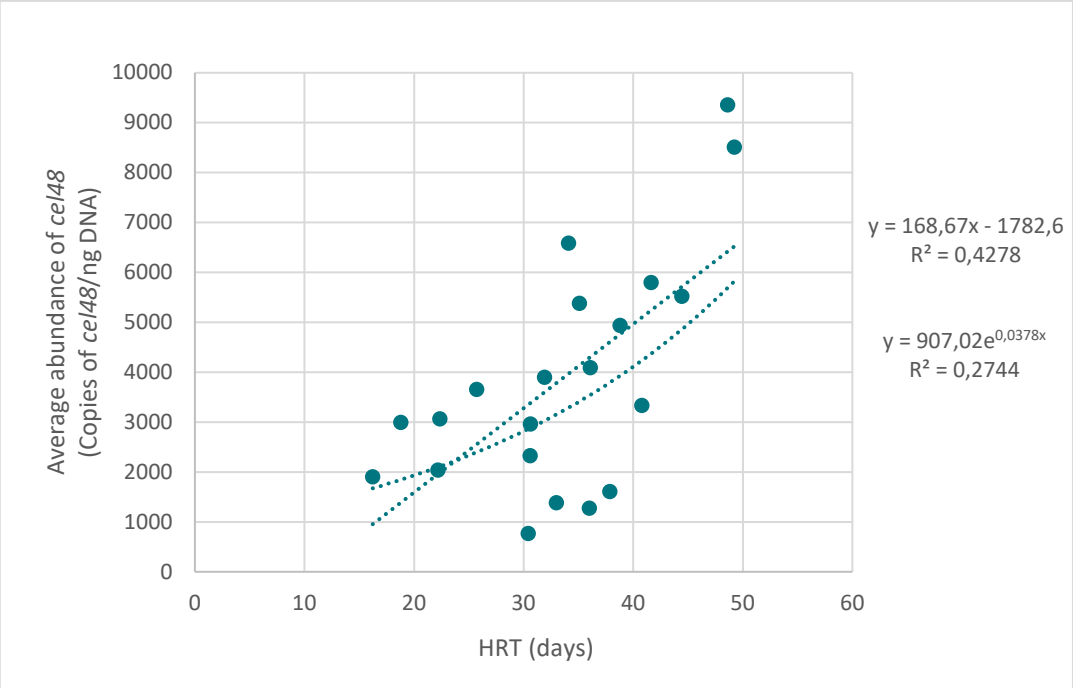

Figure 12. Relative abundance of cel48 in the farm-based biogas samples in relation to HRT (biogas plant E excluded).

4. Discussion

This study investigated the cellulose-degrading bacterial community in biogas systems using two different methods. In the first part of the study, the cellulose-degrading bacterium Dc1 was isolated and characterised using culture-dependent methods. In the second part of the study, the cellulose-degrading bacterial community was studied from a molecular point of view where a functional gene marker, *cel48*, was quantified and correlated to process parameters in a set of Swedish farm-based biogas plants.

4.1 Characterisation of the isolate Dc1

4.1.1 Substrate utilisation screening

The results from the substrate utilisation screening suggest that Dc1 is mainly a cellulolytic and saccharolytic species, with some ability to degrade amino acids (methionine and tryptone) and carboxylic acids (fumarate). Growth of the studied culture on cellulose produced a yellow colour, typical for the yellow affinity substance (YAS), involved in cellulose degradation (Ljungdahl et al. 1988; Kopečný & Hodrová 1997). This supports the fact that Dc1 can degrade cellulose.

However, due to the contamination of the culture, the results for the substrates in which growth was observed must be validated with a pure culture of Dc1, as the growth could be attributed to the contaminant and not Dc1 (see section 4.1.2). The growth of Dc1 on serine, betaine and glycerol must also be validated with a pure culture of Dc1 due to inconsistent results between the duplicates. No growth of the culture was observed with the other substrates tested, indicating that Dc1 does not degrade the investigated alcohols, most amino acids and carboxylic acids, as well as the methoxybenzenes and the amine.

Comparison of substrate utilisation to other cellulolytic bacteria

The substrate utilisation results should be compared to those of species closely related to the strain of interest (based on the 16S rRNA gene sequence). As mentioned in the results, the 16S rRNA gene sequence for Dc1 was closely similar

to several species within the genus *Acetivibrio*. This genus contains several species that produce cellulosomes (Minor et al. 2024).

A. cellulolyticus, the type species for the *Acetivibrio* genus, is a purely cellulolytic species that can degrade cellulose, cellobiose and salicin (Patel et al. 1980). Simpler sugars like glucose and fructose do not support its growth. For growth on both cellulose and cellobiose, fermentation products are H_2, CO_2 and acetic acid. Degradation of cellulose also produces ethanol, while for cellobiose, propanol and butanol are produced.

The other species to which Dc1 exhibits the closest similarity also only degrade cellulose and cellobiose, such as *A. clariflavus* (Shiratori et al. 2009) and *A. mesophilus* (Rettenmaier et al. 2019). Other species within the *Acetivibrio* genus degrade additional compounds, such as laminarin (*A. straminisolvens* (Kato et al. 2004)) and xylan (*A. aldrichii* (Yang et al. 1990)). While the ability to degrade cellulose and cellobiose of *A. thermocellus* has been proven several times (McBee 1954; Ng et al. 1977), the results regarding its utilisation of simpler sugars have been conflicting. Some studies found *A. thermocellus* unable to degrade any simpler sugars (Ng et al. 1977), while other studies found it to utilise xylose (McBee 1954) or glucose and fructose (Patni & Alexander 1971a; b). This difference may be attributed to differences in the growth media; for instance, Patni and Alexander (1971b) confirmed that *A. thermocellus* was able to degrade glucose during growth in a medium containing higher amounts of yeast extract compared to the growth medium used in previous studies.

The fermentation products of Dc1 on cellulose and sugars were never measured with HPLC due to the contamination of the culture. Therefore, no conclusions can be drawn on its production of VFAs from these substrates within this study. The HPLC results, however, showed that it produced acetate, butyrate and isovalerate from amino acids and carboxylic acids. The ability of its closest relatives to degrade these substrates is unknown due to the limited range of substrates tested in their respective characterisations.

As mentioned in the introduction (see section 1.1.5), a previous Master's thesis indicated the ability of Dc1 to grow with a range of different carbohydrates in addition to cellulose and cellobiose, *i.e.* the pentavalent and hexavalent sugars glucose, ribose, fructose and xylose (Sjöberg 2023). This ability to degrade simple sugars does not seem to occur in its closest relatives.

The observed growth of the culture on tryptone, fumarate and methionine could be attributed to the contaminant. However, it should be noted that the comparison between the substrate utilisation of Dc1 and its closest relatives is limited by the number and range of substrates included in the characterisations of Dc1's most similar relatives; most of these characterisations only investigated the growth of the isolates on a few carbohydrates. Therefore, it is possible that the Dc1's closest relatives would exhibit similar substrate utilisation characteristics to Dc1 if a wider

range of substrates were to be tested on them. The requirements for a valid characterisation of a novel species have changed and increased over time. Therefore, many older characterisations contain less information about the isolates than is required today. This could explain why much information on the substrate utilisation of Dc1's closest relatives is still missing, given that many of them were published many years ago. The suggested ability of Dc1 to degrade substrates other than cellulose and sugars shows that testing a wider range of substrates can reveal abilities of isolates to degrade unpredicted substrates. It is also interesting that Dc1 appears to be able to degrade a wider range of carbohydrates than its most closely related species, supporting the hypothesis that Dc1 is a novel species.

4.1.2 Contamination of the culture

The contamination of the culture and its impact on the characterisation results are discussed in the section below. As previously mentioned, all contaminations confirmed with 16S rRNA gene sequencing indicated that the contaminations were caused by the same contaminant species. Although the obtained 16S sequences were less than 97% similar to *T. aegyptius*, the observed level of similarity (at most 95%) still suggests that the contaminant physiologically resembles that species.

As mentioned in section 3.1.3, with only one exception, all Dc1 cultures analysed with 16S rRNA gene sequencing at least one month after incubation had been outcompeted by the contaminant (Table 2). The culture that still exhibited the highest similarity to *A. cellulolyticus* based on 16S rRNA gene sequencing after 6 weeks had a background signal in the chromatogram, indicating a contamination of that culture, as well. A possible explanation for the fact that the contaminant was only detectable a certain time after inoculation is that it utilises the mono- or oligosaccharides released after cellulose degradation by Dc1, rendering the circumstances favourable for growth only at a later stage of the incubation. This is supported by the fact that *T. aegyptius* degrades cellobiose and glucose, but not cellulose (Gössner et al. 1999). It is still possible that the contaminant degrades cellulose however, as 16S rRNA gene sequencing could not confirm that the contaminant belongs to *T. aegyptius*.

The contaminant was also found in other serum bottles that had not been inoculated with the Dc1 culture. It was later clarified that a cellobiose stock solution was contaminated. Most of the contaminated cultures had been in contact with or grown on this contaminated cellobiose. Additionally, a negative control containing this cellobiose stock solution was also contaminated. This strongly points to the cellobiose stock solution as the source of the contamination.

Yet, the contaminated cellobiose stock solution cannot explain all the observed contaminations. The cultures from the second isolation were also contaminated despite not being grown on the contaminated cellobiose stock solution, suggesting

that the contaminant was present in the culture already from the isolation of the individual colonies. This could be due to a limitation of the agar shake dilution method, where there is a risk of the needle encountering other species when single colonies are picked. Thus, the contaminant could have been present in the agar shakes and could not be separated from Dc1 during the isolation. Alternatives to the agar shake dilution method can be used for the isolation of anaerobic microorganisms (Hanišáková et al. 2022). For instance, colonies can be plated on a petri dish and inoculated in an anaerobic environment, such as an anaerobic glove box. In this method, it is easier to pick single colonies of the bacterium of interest without encountering colonies from other species compared to the agar shake dilution method, reducing the risk of contaminating the culture during the isolation.

Nevertheless, Dc1 has previously been successfully isolated with the agar shake dilution method (Sun 2015). Therefore, it seems most plausible to conclude that the contaminant species entered the culture from the outside, perhaps from the lab environment or due to cross-contamination from previous experiments. For example, there is a risk of remaining spores from previous experiments in the rubber stoppers used to seal the serum bottles, which can be reactivated upon contact with the new medium. The rubber stoppers are autoclaved between experiments, but the current cleaning routine might not be sufficient to clean them of spores. Although *T. aegyptius* does not form spores (Gössner et al. 1999), there is still a possibility that the contaminant is sporulating. The contaminant was mostly observed in sugars, confirming the pronounced difficulty in retaining a pure culture in substrates on which many species can grow. Although the cultures and solutions were always contaminated with the same species, there was not always a clear pattern regarding which cultures were contaminated, and the reason for the contamination could not always be understood. Most probably, both the contaminated cellobiose stock solutions, together with cross-contamination from previous experiments, caused the contamination of the Dc1 culture.

Additionally, using a newly prepared cellobiose stock solution led to growth in only some negative controls but not all. Growth also occurred in two negative controls containing a glucose solution. The negative controls came from different batches of growth medium, C1 and C2 (however, they were always the same as the corresponding bottle to which it was a negative control), suggesting that contamination can also originate from other sources, such as the C1 or C2. Residual spores in the rubber stoppers may also have led to sporadic contamination, affecting individual serum bottles inconsistently. As these serum bottles were never investigated with 16S rRNA gene sequencing, the taxonomic identity of the contaminant in these bottles is unknown. If such an analysis were to show the highest identity to *T. aegyptius*, it would provide further evidence that the contaminant species entered the culture from the outside and that it did not follow

from the isolation. Intendedly, the recurring problem with contamination illustrates the challenge with anaerobic isolations and cultivations.

4.1.3 Remaining analyses for a complete characterisation of Dc1

The contamination of the culture significantly decreased the number of characterisation tests that could be performed on Dc1 within the present study, leaving most of the characterisation tests remaining for future work. To proceed with the characterisation, a pure culture of Dc1 must first be obtained. As the cultures from both isolations from the same set of agar shake dilution tubes were contaminated, it might be necessary to either prepare new agar shake tubes from which Dc1 can be isolated, or to use a different method for the isolation.

Once a pure culture of Dc1 has been established, the remaining characterisation tests can be performed. First, the growth of Dc1 on the substrates that need to be confirmed from the present study should be repeated (*i.e.,* glucose, xylose, cellulose, cellobiose, fumarate, methionine and tryptone). Additionally, growth on xylan should be tested.

Given that Dc1 exhibited a high similarity (up to 98.5%) to *A. cellulolyticu*s based on 16S rRNA gene analysis, the characterisation should focus on determining whether Dc1 belongs to the same species. This will be elucidated by repeating the same characterisation tests published in the characterisation for *A. cellulolyticus* (Patel et al. 1980) on Dc1, as well as comparing their whole genomes.

Should this comparison suggest that Dc1 represents a novel species, another set of characterisation assays will be required for a complete and valid characterisation of Dc1 according to the prevailing requirements from the IJSEM (Microbiology Society). This includes presenting the complete genome sequence and its characteristics, such as the GC-content, N50 and number of tRNA and rRNA genes. A whole-genome sequencing with Nanopore of Dc1 and two of its closest relatives (*A. cellulolyticus* and *A. clariflavus*) was planned within this work. The closest relatives were ordered from Leibniz Institute DSMZ German Collection of Microorganisms and Cell Cultures GmbH and inoculated in the appropriate medium according to the DSMZ instructions. However, the cultures were contaminated due to improper autoclaving of the growth medium, and the experiment could not be performed.

Other analyses required for the characterisation include a phylogenetic analysis based on genomic information (16S rRNA gene or the whole genome sequence), morphological characteristics (studied using microscopy), growth characteristics (temperature- and pH-optima, tolerance to oxygen and salinity), and, although not required but recommended, chemotaxonomy (such as fatty acid analysis of the cell wall). These characteristics should be compared to those of similar species (*e.g.*, *A. cellulolyticus*, *A. clariflavus*, *A. aldrichii* and *A. straminisolvens*) to elucidate

whether Dc1 is a novel species. Additionally, a viable culture of Dc1 must be deposited in at least two culture collections in different countries and be available without restrictions.

4.2 Abundance of *cel48* in a set of Swedish farm-based biogas plants

4.2.1 Evaluation of the methodological choice

This section will shortly discuss the advantages and limitations of the chosen method from a larger point of view.

The chosen method was based on the detection and quantification of a functional genetic marker for cellulose degradation. The fact that this gene is directly linked to the function of interest, *i.e.* cellulose degradation, renders the chosen method preferred over 16S rRNA gene sequencing studies with universal primers when the function of microbial communities is of interest. While providing direct taxonomic information, 16S rRNA gene-based sequencing studies remain dependent on rarely available relevant and high-quality information from databases for inferring the function of the identified taxa (Pereyra et al. 2010). This is further complicated by the fact that physiological properties can vary widely between strains with highly similar 16S rRNA gene sequences (>99%) (Ash et al. 1991; Vilas-Bôas et al. 2007), and that databases risk containing insufficient information to distinguish potentially functionally divergent sequences on lower taxonomic levels.

When the functional approach is used with qPCR, it is, however, not possible to completely secure taxonomic affiliation. This information can be gained by complementing the analysis with other techniques, such as Illumina sequencing (Rettenmaier et al. 2020), thus giving information on both the similarity to other *cel48* and an indication of the taxonomy of the sequences (Rettenmaier et al. 2020).

Instead of focusing on a larger group of cellulose-degrading bacteria, it would also have been possible to focus the study on the abundance of specific cellulose-degrading subcommunities, *i.e.* a specific species or members of a certain family, for instance. Studies have, for instance, used primers specific for Fibrobacter to study cellulose degradation in municipal landfill sites (McDonald et al. 2008, 2012). This approach would elucidate more clearly how a specific set of cellulose-degrading microorganisms reacts to different process parameters compared to the chosen functional gene approach. However, that approach can be challenging for the study of microorganisms from complex environments, requiring the development and optimisation of the primers, which is potentially both time-consuming and practically challenging, and was thus considered outside the time-scope of this study. Furthermore, given that the cellulose-degrading microbial

community can differ between biogas processes, the study of a specific subgroup of this community in a specific biogas process might not offer any insights into the more general knowledge of the cellulose-degrading bacterial community, but risks being constrained to the specific process under the specific circumstances where it was investigated.

4.2.2 Evaluation of the standard curve

Results confirmed that the plasmid insert represented the gene of interest and that the sequence resembled those from known anaerobic cellulose-degraders. However, many BLAST results illustrated closest similarity to uncultured bacteria, reiterating the need for further characterisation of the cellulose-degrading bacterial community in biogas systems.

As mentioned in the results, the quality of the standard curve was high regarding its efficiency and R^2-value. However, the standard curve had two melt peaks instead of a single one. The presence of two melt peaks indicated that the quantification by the standard curve was based on more than one DNA sequence and that the standard curve registered signal from both these sequences instead of from only *cel48*. This can have caused an erroneous quantification of *cel48* within the present study.

A few results and observations indicate that the second melt peak represents the dissociation temperature of the product of interest, while the first peak is probably caused by primer dimers (Rettenmaier et al. 2020). The first melt peak had a considerable overlap with the melt peak from the negative control (Figure 7), which was most probably caused by primer dimers. Still, the PCR products obtained from the standard curve only had one band on the agarose gel, indicating that mainly the right product was produced in the PCR and that no unspecific amplification occurred in the standard curve (Figure 10). The agarose gel showed that, with decreasing DNA amounts, the proportion of primer dimers in the samples increased. Similarly, the first melt peak decreased with increasing concentrations, while the second peak increased with decreasing concentration (Figure 7). The melt peak shows how fast DNA dissolves at the given temperature. Although there is no direct relationship between the concentration of the DNA and the height of the melt peak, it can still give a hint of the concentration of this specific sequence.

The study that designed the primers used in the current work reported a dissociation temperature of the product at 90 ºC (using primer mix cel48-Mix2), which is relatively close to the melt temperature of the second peak within the present study (87- 88.5 ºC) (Rettenmaier et al. 2020). Furthermore, that study observed dissociation of primer dimers between 76-83 ºC in all samples, further supporting the theory that the first melt peak in the current study was caused by primer dimers.

To prevent the registration of signal from the primer dimers, Rettenmaier et al (2020) added a dissociation step at 83 ºC in each cycle during the quantification. This could also be included in the present analysis to increase the accuracy of the quantification of *cel48*. Additionally, the amount of primers used in the PCR reaction could be decreased as a strategy to reduce problems with primer dimer.

4.2.3 Evaluation of the results from the farm-based biogas plants

Overall observations

As previously discussed, the quantification of *cel48* in the farm-based biogas samples might not be correct, as the standard curve was disturbed by a signal from primer dimers. Yet the obtained absolute values (copies *cel48*/mL sample) from the present study are within the same range as the previous study investigating the abundance of *cel48* in biogas plants with cel48-Mix2 (Rettenmaier et al. 2020). Additionally, all samples were located close to each other in the standard curve (Figure A2.2, Appendix 2), meaning that they were subject to the same systematic error of quantification from the standard curve. This indicates that, although the quantification might not be correct, the comparison of the gene abundance between the samples is still accurate and relevant.

Most of the samples did not have the same dissociation temperature as the standard curve. The shift in the melting peaks could be attributed to variations of the gene, caused by differences in the GC-content that alter the melting temperature of DNA (Borisova et al. 1993). The fact that some samples had multiple melting peaks could indicate that they contain several variants of *cel48*, perhaps coming from different species. However, although this is rare among cellulose-degrading microbes, studies have also found that both *A. thermocellum* and *A. straminisolvens* have two different *cel48* genes (Izquierdo et al. 2010). The multiple peaks could partly be explained by the fact that degenerated primers were used, as they can amplify a larger range of gene variants.

The most abundant peak from the samples at 84-85.5 ºC was quite far away from both peaks on the standard curve. Still, the gel electrophoresis confirmed the presence of a clear band of the desired length in all samples (Figure 10), indicating that the correct PCR product was produced and quantified. However, Sanger or Illumina sequencing of the PCR products would be necessary to confirm that the PCR product from the samples is the same as was quantified by the standard curve.

The precision was high for most samples (±<16%), except for samples G and I (high standard deviation) and K (which lacked standard deviation). The indications of unspecific amplification in samples I and K (Figure 10) can be a source of error for these samples. The presence of extra bands for these samples was disregarded

in the analysis of the qPCR, but this lowers the reliability of the results for these samples and could be a source of error.

Interestingly, the relative and absolute amounts of *cel48* were comparable between the samples. This indicates that the cellulose-degrading bacterial community represents a similar part of the total DNA in their respective systems and that the biogas plants have similar absolute quantities of *cel48*, *i.e.*, comparable cellulolytic potential (Figure 11). As previously mentioned, the relative and absolute amounts of the gene for biogas plants D, N, P and Y did not correlate to the overall trend (Figure 11; Figure A2.3 Appendix 2). No information from either any of the process parameters or the 16S data was found that could explain this. This suggests that the abundance of *cel48* is dependent on many factors together, rather than single ones.

Correlations to process parameters

Given the problems with the quantification of *cel48*, conclusions from the qPCR must be drawn with caution. Still, as the values can be compared between the samples, indications of correlations to process parameters can be analysed.

The results indicate that a higher HRT increases the relative abundance of cellulose-degraders in the biogas system. The recalcitrant structure of lignocellulosic biomass causes its microbial degradation to take considerable time. Therefore, longer HRTs (50 – 100 days) favour the breakdown of this material and prevent the washout of these microorganisms (Schnürer & Jarvis 2018). After the removal of the outlier E, the analysed HRTs were within the range of 16-49 days in the present study. Given that *cel48* is a marker gene for degradation of crystalline cellulose (Koeck et al. 2014), it seems reasonable that the abundance of this gene should be positively correlated with longer HRTs. A previous study found that a longer HRT (60 days) correlated with increased specific methane yield, process stability, and cellulose degradation in a biogas reactor operating on wheat straw, in comparison to shorter HRTs (40 and 20 days) (Shi et al. 2017). Similarly, another study on Swedish farm-based biogas plants also found a positive correlation between hydraulic retention time and the degree of degradation (Ahlberg-Eliasson et al. 2017).

There was an indication of a negative correlation of *cel48* with increased TAN within the range 1.7 - 3.9 g/L (approximated to 0.1 – 0.8 g/L NH_3 (Appendix 2)) in this study, although not statistically significant. Previously, a TAN concentration of 3.8 g/L (0.4 g NH_3/L) was found to negatively impact the cellulose degradation and the abundance of several cellulose-degrading families in the biogas system (Eliasson et al. 2023). Other studies reported inhibition of cellulose degradation at similar levels, such as TAN concentration of 4.3 g/L (NH_3 levels not available) (Wang et al. 2013), or 4.6 and 5.1 g/L (0.365 and 0.408 g NH_3/L, respectively) (Sun et al. 2016). At the same time, another study did not find any correlation

between TAN and inhibited cellulose degradation within the TAN ranges 2.4 – 7.8 g/L (0.283 – 0.957 g NH_3/L) (Fernandes et al. 2012). Although the effect of ammonia on cellulose degradation and the cellulose-degrading bacterial community remains unclear, these results collectively indicate that the cellulose-degrading community and cellulose degradation might not be significantly inhibited within the tested TAN range in the present study (1.7-3.9 g/L).

There was no clear, unified correlation between the abundance of *cel48* and the other investigated process parameters (carbon reduction, substrate carbon content and process temperature) either. This could suggest that the abundance of *cel48* is more case-by-case related, dependent on several process parameters and rather than specific ones. Carbon reduction and substrate carbon content do not, however, only include cellulose, but all types of carbohydrates in the given system, which could explain why there was no correlation to *cel48* with these parameters.

4.2.4 Limitations of the study

The study is subject to several limitations. The use of degenerated primers allows for more gene variants of *cel48* to be identified at the cost of precision. This could partly explain the shift in the samples' melt peak from the standard curve, and the fact that the peaks from some samples were a composition of several smaller peaks. The efficiency of the degenerated primers may also vary among gene variants, favouring some gene variants over others. Another important limitation of the study is that only one sample from each reactor was used; more samples over time are required to verify that the samples are representative of their environments.

Another potential source of bias between the samples is the presence of inhibitors. Biogas plants operating on manure typically contain high amounts of humic acid that can inhibit the PCR reaction (Sidstedt et al. 2020). Since the qPCR was normalised by the amount of DNA, the dilution factor of the samples differed. This means that the samples may contain different amounts of humic acid, which can introduce bias to the results between the samples. Still, the initial DNA concentrations of the samples were quite similar; therefore, the difference in the dilution factor between samples was not more than 10x. In an optimal case, however, dilution should be optimised for each sample individually to avoid PCR-inhibition from humic acid. The DNA extraction was, however, performed with an extra purification step to reduce the impact of this problem.

Another limitation of the study relates to the study of microbial consortia. The abundance of *cel48* does not say anything about the sensitivity of specific species to process parameters, nor anything about the changes in community structure as a response to the changes in process parameters. *Cel48* covers a range of different species which can have individual sensitivity to ammonia or other process parameters. It is possible, for example, that some more ammonia-tolerant species are more abundant at higher ammonia stress.

The calculation of the absolute amount of *cel48* in the samples is based on several assumptions, including that the efficiency of the DNA extraction was 100% and that the efficiency was the same for all samples. Therefore, the absolute amount of genes (copies/mL sample) in this study is only an estimation.

It is also important to note that the study only used one genetic marker for cellulose degradation, while cellulose degradation is mediated by enzymes with various functions and mechanisms (Himmel et al. 2010). Thus, the present study is not sufficient to estimate the actual effect of the process parameters on the cellulose-degrading community or the overall cellulose-degrading potential of farm-based biogas systems. In addition, the study only measured the abundance of the genetic marker, not its expression or activity. Simply finding the gene does not mean it is active or contributes to cellulose degradation in the system. Information on the activity of the gene can be obtained by studying its expression (RNA) or investigating enzyme activity, for instance.

4.3 Conclusion

This study provided some new insights into the substrate utilisation and taxonomy of Dc1, as well as some insights into how cellulose degradation correlates with process parameters in biogas systems.

The closest relatives of Dc1 do not share its putative ability to degrade simpler sugars, suggesting that Dc1 is a novel species. The ability to degrade amino acids and fumarate of its closest relatives has never been investigated before. Due to the problems with the contamination of the Dc1 culture, the present study was not able to determine the taxonomic identity of Dc1.

The results from the qPCR suggest that a longer HRT and a lower TAN could be beneficial for processes aimed at increasing the efficiency of degradation of lignocellulosic materials. However, the reliability of the quantification of *cel48* is decreased by the interference of the primer dimers.

More information is still required on the cellulose-degrading bacterial community to improve the microbial degradation of lignocellulosic biomass in the biogas system to its full potential. In general, all methods and approaches to study this have their advantages and drawbacks, but together they contribute to a growing body of knowledge that will, eventually, fully decipher the enigma of microbial lignocellulose degradation.

References

Ahlberg-Eliasson, K., Nadeau, E., Levén, L. & Schnürer, A. (2017). Production efficiency of Swedish farm-scale biogas plants. *Biomass and Bioenergy*, 97, 27–37. https://doi.org/10.1016/j.biombioe.2016.12.002

Al-Awadhi, H., Dashti, N., Khanafer, M., Al-Mailem, D., Ali, N. & Radwan, S. (2013). Bias problems in culture-independent analysis of environmental bacterial communities: a representative study on hydrocarbonoclastic bacteria. *SpringerPlus*, 2 (1), 369. https://doi.org/10.1186/2193-1801-2-369

Ash, C., Farrow, J.A.E., Dorsch, M., Stackebrandt, E. & Collins, M.D. (1991). Comparative Analysis of Bacillus anthracis, Bacillus cereus, and Related Species on the Basis of Reverse Transcriptase Sequencing of 16S rRNA. *International Journal of Systematic and Evolutionary Microbiology*, 41 (3), 343–346. https://doi.org/10.1099/00207713-41-3-343

Avfall Sverige (2008). *Den svenska biogaspotentialen från inhemska råvaror*. (2008:02). Avfall Sverige utveckling. https://www.yumpu.com/sv/document/view/31720514/200802-den-svenska-biogaspotentialen-fran-avfall-sverige [2024-08-27]

Axelsson Bjerg, M., Heino, F., Shakeri Yekta, S., Šafarič, L., Enrich Prast, A., Moestedt, J., Perman, E., Schnürer, A. & Björn, A. (2025). Enhancing biogas production from lignocellulosic digestate through priming and post-digestion. *Bioresource Technology Reports*, 30, 102168. https://doi.org/10.1016/j.biteb.2025.102168

Azman, S., Khadem, A.F., van Lier, J.B., Zeeman, G. & Plugge, C.M. (2015). Presence and Role of Anaerobic Hydrolytic Microbes in Conversion of Lignocellulosic Biomass for Biogas Production. *Critical Reviews in Environmental Science and Technology*, 45 (23), 2523–2564. https://doi.org/10.1080/10643389.2015.1053727

Bajpai, P. (2016). *Pretreatment of Lignocellulosic Biomass for Biofuel Production*. Springer Singapore. https://doi.org/10.1007/978-981-10-0687-6

Bayer, E.A., Belaich, J.-P., Shoham, Y. & Lamed, R. (2004). The Cellulosomes: Multienzyme Machines for Degradation of Plant Cell Wall Polysaccharides. *Annual Review of Microbiology*, 58 (1), 521–554. https://doi.org/10.1146/annurev.micro.57.030502.091022

Bayer, E.A., Kenig, R. & Lamed, R. (1983). Adherence of Clostridium thermocellum to cellulose. *Journal of Bacteriology*, 156 (2), 818–827. https://doi.org/10.1128/jb.156.2.818-827.1983

Benner, R., Maccubbin, A.E. & Hodson R E (1984). Anaerobic Biodegradation of the Lignin and Polysaccharide Components of Lignocellulose and Synthetic Lignin by Sediment Microflora. *Applied and Environmental Microbiology*, 47 (5), 998–1004. https://doi.org/10.1128/aem.47.5.998-1004.1984

Borisova, O.F., Shchyolkina, A.K., Chernov, B.K. & Tchurikov, N.A. (1993). Relative stability of AT and GC pairs in parallel DNA duplex formed by a natural sequence. *FEBS Letters*, 322 (3), 304–306. https://doi.org/10.1016/0014-5793(93)81591-M

Brooks, J.P., Edwards, D.J., Harwich, M.D., Rivera, M.C., Fettweis, J.M., Serrano, M.G., Reris, R.A., Sheth, N.U., Huang, B., Girerd, P., Strauss, J.F.,

Jefferson, K.K., Buck, G.A., & Vaginal Microbiome Consortium (additional members) (2015). The truth about metagenomics: quantifying and counteracting bias in 16S rRNA studies. *BMC Microbiology*, 15 (1), 66. https://doi.org/10.1186/s12866-015-0351-6

Clavel, T., Faber, F., Groussin, M., Haller, D., Overmann, J., Pauvert, C., Poyet, M., Selkrig, J., Stecher, B., Typas, A., Vehreschild, M.J.G.T., Westermann, A.J., Wylensek, D. & Maier, L. (2025). Enabling next-generation anaerobic cultivation through biotechnology to advance functional microbiome research. *Nature Biotechnology*, 43 (6), 878–888. https://doi.org/10.1038/s41587-025-02660-6

Danielsson, R., Dicksved, J., Sun, L., Gonda, H., Müller, B., Schnürer, A. & Bertilsson, J. (2017). Methane Production in Dairy Cows Correlates with Rumen Methanogenic and Bacterial Community Structure. *Frontiers in Microbiology*, 8. https://doi.org/10.3389/fmicb.2017.00226

Denman, S.E. & McSweeney, C.S. (2006). Development of a real-time PCR assay for monitoring anaerobic fungal and cellulolytic bacterial populations within the rumen. *FEMS Microbiology, Ecology*, 58 (3), 572–582. https://doi.org/10.1111/j.1574-6941.2006.00190.x

Dinsdale, R.M., Hawkes, F.R. & Hawkes, D.L. (1996). The mesophilic and thermophilic anaerobic digestion of coffee waste containing coffee grounds. *Water Research*, 30 (2), 371–377. https://doi.org/10.1016/0043-1354(95)00157-3

Directive (EU) 2023/2413 (2023). *Directive (EU) 2023/2413 of the European Parliament and of the Council of 18 October 2023 amending Directive (EU) 2018/2001, Regulation (EU) 2018/1999 and Directive 98/70/EC as regards the promotion of energy from renewable sources, and repealing Council Directive (EU) 2015/652.* Official Journal L. https://eur-lex.europa.eu/eli/dir/2023/2413/oj [2025-06-05]

Douglas, C.A., Ivey, K.L., Papanicolas, L.E., Best, K.P., Muhlhausler, B.S. & Rogers, G.B. (2020). DNA extraction approaches substantially influence the assessment of the human breast milk microbiome. *Scientific Reports*, 10 (1), 123. https://doi.org/10.1038/s41598-019-55568-y

Edwards, U., Rogall, T., Blöcker, H., Emde, M. & Böttger, E.C. (1989). Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Research*, 17 (19), 7843–7853. https://doi.org/10.1093/nar/17.19.7843

Eliasson, K.A., Singh, A., Isaksson, S. & Schnürer, A. (2023). Co-substrate composition is critical for enrichment of functional key species and for process efficiency during biogas production from cattle manure. *Microbial Biotechnology*, 16 (2), 350–371. https://doi.org/10.1111/1751-7915.14194

Fernandes, T.V., Keesman, K.J., Zeeman, G. & van Lier, J.B. (2012). Effect of ammonia on the anaerobic hydrolysis of cellulose and tributyrin. *Biomass and Bioenergy*, 47, 316–323. https://doi.org/10.1016/j.biombioe.2012.09.029

Gössner, A.S., Devereux, R., Ohnemüller, N., Acker, G., Stackebrandt, E. & Drake, H.L. (1999). Thermicanus aegyptius gen. nov., sp. nov., isolated from oxic soil, a fermentative microaerophile that grows commensally with the thermophilic acetogen Moorella thermoacetica. *Applied and Environmental Microbiology*, 65 (11), 5124–5133. https://doi.org/10.1128/AEM.65.11.5124-5133.1999

Hanišáková, N., Vítězová, M. & Rittmann, S.K.-M.R. (2022). The Historical Development of Cultivation Techniques for Methanogens and Other Strict Anaerobes and Their Application in Modern Microbiology. *Microorganisms*, 10 (2), 412. https://doi.org/10.3390/microorganisms10020412

Hansen, K.H., Angelidaki, I. & Ahring, B.K. (1998). Anaerobic Digestion of Swine Manure: Inhibition by Ammonia. *Water Research*, 32 (1), 5–12. https://doi.org/10.1016/S0043-1354(97)00201-7

Heller, H.H. (1921). Principles concerning the isolation of anaerobes: Studies in Pathogenic Anaerobes II. *Journal of Bacteriology*, 6 (5), 445–470. https://doi.org/doi: 10.1128/jb.6.5.445-470.1921

Himmel, M.E., Xu, Q., Luo, Y., Ding, S.-Y., Lamed, R. & Bayer, E.A. (2010). Microbial enzyme systems for biomass conversion: emerging paradigms. *Biofuels*, 1 (2), 323–341. https://doi.org/10.4155/bfs.09.25

Hugerth, L.W. & Andersson, A.F. (2017). Analysing Microbial Community Composition through Amplicon Sequencing: From Sampling to Hypothesis Testing. *Frontiers in Microbiology*, 8. https://doi.org/10.3389/fmicb.2017.01561

Izquierdo, J.A., Sizova, M.V. & Lynd, L.R. (2010). Diversity of Bacteria and Glycosyl Hydrolase Family 48 Genes in Cellulolytic Consortia Enriched from Thermophilic Biocompost. *Applied and Environmental Microbiology*, 76 (11), 3545–3553. https://doi.org/10.1128/AEM.02689-09

Kato, S., Haruta, S., Cui, Z.J., Ishii, M., Yokota, A. & Igarashi, Y. (2004). Clostridium straminisolvens sp. nov., a moderately thermophilic, aerotolerant and cellulolytic bacterium isolated from a cellulose-degrading bacterial community. *International Journal of Systematic and Evolutionary Microbiology*, 54 (6), 2043–2047. https://doi.org/10.1099/ijs.0.63148-0

Koeck, D.E., Pechtl, A., Zverlov, V.V. & Schwarz, W.H. (2014). Genomics of cellulolytic bacteria. *Current Opinion in Biotechnology*, 29, 171–183. https://doi.org/10.1016/j.copbio.2014.07.002

Kopečný, J. & Hodrová, B. (1997). The effect of yellow affinity substance on cellulases of Ruminococcus flavefaciens. *Letters in Applied Microbiology*, 25 (3), 191–196. https://doi.org/10.1046/j.1472-765X.1997.00202.x

Li, J., Rui, J., Pei, Z., Sun, X., Zhang, S., Yan, Z., Wang, Y., Liu, X., Zheng, T. & Li, X. (2014). Straw- and slurry-associated prokaryotic communities differ during co-fermentation of straw and swine manure. *Applied Microbiology and Biotechnology*, 98 (10), 4771–4780. https://doi.org/10.1007/s00253-014-5629-3

Li, Y., Zhang, R., Liu, G., Chen, C., He, Y. & Liu, X. (2013). Comparison of methane production potential, biodegradability, and kinetics of different organic substrates. *Bioresource Technology*, 149, 565–569. https://doi.org/10.1016/j.biortech.2013.09.063

Liu, T. (2019). *Biogas production from lignocellulosic agricultural residues*. (Diss). Swedish University of Agricultural Sciences. https://res.slu.se/id/publ/104237

Ljungdahl, L.G., Coughlan, M.P., Mayer, F., Mori, Y., Hon-nami, H. & Hon-nami, K. (1988). Macrocellulase complexes and yellow affinity substance from *Clostridium thermocellum*. In: *Methods in Enzymology*. Academic Press. 483–500. https://doi.org/10.1016/0076-6879(88)60158-3

McBee, R.H. (1954). The characteristics of Clostridium thermocellum. *Journal of Bacteriology*, 67 (4), 505–506. https://doi.org/10.1128/jb.67.4.505-506.1954

McDonald, J.E., Houghton, J.N.I., Rooks, D.J., Allison, H.E. & McCarthy, A.J. (2012). The microbial ecology of anaerobic cellulose degradation in municipal waste landfill sites: evidence of a role for fibrobacters. *Environmental Microbiology*, 14 (4), 1077–1087. https://doi.org/10.1111/j.1462-2920.2011.02688.x

McDonald, J.E., Lockhart, R.J., Cox, M.J., Allison, H.E. & McCarthy, A.J. (2008). Detection of novel Fibrobacter populations in landfill sites and determination of their relative abundance via quantitative PCR.

Environmental Microbiology, 10 (5), 1310–1319. https://doi.org/10.1111/j.1462-2920.2007.01544.x

Microbiology Society. *International Journal of Systematic and Evolutionary Microbiology, Publication of new taxa*. https://www.microbiologyresearch.org/content/journal/ijsem?page=about-journal#2 [2025-07-02]

Minor, C.M., Takayesu, A., Ha, S.M., Salwinski, L., Sawaya, M.R., Pellegrini, M. & Clubb, R.T. (2024). A genomic analysis reveals the diversity of cellulosome displaying bacteria. *Frontiers in Microbiology*, 15. https://doi.org/10.3389/fmicb.2024.1473396

Moestedt, J., Påledal, S.N., Schnürer, A. & Nordell, E. (2013). Biogas Production from Thin Stillage on an Industrial Scale—Experience and Optimisation. *Energies*, 6 (11), 5642–5655. https://doi.org/10.3390/en6115642

Monlau, F., Barakat, A., Trably, E., Dumas, C., Steyer, J.-P. & Carrère, H. (2013). Lignocellulosic Materials Into Biohydrogen and Biomethane: Impact of Structural Features and Pretreatment. *Critical Reviews in Environmental Science and Technology*, 43 (3), 260–322. https://doi.org/10.1080/10643389.2011.604258

Ng, T.K., Weimer, P.J. & Zeikus, J.G. (1977). Cellulolytic and physiological properties of Clostridium thermocellum. *Archives of Microbiology*, 114 (1), 1–7. https://doi.org/10.1007/BF00429622

Noike, T., Endo, G., Chang, J.-E., Yaguchi, J.-I. & Matsumoto, J.-I. (1985). Characteristics of carbohydrate degradation and the rate-limiting step in anaerobic digestion. *Biotechnology and Bioengineering*, 27 (10), 1482–1489. https://doi.org/10.1002/bit.260271013

Okonechnikov, K., Golosova, O., Fursov, M., & the UGENE team (2012). Unipro UGENE: a unified bioinformatics toolkit. *Bioinformatics*, 28 (8), 1166–1167. https://doi.org/10.1093/bioinformatics/bts091

Olatunji, K.O., Ahmed, N.A. & Ogunkunle, O. (2021). Optimization of biogas yield from lignocellulosic materials with different pretreatment methods: a review. *Biotechnology for Biofuels*, 14 (1), 159. https://doi.org/10.1186/s13068-021-02012-x

Patel, G.B., Khan, A.W., Agnew, B.J. & Colvin, J.R. (1980). Isolation and Characterization of an Anaerobic, Cellulolytic Microorganism, Acetivibrio cellulolyticus gen. nov., sp. nov.†. *International Journal of Systematic and Evolutionary Microbiology*, 30 (1), 179–185. https://doi.org/10.1099/00207713-30-1-179

Patni, N.J. & Alexander, J.K. (1971a). Catabolism of Fructose and Mannitol in Clostridium thermocellum: Presence of Phosphoenolpyruvate: Fructose Phosphotransferase, Fructose l-Phosphate Kinase, Phosphoenolpyruvate: Mannitol Phosphotransferase, and Mannitol l-Phosphate Dehydrogenase in Cell Extracts. *Journal of Bacteriology*, 105 (1), 226–231. https://doi.org/10.1128/jb.105.1.226-231.1971

Patni, N.J. & Alexander, J.K. (1971b). Utilization of Glucose by Clostridium thermocellum: Presence of Glucokinase and Other Glycolytic Enzymes in Cell Extracts. *Journal of Bacteriology*, 105 (1), 220–225. https://doi.org/10.1128/jb.105.1.220-225.1971

Pereyra, L.P., Hiibel, S.R., Prieto Riquelme, M.V., Reardon, K.F. & Pruden, A. (2010). Detection and Quantification of Functional Genes of Cellulose-Degrading, Fermentative, and Sulfate-Reducing Bacteria and Methanogenic Archaea. *Applied and Environmental Microbiology*, 76 (7), 2192–2202. https://doi.org/10.1128/AEM.01285-09

Perman, E., Westerholm, M., Liu, T. & Schnürer, A. (2024). Comparative study of high-solid anaerobic digestion at laboratory and industrial scale – Process performance and microbial community structure. *Energy Conversion and*

Management, 300, 117978. https://doi.org/10.1016/j.enconman.2023.117978
Rettenmaier, R., Gerbaulet, M., Liebl, W. & Zverlov, V.V. (2019). Hungateiclostridium mesophilum sp. nov., a mesophilic, cellulolytic and spore-forming bacterium isolated from a biogas fermenter fed with maize silage. *International Journal of Systematic and Evolutionary Microbiology*, 69 (11), 3567–3573. https://doi.org/10.1099/ijsem.0.003663
Rettenmaier, R., Lo, Y.K., Schmidt, L., Munk, B., Lagkouvardos, I., Neuhaus, K., Schwarz, W., Liebl, W. & Zverlov, V. (2020). A Novel Primer Mixture for GH48 Genes: Quantification and Identification of Truly Cellulolytic Bacteria in Biogas Fermenters. *Microorganisms*, 8 (9), 1297. https://doi.org/10.3390/microorganisms8091297
Sawatdeenarunat, C., Surendra, K.C., Takara, D., Oechsner, H. & Khanal, S.K. (2015). Anaerobic digestion of lignocellulosic biomass: Challenges and opportunities. *Bioresource Technology*, 178, 178–186. https://doi.org/10.1016/j.biortech.2014.09.103
Schlüter, A., Bekel, T., Diaz, N.N., Dondrup, M., Eichenlaub, R., Gartemann, K.-H., Krahn, I., Krause, L., Krömeke, H., Kruse, O., Mussgnug, J.H., Neuweger, H., Niehaus, K., Pühler, A., Runte, K.J., Szczepanowski, R., Tauch, A., Tilker, A., Viehöver, P. & Goesmann, A. (2008). The metagenome of a biogas-producing microbial community of a production-scale biogas plant fermenter analysed by the 454-pyrosequencing technology. *Journal of Biotechnology*, 136 (1), 77–90. https://doi.org/10.1016/j.jbiotec.2008.05.008
Schnürer, A. (2016). Biogas Production: Microbiology and Technology. In: *Anaerobes in biotechnology*. Springer, Cham. 195–234. https://doi.org/10.1007/10_2016_5
Schnürer, A. & Jarvis, Å. (2018). *Microbiology of the biogas process*. Swedish University of Agricultural Sciences.
Schwarz, W. (2001). The cellulosome and cellulose degradation by anaerobic bacteria. *Applied Microbiology and Biotechnology*, 56 (5), 634–649. https://doi.org/10.1007/s002530100710
Shiratori, H., Sasaya, K., Ohiwa, H., Ikeno, H., Ayame, S., Kataoka, N., Miya, A., Beppu, T. & Ueda, K. (2009). Clostridium clariflavum sp. nov. and Clostridium caenicola sp. nov., moderately thermophilic, cellulose-/cellobiose-digesting bacteria isolated from methanogenic sludge. *International Journal of Systematic and Evolutionary Microbiology*, 59 (7), 1764–1770. https://doi.org/10.1099/ijs.0.003483-0
Sidstedt, M., Rådström, P. & Hedman, J. (2020). PCR inhibition in qPCR, dPCR and MPS—mechanisms and solutions. *Analytical and Bioanalytical Chemistry*, 412 (9), 2009–2023. https://doi.org/10.1007/s00216-020-02490-2
Sjöberg, C. (2023). *Anaerobic cellulose degrading bacteria isolated from biogas plants*. Swedish University of Agricultural Sciences. Department of Molecular Sciences. http://urn.kb.se/resolve?urn=urn:nbn:se:slu:epsilon-s-18800
Sun, L. (2015). *Biogas Production from Lignocellulosic Materials*. (Diss). Swedish University of Agricultural Sciences. https://res.slu.se/id/publ/77463
Sun, L., Liu, T., Müller, B. & Schnürer, A. (2016). The microbial community structure in industrial biogas plants influences the degradation rate of straw and cellulose in batch tests. *Biotechnology for Biofuels*, 9 (1), 128. https://doi.org/10.1186/s13068-016-0543-9
Tang, H., Ou, J.F. & Zhu, M.J. (2015). Development of a quantitative real-time PCR assay for direct detection of growth of cellulose-degrading bacterium Clostridium thermocellum in lignocellulosic degradation. *Journal of*

Applied Microbiology, 118 (6), 1333–1344. https://doi.org/10.1111/jam.12801

The European Green Deal (2019). *Communication from the Commission to the European Parliament, the European Council, the Council, the European Economic and Social Committee and the Committee of the Regions.* COM/2019/640 final. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52019DC0640

Tindall, B.J., Rosselló-Móra, R., Busse, H.-J., Ludwig, W. & Kämpfer, P. (2010). Notes on the characterization of prokaryote strains for taxonomic purposes. *International Journal of Systematic and Evolutionary Microbiology*, 60 (1), 249–266. https://doi.org/10.1099/ijs.0.016949-0

Tsavkelova, E.A. & Netrusov, A.I. (2012). Biogas production from cellulose-containing substrates: A review. *Applied Biochemistry and Microbiology*, 48 (5), 421–433. https://doi.org/10.1134/S0003683812050134

Vanwonterghem, I., Jensen, P.D., Ho, D.P., Batstone, D.J. & Tyson, G.W. (2014). Linking microbial community structure, interactions and function in anaerobic digesters using new molecular techniques. *Current Opinion in Biotechnology*, 27, 55–64. https://doi.org/10.1016/j.copbio.2013.11.004

Vilas-Bôas, G.T., Peruca, A.P.S. & Arantes, O.M.N. (2007). Biology and taxonomy of Bacillus cereus, Bacillus anthracis, and Bacillus thuringiensis. *Canadian Journal of Microbiology*, 53 (6), 673–687. https://doi.org/10.1139/W07-029

Wang, X., Yang, G., Feng, Y., Ren, G. & Han, X. (2012). Optimizing feeding composition and carbon–nitrogen ratios for improved methane yield during anaerobic co-digestion of dairy, chicken manure and wheat straw. *Bioresource Technology*, 120, 78–83. https://doi.org/10.1016/j.biortech.2012.06.058

Wang, Z., Xu, F. & Li, Y. (2013). Effects of total ammonia nitrogen concentration on solid-state anaerobic digestion of corn stover. *Bioresource Technology*, 144, 281–287. https://doi.org/10.1016/j.biortech.2013.06.106

Westerholm, M., Roos, S. & Schnürer, A. (2010). Syntrophaceticus schinkii gen. nov., sp. nov., an anaerobic, syntrophic acetate-oxidizing bacterium isolated from a mesophilic anaerobic filter. *FEMS Microbiology Letters*, 309 (1), 100–104. https://doi.org/10.1111/j.1574-6968.2010.02023.x

Westerholm, M., Schnürer, A., Westerholm, M. & Schnürer, A. (2019). Microbial Responses to Different Operating Practices for Biogas Production Systems. In: *Anaerobic Digestion*. IntechOpen. https://doi.org/10.5772/intechopen.82815

Yang, J.C., Chynoweth, D.P., Williams, D.S. & Li, A. (1990). Clostridium aldrichii sp. nov., a Cellulolytic Mesophile Inhabiting a Wood-Fermenting Anaerobic Digester. *International Journal of Systematic Bacteriology*, 40 (3), 268–272. https://doi.org/10.1099/00207713-40-3-268

Zakrzewski, M., Goesmann, A., Jaenicke, S., Jünemann, S., Eikmeyer, F., Szczepanowski, R., Al-Soud, W.A., Sørensen, S., Pühler, A. & Schlüter, A. (2012). Profiling of the metabolically active community from a production-scale biogas plant by means of high-throughput metatranscriptome sequencing. *Journal of Biotechnology*, 158 (4), 248–258. https://doi.org/10.1016/j.jbiotec.2012.01.020

Zarraonaindia, I., Smith, D.P. & Gilbert, J.A. (2013). Beyond the genome: community-level analysis of the microbial world. *Biology & Philosophy*, 28 (2), 261–282. https://doi.org/10.1007/s10539-012-9357-8

Zeng, Y., Zeng, D., Zhang, Y., Ni, X., Tang, Y., Zhu, H., Wang, H., Yin, Z., Pan, K. & Jing, B. (2015). Characterization of the cellulolytic bacteria communities along the gastrointestinal tract of Chinese Mongolian sheep by using PCR-DGGE and real-time PCR analysis. *World Journal of*

Microbiology and Biotechnology, 31 (7), 1103–1113. https://doi.org/10.1007/s11274-015-1860-z

Zheng, Y., Zhao, J., Xu, F. & Li, Y. (2014). Pretreatment of lignocellulosic biomass for enhanced biogas production. *Progress in Energy and Combustion Science*, 42, 35–53. https://doi.org/10.1016/j.pecs.2014.01.001

Popular science summary

Today, society is facing many challenges caused by emissions of greenhouse gases leading to climate change. Energy from renewable sources, such as biogas, play an important role to develop a more sustainable society.

Biogas is produced through the degradation of organic material by a community of microorganisms in the absence of oxygen. Different organic materials can be used to produce biogas, but agricultural wastes and other fibre-rich materials (crop residues and manures, for instance) are especially interesting because they are both highly available and abundant. The amount of biogas that can be produced would significantly increase if more of these materials (collectively named "lignocellulosic biomass") could be used for biogas production. Unfortunately, this is not so easy to achieve, as the complex chemical structure of lignocellulosic biomass is difficult for the microorganisms in the biogas system to degrade. The degradability of lignocellulosic biomass in the biogas system can, however, be improved by making use of the community of cellulose-degrading bacteria that are already present in the biogas system. This could, for instance, mean adding more of these bacteria into the biogas reactor, or optimising the reactor conditions to favour this community. At present, the possibility of developing such methods is limited because many species in this community and their preference for process parameters are still unknown. To help gain information on this, the purpose of this study was to examine the cellulose-degrading bacterial community in biogas systems and its correlation to process parameters.

To do so, this study investigated the biochemical properties of a cellulose-degrading bacterium (named Dc1), isolated from an industrial biogas-plant. This information is required to understand whether Dc1 represents a new species. To obtain information on the preference of the cellulose-degrading bacterial community to process parameters in the biogas-system, a biomarker for cellulose-degradation (*cel48*) was quantified in a set of biogas samples from small-scale biogas plants on Swedish farm-baseds. This was done using a method called quantitative PCR, which can detect and quantify specific genes of interest. The more of the gene found, the more cellulose-degrading bacteria are present there.

The results showed that Dc1 mainly degrades cellulose and carbohydrates, but also had some ability to degrade amino acids. The sequence of Dc1 was similar to *Acetivibrio cellulolyticus* (98.5%), an anaerobic cellulose-degrading species that

occur in the biogas-system. However, not enough biochemical tests were performed on Dc1 to determine whether it represents a new species or whether it belongs to *A. cellulolyticus* too.

The quantitative PCR showed that the amount of *cel48* increased with increasing hydraulic retention time (HRT). HRT refers to the time it takes to exchange the full reactor volume, *i.e.* a longer HRT means that the material stays in the reactor for a longer time. There was also a trend of decreasing amount of *cel48* with increased total ammonium-concentration (TAN). These results indicate that a longer HRT and a lower amount of TAN can be beneficial for the cellulose-degrading bacteria in the biogas system, which may increase the degradation of lignocellulose. Some other process parameters were also investigated, but no correlations or trends could be seen. This could be due to the fact that the cellulose-degrading bacteria is not only dependent on single process parameter, but on the whole environment created by many process parameters together.

The results from both parts of the study must be interpreted with care, as they were subject to shortcomings that decrease the reliability of the results. Nevertheless, the indications from the results are still interesting and relevant, and contribute to a better understanding of the microbial cellulose-degrading community in the biogas system.

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Appendix 1

VFA production from the substrate utilisation screening, analysed with HPLC, is presented below (Table A1). All values below 0.1 g/L were excluded from the analysis, as this corresponds to the lowest value of the standards. If only one duplicate from the same substrate had a value above this threshold, only this value was included in the analysis. Some sample runs were disturbed by substances from previous runs. In such cases, only the duplicate without disturbance was included in the analysis.

Table A1. HPLC-raw data from the substrate utilisation screening showing the produced VFA as products of degradation from all testes substrates in the substrates utilisation screening.

Sample	Nr	Lactate	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate
Asparagine (negative control)	1	0.000	0.000	0.027	0.000	0.000	0.000	0.000
Asparagine	2	0.005	0.034	0.032	0.000	0.000	0.000	0.000
Asparagine	3	0.002	0.036	0.040	0.000	0.000	0.000	0.000
Acetoine	4	0.020	0.040	0.109	0.000	0.000	0.000	0.000
Acetoine	5	0.025	0.035	0.062	0.000	0.000	0.000	0.000
Ethanol	6	0.007	0.030	0.136	0.000	0.000	0.000	0.000
Ethanol	7	0.021	0.034	0.038	0.000	0.000	0.000	0.000
Formate	8	0.020	0.039	0.098	0.000	0.000	0.000	0.000
Formate	9	0.012	0.040	0.074	0.000	0.000	0.000	0.000
Pyruvate	10	0.015	0.113	0.038	0.000	0.000	0.000	0.000
Pyruvate	11	0.012	0.092	0.036	0.000	0.000	0.000	0.000
Acetate	12	0.020	1.470	0.091	0.000	0.000	0.000	0.000
Acetate	13	0.008	1.476	0.073	0.000	0.000	0.000	0.000

Fumarate	14	0.000	0.195	0.023	0.000	0.000	0.000	0.000
Fumarate	15	0.000	0.258	0.025	0.000	0.000	0.000	0.000
Vanillate	16	0.020	0.032	0.069	0.000	0.000	0.000	0.000
Vanillate	17	0.000	0.017	0.106	0.000	0.000	0.000	0.000
1-butanol	18	0.000	0.030	0.028	0.000	0.013	0.000	0.000
1-butanol	19	0.018	0.027	0.081	0.000	0.010	0.000	0.000
Citrate	20	0.000	0.031	0.056	0.000	0.000	0.000	0.039
Citrate	21	0.003	0.028	0.058	0.000	0.000	0.000	0.000
Glycerol	22	0.009	0.078	0.039	0.000	0.000	0.000	0.000
Glycerol	23	0.011	0.067	0.297	0.000	0.000	0.000	0.000
Betaine	24	0.000	0.038	0.036	0.000	0.000	0.000	0.000
Betaine	25	0.000	0.028	0.284	0.000	0.004	0.000	0.000
Syringate	26	0.031	0.042	0.047	0.000	0.000	0.000	0.000
Syringate†	**27**	**0.017**	**0.043**	**0.046**	**0.000**	**0.000**	**67.055**	**2.123**
Leucine†	**28**	**0.064**	**0.029**	**0.088**	**0.000**	**0.000**	**67.753**	**1.658**
Leucine	29	0.041	0.033	0.040	0.000	0.000	0.000	0.000
Tryptophan	30	0.007	0.020	0.067	0.000	0.000	0.000	0.000
Tryptophan	31	0.000	0.021	0.059	0.000	0.000	0.000	0.000
Ethylene glycol	32	0.002	0.033	0.109	0.000	0.000	0.000	0.000
Ethylene glycol	33	0.004	0.028	0.150	0.000	0.000	0.000	0.000
Serine	36	0.115	0.134	0.000	0.000	0.000	0.150	0.000
Serine	37	0.126	0.856	0.000	0.094	0.214	0.156	0.000
2,3-butanediol	38	0.094	0.105	0.000	0.000	0.000	0.138	0.000
2,3-butanediol	39	0.101	0.104	0.000	0.094	0.114	0.137	0.000
Malate	40	0.096	0.074	0.000	0.096	0.000	0.137	0.000
Malate	41	0.075	0.107	0.000	0.096	0.000	0.139	0.000
Lactate	42	0.924	0.115	0.000	0.000	0.000	0.138	0.000

Lactate	43	0.915	0.123	0.000	0.093	0.000	0.137	0.000
1,2-propanediol	44	0.102	0.101	0.000	0.000	0.000	0.136	0.000
1,2-propanediol	45	0.095	0.102	0.000	0.000	0.000	0.136	0.000
Growth medium (inoculated)	46	0.079	0.122	0.000	0.000	0.000	0.136	0.000
Growth medium (inoculated)	47	0.093	0.100	0.000	0.000	0.000	0.000	0.000
Growth medium (inoculated)	48	0.090	0.105	0.000	0.000	0.000	0.136	0.000
Growth medium (inoculated)	49	0.105	0.102	0.000	0.000	0.116	0.134	0.000
Growth medium (not inoculated)	35	0.000	0.000	0.049	0.000	0.000	0.000	0.000
Growth medium (not inoculated)	51	0.078	0.075	0.000	0.095	0.000	0.000	0.000
Growth medium (not inoculated)	52	0.000	0.089	0.000	0.093	0.000	0.000	0.000
Proline	53	0.091	0.091	0.000	0.000	0.000	0.133	0.000
Proline	54	0.119	0.093	0.000	0.000	0.000	0.134	0.000
Methanol	55	0.094	0.101	0.000	0.000	0.000	0.136	0.000
Methanol	56	0.097	0.100	0.000	0.000	0.000	0.133	0.000
Tryptone	57	0.105	0.277	0.000	0.130	0.176	0.258	0.000
Tryptone	58	0.099	0.206	0.000	0.000	0.198	0.297	0.000
Benzoic acid	59	0.090	0.091	0.000	0.000	0.000	0.133	0.000
Benzoic acid†	**60**	**11.411**	**0.065**	**0.000**	**0.000**	**0.000**	**0.133**	**0.000**
2-propanol†	**61**	**11.304**	**0.073**	**0.000**	**0.000**	**0.000**	**0.134**	**0.000**
2-propanol	62	0.088	0.097	0.000	0.000	0.000	0.000	0.000
Histidine	63	0.119	0.094	0.000	0.000	0.118	0.134	0.000

Histidine	64	0.124	0.103	0.000	0.000	0.114	0.134	0.000
Methionine	65	0.097	0.099	0.000	0.000	0.247	0.000	0.000
Methionine	66A	0.113	0.100	0.000	0.089	0.209	0.000	0.000
Methionine	66B	0.121	0.103	0.000	0.000	0.239	0.000	0.000
L-isoleucine	67	0.090	0.112	0.000	0.000	0.114	0.136	0.000
L-isoleucine	68	0.087	0.110	0.000	0.000	0.113	0.148	0.000
Cysteine	69	0.135	0.113	0.000	0.000	0.115	0.134	0.000
Cysteine	70	0.151	0.108	0.000	0.000	0.114	0.133	0.000
Dimethyl-amine	71	0.101	0.102	0.000	0.000	0.114	0.134	0.000
Dimethyl-amine	72	0.120	0.095	0.000	0.000	0.114	0.000	0.000
Casamino-acids	73	0.119	0.108	0.000	0.000	0.114	0.134	0.000
Casamino-acids	74	0.123	0.105	0.000	0.000	0.114	0.135	0.000

† Excluded from the analysis due to disturbance from previous runs in HPLC.

Appendix 2

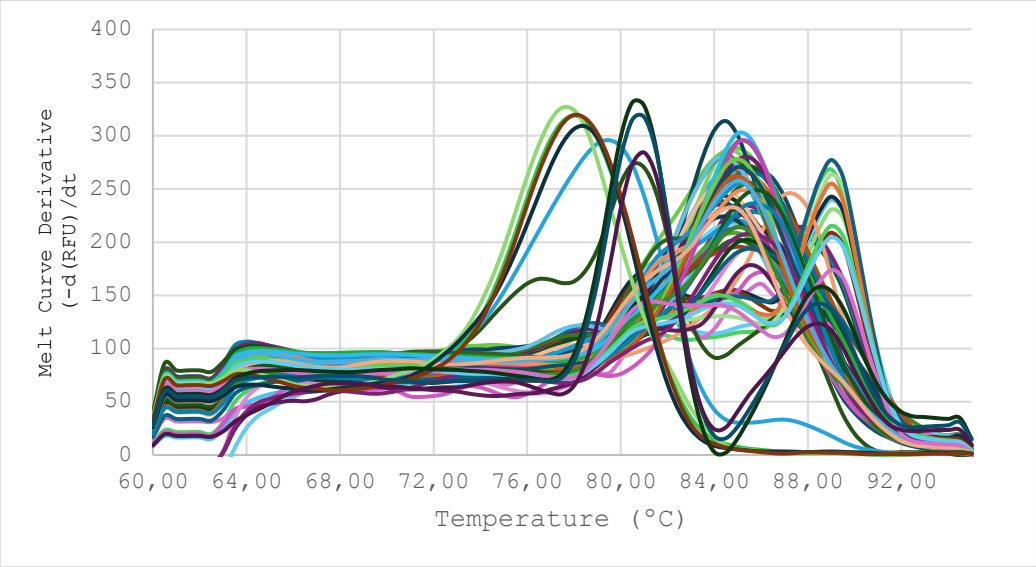

Figure A2.1. All melt peaks from the qPCR from the standard curve, negative control and the farm-based biogas samples.

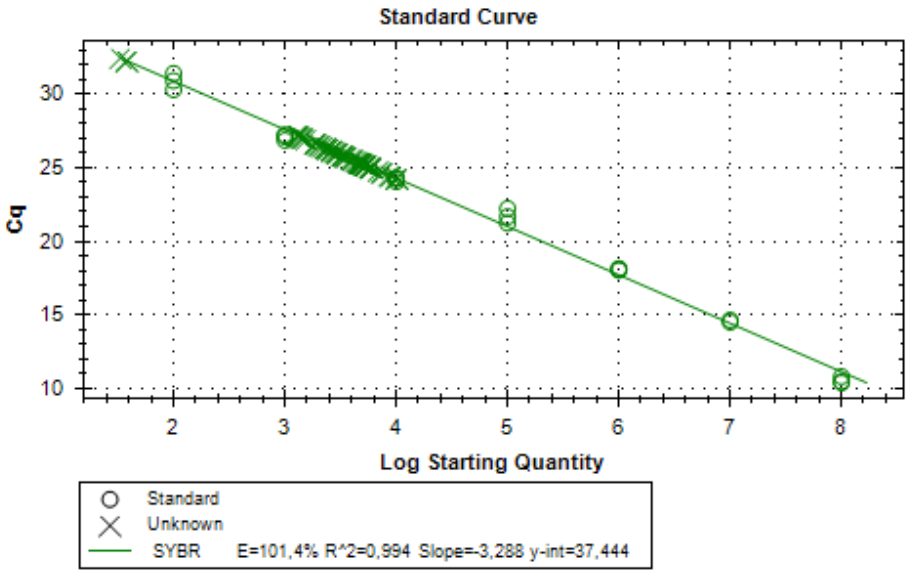

Figure A2.2. Standard curve together with the results from the farm-based biogas plant samples.

*Table A2.1. Operating parameters of the Swedish farm-based biogas plants used in this study. *Levels of ammonia (NH_3) were approximated within the pH range 7.5 – 8 (Hansen et al. 1998).*

Biogas plant	**Hydraulic retention time (HRT) (days)**	**Process temperature (ºC)**	**Total carbon content substrate (g/L)**	**TAN digestate (g/L)**	**NH_3 digestate* (g/L)**	**Carbon reduction (%)**
A	31	38.0	34.4	2.9	0-1 - 0.3	17
C	36	41.0	26.3	1.7	0-1 - 0.2	26
D	49	38.0	26.5	2.6	0-1 - 0.3	36
E	176	39.0	49.4	2.7	0-1 - 0.3	52
F	49	38.0	37.8	2.3	0-1 - 0.3	40
G	32	38.0	38.1	2.0	0-1 - 0.2	41
I	30	38.0	42.0	2.1	0-1 - 0.3	23
K	22	39.0	38.6	3.3	0-1 - 0.4	40
L	16	37.0	35.5	3.4	0-1 - 0.4	70
M	38	38.0	28.1	3.9	0-1 - 0.5	66
N	26	38.0	N/A	N/A	N/A	N/A
O	34	55.0	20.6	2.6	0-1 - 0.7	29
P	39	40.0	48.1	2.3	0-1 - 0.3	65
S	44	38.0	20.8	3.1	0-1 - 0.4	21
U	26	42.0	35.4	2.5	0-1 - 0.4	26
V	35	40.0	22.7	3.3	0-1 - 0.4	36
X	19	38.0	27.3	2.1	0-1 - 0.3	44
Y	22	52.0	99.7	3.1	0-1 - 0.8	90
Z	41	40.0	38.7	2.9	0-1 - 0.4	66
Å	33	38.0	39.2	2.6	0-1 - 0.3	32
BB	31	38.0	47.0	2.0	0-1 - 0.2	52
Ö	42	55.0	37.9	2.7	0-1 - 0.8	3

Table A2.2. Average relative and absolute abundance of cel48 in the farm-based biogas plant samples, together with the standard deviation. Average values are based on triplicates from the qPCR.

Biogas plant	**Average relative abundance of gene (copies of cel48/ng DNA)**	**Standard deviation relative amount**	**Average volumetric abundance of gene (copies of cel48/mL sample)**	**Standard deviation absolute amount**
A	2326	60	2128544	67244
C	4091	200	7076616	422766
D	8511	1096	7745338	1221202
E	4773	266	6031398	411957
F	9353	557	14775262	1078566
G	3897	890	5811900	1626002
I	769	1037	730833	1206490
K	3065	N/A	5589506	N/A
L	1903	19	1897020	22615
M	1606	174	2339162	310351
N	1275	40	9436233	363505
O	6585	669	9408190	1171328
P	4936	201	14874446	607185
S	5518	267	5285184	255560
U	3653	324	5124814	556213
V	5380	604	8238239	1132106
X	2992	323	3959815	523484
Y	2039	208	6629912	829308
Z	3332	257	3552152	335540
Å	1383	78	2500829	173613
BB	2959	256	3134161	332344
Ö	5795	410	6973536	603574

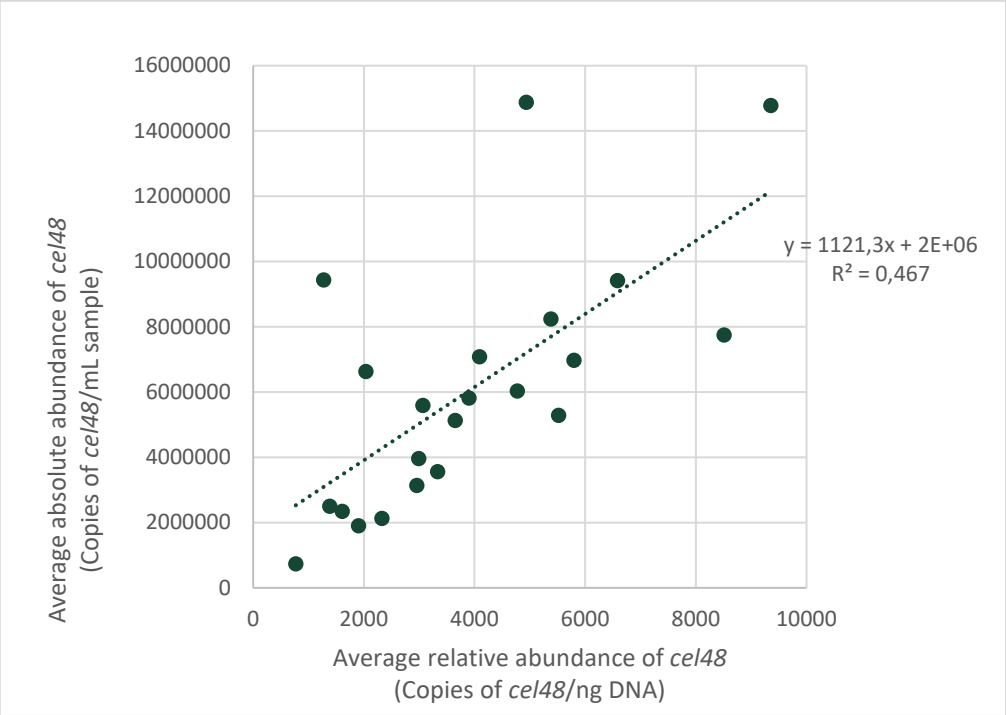

Figure A2.3. Correlation between the average relative and absolute abundance of cel48 in the farm-based biogas samples.

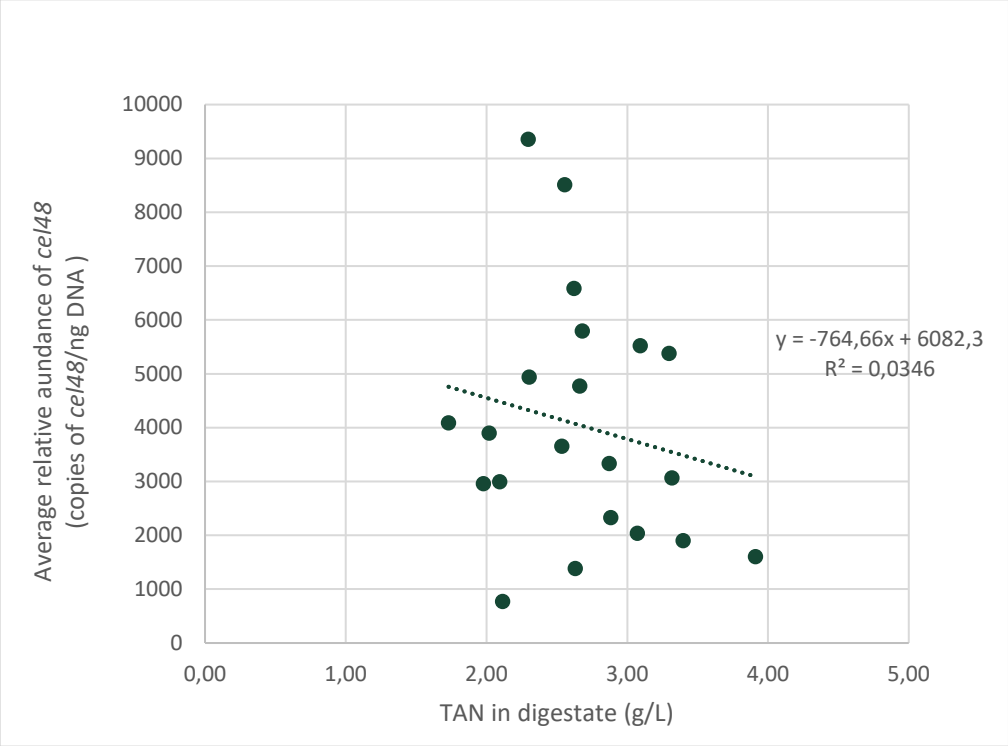

Figure A2.4. Average relative abundance of cel48 in relation to TAN in digestate.

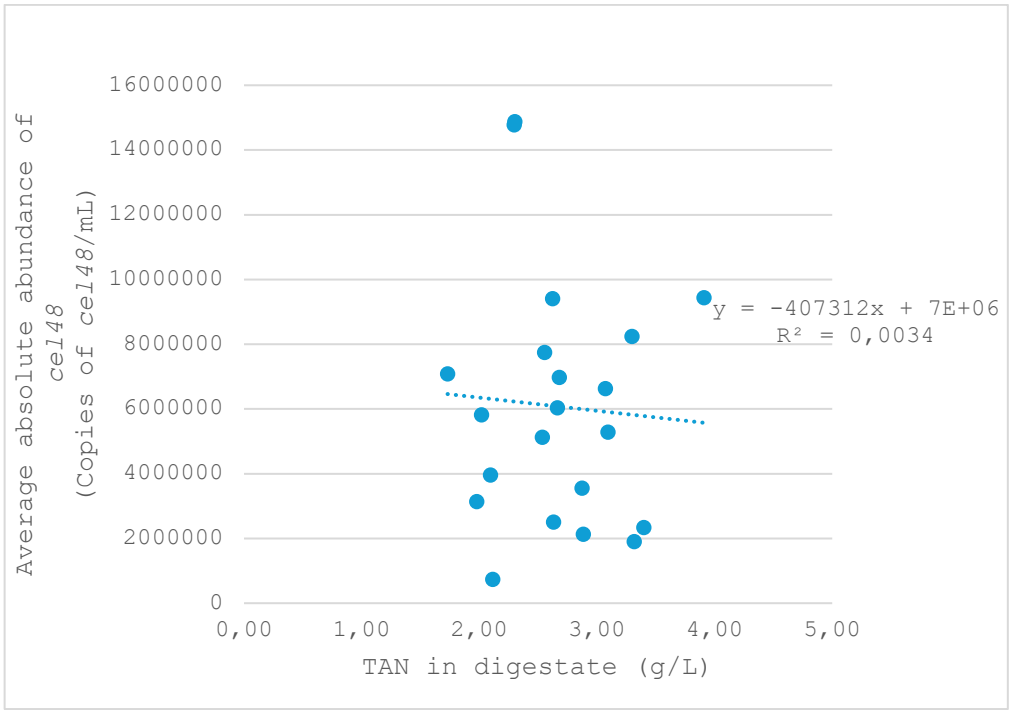

Figure A2.5. Average absolute abundance of cel48 in relation to TAN in digestate.

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