Characterization of a novel bacterium originating from a biogas process

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Abstract

An anaerobic bacterium, isolated from an enrichment culture originating from a biogas reactor operating at high ammonia conditions, was characterised via various tests. An analysis of the 16S rRNA gene confirmed that the closest relative was *Desulfotomaculum halophilum* with 92,5 % similarity. The isolated bacterium had a narrow substrate range and only used three compounds for growth, which of pyruvate was the optimal, used as a substrate for most inoculations in this study. Lactate was also tested as substrate, together with sulphate to evaluate if the isolate could use it as an electron acceptor. Neither of those inoculations formed growth. The bacterium ferments pyruvate and acetate are formed. It is a gram positive mesophilic bacterium and growth was measured between 25 °C and 38.5 °C. It grows well in high concentrations of ammonia, tested up to 0.24 M, and can also grow in high sodium salinity, here tested until 0.18 M. Microscopic analyses showed that the cells are rod shaped and they move by twisting around. The described bacterium is most likely a new species.

Keywords: biogas, ammonia-tolerance, mesophilic, characterization, bacterium

Table of contents

List of tables

List of figures

Abbreviations

HPLC	High Performance Liquid Chromatography
OD	Optical density
VFA	Volatile fatty acids

1. Introduction

1.1 The biogas process

In the biogas process organic matter, such as food waste or manure, are degraded by microorganisms. They do so in an anaerobic environment, where large organic compounds are degraded step by step to form biogas, a mix of methane and carbon dioxide. Large organic compounds, for example proteins and polysaccharides, undergo hydrolysis via extracellular enzymes secreted by hydrolytic bacteria. The large complex compounds are too big for microorganisms to use as nourishment and are therefore broken apart by the extracellular enzymes outside of the cells. After hydrolysis smaller compounds are formed, such as sugars, amino acids or glycerol, and they are taken up by the cell and used by the microorganisms as substrate in a process called fermentation (Schnürer and Jarvis 2018).

The fermentation is a process where molecules are broken down anaerobically. In the glycolysis glucose are broken down to pyruvate. Pyruvate can then either be oxidised, aerobically, via tricarboxylic acid cycle or it can be reduced, anaerobically, for example through fermentation in which lactate, acetate etc can be formed (Britannica 2023). The fermentation from pyruvate to acetate gives one ATP per pyruvate (Sawers and Clark 2004). In fermentation no external electron acceptor is used and products such as short chain organic acids, alcohols etc are formed when the organism place electrons on internal compounds. There are many different fermenting bacteria in the biogas process and their substrate specificity vary. The variation of fermenting bacteria and substrates leads to a variety of products (Schnürer and Jarvis 2018).

In a biogas process the products from fermentation are used by microorganisms that convert them through reactions called anaerobic oxidation. The anaerobic oxidations mainly form hydrogen gas, carbon dioxide and acetate. A regulating factor for the anaerobic oxidation is the concentration of hydrogen gas in the surrounding environment. It is therefore important that hydrogen-consuming organisms are present and keep the concentration of hydrogen gas low in the anaerobic degradation process (Ahring et al. 2003). In biogas systems there are two types of methanogens, hydrogenotrophic methanogens and acetoclastic methanogens. Hydrogenotrophic methanogens use hydrogen gas and carbon

dioxide to form methane. The hydrogenotrophic methanogens regulate the hydrogen concentration in the environment and are therefore important for a stable process (Demirel and Scherer 2008). Aceticlastic methanogens use acetate to form methane and carbon dioxide (Schnürer and Jarvis 2018). If the methanogens are unactive in the biogas process their substrates, H_2, CO_2 and acetate accumulates, which leads to build up of organic acids such as volatile fatty acids (VFA). Accumulation of acids might result in a pH drop to low levels and there is a risk that the complete process stops (Bangsø Nielsen and Angelidaki 2008).

One common cause for VFA accumulation is inhibition of methanogens in processes fed protein-rich materials, such as slaughter house waste, chicken manure or food waste. These materials are commonly used as substrates in biogas processes, since they give high methane yields and the generated digestate has a high nutrient content, which makes it a good fertilizer (Kovács et al. 2013). However, proteins and amino acids contain amino groups, which are released as either ammonia or ammonium during the degradation. Ammonia and ammonium are in equilibrium and which one is dominant depends on the pH and temperature of the process. High levels of ammonia can inhibit microorganisms and, in the biogas process, the aceticlastic methanogens are particularly negatively affected by rising levels of ammonia (Schnürer and Jarvis 2018), which leads to high VFA levels and lower methane yields (Wang et al. 2009). However, bacteria can also be inhibited by ammonia and it is therefore important that the complete microbial community in the process can tolerate high ammonia levels so the organic materials can be efficiently transformed into methane. The purpose of this study was to characterize an ammonia tolerant bacterium. This will lead to better understanding of the microbial community within the biogas process, especially with high ammonia. In long-term, increased knowledge of ammonia-tolerant species will make it possible to operate a biogas process to achieve the highest possible methane yield under high ammonia conditions.

Today, there are many blank spots regarding the knowledge of the microbial community within a biogas process. Via molecular analyses a great diversity of microorganisms is seen but their role in the biogas process is unknown. Isolation and characterization of microorganisms is important to broaden the knowledge and gain understanding regarding how a biogas process best is operated.

1.2 Characterization of a novel bacterium from a biogas process

When isolating a bacterium, it is important to characterize it to decide its species or if it is a new unknown bacterium. A characterization will give a description of the bacterium and provide information on which role it has in a microbial community.

If they are also found in the biogas process the characterization gives information on the role of these bacteria in the biogas process and thus also provides information on how the total degradation works. To characterize a new bacterium various analysis should be done, including sequencing of the 16S rRNA gene, determination of oxygen sensitivity, substrate specificity and the use of electron acceptors. Other growth conditions such as temperature range, pH range and NaCl tolerance should also be determined. Furthermore, cell morphology (shape and motility), C+G DNA content and cellular fatty acid patterns should be determined. growth conditions. Other required analysis is the sequencing of the whole genome and comparison with the closest relative in order to determine the phylogenetic placement of the novel species (Schnürer, Singh, et al. 2022)

1.2.1 16S rRNA gene

To identify bacteria and systematically decide their taxonomic placement, sequencing of the 16S rRNA gene is often used. It is a housekeeping gene that have conserved regions, and can be amplified using PCR primers that are designed to target this gene. Therefore, the use of correct primers is important to get a good result from the PCR. In the 16S rRNA gene there are also variable sites that identify the taxonomy of different bacteria (Fuks et al. 2018). There are other ways to identify a bacterium, for example by sequencing the whole genome. According to Fuks et al. (2018) one advantage of sequencing the 16S rRNA gene is that there are developed databases with 16S rRNA genes sequences. In order to get a correct classification, the characterization of a new bacterium contains sequencing of both the 16S rRNA gene and the whole genome.

1.2.2 Oxygen sensitivity

Another parameter to investigate during characterization of anaerobic bacteria is their tolerance to oxygen. During the oxygen metabolism superoxide is released, which is a strong oxidant and will damage the cell if not scavenged. Aerobic organisms have superoxide dismutase that protects the cell from the damaging superoxide. Anaerobic organisms have little to no superoxide dismutase and are therefore sensitive to oxygen (Pan and Imlay 2001). There is a gradient in how sensitive different bacterium are, some anaerobes die within an hour of contact with molecular oxygen while other can survive longer in the presence of oxygen (Rolfe et al. 1978).

1.2.3 Substrate and electron acceptors

To grow, microorganisms use different substrates to produce energy and for biosynthesis. Bacteria can use a variety of substrates and bacteria that perform anaerobic oxidation needs an external electron acceptor, such as nitrate, sulphate

etc. During characterization of the bacterium, it is important to decide which substrate support the growth of the bacterium and if they need an electron acceptor for degradation of some substrates.

2. Methods and Materials

2.1 Source of organism

The isolated bacterium investigated in the present study (here referred to as isolate 22), was previously isolated from an enrichment culture that had been obtained by inoculating a reactor with sludge from a high ammonia biogas process. The reactor was thereafter continuously fed with propionate under high ammonia conditions. In order to isolate species from the culture, samples from the enrichment culture was then transferred to agar-shakes containing pyruvate as carbon source as done by Westerholm, Roos, and Schnürer (2010).

2.2 General preparation of incubation bottles and tubes

For cultivation of the isolate, basal media was prepared as explained by Westerholm, Roos, and Schnürer (2010). The basal medium contained phosphate buffer in order to maintain the pH around 7. Resazurin was one of the components in the basal media, which has a bright pink colour when in contact with oxygen. To make the media anaerobic, it was boiled to remove oxygen and then cooled on ice while flushing with N_2 to avoid oxygen getting back into the medium. For incubation in 118 ml bottles, 18 ml of basal media containing 0.2 g/L yeast was added to each bottle under flushing with N_2. The bottles were closed with butyl stoppers and sealed with aluminum capsules. The gas in the bottles was switched from N_2 to N_2/CO_2 (80/20) gas mixture by creating vacuum using a vacuum pump and the bottles were then filled up with the gas mixture. Vacuum was created a total of three times. The bottles were then autoclaved for 20 minutes at 125 °C. After sterilization 1 ml of C1 (a vitamin solution) and 1 ml of C2 (a solution containing sodium sulphide and cystein to reduce the last O_2 in the media and in the gas phase) were added, as they cannot be autoclaved, with sterile syringes resulting in a total of 20 ml of media in each bottle. After adding the C2 solution the resazurin in the basal media turned clear, indicating that there is no oxygen in the media.

For studies of optimized growth conditions, 4,5 ml media was added to glass tubes during N_2 flush. The tubes were closed with butyl stoppers and sealed with plastic screw corks. The gas in the bottles was changed from N_2 to N_2/CO_2 (80/20) as described above. The tubes were then autoclaved for 20 minutes at 125 °C. After sterilization 0.25 ml C1 and 0.25 ml C2 was added to each tube with sterile syringes giving a total volume of 5 ml.

For incubations 10 mM substrate was added and inoculation of 5% volume growing culture of isolate 22 were used, incubations were places in 37 °C if nothing else is mentioned.

2.3 Preferred substrate

Previous studies to find growth substrates for isolate 22 have tested different types of sugars and amino acids. In these tests, isolate 22 was grown in medium containing yeast extract and 10 mM of the substrate. These tests demonstrated that pyruvate was a good substrate for the bacterium and this substrate was therefore used in cultivations of the present study (if not stated otherwise).

2.4 Determination of growth

Growth was determined by visual examination of turbidity in the anaerobic bottles and by analyzsis of the products formed using a High Performance Liquid Chromatography (HPLC) as described by Westerholm, Roos, and Schnürer (2010). For preparation of HPLC analyses, 0.7 ml from the culture was taken out and transferred to an Eppendorf tube. To the tube 70 µl of 5 M H_2SO_4 was added to avoid precipitation of compounds in the HPLC. The solution was then mixed and transferred, with a syringe via sterile filter, to HPLC glass vials. Both tubes and bottles were tested from different inoculation conditions.

2.5 Growth conditions

To examine the maximum and minimum temperature at which the isolate can grow, seven 118 ml bottles with basal media and pyruvate were inoculated with isolate 22. The bottles were incubated at different temperatures according to table 1.

Table 1: The different temperatures that the isolate 22 were incubated at to find the minimum and maximum temperature that supports its growth.

Bottles	Temperature (°C)
1	15
2	20
3	25
4	30
5	38,5
6	45
7	52

The optimal temperature was measured by incubation in glass tubes. A total of 15 glass tubes with basal-medium were prepared, in which 0,5 ml of isolate 22 was added. An additional tube without inoculum was prepared as a negative control. Triplicate tubes were incubated at each temperature and one negative control was placed at 37 °C (Table 2). The growth in the incubations was monitored by analyzing the optical density (OD at 600nm) of the cultures in the tube once a day.

Table 2: Triplets of inoculated tubes were placed under following conditions to examine the optimal growth temperature, growth was measured with optical density at 600 nm.

Tubes (nr)	Temperature (°C)
3	35
3	37
3	38,5
3	41
3	45
Negative control	37

The tolerance to high ammonium concentration was tested by cultivating the isolate at gradually increasing levels of NH_4Cl according to table 4. The cultivations were done in nine 118 ml bottles with basal media and the first bottle contained 0.05 M NH_4Cl and the subsequent step was performed with an increase of 0.05 M. The increases were thereafter made with 0.02 M intervals. One bottle with 0.05 M NH_4Cl without inoculum was used as a negative control. Every time visible growth was noticed, 1 ml of microbial culture from the current concentration was transferred to the next step according to table 3.

Table 3: Presentation of the different concentrations of NH4Cl that isolate 22 has been tested in. The concentration was increased as growth appeared in the previous concentration.

Bottle	NH_4Cl (M)	Isolate 22
1	0,05	+
2	0,10	+
3	0,12	+
4	0,14	+
5	0,16	+
6	0,18	+
7	0,20	+
8	0,22	+
9	0,24	+
Control	0,05	-

To further investigate which conditions this bacterium tolerate, the isolate was inoculated in increasing sodium chloride concentrations. Bottles with basal media and pyruvate was prepared with NaCl and isolate 22 according to table 4. To one of the bottles with 0.05 M NaCl isolate 22 was added. When visible growth appeared in that bottle, 1 ml was transferred to the next concentration according to table 4.

Table 4: Presentation of the different concentrations of NaCl that isolate 22 has been tested in. The concentration of NaCl was gradually increased as growth appeared in the previous concentration.

Bottle	NaCl (M)	Isolate 22
1	0,05	+
2	0,10	+
3	0,12	+
4	0,14	+
5	0,16	+
6	0,18	+
Control	0,05	-

All previous inoculations have been strictly anaerobic. To test the isolates resistance to oxygen, five bottles were prepared with 0, 1, 2, 3 and 5 % oxygen content. The oxygen was added from the room air with sterile syringes. It was assumed that the air inside the room had the same composition as the atmosphere, i.e. ~ 21% oxygen.

2.6 Lactate and sulphate growth

To complement the previous results of the preferred substrate and to further investigate the use of external electron acceptors during growth, three 118 ml bottles with basal media were prepared with 0.2 ml of 1 M lactate to a total concentration of 10 mM. In two of them 0.4 ml 1 M Na_2SO_4 was added to a total concentration of 20 mM. To the bottle with only lactate and to one of the bottles with lactate and sulphate 1 ml of isolate 22 each was added (Table 5). The third bottle was a negative control. The bottles were incubated for 22 days.

Table 5: Presentation of added substances to each bottle when lactate was tested as substrate and sulphate as electron acceptor for isolate 22. Lactate itself was tested as substrate but also together with sulphate as electron acceptor. One negative control with lactate and sulphate without isolate 22 was also incubated.

Bottles	Lactate	Sulphate	Isolate 22
1	+	-	+
2	+	+	+
Control	+	+	-

2.7 DNA extraction

To extract DNA from isolate 22 the DNeasy Blood and Tissue Kit from Qiagen was used. First 2 ml of the isolate was added to a 2 ml microcentrifuge tube, with a sterile syringe, and centrifuged at 5000 g for 10 minutes. This procedure was repeated to a total of 4 centrifugations until a bacterial pellet was visible. After the fourth centrifugation the supernatant was totally removed. The bacterial pellet was resuspended in 180 µl enzymatic lysis buffer and then the tube was incubated at 37°C. After 30 minutes incubation 25 µl proteinase K and 200 µl Buffer AL was added to the solution and the tubes was incubated at 56°C for 30 minutes. Then 200 µl of 99 % ethanol was added to the sample and the tubes were mixed by vortexing.

The solution was then transferred, by pipet, to a DNeasy Mini spin column placed in a 2 ml collection tube and spun at ≥ 6000 g for 1 min. The flow through and collection tube was discarded. The DNeasy Mini spin column was places in a new 2 ml collection tube and 500 µl Buffer AW1 was added and the tube was again spun at ≥ 6000 g for 1 min. Discard the flow through and collection tube and place the DNeasy Mini spin column in a new 2 ml collection tube. 500 µl of Buffer AW2 was added and spun at 20000 g for 3 minutes. The flow through and collection tube was discarded. The DNeasy Mini spin column was placed in a 1.5 ml Eppendorf tube and 100 µl Buffer AE was added to the DNeasy membrane. The tube was incubated at room temperature for 1 minute before spun at ≥ 6000 g for 1 min. The DNeasy Mini spin column was discarded.

2.8 PCR and sequencing of 16S rRNA gene

For 16S rRNA gene sequencing the forward primer was Bakt341f (CCTACGGGNGGCWGGAG) and the reverse primer was Bakt805r (GACTACHVGGGTATCTAATCC) and they were degenerated from the primers by Herlemann et al. (2011).

To a master mix (2x Phusion Mastermix) with 1 µl primer Bakt341f, 1 µl primer Bakt805r and 22 µl water, 1µl of DNA extract from isolate 22 were added. A control of only the master mix was also included. The two solutions were set in a PCR machine and the amplification was run under following conditions: initial denaturation at 95°C for 15 min followed by 30 cycles of 95°C for 1 min, 61°C for 1 min and 72°C for 1 min and a final elongation step for 7 minutes at 72°C.

The PCR products were confirmed using agarose gel electrophoresis. 5 µl of the DNA PCR sample was dyed with 1 µl 6X DNA loading dye and then put in a well on the gel plate. 5 µl of the negative control was dyed and added to a well next to the DNA sample. In another well 4 µl of a DNA ladder (GeneRuler 100 bp DNA Ladder) was added.

The 20 µl of DNA sample left from the PCR reaction was then treated, and the amplified PCR product was purified from the remaining primers, genomic DNA and components of the mastermix. 16 µl of Agencourt AMPure was added to the PCR tube and the solution was pipetted up and down a few times to get mixed. The tube was placed on a magnetic board and after five minutes the clear solution was pipetted out. To rinse 180 µl of 70 % EtOH was added for 30 seconds before it was pipetted out. A total of two washes were performed. Once the EtOH was eluted the tube was removed from the magnetic board and the last EtOH was let to air dry. 20µl of nuclease free water was added and mixed with the magnetic beads by pipetting up and down a few times. The tube was places back on the magnetic tray and after five minutes the clear solution was pipetted into a new sterile PCR tube. The solution was sent for sequencing by Macrogen. The final sequence was analyzed using nucleotide BLAST (National Library of Medicine, 2023).

2.9 Microscopy

The morphology of the isolate was visually examined with the microscope Lumascope LS720 by Etaluma.

2.10 Gram staining

To find out if the bacterium is gram positive or gram negative, it was stained using conventional staining methods (Smith and Hussey 2005).

3. Results

3.1 Preferred substrates

The previously tests to find substrates used by isolate 22 gave the results presented in table 6. The incubation with only yeast extract from the media gave a reading of 0.042 g/l acetate on the HPLC analysis. The average of acetate produced from yeast was therefore subtracted from the acetate reading of each substrate, to find out how much acetate was produced from each substrate. To be able to draw a conclusion that the microorganism has grown on a substrate it needs to produce more than 0.2 g/L acetate, which is marked with (+) in table 6. If there was more acetate produced from a substrate than only the yeast, but less than 0.2 g/L, that is marked with (*) in table 6. There were three substrates, according to these previously done tests, that support growth for isolate 22. The substrates is fructose, mannose and pyruvate. The highest acetate reading was for pyruvate. The isolate had a narrow substrate specificity and could only use sugars to grow.

*Table 6: Results from the test with isolate 22 grown on different substrates. The substrates presented in this table gave production of acetate, the extended list of tested substrates is found in appendix 1. + = growth and * = some growth.*

Substrate	Growth
Glycerol	*
Fructose	+
D,L-laktat	*
Mannose	+
Methionine	*
Pyruvate	+
Raffinose (pentahydrate)	*
Ribose	*
Sorbitol	*

3.2 Lactate and sulphate growth

When analyzed with HPLC there was less than 0.2 g/L acetate in both the incubation with and without sulphate together with lactate. There were also equal amounts of propionate as acetate in both incubations, but less than 0,2 g/L. Lactate, both with and without additional sulphate, did not support growth for isolate 22.

3.3 PCR

The gel electrophoresis of the PCR products when using DNA extracted from isolate 22 indicated formation of one PCR product around 400-500 base pairs when viewed under UV-light and compared to the DNA ladder next to it. The gel electrophoresis indicated that no PCR-product was formed in the negative control (Figure 1). The PCR product was sent for sequencing and the result was analyzed with BLAST. This showed that the closest described relative were *Desulfotomaculum halophilum* with 92,54 % identity.

Figure 1: The gel electrophoresis plate from analysis of the PCR products from the 16S rRNA gene viewed under UV-light. To the left (1) is the DNA reference ladder and the single band right next to the ladder (2) are the isolate 22 PCR sample with a DNA sequence of app. 450 bp. From the third well the negative control gave no results under UV-light.

3.4 Growth conditions

3.4.1 Maximum and minimum temperature

There was no growth visible at 20°C or below, or at 45°C or above but in all the bottles between there was visible growth. A HPLC analysis of all bottles except the 15 °C strengthened the result that there was no growth (<0.2g/L acetate) when incubated at or below 20 °C and at or above 45°C. The bottles incubated at 25°C, 30°C and 38,5°C had formed between 0,45 g/L to 0,51 g/L acetate. Consequently, growth of isolate 22 is, from tested temperature intervals, supported between 25°C and 38,5°C.

3.4.2 Optimum temperature

To determine the optimum temperature, isolate 22 was grown in glass tubes and analysed by testing the optical density of the solution. The growth of isolate 22 in the glass tubes were not satisfying and the readings were not consistent. Therefore, the optical density tests were terminated and the production of acetate in the tubes were instead tested with HPLC analysis eight days after incubation to test if there were more acetate formed in one temperature than the others. The amount of formed acetate ranged between 0.46 g/L to 0.49 g/L in the incubations at 37, 38.5 and 41 °C. To complement the HPLC analysis from the glass tubes, and to further investigate the optimum temperature, HPLC analysis were made on the bottles incubated at 20°C, 25°C, 30°C, 37°C, 38.5°C, 45°C and 52°C, 21 days after incubation, to get an overview of how much acetate is produced by the isolate in each temperature. From the bottles with growth (25°C, 30°C, 37°C, 38.5°C) the amount of formed acetate was between 0.45 g/L to 0.51 g/L. There was no distinct difference between the different temperatures, neither in the glass tubes nor the bottles, and no optimum temperature were possible to determine during this project.

3.4.3 Ammonium concentration

When incubated with ammonia, isolate 22 grew until the concentrations reached 0.24 M. Further increase in concentration were not tested due to time constrains. Visually, isolate 22 seemed to grow better and form more cells quicker when there was some NH_4Cl added to the medium than without it.

3.4.4 Sodium Chloride concentration

The growth in the bottles with sodium chloride was normal at 0.05 M but already at 0.10 M there were a visible decline in growth. When compared to growth in NH_4Cl enriched media the growth of isolate 22 is slightly inhibited when grown with NaCl. In this project it was possible to grow isolate 22 in up to 0.18 M NaCl, further increases in NaCl concentration were not made due to time constraints.

3.4.5 Oxygen resistance

After two days incubation the control, with 0 % oxygen, had grown and formed cells that formed turbidity to a visible amount. The incubation with 1 % oxygen in the gas phase had visibly less growth than the control bottle. Likewise, the incubation with 2, 3 and 5 % oxygen had gradually lower to no growth at all. The media in the bottles with oxygen added had turned pink, an indication that there is oxygen in the incubation. After another 3 days there was growth in all bottles and white flakes had formed in all bottles with added oxygen. The medium had turned clear again, indicating an anaerobic environmont. The amount of flakes increased with the oxygen content.

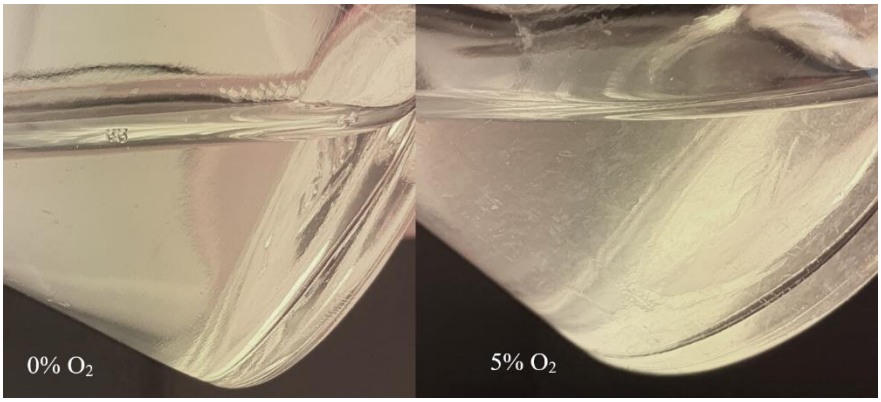

Figure 2: The bottles with 0% oxygen (left) and 5 % oxygen (right). In the left bottle there are normal growth for isolate 22 and in the right bottle there are flakes formed, that makes the solution unclear, indicating that something happens within the bottle when oxygen is present.

3.5 Microscopy

From microscope images of isolate 22 the bacterium can be viewed as a rod shaped slightly curved cell. There was some movement of the cells and the movement could be described as twisting. The size of the cells was not possible to determine. In figure 3 the cells are visible as dark rods.

Figure 3: Microscope image of the isolate 22. The bacteria are the dark rod shaped cells.

3.6 Gram staining

The bacteria stained positive, see figure 4.

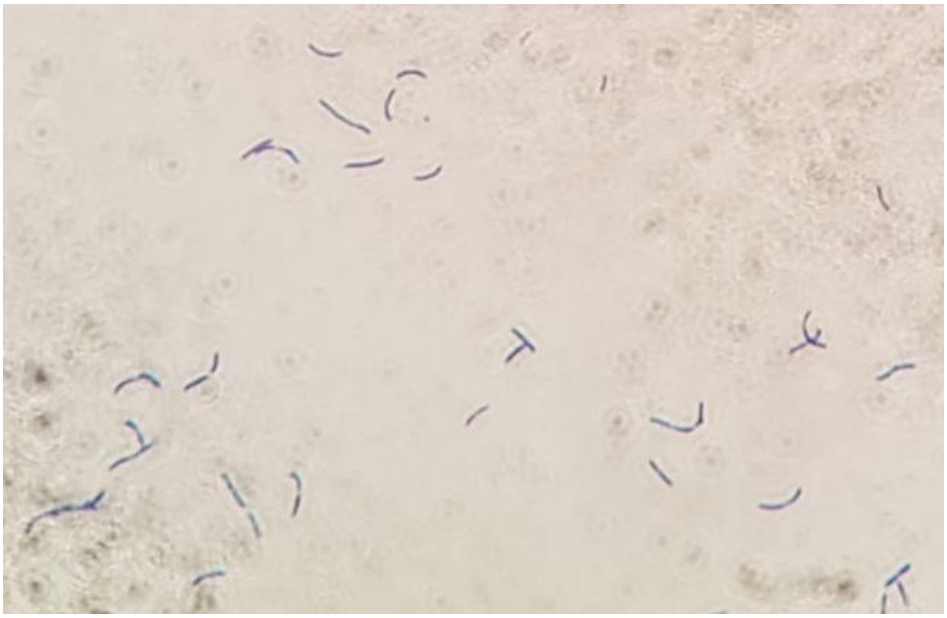

Figure 4: Microscope image of isolate 22 after gram staining. The blue colour indicates gram positive cell walls.

4. Discussion

When analyzing the PCR product, from the 16S rRNA gene, with gel electrophoresis there was one band showing in the range of approximately 500 bp. That is reasonable because the primers are placed on the gene forming a sequence of 464 bp. After sequencing the 16S rRNA gene was analyzed using BLAST and the organism with highest similarity in that sequence was *D. halophilum*. A previous sequencing with primers giving a larger product (1500 bp) had uncertainties. Those uncertainties could arise from primers that don´t fit properly or a PCR protocol that is not optimal for this gene. Nevertheless, the sequencing of the sequence from the present study was certain and showed that the closest described organism was *D. halophilum* which is a halophilic bacterium collected from an oilfield. *D. halophilum* is a sulphate reducing bacterium and grows on various substrates if sulphate is present as external electron acceptor. For example, it does not grow on lactate if sulphate is not present, but do grow on lactate if sulphate is present (Tardy-Jacquenod et al. 1998). This is in contrast to isolate 22 that do not grow on lactate either with or without sulphate. 92.5 % similarity is relatively low and isolate 22 is therefore most likely a new species. Traditionally the limit for a new species lies at 97 % (Stackenbrandt and Goebel 1994). Differences in substrate use between the two bacteria are furthermore support that isolate 22 and *D. halophilum* are two different species. To get a more certain description of similarities and differences between the two species a whole genome sequencing of isolate 22 needs to be complete in order to compare them.

Isolate 22 is a mesophilic bacterium and should therefore have a growth maximum at or around 37 °C. To test the optimal growth temperature optical density was analyzed of the incubations in glass tubes. Those incubations did not go as planned and gave inconsistent results due to low cell formation and problems measuring the OD in the glass tubes, probably due to conditions that were not satisfying for supporting growth of isolate 22. Those test where therefore terminated and another approach to find the optimal growth temperature were to test the amount of acetate produced in the tubes and bottle incubations. Analyses of the acetate was done once for every incubation at a certain time after inoculation. Since there is the same concentration of substrate in all incubations the amount of formed acetate should be the same in the end. The results from HPLC analysis of acetate concentration showed that there were about the same amount of acetate in

all tubes and bottles. To find the optimum growth temperature the acetate would have had to be analysed over time for the incubations, to see which temperature grew fastest and therefore formed acetate first, in the same way optical density were tested.

Nevertheless, isolate 22 grew in the range between 25°C and 38,5°C which is normal for a mesophilic bacteria. *D. halophilum* had an optimum growth at 35°C and slow growth at 30°C and 40°C. That is similar to the growth for isolate 22. Both the tests on the optimum growth temperature and the maximum and minimum temperature is possible to extend to get more detailed results. Incubations with smaller temperature intervals would give smaller gaps than this study. The lack of data between 38,5°C and 45°C leaves a big gap on where the actual maximum temperature that supports growth are. To decrease the temperature steps between the incubations would make it possible to get a more precise value on where the maximum and minimum temperatures are. To once again try to measure he optimum temperature, inoculation with ammonia, preferably the optimum amount, could give better results.

The isolate tolerated high concentrations of ammonia as was expected since it originates from a biogas system with nitrogen rich substrates. There are some bacteria that lack active pumps to transport ammonia into the cells. Lack of those pumps could be a coping mechanism when growing in ammonium rich environments (Manzoor et al. 2016). It is possible that isolate 22 also lacks those pumps, but this have to be further investigated by genome sequencing to be able to make a correct conclusion. It could therefore be an advantage for those bacteria to grow in an environment with high ammonia levels because that gives them the opportunity to get a viable amount of nitrogen into the cell without the active pumps (personal comment Maria Westerholm). A continuation of this experiment would be to investigate the ammonia levels being most optimal for this bacterium. Interestingly the growth of the bacterium when inoculated with sodium chloride was restricted, after 2 days incubation, already at 0,10 M NaCl. After another three days the growth had improved indicating that it needed some time to adapt to higher NaCl. Although the growth with NaCl had been improved, the slight inhibition at start was not detected for incubation with NH_4Cl. It is hard to determine if there is a difference, by visual analyzis, when comparing the NaCl incubations with incubations without added compounds. To be able to draw a conclusion whether the NaCl increase or decrease growth, in comparison to incubations without it, further testing needs to be done. The closest relative, *D. halophilum*, is a halophile and had an optimum growth at 4-6% NaCl (approximately 0.85 M) which is a very high salinity. To find the concentration of ammonia and sodium chloride at which isolate 22 can grow, increasing concentrations could have been made with larger intervals to shorten the total time to reach higher concentrations. There was no information to support a hypothesis on how high ammonia concentration that the

isolate could tolerate since the characterization of *D. halophilum* only tested sodium salinity tolerance. Based on that characterization, that the *D. halophilum* had an optimal growth around 0.85 M NaCl, the intervals could have been bigger. On the other hand, the similarity between the two are quite low and could therefore differ a lot in characteristics. Regardless, isolate 22 is tolerant to high concentrations and here it grew to as much as 0.24 M NH_4Cl and quite high concentrations of NaCl, here tested up until 0.18 M. Further cultivations will be performed in the lab to determine the upper limit for which concentrations of NH_4Cl and NaCl isolate 22 can tolerate.

The oxygen tolerance test, after 2 days, resulted in less growth in the bottles with oxygen in the gas phase. Already at 1 % O_2 the growth of isolate 22 decreased. Therefore, it is likely that this bacterium is very sensitive to oxygen and prefer a strictly anaerobic environment. Since the inoculations in the glass tubes did not grow as expected, or not at all in some of them, there is a possibility that those tubes do not give a totally anaerobic environment. That could be the reason the incubations did not increase as hoped and gave unclear results. The glass bottles that were crimp sealed were probably more airtight than the plastic screw caps on the glass tubes since the isolate grew well in those. After 5 days incubation with oxygen in the gas phase the medium had turned clear, indicating no oxygen, and white flakes had formed in the bottle. One possible explanation is that the oxygen had somehow reacted with something in the medium (for instance the reducing agents) and precipitated. Therefore, the molecular oxygen levels in the bottles dropped and the environment turned anaerobic. The new anaerobic environment was suitable for isolate 22 and it started to grow. After visual examination the amount of flakes in the bottles increased with increasing amount of oxygen added, that supports the idea of it being a precipitate from a chemical reaction with oxygen. It could also be a stress reaction from the bacterium, that they start to grow close together when they are exposed to a stress, such as oxygen, and therefore form what is perceived as flakes.

Isolate 22 only used three of the substrates in a previous study, and all of those were sugars. The bacterium has a narrow range of suitable substrates. When once again inoculated with lactate it did not grow, neither together with sulphate. Both inoculations formed trace amounts of acetate and the lactate and sulphate incubation also produced trace amounts of propionate. Since neither of the other tested substrates supported growth better than pyruvate, that was chosen for all other inoculations with isolate 22. The fact that the isolate did not grow on lactate when sulphate is present makes it possible to draw the conclusion that the bacteria does not use sulphate as electron acceptor when lactate is present as electron donor. In contrast to the closest relative, *D. halophilum*, that grows on lactate if sulphate is present in the medium as an electron acceptor. It would be interesting in a full

characterization to test if isolate 22 can use other molecules as electron acceptors, such as sulphite or thiosulphate, in contrast to sulphate.

The bacteria form isolate 22, is gram positive which is the same as *D. halophilum* they are also the same shape, rod shaped that is slightly curved (Figure 3). Over all the two bacteria have some similar characteristics and some that make them different. Based on this characterization of isolate 22 it is most likely a new species, a mesophilic pyruvate fermenting bacteria that forms acetate. It is gram positive, rod shaped and can grow in both high concentrations of ammonia and high salinity. To get a full characterization of this bacterium there is more tests to be done, and one of them are a sequencing of the whole genome.

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Appendix 1

Table 1: In an earlier study the substrate specifity were tested. In this appendix the extended list of tested substrates are presented. (+) = >0,2 g/L acetate formed, (*) = <0,2 g/L actete formed and (-) = no acetate formed.

Substrate	Growth
Acetate	-
Acetoin	-
Arabinose	-
Asparagine	-
Benzoic acid	-
Betaine	-
1-Butanol*	-
2,3-Butandiol	-
Casaamino acids	-
Cellobiose	-
Citrate	-
L-Cysteine	-
Dimethylamine	-
D-Galactose	-
D-Glucose	-
Glycerol	*
Ethanol	-
Ethanolamin	-
Etylenglycol	-
Format (sodium format)*2	-
Fructose	+
Fumaric acide	-
Histidine	-
Lactose	-
D,L-laktat	*

Substrate	Growth
Leucine	-
Malic acid	-
Maltose	-
Mannitol	-
Mannose	+
Methanol	-
Methionine	*
Methylamine	-
Phenylalanine	-
Proline	-
1,2-Propanediol	-
2-Propanol	-
Pyruvate	+
Pyruvate	+
Raffinose (pentahydrate)	*
Ribose	*
Salicin	-
Serine	-
Sorbitol	*
Sucrose	-
Syringate	-
Tryptone	-
Tryptofan	-
Vanillic acid	-
Xylose	-

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