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**Faculty of Natural Resources and Agricultural Sciences**

# Anaerobic cellulose degrading bacteria isolated from biogas plants

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Department of Molecular Sciences
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**Anaerobic cellulose degrading bacteria isolated from biogas plants**

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// Abstract
Two cellulose degrading bacteria, named BCISO-4 and DC1-18-4, were isolated from samples retrieved from two large scale biogas-plants. In order to investigate and determine information regarding their growth preferences multiple tests were performed.

Growth for the two isolates was tested on the carbon sources: cellobiose, starch, hemicellulose, straw, cellulose, fructose, glucose, ribose, mannose, xylose and sorbitol (0.5w%/v.). Strong growth was documented on cellulose and cellobiose for both organisms and for BCISO-4 weak growth was possible with xylose, starch, sorbitol, glucose, fructose and ribose. For DC1-18-4 weak growth was observed with xylose, straw, glucose and ribose. Growth was also tested on a more complex nutrient medium, reinforced clostridial medium (RCM), no growth was however documented.

The major products produced during growth, as analyzed by HPLC, were determined to be acetate and butyrate for both organisms. For BCISO-4 and DC1-18-4 the growth span for temperature and pH was determined to 25-54 ° C and pH 6-10, and 25-51 ° C and pH 6-9, respectively. Enzyme activity for free enzymes as well as cell-bound was tested on glucose and xylose, and all tests indicated activity.

Terminal restriction fragment length polymorphism analysis (TRFLP) targeting the cel 5 gene was performed on samples from the biogas-plants to investigate presence of the isolated species, which confirmed their presence.

Keywords: anaerobic bacteria, biogas, cellulose, cellobiose, VFA, enzyme activity, TRFLP

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# 1. Introduction

In today's fossil fuel dependent society there is a need for alternative energy sources that are renewable, environmentally friendly and dependable. An example of such energy source is methane derived through anaerobic digestion (AD) of organic material (manure, household waste etc.). AD is a natural process occurring in places such as a cow's stomach or swamps but also in constructed reactors degrading different organic waste streams to the final end product biogas (methane and carbon dioxide). Highly advantageous with this process is that the rest product is nutrient rich and can be used for fertilization, thus contributing to closing the carbon and nutrient cycle by bringing this back to the soil. A renewable biomass source that is cheap, highly available and can be used for the AD-process is cellulose based materials, such as agricultural waste and manure. Under anaerobic conditions these materials can be digested to biogas, a mixture of carbon dioxide and methane gas. This gas can be used for heating purposes, for creating electricity and as fuel. The biomethanation process is however rather complex and "copying" it from a natural to an industrial process is not accomplished without complications. The biomethanation process is relying on synergistic actions between multiple microorganisms, and due to this complexity, many problems can easily arise. To prevent such complications in advance, or have the ability to counteract them, a database of information about the major microorganisms could be highly advantageous.

This master thesis project was carried out at the Swedish University of Agricultural Sciences (SLU) in Uppsala at the department of molecular sciences where PhD student Li Sun prior to the start of this thesis had isolated two cellulose degrading bacteria from samples from two different large-scale biogas plants. Cellulose degraders are the organisms responsible for the first step in the biomethanation process, where the complex biopolymers are degraded to monomers or oligomers. This hydrolytic step is often rate limiting when cellulose-based materials are degraded. An improved effectiveness/velocity of this step is highly interesting as the entire process would be positively affected. Consequently, it is highly interesting to investigate these bacteria further.

The short time purpose and focus of this project was to collect information regarding the two isolates. Information that would allow us to learn more about their carbohydrate degrading capacity, physiological parameters optimal for growth, enzyme production etc. The long-term purpose was to apply this information at biogas producing facilities with the hope to maximize their biogas production. This could be accomplished either through applying these bacteria as a hydrolyzing pretreatment or by optimizing the conditions in the biogas tank for the benefit of these bacteria. To achieve this, knowledge concerning substrates preference, optimal pH and temperature spans is of great importance. It is also valuable to know to what abundance the isolates exist in the biogas plants; tests to determine this were therefore made.

# 2. Literature review

## *Cellulosic material and their potential*

Degradation of plants naturally takes place in nature in a variety of manners and performed by a diverse community of organisms. To understand and describe the processes during which plants are degraded one must first understand what plants are made of. Through the process of photosynthesis, and by the aid of chlorophyll, light energy can be stored as a high potential energy molecule: glucose. These glucose molecules are the building blocks of cellulose. When the glucose is assembled ($\beta \rightarrow 1,4$ bonds) it turns into linear chains, if these are bundled up they create elementary fibrils, and further assembly makes microfibrils and finally cellulose fibers. A plant is made of cellulose fibers which are intertwined with hemicellulose and lignin (Tsavkelova & Netrusov, 2012). The cellulose fiber and hemicellulose connection is physical, whereas the lignin connection is both physical and chemical (Mussatto, et al., 2008). The presence of lignin results in a rigid cell wall due to the increase of bonding within the wall (Leschine, 1995). Lignin also surrounds the cellulose and hemicellulose, like a coat, making the other polymers less accessible (Mussatto, et al., 2008). The monomeric structure and functional groups of lignin also contributes to the difficulties of degradation (Sewalt, et al., 1997). Cellulosic materials have great potential; the depot of materials is renewable and can be acquired in large amounts. It can be obtained from industrial and municipal waste or produced by dedicated energy crops.

A method utilizing the energy from the plants and its carbohydrates in an effective way will allow a very large amount of energy to become accessible. Synergetic actions between microorganisms as well as production of various enzymes are essential for the break down. These degrading processes can take place under different environmental conditions. The degrading organisms have diverse requirements and degrade the material into different products. Such a degrading process is biomethanation, an anaerobic digestion (AD)-process with methane and carbon dioxide as final products (Angelidaki, et al., 2011).

AD can pose difficulties, the microorganisms may not be able to access all the material (Linné, et al., 2008) or nutrient can be limited. To increase the accessibility different pretreatment methods can be applied, such as: thermal, physical (mechanical as well as chemical) or ultrasound (Nizami, 2012) (Linné, et al., 2008). Thermal pretreatment is heating the substrate prior to digestion, partially breaking up the substrate. Grinding is an example of physical pretreatment. By adding enzymes or certain microorganisms the degradation can also be improved. Problematic with this strategy is however that different enzymes are required for different macromolecular structures. The addition of certain microorganism is called bioaugmentation and its effects for degrading carbohydrates (straw) to increase methane production have been documented (Peng, et al., 2014). Nutrient limitation can be solved by co-digestion, which may optimize the biogas process. During co-digestion a variety of substrates are mixed to get an optimal nutrient composition and a more stable and effective process (J. Mata-Alvarez, 2014). Co-digestion has

in some cases been shown to increase the methane production with up to 60% (Parawira, et al., 2008).

## Biomethanation

The biomethanation process can occur spontaneously in anaerobic environments, for example during degradation of organic materials in a cow's stomach, swamps or landfills (Massé, et al., 2012). This method is also used by humans, under controlled conditions, to harvest the produced methane and carbon dioxide (biogas). The biogas can be used for heating purposes, for production of electricity or as vehicle fuel (Nizami, 2012).

The biomethanation of organic materials can be divided into multiple steps performed by a variety of organisms with different metabolic abilities. The different reactions are grouped into four major steps named: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Chaudhari, et al., 2012). The key groups of microorganisms required are: fermenting and hydrolyzing bacteria, hydrogen producing acetogenic bacteria and two types of methanogenic archaea (Ahring, 2003). The microbial population can differ greatly in different biogas processes. The structure of the microbial community is shaped by different environmental parameters, such as pH, temperature, substrate composition etc. (Tsavkelova & Netrusov, 2012).

The hydrolytic and fermenting bacteria transform the complex biopolymers to mono and oligomers and further on to hydrogen plus carbon dioxide, acetate and other volatile fatty acids (VFA) and alcohols (figure 1). The biopolymers are most commonly carbohydrates, lipids and proteins. The hydrolytic step is generally regarded as rate limiting when the substrate consists of high amounts of lignocellulose (Demain, et al., 2005). Examples of bacterial genera commonly involved in the hydrolysis of cellulose are *Clostridium*, *Acetivibrio*, *Bacteroides* and *Ruminococcus* (Tsavkelova & Netrusov, 2012).

The hydrogen producing acetogenic bacteria are responsible for converting different VFA to acetate and hydrogen plus carbon dioxide. The removal of hydrogen proceeds in the final step where hydrogen plus carbon dioxide and acetate are converted to methane. This requires two types of methanogenic archaea, hydrogenotrophic and aceticlastic methanogens (Ahring, 2003) (Angelidaki, et al., 2011). For the methanation to take place the environment must be strictly anaerobic and the presence of other electron acceptors, then carbon dioxide, limited (Angelidaki, et al., 2011).

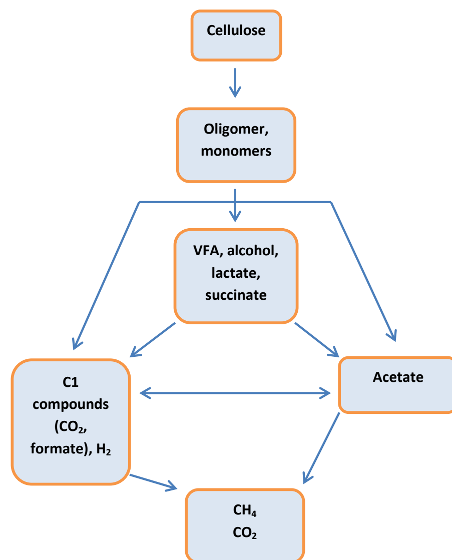


Figure 1. The biomethanation process. Inspiration from (Tsavkelova & Netrusov, 2012)

An advantage with AD in comparison to aerobic digestion (composting) is that there is no need for aeration, which is costly and can be troublesome (Demain, et al., 2005). Advantageous with AD is also the closed layout of the process, making it possible to harvest products like biogas, which else would diffuse away. The potential in the excess of possible substrates, such as lignocellulose, is large. Following a completed degradation, the rest material is rich in nutrients and can after some further processing be used as fertilizer in agriculture (Anon., 2021). In anaerobic digestion the fractions of mineralized nitrogen and phosphorus is also increased compared to the original input material (Massé, et al., 2007). Disadvantageous with AD is high capital cost and rather high failure rate (Massé, et al., 2012).

## Analysis of genetic material through 16s rRNA and functional genes

Following the isolation of an organism two different approaches are common for studying them: 16s rRNA and functional gene sequencing.
For the identification of an organism the 16s rRNA gene can be sequenced and compared in a database with other sequenced and characterized microorganisms. This might enable gathering of information regarding the isolates taxonomic placement and phenotype. There is however a chance that the organisms have not been isolated and characterized prior to this event and therefore does not exist in the database. Also, even with high similarity of 16s rRNA between two organisms they might not be the same and display phenotypic differences (Pereyra, et al., 2010). By examining specific genetic markers (functional genes) a specific trait can be searched for. An example of such functional gene is related to cellulose degrading capacity. An interesting gene in this regard is the glycoside hydrolase, which in anaerobic bacteria

most commonly belong to the families 5, 9 and 48. (Tsavkelova & Netrusov, 2012) (Pereyra, et al., 2010) (Henrissat & Bairoch, 1996). In this master thesis the genes coding for glycoside hydrolases of family 5 and 48 have been used since degenerated primers were available for those genes.

TRFLP is a method that can be used in combination with functional genes to investigate the microbial community and see the changes over time. The method works through using DNA (the desired gene amplified with PCR) that is cleaved with restriction enzymes (Walker, et al., 2017). The resulting pattern can give some suggestion of the community profile if compared to database of known species. Over time the pattern can be compared to see how the community changes, such as for example the cellulose degrading community in a biomethanation process. If subjecting the community to changes in physiological parameters the results could be followed and documented by this method. For example, in a previous work the cel5 and 48 containing microbial community and its change caused by different substrates and changing temperature was evaluated using TRFLP (Sun, et al., 2012).

### *Cellulosomes and free enzymes*

The enzymes responsible for cellulose degradation is either secreted into the medium or bound to the organism. The organisms, cellulose degraders, produce a variety of enzymes with different preferences in regards to: degrading mechanism (endo or exo), structural region of carbohydrate and chain length. The structure of the cellulose changes during the degrading process therefor requiring different enzymes. (Béguin & Aubert, 1994)

Organic materials differ in their toughness of degradability depending on their component composition. Lignocellulose rich materials pose difficulty to many organisms. As a way of handling that problem some organisms not only produce enzymes that are free in solution but also enzyme complexes that are bound to the membrane of the organism (Tsavkelova & Netrusov, 2012). These large, membrane bound enzyme complexes are named cellulosomes. Their task is to, through synergistic interactions and optimal distance to, make the degradation more effective. Their effectiveness is also a result of the large variety of enzymes assembled in the units. The attack is more comprehensive due to the many enzymes attacking the substrate at different positions. Collecting many enzymes in one complex and therefor increasing their local concentration is also an effective way for the organism to preserve energy (Vodovnik & Logar, 2010).

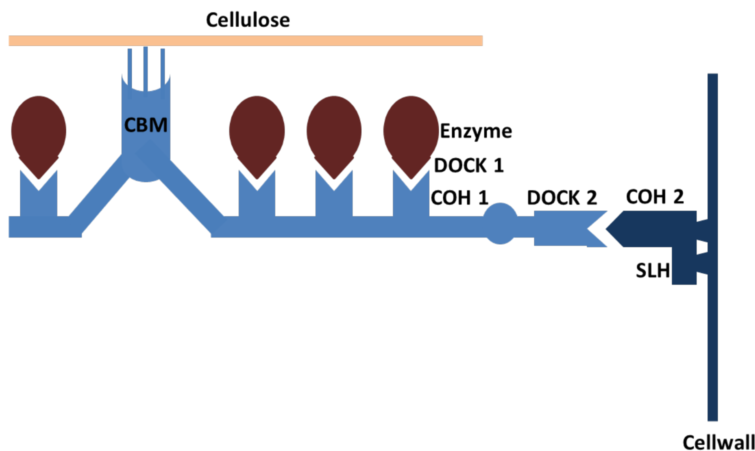


**Figure 2. The cellulosome composition for *Clostridium thermocellum*. The light blue unit is the scaffolding with a carbohydrate binding module, CBM. COH 1 and 2 are cohesins, DOCK 1 and 2 are dockerins and SLH, surface-layer homologous module. Inspiration from (Tsavkelova & Netrusov, 2012)**

The cellulosome is made up of multiple subunits with different functions (figure 2); some provide structure and other catalytic activity (Vodovnik & Logar, 2010). The backbone of the cellulosome is a scaffolding with attached cohesins. The cohesins are modules to which other subunits can attach with dockerin modules. In order to enable a high proximity between substrate and enzyme a carbohydrate binding module (CBM) is often present, this module can recognize and attach to the carbohydrate and as a result closing the distance between substrate and enzyme. An anchoring protein binds the cellulosome to the bacterial cell wall. The catalytic enzymes attached to the scaffolding are not only cellulases but also xylanases, mannanases etc..since their presence are required for the degradation of the plant cell wall (Bayer, et al., 2004). Cellulosomes of different bacteria can be different; the number of scaffoldings may for example differ as well as the nature of the cohesins (Bayer, et al., 2004).

The glycoside hydrolases that are responsible for the cellulose degradation work in a diverse manner; some have a retaining mechanism and other inverting (Koshland, 1953). Due to the structure of the cellulose, there is a large variety of glycoside hydrolases. The active site topologies for most glycoside hydrolases are pocket, cleft or tunnel. This  allows binding of different types of carbohydrates (Davies & Henrissat, 1993).

## 2. Materials and Methods

The two organisms, named DC1-18-4 and BCISO-4, were isolated from samples taken from two large scale biogas-plants. The organisms were isolated by PhD student, Li Sun.

### *Medium*

In all tests, if not otherwise stated, the medium used was a basal medium (BM) with the addition of cold 1 (C1) and cold 2 (C2) as described in (Westerholm, et al., 2012). C1 is a vitamin, salt and trace element solution that may be required for growth. C2 is a reducing agent as well as part of the buffer system. Both supplements were added in 5 v%, pH was kept at 7.2. The BM was prepared through mixing a phosphate buffer, yeast extract and resazurin (redox indicator) and 865 ml of distilled water; plus some extra to compensate for the evaporation during boiling. After approximately 20 minutes of boiling the medium was cooled under $N_2$-gas flushing. The medium was subsequently divided into bottles, 36 mL into each 118 mL serum bottle whilst flushing with $N_2$ –gas. While still flushing with gas, butyl rubber stoppers were put on and the bottles were effectively sealed with al-caps. The gas phase finally exchanged to $N_2/CO_2$ (80:20) with a final pressure of 0.2 atmospheres over pressure. Finally, the bottles were autoclaved at 120°C for 20 minutes. The BM medium contained resazurin which enabled the state of oxidation to be determined by color (colorless below -60mV).

The C1 solution was prepared by mixing the ingredients (salt, vitamins and trace metals) with 35.5 mL of pre boiled distilled water that had cooled whilst flushing with $N_2$-gas (Westerholm, et al., 2012). The mixture was then sterile filtered (0.25 µm) into pre autoclaved serum bottles filled with $N_2$-gas phase. The bottles were then pressurized up with $N_2$-gas until 0.2-0.5 atmospheres above pressure was reached. 2.2 mL of C1 was added to the sealed serum bottles of BM using a syringe. The addition was made after the medium had cooled. C1 was added in a larger volume than stated in (Westerholm, et al., 2012) in order to compensate for the larger final volume.

The C2 solution was prepared by mixing all ingredients and sterile filtered into pre autoclaved bottle an $N_2/CO_2$-gas phase (Westerholm, et al., 2012). Pressure was set to 0.2-0.5 atmospheres above pressure. 2.2 mL of C2 was added to the sealed serum bottles of BM using a syringe. The addition was made after the medium had cooled. C2 was added in a larger volume than stated in (Westerholm, et al., 2012) in order to compensate for the larger final volume.

A reinforced clostridial medium (RCM) was used for some of the experiments. RCM is very rich in nutrients and can therefore be used as a control, ensuring that growth is not limited in the BM medium due to non-optimal nutrient conditions. This medium was purchased from MERCK (Germany) and prepared according to description provided by the manufacturer. The medium was, if not stated otherwise, used in a total volume of 36 mL in 118 mL serum bottles. The medium was added to the bottles whilst flushing with $N_2$-gas after which they were closed with butyl rubber and al-caps. The gas phase was subsequently changed to $N_2/CO_2$ (80:20) with

a final pressure of 0.2 atmospheres above pressure and finally autoclaved at 120 °C for 20 minutes.

## Substrate utilization

The growth of both isolated bacteria, DC1-18-4 and BCISO-4, was evaluated using different carbohydrates and BM medium. The carbon sources tested for growth were: cellobiose, starch, hemicellulose, ground wheat straw, cellulose, fructose, glucose, ribose, mannose, xylose and sorbitol (an alcohol). Growth with hemicelluslose as a carbon source was only tested for organism BCISO-4 due to shortage of substrate.

Carbohydrates were added to a final concentration of 0.5 w/v %. The bottles were inoculated with 5-10% of inoculum, inserted into the bottles with a syringe, and incubated in a dark room at 37 °C. Each test was performed in duplicates with a negative control. The carbohydrates were pre mixed in stock solutions with a concentration of 50 mg/mL (using cooled pre boiled water) and sterile filtrated into the bottles. For the substrates straw, starch and cellobiose stock solutions could not be prepared due to low solubility. Instead 0.2 g (reaching a final concentration of 0.5w/v % in each bottle) of the carbon source were added directly to the bottles prior to the medium being added.

Growth on RCM was tested in multiple combinations: RCM on its own, with the addition of cellobiose, combined with BM and combined with BM and cellobiose. Cellobiose was chosen since it had already proven to be a sufficient carbon source. The BM and RCM combination was tested in order to ensure sufficiently reduced environment (BM) in combination with high nutrients (RCM).

## Fermentation products

The type and amount of fermentation products formed during growth was determined through high performance liquid chromatography (HPLC) analysis according to the protocol published by (Westerholm, et al., 2012). 2 mL liquid were removed from the growth bottles after 9 days of incubation and prepared for the analysis. Negative controls were analyzed as well; these cell samples were removed prior to inoculation for the substrate utilization test.

## Physiological test: temperature

The temperature span within which the two organisms could grow was evaluated by growing the bacteria with cellulose (50 mg/ml) in BM at temperatures ranging within the span of 15 and 56° C (56, 54, 51, 37, 30, 25, 20 and 15° C). When growth had been observed through visual examination (changes in color of the medium to yellow and turbidity) the test was repeated at observed maximum and minimum temperatures, including a negative control in order to verify the result. The yellow color of the medium is a sign of cell density.

## *Physiological test: pH*

The pH span, for successful growth and cellulose degradation, was evaluated by inoculation of the two isolates in BM set at different pH-values, using hydrochloric acid (2M) and sodium carbonate (2M). The pH was set at the beginning of the test (measured through subtracting 2 ml for measurements with the pH electrode) but no attempts were made to keep the pH at a constant level during the incubation. Measurements of pH were made at the end of the test when growth had been observed. This measurement was performed in the same manner as the measurements conducted before start.

The chosen pH-values were: 5, 6, 7, 8, 9 and 10 (table 1 and 2).

**Table 1. Summary of the pH study for DC1-18-4. Information regarding the sought pH value and the actual reached value as well as the volume (mL) of acid or base that was added.**

| Organism: DC1-18-4 | | | | | | |
|---|---|---|---|---|---|---|
| **pH:** | 5 | 6 | 7 | 8 | 9 | 10 |
| **Addition of base** | - | - | initial pH | 0.2 | 0.5 | 4.0 |
| **Addition of acid (mL):** | 1.0 | 0.6 | initial pH | - | - | - |
| **Actual pH at start:** | 5.1 | 6.2 | initial pH | 7.9 | 8.8 | 9.9 |

**Table 2. Summary of the pH study for BCISO-4. Information regarding the sought pH value and the actual reached value as well as the volume (mL) of acid or base that was added.**

| Organism: BCISO-4 | | | | | | |
|---|---|---|---|---|---|---|
| **pH:** | 5 | 6 | 7 | 8 | 9 | 10 |
| **Addition of base (mL):** | - | - | initial pH | 0.2 | 0.5 | 4 |
| **Addition of acid (mL):** | 1.0 | 0.6 | initial pH | - | - | - |
| **Actual pH at start:** | 5.3 | 6.2 | initial pH | 8 | 9.0 | 9.9 |

In order to determine how much acid or base that should be added to the bottles to reach the desired pH two separate BM bottles (with added C1 and C2) were used for a pre-test. During the pre-test the caps were removed and bottle flushed with $N_2/CO_2$ (80:20) and pH measured whilst slowly adding acid into one bottle and base into the other. The amounts, and pH, were documented and the information was used to determine the approximate volume needed for the actual test. The acid and base were sterile filtered into the bottles using a syringe. In order to confirm the pH, 1 mL was removed from the bottles, through syringe and transferred to an Eppendorf tube. Using an electrode whilst flushing with $N_2/CO_2$ (80:20) pH could be measured. As seen in table 1 and 2 the pH reached was close to that aimed for.

### *Enzyme activity*

The enzyme activity for cleavage of glucose and xylose was investigated by an activity test using both cell pellet, supernatant and cell mix (mixture where the cells were in suspension). The main purpose was to determine in which fraction enzyme activity would be presented. It was also tested if the bacteria produced enzymes towards a carbon which it had not been grown on. In the test a substrate, e.g. a sugar molecule coupled to a fluorophore was used. If this compound was cleaved a color reaction occurred. The substrates used were 4-Nitrophenyl beta-D-glucuronide (PNPG which simulated glucose) and 4-Nitrophenyl beta-D-xylopyranoside (PNPX which simulated xylose). Required for the test was: 100 µL of cell sample, 100 µL sodium carbonate and 5 µL of PNPG (20mM)/ PNPX (20 mM). For the first test, cells were collected from cultures grown on glucose (table 3). The glucose cleaving activity was investigated to evaluate if the enzyme activity could be connected to soluble or membrane bound enzymes**.** For the second test (table 4) the cultures were grown on cellulose or xylose and tested for enzyme activity on xylose and glucose respectively. Both tests were performed with different reaction times.

The cell mixture was retrieved by removing a sample from the cell cultures (using a syringe). The sample were subjected to centrifugation at 1000 RCF for 3 minutes whereafter the supernatant was removed. The resulting pellet was washed 2 times with a sodium acetate buffer (50 mM) and afterwards resuspended in 150 µL of the same buffer. The buffer had a pH of 5.6. Tests on all fractions were performed according to the recipe: 100 µL cell sample, 5 µL substrate (20 mM PNPG or PNPX) and 100 µL sodium carbonate (0.4 M). All test tubes were incubated at 37 ° C for the entire reaction time. The different reaction times as well as the outline of the tests can be seen in tables 3 and 4. The reaction times for the cell mix and supernatant were chosen with the aim to establish if time was an important factor for the results. The pellet was only run for one reaction time (16 hours) due to difficulties in achieving a good enough size pellet. Reactions were stopped by the addition of sodium carbonate (0.5 M), increasing the pH and incapacitating the enzymes. The sodium carbonate also made the fluorophore give off a yellow color which was used, through visual observation, to determine the activity.

**Table 3. The outline of the enzyme activity test with supernatant, cell mixture and pellet for both investigated bacteria, BCISO-4 and DC1-18-4. The culture was grown on glucose and its activity tested on PNPG. The reaction time symbolizes the time before the addition of sodium carbonate.**

| Organism: | Culture grown on: | Substrate: | Fraction: | Reaction time: |
|---|---|---|---|---|
| BCISO-4 | Glucose | PNPG | Supernatant | 30 min |
| BCISO-4 | Glucose | PNPG | Supernatant | 5 hours |
| BCISO-4 | Glucose | PNPG | Supernatant | 23 hours |
| BCISO-4 | Glucose | PNPG | Cell mixture | 30 min |
| BCISO-4 | Glucose | PNPG | Cell mixture | 5 hours |
| BCISO-4 | Glucose | PNPG | Cell mixture | 23 hours |
| BCISO-4 | Glucose | PNPG | Pellet | 16 hours |
| DC1-18-4 | Glucose | PNPG | Supernatant | 30 min |
| DC1-18-4 | Glucose | PNPG | Supernatant | 5 hours |
| DC1-18-4 | Glucose | PNPG | Supernatant | 23 hours |
| DC1-18-4 | Glucose | PNPG | Cell mixture | 30 min |
| DC1-18-4 | Glucose | PNPG | Cell mixture | 5 hours |
| DC1-18-4 | Glucose | PNPG | Cell mixture | 23 hours |
| DC1-18-4 | Glucose | PNPG | Pellet | 16 hours |

**Table 4. The outline of the enzyme activity test with supernatant, cell mixture and pellet for both investigated bacteria, BCISO-4 and DC1-18-4. The cultures were grown on cellulose or xylose and activity tested on PNPX or PNPG. The reaction time symbolizes the time before the addition of sodium carbonate.**

| Organism: | Culture grown on | PNPG/PNPX | Fraction: | Reaction time: |
|---|---|---|---|---|
| BCISO-4 | Cellulose | PNPX | pellet | 16 hours |
| BCISO-4 | Xylose | PNPG | pellet | 16 hours |
| BCISO-4 | Xylose | PNPG | Cell mixture | 2 hours |
| BCISO-4 | Xylose | PNPG | Cell mixture | 20 hours |
| BCISO-4 | Cellulose | PNPX | Cell mixture | 2 hours |
| BCISO-4 | Cellulose | PNPX | Cell mixture | 20 hours |
| BCISO-4 | Xylose | PNPG | Supernatant | 2 hours |
| BCISO-4 | Xylose | PNPG | Supernatant | 20 hours |
| BCISO-4 | Cellulose | PNPX | Supernatant | 2 hours |
| BCISO-4 | Cellulose | PNPX | Supernatant | 20 hours |
| DC1-18-4 | Cellulose | PNPX | pellet | 16 hours |
| DC1-18-4 | Xylose | PNPG | pellet | 16 hours |
| DC1-18-4 | Xylose | PNPG | Cell mixture | 2 hours |
| DC1-18-4 | Xylose | PNPG | Cell mixture | 20 hours |
| DC1-18-4 | Cellulose | PNPX | Cell mixture | 2 hours |
| DC1-18-4 | Cellulose | PNPX | Cell mixture | 20 hours |
| DC1-18-4 | Xylose | PNPG | Supernatant | 2 hours |
| DC1-18-4 | Xylose | PNPG | Supernatant | 20 hours |
| DC1-18-4 | Cellulose | PNPX | Supernatant | 2 hours |
| DC1-18-4 | Cellulose | PNPX | Supernatant | 20 |

## *DNA extraction and PCR*

The DNA from the bacteria was extracted using QIAGEN DNeasy Blood&Tissue according to producer's manual.

Preparations for the PCR were made by mixing the ingredients in table 5. The preparations were made shortly before the start of the PCR and were mixed in a PCR-tube. Primers (Pereyra, et al., 2010) (table 6) were delivered from Invitrogen. All PCR programs were the same as described by (Sun, et al., 2012). Total bacteria were analyzed targeting the 16S rRNA gene and the cellulose degrading bacteria were analyzed targeting both Cel5 and Cel48 genes.

**Table 5. Recipe for the PCR reaction for amplification of Cel5 and Cel48 genes as well as total bacteria.**

| PCR recipe | | |
|---|---|---|
| **Ingredients:** | Volumes (µL) | Concentration |
| **Dream Taq Green PCR Master mix (2x)** | 12.5 | |
| **Primer forward** | 1 | 10 pmol/µL |
| **Primer reverse** | 1 | 10 pmol/µL |
| **Water, nuclease free** | 9.5 | |
| **DNA template** | 1 | |

**Table 6. Primers used to detect glycosidase family 5 and 48 and total bacteria by PCR (Pereyra, et al., 2010).**

| Primers: | | | |
|---|---|---|---|
| **Forward** | Cel5_392F | Cel48_490F | Ba-T-8F |
| **Reverse** | Cel5_754R | Cel48_920R | Ba-T-1522R |

Negative controls were run on the PCR to verify that no contaminations had entered the samples during the preparation process. This sample was run with additional water as a substitute to DNA template. For the negative control the Ba-primers were used.

## *Sequence analysis*

To validate a successful amplification the PCR product was run on an agarose gel (1% agarose), with the addition of GelRed Nucleic Acid (from BIOTIUM) and a ladder. The presence of bands indicated product and their location, in comparison with the ladder, show the size of the amplified fragments (table 7). A successful run would also be signified with no band in the negative control.

Table 7. The genes Cel5 and Cel48, their estimated size measured in number of base pairs.

| Fragment | Cel5 | Cel48 |
|---|---|---|
| Number of base pairs | 362 | 430 |

Following a successful PCR, samples were purified using QIAGEN MinElute PCR Purification Kit, according to the producer manual, and sent to sequencing analysis at Uppsala genome center.

For the construction of a clone gene library, DNA was extracted from both bacteria, as described above. The PCR reaction was run as described above targeting cel5 and cel48 for both organisms. Gel electrophoresis was performed where the correct length of the amplification product were confirmed (see table 7). The bands were then cut from the gel and placed in eppendorf tubes. QUIAGEN gel extraction kit was used to extract and purify the DNA fragments from the gel. The fragments were then ligated overnight into pGEM-Teasy plasmids with T4 DNA ligase and 2X Rapid Ligation buffer. The plasmids used contained a gene for antibiotic tolerance (amplicillin). The plasmids were thereafter transferred to freeze competent *Escherichia coli* (*E. coli*) cells that had been thawed from the -70 freezer. Plasmids and *E. coli* mixture were then placed on a shaking table in SOC medium to ensure that the *E. coli* would take up the plasmid. The cell mixture was incubated at 37 ° C for an hour and a half. Each sample was then spread on three agar plates supplemented with ampicillin. The ampicillin ensured that only the cells that had a plasmid could grow, due to antibiotic tolerance on the plasmid. Different volumes were spread on the plates (10, 20 or 50 µL). The agar plates were incubated at 37 ° C overnight. Single colonies were chosen and spread on new agar plates. The plates were then placed at 37 ° C overnight. A second PCR reaction was prepared for colony PCR e g a single colony was transferred to the PCR tubes. These PCR reactions were run with three different primers: the cel5, cel48 (table 6) and M13. The PCR reaction with the cel5 and cel48 primer was run as described previously and for the M13 primer the same procedure was followed with the exception that twice the volume was used.

## *Detection and importance in environmental samples*

Terminal restriction fragment length polymorphism (TRFLP) analysis was conducted on samples collected from three biogas plants. The samples had been kept in the freezer. For extraction of DNA a sample of 200 µL were removed for each test and the protocol from MP FastDNA Spin Kit for Soil was used. The extractions were prepared in triplicates.

Table 8. PCR recipe for amplification of DNA from environmental samples for TRFLP analysis.

| PCR recipe for environmental samples | |
|---|---|
| **ingredients:** | Volumes (µL) |
| **Primer forward** | 1 |
| **Primer reverse** | 1 |
| **Water, nuclease free** | 9.5 |
| **DNA template** | 12.5 |

A PCR reaction was prepared as described in table 8 and conducted as previously described under the section above, DNA extraction and PCR. It was run with different initial DNA concentrations in order to reach the most optimal PCR amplification. The reverse primer had a fluorophore added to enable later detection. After the entire sample volume had been run on the agarose gel (2% agarose) the bands were cut and DNA extracted and purified through the use of QIAGEN gel extraction kit. Following the extraction, another gel electrophoresis was run, to verify that the sample was pure. The restriction enzymes MobI (for cel5) and AluI (for cel48) were added and left overnight to digest the samples. The restriction enzyme cuts the sample at specific sites. The enzymes were inactivated the following morning by a large change in temperature, from approximately 50 to 4 ° C. The samples were then prepared, according to the instructions, provided by the DNA sequencer company. The data collected from the analysis was analyzed using Peak Scanner Software and Microsoft Excel.

# 3. Results

## *Growth pattern*

The interpretation regarding growth in the different tests was made based on a color change noted through visual observation. As the bacteria grow the color of the medium turned yellow/orange, caused by the anchor protein in the cellulosome. Both organisms grow in a similar manner and appeared almost the same. Examples of visual confirmation of growth can be seen in figure 3. In both pictures the cultures are grown on BM and cellulose. The culture in the right figure is considerably older than those exhibited in the figure to the left, explaining the stronger coloring of the liquid. A slight difference in color between DC1-18-4 and BCISO-4 can also be seen in figure 3.

**Figure 3. Left picture. The bacteria display slightly different colors during growth. DC1-18-4 is cultivated in the bottle to the left, and BCISO-4 is in the bottle to the right. Growth on BM with cellulose. Right picture. Growth of BCISO-4 in BM with cellulose. The culture is approximately two months old.**

Based on the properties of the sugar the bacteria grew differently, as can be seen in figure 4. The culture shown in the bottles from left to right is growth on cellulose, ribose and cellobiose, respectively. These 3 carbon sources represent 3 different categories of investigated carbons; insoluble, low solubility and soluble. The growth pattern on the insoluble cellulose consisted of two phases: growth on the sediment of the cellulose following growth in solution. In the cultures shown in figure 3, these phases were also evident. These two phases differed (color and growth speed) somewhat between the bacteria, growth temperature also affected the growth pattern.

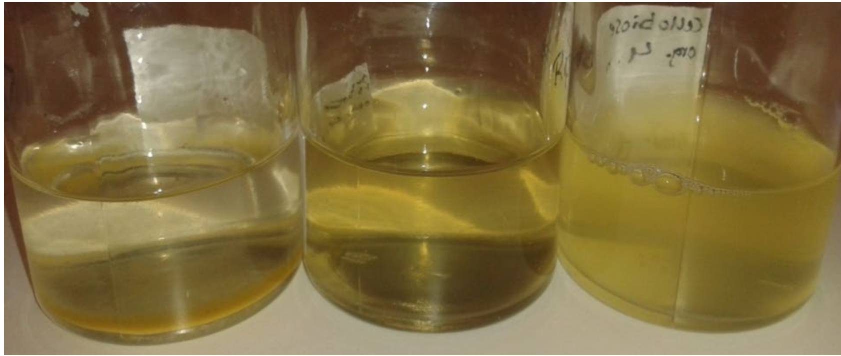

Figure 4. Comparison of the different growth patterns for BCISO-4 on the carbon sources cellulose, ribose and cellobiose (left to right).

## *Substrate utilization*

When testing the growth ability in BM on different carbon sources the test was allowed to run for approximately two months. The results were monitored through visual observation. Results for both bacteria are summarized in table 9.

Table 9. Result from the substrate utilization test on 11 carbon sources for both isolates. A double plus symbol (++) indicates fast growth, the single plus (+) indicates growth (however limited to some extent) and the minus symbol (-) indicates that no growth was evident. A ND indicates that growth could not be determined.

| | **Organism:** | |
|---|---|---|
| **Carbon source:** | BCISO-4 | DC1-18-4 |
| **Xylose** | + | + |
| **Cellobiose** | ++ | ++ |
| **Starch** | + | ND |
| **Sorbitol** | + | - |
| **Straw** | ND | + |
| **Glucose** | + | + |
| **Fructose** | + | - |
| **Mannose** | - | - |
| **Cellulose** | ++ | ++ |
| **Ribose** | + | + |
| **Hemicellulose** | ND | ND |

Due to difficulties, through visual observation, to determine if the test had a positive or negative outcome some of the results were not determined, ND. The straw and hemicellulose colored the medium, making it difficult to identify growth through visual observation. The straw was also insoluble and significantly lowered the visibility in the bottle. Growths on some substrates were also ND due to a shortage of medium and substrate, limiting the number of tests.

Over time, through visual observation, it was evident that BCISO-4 grew faster than DC1-18-4; under the given conditions. The carbon sources cellulose and cellobiose were however preferred by both organisms and

resulted in a faster growth for both of them as compared to other substrates. In some of the less preferable carbon sources, it took over a month for growth to appear and even then growth was not strong. For some of the carbon sources no growth appeared at all (table 9).

All growth tests on RCM were unsuccessful, e g no growth was observed.

## HPLC analysis

HPLC analysis of liquid samples from both bacteria illustrated that the two main products from the investigated substrates were acetate and butyrate (table 10 and 11). Other peaks were also identified in the chromatogram, suggesting additional products (as can be seen in table 10 and 11), however at very low concentrations, lower than 0.1 g/L. Thus, these values were not reflected on further.

Interpretation of the HPLC result was made after comparison to the negative control, so that the VFA production could be separated from the compounds produced from the medium only and not from the substrate. Any amount of VFA found in the negative control was subtracted from the values obtained from cultures supplemented with substrate. The measured amount of glucose in the negative control was 3.6 g/L. The glucose concentration given in table 10 and 11 are therefore the remaining levels from the original additive, illustrating that not all glucose was consumed during growth. Glucose accumulation can be observed from growth on cellobiose and cellulose.

**Table 10. VFA production during growth of DC1-18-4 on different carbon sources. Only peaks identified in the HPLC are listed. The concentrations are levels reached above those achieved in a negative control.**

| DC1-18-4 | Carbon source: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| VFA (g/L): | Ribose | Fructose | Mannose | Glucose | Xylose | Cellobiose | Sorbitol | Cellulose |
| Glucose | | | | 3.06 | | 0.26 | | 0.17 |
| Acetate | 0.31 | | 0.02 | 0.04 | 0.32 | 0.43 | 0.05 | 0.25 |
| Propionate | | 0.17 | | | | | | |
| I-Butyrate | | 0.08 | | | | | | |
| Butyrate | 0.26 | | | | 0.17 | 0.45 | | 0.23 |
| I-Valerate | | | | | | | 0.21 | |
| Valerate | | | | | | | | |

**Table 11. VFA production during growth of BCISO-4 on different carbon sources. Only peaks identified in the HPLC are listed. The concentrations are levels reached above those achieved in a negative control.**

| BCISO-4 | Carbon source: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **VFA (g/L):** | Ribose | Fructose | Mannose | Glucose | Xylose | Cellobiose | Sorbitol | Cellulose |
| **Glucose** | | | | 0.61 | | 0.66 | | 0.40 |
| **Acetate** | 0.02 | 0.02 | | 0.31 | 0.20 | 0.22 | | 0.26 |
| **Propionate** | | | | | | | | |
| **I-Butyrate** | | | | | | | | |
| **Butyrate** | | | | 0.20 | 0.10 | 0.10 | | 0.23 |
| **I-Valerate** | | | | | | 0.16 | | |
| **Valerate** | | | | | | | | |

Calculation a carbon balance for DC1-18-4 on substrate ribose with the values in table 10 gives:

Ribose:
0.05 g/mL (original concentration) divided by ribose's molecular weight of 150 equals 0.33 mol/L.
Number of carbons per 100 molars of substrate: 5 carbons 5*100=500

Acetate:
0.31 g/L of acetate from table 10
0.31 g/L divided by acetates molecular weight of 59 equals 0,005 molars/L
Per 100 molars of substrate: 0.005*300=1.5 molars
Number of carbons per 100 molars of substrate: acetate has 2 carbons 1.5*2=3

Butyrate:
0.26 g/L of butyrate from table 10
0.26 g/L divided by butyrate's molecular weight of 87 equals 0.002 molars/L
Per 100 molars of substrate: 0.002*300=0.86 molars
Number of carbons per 100 molars of substrate: butyrate has 4 carbons 0.86*4= 3.44

If 10% of the ribose is consumed: (3+3.44)/(500*0.1)*100= 12.88%
If 50% of the ribose is consumed: (3+3.44)/(500*0.5)*100= 2.58%
If 100% of the ribose is consumed: (3+3.44)/(500)*100= 1.29%

## *Physiological test: temperature*

The temperature test was run for approximately five weeks and results are summarized in table 12. To summarize, none of the organisms grew at a temperature below 25 ° C and maximum temperature for observed growth was at 54 ° and 51° C for BCISO-4 and DC1-18-4, respectively. Cultures incubated at lower temperatures grew significantly slower than the ones incubated at higher temperatures.

Table 12. Growth of BCISO-4 and DC1-18-4 at different temperatures. A double plus symbol (++) indicates fast growth, the single plus (+) indicates growth (however limited to some extent), the minus symbol (-) indicates that no growth was evident.

| Temperature (°C): | Organism: | |
|---|---|---|
| | BCISO-4 | DC1-18-4 |
| **56** | - | - |
| **54** | ++ | - |
| **51** | ++ | + |
| **37** | ++ | ++ |
| **30** | + | + |
| **25** | + | + |
| **20** | - | - |
| **15** | - | - |

An observation made, regarding the growth pattern at 51 ° C, was that growth during the first weeks was plentiful; coloring both medium and cellulose orange. However, as the test continued, the coloring lessened significantly. This phenomenon appeared for both organisms. When comparing this growth pattern to the other temperatures, they did not behave in the same way. An image of the growth for BCISO-4 at 37 ° C and at 51 ° C can be seen in figure 5. The two bottles in the image were inoculated at the same day and at start displayed the same growth pattern.

Figure 5. Growth of BCISO-4 in BM on cellulose at 37 and 51˚C (the culture at 51˚C initially had high turbidity which decreased over time and in line with this the yellow color was reduced).

## *Physiological test: pH*

The pH growth test was run for approximately one month. Measurement of pH at the end of the test illustrated pH changes over time in the bottles where growth occurred (Table 13 and 14). DC1-18-4 showed growth between pH 6.2-8.8 and BCISO-4 between 6.2-9.9. The pH value changed over time as the bacteria grew and the final value was not the same as initial. To be noted was that to some bottles quite a large volume of base/acid had to be added to

reach the desired pH (table 1 and 2) and thus a dilution of growth medium might have influenced the results.

Table 13. Growth test for DC1-18-4 conducted at different initial pH, spanning from pH 5-10. Growth is documented as a plus symbol (+) and no growth as a minus symbol (-).

| Organism: DC1-18-4 | | | | | | |
|---|---|---|---|---|---|---|
| **pH:** | 5 | 6 | 7 | 8 | 9 | 10 |
| **Actual pH at start:** | 5.1 | 6.2 | initial pH | 7.9 | 8.8 | 9.9 |
| **Growth:** | - | + | + | + | + | - |
| **pH at end** | | 5.8 | 5.8 | 6.9 | 6.8 | |

Table 14. Growth test for BCISO-4 conducted at different initial pH, spanning from 5-10. Growth is documented as a plus symbol (+) and no growth as a minus symbol (-).

| Organism: BCISO-4 | | | | | | |
|---|---|---|---|---|---|---|
| **pH:** | 5 | 6 | 7 | 8 | 9 | 10 |
| **Actual pH at start:** | 5.3 | 6.2 | initial pH | 8.0 | 9.0 | 9.9 |
| **Growth:** | - | + | + | + | + | + |
| **pH at end** | | 5.6 | 5.7 | 6.3 | 6.8 | 9.5 |

## *Enzyme activity*

A summary of the enzyme activity test results performed on pellet, cell mixture and supernatant can be seen in table 15 and 16.

The results for DC1-18-4 displayed an overall higher activity than BCISO-4, meaning that when comparing the yellow color reaction (cleavage of substrate and fluorophore) of DC1-18-4 to BCISO-4 it occurred faster and with a stronger color. This was observed in all fractions after 5 hours (table 15), indicating a larger production, or higher activity, of enzymes. Since both organisms had activity in supernatant as well as pellet it is evident that the bacteria produced free enzymes as well as cell bound. DC1-18-4 displayed a rather high and similar activity in all fractions (table 15 and 16).

For BCISO-4 a higher activity, when comparing to the other tests, was only accomplished in the cell pellet. After 23 hours, only medium activity had been reached in all other fractions. For BCISO-4 it can be observed that when grown on xylose and tested for PNPG (cell mix and supernatant) no activity was observed after 2 hours. However, activity was measured at 20 hours. For BCISO-4 grown on cellulose and tested on PNPX the activity also increases over time, in comparison to DC1-18-4 which displayed the same amount of activity throughout the test. For pellet, the reaction time of 16 hours was the only one tested, making it more difficult to draw conclusions. It could only be observed that the organism showed a high activity.

Table 15. Activity test for BCISO-4 and DC1-18-4 originally grown on glucose and given the substrate PNPG. Results are measured in (-) no activity, (+) low activity, (++) medium activity and (+++) high activity.

| Organism: | Culture grown on: | Substrate: | Fraction: | Reaction time: | Result: |
|---|---|---|---|---|---|
| **BCISO-4** | Glucose | PNPG | Supernatant | 30 min | + |
| **BCISO-4** | Glucose | PNPG | Supernatant | 5 hours | ++ |
| **BCISO-4** | Glucose | PNPG | Supernatant | 23 hours | ++ |
| **BCISO-4** | Glucose | PNPG | Cell mixture | 30 min | + |
| **BCISO-4** | Glucose | PNPG | Cell mixture | 5 hours | ++ |
| **BCISO-4** | Glucose | PNPG | Cell mixture | 23 hours | ++ |
| **BCISO-4** | Glucose | PNPG | Pellet | 16 hours | +++ |
| **DC1-18-4** | Glucose | PNPG | Supernatant | 30 min | + |
| **DC1-18-4** | Glucose | PNPG | Supernatant | 5 hours | +++ |
| **DC1-18-4** | Glucose | PNPG | Supernatant | 23 hours | +++ |
| **DC1-18-4** | Glucose | PNPG | Cell mixture | 30 min | ++ |
| **DC1-18-4** | Glucose | PNPG | Cell mixture | 5 hours | +++ |
| **DC1-18-4** | Glucose | PNPG | Cell mixture | 23 hours | +++ |
| **DC1-18-4** | Glucose | PNPG | Pellet | 16 hours | +++ |

**Table 16. Activity test for BCISO-4 and DC1-18-4 originally grown on cellulose and xylose, given the substrate PNPX or PNPG respectively. Results are measured in (-) no activity, (+) low activity, (++) medium activity and (+++) high activity.**

| Organism: | Culture grown on | PNPG/PNPX | Fraction: | Reaction time (h) | Result: |
|---|---|---|---|---|---|
| **BCISO-4** | Cellulose | PNPX | Pellet | 16 | ++ |
| **BCISO-4** | Xylose | PNPG | Pellet | 16 | ++ |
| **BCISO-4** | Xylose | PNPG | Cell mixture | 2 | - |
| **BCISO-4** | Xylose | PNPG | Cell mixture | 20 | ++ |
| **BCISO-4** | Cellulose | PNPX | Cell mixture | 2 | + |
| **BCISO-4** | Cellulose | PNPX | Cell mixture | 20 | ++ |
| **BCISO-4** | Xylose | PNPG | Supernatant | 2 | - |
| **BCISO-4** | Xylose | PNPG | Supernatant | 20 | ++ |
| **BCISO-4** | Cellulose | PNPX | Supernatant | 2 | + |
| **BCISO-4** | Cellulose | PNPX | Supernatant | 20 | ++ |
| **DC1-18-4** | Cellulose | PNPX | pellet | 16 | ++ |
| **DC1-18-4** | Xylose | PNPG | pellet | 16 | ++ |
| **DC1-18-4** | Xylose | PNPG | Cell mixture | 2 | ++ |
| **DC1-18-4** | Xylose | PNPG | Cell mixture | 20 | ++ |
| **DC1-18-4** | Cellulose | PNPX | Cell mixture | 2 | ++ |
| **DC1-18-4** | Cellulose | PNPX | Cell mixture | 20 | ++ |
| **DC1-18-4** | Xylose | PNPG | Supernatant | 2 | ++ |
| **DC1-18-4** | Xylose | PNPG | Supernatant | 20 | ++ |
| **DC1-18-4** | Cellulose | PNPX | Supernatant | 2 | ++ |
| **DC1-18-4** | Cellulose | PNPX | Supernatant | 20 | ++ |

## *Sequence analysis*

A successful extraction and PCR was confirmed by gel electrophoresis. The samples amplified presented bands in the correct size and a blank negative control.

The PCR product, amplified with the total bacteria primer, was sent for sequencing and showed results verifying that the bacteria was still the same which originally had been isolated from the biogas plant. Mutations can occur or accidental contamination when inoculating. The sequencing also confirmed that only one organism was present in the culture. If the results from the sequencing would have come back inconclusive that would have indicated presence of more than one bacteria. Samples were sent for sequencing at multiple times.

In the gel electrophoresis run after using the primers for cel5 and cel48, on extracted material for both BCISO-4 and DC1-18-4, bands could be seen corresponding to the theoretical length of the cel5 and cel48 fragments. A confirmation that the carbohydrate cleaving ability coupled to cel5 and cel48 were present in both bacteria.

The sequencing of the cel5 and cel48 genes after insertion into the pGEM-Teasy plasmid provided the entire and undamaged sequence of the genes (table 17). Being inserted into the plasmids and thereafter amplified with the M13 primer gave a larger piece of DNA that enabled a more accurate sequencing where no bps from cel5 or cel48 were lost at the ends of the fragment. This DNA sequence was analyzed providing information necessary for the TRFLP, e g at which sites the restriction enzymes would cut and the length of the obtained fragments would be obtained for DC1-18-4 or BCISO-4. The theoretical number of bps in each fragment can be seen in table 17 below. For BCISO-4 not enough PCR product were obtained for the analysis and thus for the cel5 gene no clone library could be made.

**Table 17. Insertion into the pGEM-Teasy plasmid and thereafter sequenced provided the lengths (number of bp) of Cel5 and Cel48 fragments in both isolates.**

| | Cel5 | Cel48 |
|---|---|---|
| **DC1-18-4** | 386 bp | 296 bp |
| **BCISO-4** | - | 319 bp |

## *Detection and importance in environmental samples*

Data for the TRFLP analysis of environmental samples are assembled in figure 6 and 7. For the analysis of the cel48 genes only samples from two biogas plants were successful. For the third sample the DNA amplification in the PCR was unsuccessful and no results were obtained.

The TRFLP of three biogas plants, are in the graph represented through different colors. Their location on the x-axis indicate the size of the fragment (measured in bps) and their height is the abundance found in the environmental sample. When comparing the TRFs from the cel5 TRFLP plot (figure 6) with the theoretical size of the DC1-18-4 cel5 fragments (table 17) there is a match at 386 bps. The small peak from biogas plant 1 is most likely the DC1-18-4 cel5 fragment. The TRF at 386 bp is however very small, representing 1.6%, signifying that among other bacteria with cel 5 it represented a very small fraction. DC1-18-4 was only present in biogas plant 1. Since only the size of one cel5 fragment had been identified there was no possibility to determine the presence of cel5 from BCISO-4.

For cel 48 (fig 7) the presence of TRFs at 296 or 319 bp was investigated. However, no perfect match was found. The closest match was the TRF at 328 bps from biogas plant 1.

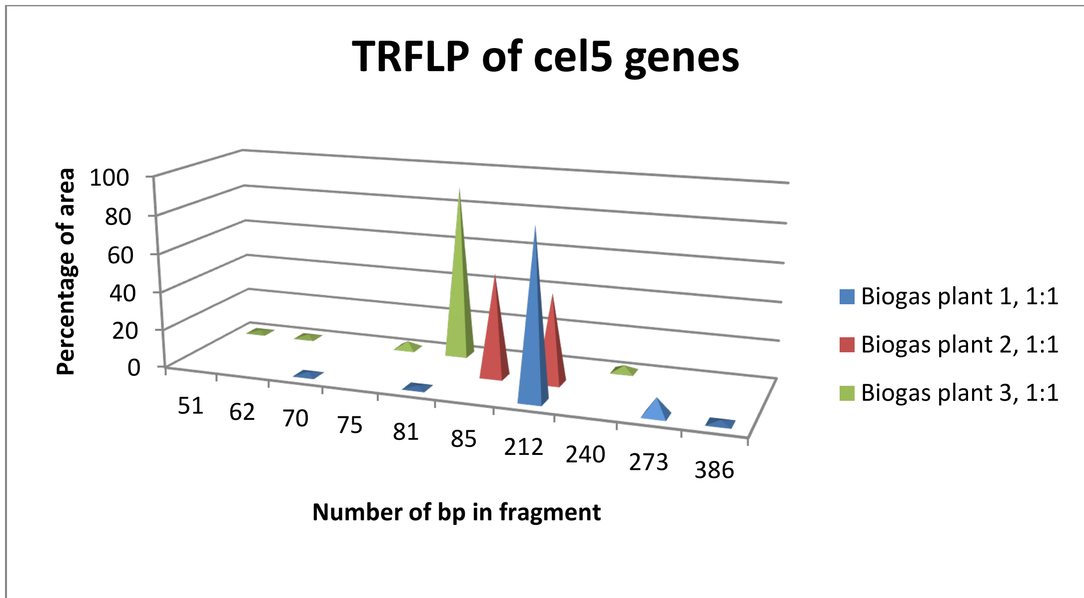


**Figure 6. Results from the TRFLP analysis of the cel5 genes. Samples were collected from three large scale biogas plants.**

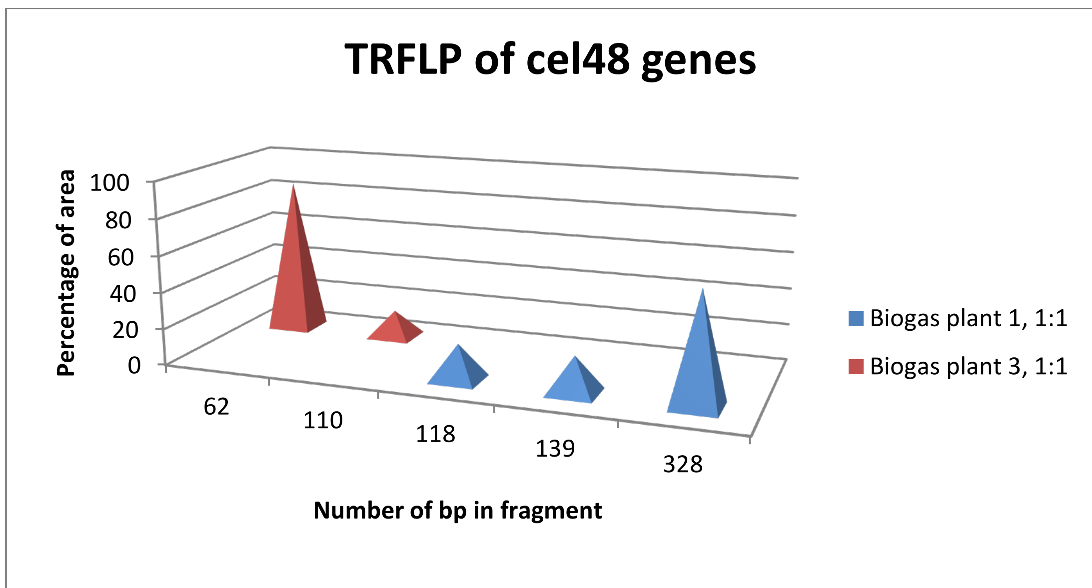


**Figure 7. Results from the TRFLP analysis of the cel48 genes. Samples were collected from three large scale biogas plants.**

# 4. Discussion

## *Genetic similarities*

The 16s rRNA gene of both bacteria had prior to this thesis project been extracted and sent on sequencing in order to determine if they had, by others, already been characterized. The 16s rRNA sequences were run in the NCBI BLAST database and comparisons were made to already uploaded sequences and the similarity calculated. Plenty of closely related microorganisms were found but only the hits with high enough similarity, higher than 95%, were further investigated. Both isolates displayed a 97% 16s rRNA similarity to other microorganisms suggesting that our isolates could be either one. When the displayed 16s rRNA similarity is high there is potential match with a known organism. If the percentage is lower than 95-97%, the dissimilarity is probably too high and then the bacterium might represent a novel species. (Stackbrandt & Ebers, 2006) (Stackebrandt, et al., 2002) (Ramasamy, et al., 2014). A case such as this, with a similarity at 97%, makes it difficult to judge if the species is novel or not. A complementary way of determination would be a comparison of physiological characteristics and see how well these correspond and a whole genome comparison. Traits for comparison could be: substrate preference, growth patterns, morphology, pH, temperature range etc. Below is a comparison based on VFA production and growth preference on carbon sources, pH and temperature.

*Clostridium straminisolvens* and BCISO-4 display a 97% 16s rRNA likeness as well as some characteristic similarities; such as their substrate preference for cellulose and cellobiose and fermentation production of acetate. A dissimilarity is however butyrate production by BCIOS-4. *C. straminisolvens* also produce lactate, ethanol, hydrogen and carbon dioxide. Those additional fermentation products were not produced by BCIOS-4. For *C. straminisolvens* the temperature span for optimum growth is 50 to 55 °C and the pH interval between 6.0 and 8.5 (Kato, et al., 2004). These values do not correspond with BCIOS-4, which had a wider pH and temperature interval. *C. straminisolvens* was isolated from a bred microbial cellulose degrading community.

*Acetivibrio cellulosolvens* was calculated to have a 97% likeness to DC1-18-4, however the bacteria displayed multiple dissimilarities. The pH and temperature growth interval is smaller for *A. cellulosolvens* compared to DC1-18-4, (pH 6.5-7.5, temperature 35-37 °C) and no production of butyrate as fermentation product. DC1-18-4 displays a characteristic yellow color when growing on cellulose, *A. cellulosolvens* does not. A larger number of fermentation products have been documented for *A. cellulosolvens* (acetic acid, ethanol, hydrogen and carbon dioxide) however the production of these was not measured for DC1-18-4. For both bacteria growth was most prominent for cellulose and cellobiose. *A. cellulosolvens* was isolated from sludge, supplemented with cellulose. (Khan, et al., 1984)

*Clostridium clariflavum* has a 97% 16s rRNA likeness to DC1-18-4. *C. clariflavum* displays a bright yellow color during growth on agar containing

cellulose or cellobiose. It has a temperature preference at 45-65 °C, pH preference at 6-8 and major fermentation products are lactate, ethanol, hydrogen, acetate, formate and carbon dioxide. DC1-18-4 prefers much lower temperatures, can tolerate a slightly higher pH and produce acetate and butyrate. *C. clariflavum* was isolated from sludge from a municipal waste fed bioreactor (Shiratori, et al., 2009).

*Clostridium aldrichii* has a 97% 16s rRNA likeness to DC1-18-4. It can utilize the substrates cellulose, cellobiose and xylan, which corresponds well with the substrate pattern of DC1-18-4, with the exception of xylan which was not tested. Fermentation products produced for *C. aldrichii* are hydrogen, carbon dioxide, acetic-, propionic-, isobutyric-, isovaleric-, lactic- and succinic acid. Temperature span favored by DC1-18-4 was slightly higher than *C. aldrichii* and the same can be said about the pH. The isolation of *C. aldrichii* was made from a wood fermenting digester (Yang, et al., 1990).

In conclusion none of the organisms matched DC1-18-4 or BCISO-4 perfectly. Overall, they displayed quite many similar characteristics, but not identical. This shows that even though high similarity on 16S rDNA (>97%) the physiological characteristics might still be very different. The comparison made was however largely based only on, pH, temperature, substrate preference and fermentation products. A wider range of tests would have made this comparison more comprehensive. Tests that would have been valuable are gram staining, microscopy (spores, mobility and size) and whole genome sequencing (and comparison to known isolates). Also, an analysis of VFA larger than 6 carbons, $H_2$ or other substrates would have set for a better comparison. Worth mentioning is that it can be difficult when comparing features measured solely through visual observation. Different observers will measure by different standards and determining growth can thus be difficult. If repeating the tests, methods such as optical density (OD) could be used to measure biomass turbidity during growth on soluble substrates.

### *Substrate utilization and physiological properties*

The substrates that gave the most plentiful growth for both bacteria were cellulose and cellobiose, these were favored above all other substrates (table 9). Both cellulose and cellobiose have in common that they consist of glucose molecules with β (1→4) bonds. However, none of the bacteria showed d good visible growth on glucose. This is a bit perplexing but could present information regarding the substrate uptake or enzyme activity of the bacteria.

From the substrate utilization test it was evident that the bacteria grew on approximately half of all the tested substrates even though plentiful growth only occurred on cellulose and cellobiose. It appeared as if the growth was somewhat inhibited on the other substrates- as only low cell density was achieved. Inhibition could be a result of absence/shortage of a trace element or nutrient buildup of a certain molecule. Growth inhibited by absence of nutrients is contradicted by the fact that no growth was apparent when using RCM, which contains a lot of nutrients. A more likely theory is that growth was inhibited by fermentation products or substrates (Kruus, et al., 1995) (Chen, et al., 2016) (Atreya, et al., 2016). Inhibition due to high substrate

concentration has previously been documented, such as for *Clostridium thermocellum* (Kruus, et al., 1995). *C. thermocellum* was shown to be inhibited by glucose and cellobiose (competitive inhibition) at high concentrations. End-product inhibition has also been documented (Chen, et al., 2016) where the authors discuss if the end-products may bind to the enzyme in a way that inhibit its function.

DC1-18-4 and BCISO-4 grew better on cellulose and cellobiose than glucose. Not all of the glucose was consumed in the bottles where glucose was the carbon source. The bacteria did not adapt during the weeks of growth on monosaccharides, meaning that the bacteria's poor ability to degrade the presented monosaccharide did not change during the period of growth. Their growth continued to be slow and at such a minimum that comparison to the negative controls had to be made in order to confirm growth. That these bacteria show a high preference towards degradation of cellulose instead of monomers makes it highly applicable in industrial processes where it is important to keep the costs low. The growth on straw by DC1-18-4 makes it highly interesting since it is a degrader of a highly available and cheap energy source. The growth was however not very strong and the production of VFA low.
Another factor which is advantageous with hydrolytic and fermentative bacteria is if they produce VFA or hydrogen gas, products which could later be used for methane production in a biogas process. The production of some VFA was measured in the cultures, however their levels were very low. A carbon balance for DC1-18-4 with ribose as substrate and assuming that 100, 50 or 10% of the substrate had been consumed showed that the produced amounts of VFA (acetate and butyrate) represented 13, 2.6, and 1.3% respectively of the consumed carbon. This indicated that a very large amount of carbon was used for production of molecules that were unidentified. It might be production of fermentation products which were not measured. However, it was noticeable that the pressure had increased, merely when handling the cultures in the bottles, indicating gas production.

In the measurement of VFA from the cultures grown on cellobiose and cellulose it was noticeable that the glucose concentration becomes rather high. Especially in comparison to the produced acetate, which was one of the major fermentation products. That the glucose molecules are piling up brings us back to the previous discussion concerning substrate inhibition or inability for substrate uptake. The inability to utilize all sugars could perhaps indicate that the bacteria thrive- when living in symbiosis with other bacteria. In a biogas plant many microorganisms cooperate to reach full degradation. The inability for glucose uptake could also indicate a feedback inhibition on enzymes.

The enzyme activity test had two parts, one part was to examine where the bacteria produced enzymes: attached to the cells (pellet), excreted to the medium (supernatant) or both (cell mixture). The other part was to examine if the bacteria would produce enzymes towards a carbon which it had not been exposed to in the last months. The results indicate that both bacteria produce enzymes that are free and cell bound. The presence of cell bound enzymes

(indicating cellulosomes) is highly advantageous since that enables for a more effective degradation of organic material. Enzymes in all fractions also indicate efficient degradation independent on carbon structures. Since the organisms originate from a bioreactor where the substrates are diverse, they most likely produce enzymes towards a large amount of carbons at all time.

The results from the pH and temperature test, for both organisms, show growth in a rather wide range. This suggest the bacteria are endurable to fluctuations in pH and temperature. The bacteria did however have preferences within those intervals and the growth velocity greatly differed within different settings.

### *Environmental samples*

The TRFLP analysis was made in order to investigate to what degree the studied isolates existed in samples from the biogas plants. Would DC1-18-4 and BCISO-4 present a large or small portion of the bacterial life in the reactor? Unfortunately, the TRFLP analysis did not go as planned; which can be seen in the results section (figure 6 and 7). The samples should have consisted of a large variety of microorganisms and the plotted data should have given many TRFs, but this was not the case. The exact reason for the state of the results is unsure.

The plan for TRFLP analysis was to make more than one analysis and see if the composition of microbes changed over time in the bioreactor. However, due to a shortage of time, only one TRFLP analysis was made. From this single analysis we can tell, with the use of the clone library, that the isolated bacteria were present. Their abundance was however very low.

# 5. Conclusion

This master thesis presents two cellulose and cellobiose degraders, producing the main fermentation products acetate and butyrate. The easy conversion of acetate into methane and carbon dioxide makes it a very desirable products. The easy access of cellulose creates endless possibilities for its degradation to create biogas.

The long-term purpose of this thesis, as described in the introduction, was to gather information possible to use for steering of a biogas process towards a larger production of methane. More specifically, increasing methane production through creating an environment where DC1-18-4 and BCISO-4 would thrive. The environment would have focused on optimum pH, temperature, etc. However as has been stated previously, the bacteria represented a small fraction of the biogas community, which could indicate that they are not resilient enough to be used for such a purpose. However, it could also indicate that the environment in the biogas system was not optimal for DC1-18-4 and BCISO-4.

Based on the facts gathered in this work I would say that BCISO-4 and DC1-18-4 are bacteria which have not, in the past, been characterized. This conclusion is based on the 16s rRNA sequencing results in comparison to the gathered information about fermentation products as well as substrate, pH and temperature preference.

The gathering of information, which was the primary focus of this project, was successfully accomplished. However, if further tests were assembled additional VFA analysis could have been of interest, such as analyses of VFA over the growth period and with additional growth substrates. TRFLP is another interesting aspect, and primarily the environmental TRFLP should have been rerun to confirm or contradict the results. The results are questionable since so few peaks (TRFs) are present, for cel5 as well as cel48. Can there really be so few bacteria with carbohydrate degrading capacity present? Having to draw conclusion from a single run makes the risk for error large. Subtracting samples from the bioreactor at multiple times would also have been interesting in order to answer questions like; does the proportions of DC1-18-4 and BCISO-4 change over time in the bioreactor? However, if assuming that the results are correct we are presented with the question: why are the levels comparably low? The result from enzyme activity analysis concludes that the bacteria have good enzymatic activity, presenting enzymes free in solution as well as cell bound. The pH and temperature span for both bacteria are rather wide, which tells of rather adaptable bacteria. The substrate test however indicates a rather slim growth preference; of the tested substrates the bacteria preferred cellulose and cellobiose (however, only 12 growth substrates were tested). Perhaps this is the reason why the level of these bacteria appears to be low in the tested biogas process. Further testing would be required to fully understand these bacteria and the possibilities they pose for optimization of bioreactors.

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