Metabolic and molecular characterization of an acetate and lactate-degrading microbial consortium

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ABSTRACT

Acetate is one of the most important intermediaries in the degradation of organic matter in anaerobic environments where carbon dioxide is the dominant electron acceptor. In these environments, acetate is recycled by acetoclastic methanogens or by syntrophic couples. In this thesis, is described a defined culture capable of acetate and lactate oxidation under methanogenic conditions. Combinations of microbiological and analytical methods as well as molecular techniques were used to characterize this culture. This included the construction of anaerobic microcosms that were subsequently characterized by serial dilutions, gas chromatography, high performance liquid chromatography, PCR, qPCR and sequencing. Our results demonstrate that the bacterial population within our enrichment is limited to a single sulfate reducing bacterium as evidenced by qPCR and DNA sequencing analyses of 16S rRNA genes. Furthermore, the highly purified culture is capable of oxidizing both acetate and lactate in the absence of sulfate when coupled with a methanogen. The qPCR data, using universal 16S rRNA primers of Eubacteria and *Archaea,* suggest that the methanogenic population comprises nearly is 25 % of the total population of microorganisms and is dominated by hydrogentrophic methanogens belonging to the genera *Methanospirillum* and *Methanobacterium*. In contrast, analyses of qPCR data using primers to universal 16S rRNA (Eubacteria), specific *Syntrophus* genus, and for the cap protein of *S. aciditrophicus* suggests that the bacterium in the culture belongs to the genus *Syntrophus.* However, these results are extremely interesting since they suggest either a bacterium belonging to the genus *Syntrophus* is capable of reducing sulfate (novel organism, yet un-described) or that a true sulfate reducing bacterium shares the same cap protein as SB. Unfortunately at this time we do not know which of these two possibilities is correct. We recommend further characterization of the mixed culture to elucidate these important findings; as future work.

RESUMEN

Acetato es uno de los intermediarios más importantes en la degradación de materia orgánica en ambientes anaeróbicos donde el dióxido de carbono es el principal aceptador de electrones. En estos ambientes, acetato es reciclado por metanogénicos acetoclásticos o por parejas sintróficas. En esta tesis, se describe un cultivo definido capaz de degradar acetato y lactato bajo condiciones metanogénicas. Una combinación de técnicas microbiológicas y analíticas así como de técnicas moleculares fueron utilizadas para caracterizar el cultivo. Esto incluyó la combinación de un microcosmos anaeróbico que se caracterizó posteriormente por diluciones en serie, cromatografía de gas, cromatografía líquida de alta afinidad, PCR, qPCR, clonación y secuenciación. Nuestros resultados demuestran que la población de bacterias en nuestro enriquecimiento está limitada a una bacteria reductora de sulfato que se evidencia por qPCR y análisis de secuenciación del ADN de los genes del 16S rRNA. Por otro lado, este enriquecimiento altamente purificado es capaz de oxidar acetato y lactato en ausencia de sulfato cuando forma una relación sintrófica con un metanogénico. La data de qPCR utilizando primers universales del 16S rRNA de Eubacteria y *Arquea* sugieren que la población de metanogénicos presentes en nuestro enriquecimiento es 25% del total de la población de microorganismos y está dominada por metanogénicos hidrogenotróficos pertenecientes a los géneros *Methanospirillum* y *Methanobacterium*. En contraste, los análisis de qPCR utilizando primers uniersales del 16S rRNA (Eubaacteria), específicos del género (*Syntrophus*) y primers para la proteína del Cap de *S. aciditrophicus* sugieren que la bacteria en el cultivo está relacionada al género *Syntrophus*. Sin embargo, estos resultados son extremadamente interesantes ya que sugieren que existe una bacteria que pertenece al género *Syntrophus* la cual es capaz de reducir sulfato (un nuevo organismo, aún no descrito) o una bacteria reductora de sulfato que muestra tener la misma

proteína del cap de SB. Desafortunadamente a este tiempo nosotros no sabemos cuál de estas dos posibilidades es la correcta. Recomendamos la caracterización del cultivo mixto para dilucidar estos importantes hallazgos; como un trabajo futuro.

DEDICATION

I dedicate this thesis to my family; they have supported me unconditionally. Also, to all those friends who stood by my side during the good and bad times throughout this experience.

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First of all, I want to thank the Lord, because I believe that without him nothing in my life would have been possible. I want to thank all of my family, specially my parents for all their support and to my husband, Efrain Villanueva, which supported me during the difficult and frustrating periods of this thesis.

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Finally and not least important, I want to thank all those that supported me academically. I thank the Graduate Studies Office for their kindness, to the National Science Foundation for the funding and the resources for the development of this research, Dr. Mostafa ElShahed from Oklahoma State University and his laboratory personnel for all their support, and to the members of my committee for their support and providing me with their guidance. I want to thank especially my chairman, Dr. Luis A. Ríos Hernández, for his support in both, at the academic and emotional level during this process.

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Introduction, Literature Review and Objectives

1. 1 Introduction

For many years, scientists have been focused on the study of syntrophic metabolism due to their key role in the anaerobic digestion of organic matter coupled to the production of methane (McInerney et al., 2008). Syntrophic metabolism occurs in a symbiotic relationship between two microorganisms to degrade a substrate, in order to overcome thermodynamic constraints (Schink, 1997). These syntrophic relationships form a recycling process in which they can degrade large amounts of organic matter, and about 90% or more of the substrate energy is converted to CH_4 (Bryant, 1979). The microbial activities in anaerobic environments are the primary source of global methane emissions (Kato et al., 2010). In these settings, acetate is a major source of methane because it acts as an important intermediary in various steps of the degradation of organic material when CO_2 is the final electron acceptor. There are two known anaerobic pathways to obtain methane from acetate: the acetoclastic and the syntrophic pathway. At present, two genera of mesophilic methanogenic archaea (*Methanosarcina* and *Methanosaeta*) are able to use the acetoclastic route which of breaks acetate into a methyl and a carboxyl group and these in turn are converted to CH4 and CO2, respectively (Ferry, 1992). On the other hand, we only know six species of bacteria that in cooperation with methanogens possess the ability to degrade acetate syntrophically. These are *Thermotoga lettingae*, *Thermacetogenium phaeum*, *Clostridium ultunense*, Acetate-Oxidizing Rod (AOR strain) (Hattori, 2008), *Tepidanaerobacter acetatoxydans* and *Syntrophaceticus schinkii* (Westerholm et al, 2010). However, recent studies provide the possibility that a fermentative bacterium of the genus *Syntrophus* also possess this ability (Chauhan, 2006). At this time, it is not known whether *Syntrophus aciditrophicus* (SB) can perform this activity, which prompts us to investigate this process in anaerobic environments. Furthermore, this research could lead us to understand and suggest which natural partner or

partners are used to syntrophically degrade this substrate. Moreover, very few microorganisms are known to carry out this function and the study or discovery of others will help us to understand the role of specific partners, the ecology of these mutualistic associations, and explore the possibility of developing an alternative energy source.

1.2 Literature review

The degradation of complex organic matter to CO_2 and CH_4 occurs in anoxic environments where electron acceptors are limited (Schink, 1997). Some of these environments are freshwater sediments, flooded soils, wet wood of trees, rice paddies, tundra, landfills, and sewage digesters (Ferry, 1992). In these environments, the degradation of organic matter is carried out by the interaction of different communities of microorganisms such as primary fermentative bacteria, secondary fermentative bacteria and two different groups of methanogenic archaea (Hattori, 2008). In this process, the primary fermentative bacteria hydrolyze carbohydrates, lipids, and proteins to sugars, fatty acids, and amino acids. Then these fermentative bacteria convert those monomers to fatty acids, succinate, acetate, and alcohols or H_2/CO_2 (Schink, 1997). In a syntrophic relationship, the secondary fermentative bacteria degrade these compounds to H_2/CO_2, acetate, or both. In the final step, the hydrogenotrophic methanogens convert the H_2/CO_2 to methane while the acetate is degraded by the acetoclastic methanogens (Hattori, 2008).

However, in anoxic environments specifically under methanogenic conditions, acetate is recognized as a principal intermediary in the degradation of organic matter and is typically degraded by two pathways: the acetoclastic or the syntrophic (Hattori, 2008). On the acetoclastic pathway, the methanogen breaks acetate into a methyl and a carboxyl group and these in turn are converted to CH_4 and CO_2, respectively (Ferry, 1992). The second pathway, syntrophic acetate

degradation, requires a special kind of symbiosis and consists of two steps. In this mutualistic symbiosis, the syntrophic bacteria oxidize both methyl and carboxyl groups to CO_2 and H_2. However, this reaction is thermodynamically unfavorable and needs to remove hydrogen to overcome the thermodynamic constraint. Therefore, in the second step a syntrophic relationship is created. This relationship is the interaction of two different types of microorganisms, which depend on each other to degrade acetate and obtain energy to survive (Schink, 1997). The hydrogenotrophic methanogen removes H_2 and produces CH_4, turning this into a thermodynamically favorable reaction (Hattori, 2008). See equation (1).

$$CH_3COO^- + 4H_2O \rightarrow 2HCO_3^- + 4\ H_2 + H^+ \qquad \Delta G0' = +104.6\ kJ/mol \quad (1)$$

In this reaction the syntrophic bacteria cannot oxidize acetate since it will not generate enough energy to sustain viability. Therefore, it forms a syntrophic relationship with a microorganism that can remove H_2, in this case a methanogenic hydrogenotroph as shown in equation (2).

$$4H_2 + HCO_3^- + H^+ \rightarrow \quad CH_4 + 3H_2O \qquad \Delta G0' = -135.6\ kJ/mol \quad (2)$$

The equivalence of energy production of equation (1) and (2) is shown in the equation (3).

$$CH_3COO^- + H_2O \rightarrow HCO_3^- + CH_4 \qquad \Delta G0' = -31.0\ kJ/mol \quad (3)$$

Furthermore, research suggests that Syntrophic Acetate Oxidation (SAO) is favored in high temperature for thermodynamic reasons; actually, the first microorganisms discovered with this capacity were isolated from thermophilic environments (Valentine, 2004). They suggest that acetate is consumed by syntrophic acetate oxidizers at high temperatures and by acetoclastic methanogens at low temperatures (Conrad, 1999). This occurs because at low temperatures the methanogenic archaeal community is more diverse than high temperatures. While at high

temperatures, the methanogenic archaeal community is favored by hydrogenotrophic methanogens, creating the perfect atmosphere for SAO (Conrad, 1999). However, microorganisms with this metabolic capacity have been found in mesophilic and thermophilic reactors, and in natural environments (Zinder and Koch, 1984; Schnürer et al., 1994; Nazina et al., 2006; McInerney et al., 2008; Westerholm et al., 2010). Moreover, Schnürer and Nordberg (2008), observed that in mesophilic digesters the SAO occurred under high levels of free ammonia. Based on their study at a mesophilic temperature, ammonia is a selective agent for SAO because it possesses an inhibitory effect on methanogens, especially on the acetoclastic methanogens. For which, the methane production derived from acetate in the bioreactor with a high concentration of ammonia was carried out by the syntrophic pathway (Schnürer et al., 2008).

In environments such as wetlands, approximately 70% of the methane generated is produced from acetate (Conrad, 1999) whereas in anaerobic digesters this can be as high as 90% (Smith et al., 1966), which demonstrates the importance of this intermediary in the anaerobic degradation of organic matter. Many scientists have dedicated their careers to study these communities in different environments including; methanogenic reactors, leachate and oil reservoirs (Koch and Zinder, 1984). Recent molecular studies in wetlands (Florida Everglades) suggest that SAO is carried out by two possible syntrophic partners, either a sulfate-reducing bacteria in association with methanogenic hydrogenotrophs or a syntrophic bacteria from the genus *Syntrophus* in association with *Desulfovibrio* spp. (Chauhan and Ogram, 2006). These authors came to these conclusions based on their clone library results where 25 to 45% of the sequences were similar to the syntrophic bacteria belonging to the genus *Syntrophus* (Chauhan and Ogram, 2006). These conclusions are interesting because this genus is not known to syntrophically oxidize acetate; furthermore, this capacity has not been observed in controlled

laboratory experiments using *Syntrophus* thus far. On the other hand, this conclusion is solemnly based on the fact that members of the genus *Syntrophus* are known to form syntrophic associations with hydrogen-using sulfate reducer or methanogens (Hopkins et al., 1995), and not actual controlled experiments in the laboratory.

The genus *Syntrophus* belongs to the Phylum Proteobacteria and the family *Syntrophaceae*. The cells are gram-negative rods grow at an optimal temperature of 28 to 37ºC; and are capable of degrading aromatic compounds in a syntrophic association with hydrogenotrophic methanogens (Bryant, et al. 1984; McInerney et al. 2008). This genus has only three species; *S. gentianae*, *S. buswellii* and *S. aciditrophicus*, which were isolated from anoxic environments such as freshwater sediments or sewage sludge treatments plants. *S. gentianae* grows as a pure culture with crotonate, while syntrophically metabolizes benzoate, gentinate and 3-phenylpropionate (Schink, et al. 1997). However, *S. buswelli* metabolizes crotonate in pure culture and in syntrophic co-culture can metabolize benzoate (McInerney et al., 2008). On the other hand, *S. aciditrophicus* has the ability to syntrophically metabolize certain saturated and unsaturated fatty acids and methyl esters of butyrate; hexanoate and benzoate (Jackson et al. 1999). The metabolic capacity of *S. aciditrophicus* under mesophilic conditions is restricted to ferment crotonate or benzoate as a pure culture, whereas this microorganism in a co-culture with a methanogenic hydrogenotroph (or sulfate-reducer) can oxidize benzoate and fatty acids of 4 to 8 carbons in length. So far, only two hydrogenotrophs are known to serve as syntrophic partners of *S. aciditrophicus*, which are *Desulfovibrio vulgaris* G11 (sulfate reducer) and *Methanospirillum hungatei* (methanogenic hydrogenotroph) (McInerney et al., 2007). In contrast, *S. buswellii* and *S. gentianae* can syntrophically oxidize only certain aromatic compounds but not fatty acids (Mountfort et al., 1982), which leads to predict that the only species that can

potentially fulfill this niche is *S. aciditrophicus*, a new strain of this species or another species yet to be described.

Therefore, we enrich in nature to obtain a pure culture that can make the SAO. In this study we apply a cross feeding technique in an attempt to study the dynamics and the metabolic role of each member of a highly enriched microbial community. Hence, in this research we selected lactate as our primary substrate; under methanogenic conditions we anticipate that lactate would be fermented giving rise to low concentrations of acetate, which in turn would be degraded by a syntrophic couple. This approach exploits the low rates of lactate consumption and acetate production favoring the cooperation between producers and consumers, promoting physical contact favoring syntrophic interactions within the populations.

1.3 Objectives

The objective of this research was:

1. Demonstrate lactate oxidation and syntrophic acetate oxidation (SAO) by an enrichment consortium from an environmental soil sample.
2. Design specific PCR primers to identify and quantify possible syntrophic interactions in complex environmental samples using *S. aciditrophicus* as a model.
3. Isolate and identify the syntrophic acetate-degrading consortium from natural habitats.

Chapter Two
Analysis of Lactate and Acetate degradation

2.1 Introduction

A sediment free mixed culture capable of producing methane from acetate was enriched from enriching sediments from a mechanic workshop using acetate as the sole source of carbon and energy. To observe the dynamics of the enriched microbial population on acetate, we changed the substrate to lactate, a non-syntrophic substrate. An example of this is the members of the genus *Desulfovibrio* that in presence of sulfate, an electron acceptor, can degrade lactate to acetate (equation 1). However, lactate could be oxidized syntrophically by a co-culture of a methanogen and a syntrophic bacterium producing acetate, HCO_3^- and H_2 (equation 2). Is in this step when the methanogen utilize these products to produce methane (equation 3) (Bryant and McInerney, 1981). The energy-yielding process of these two reactions is shown in the equation 4.

$2CH_3CHOCOO^- + SO_4^{2-} \rightarrow 2CH_3COO^- + HS^- + 2HCO_3^-$ $\Delta G^0 = -160.3$ kJ (1)

$2CH_3CHOCOO^- + 2H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 2H^+ + 4H_2$ $\Delta G^0 = -8.4$ kJ (2)

$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$ $\Delta G^0 = -135.6$ kJ (3)

$2CH_3CHOCOO^- + H_2O \rightarrow 2CH_3COO^- + CH_4 + H^+ + HCO_3$ $\Delta G^0 = -144.0$ kJ (4)

To demonstrate that syntrophic metabolism was a dominant metabolic route in our enrichment we performed several manipulations: these include addition of an electron acceptor (sulfate), an inhibitor of methanogenesis (2-bromo-ethanesulfonate or BESA), lactate only, acetate only, and all permutations of the four basic conditions.

2.2 Materials and Methods

2.2.1 Samples

The study was performed with a sediment sample previously collected from the side of the road in front of mechanical workshop. The sediments were incubated for 3 or 4 weeks in a serum bottle with Nitrogen: Carbon dioxide (80:20 v/v) atmosphere and a previously described medium (Jackson, 1999) at room temperature without shaking in the dark. In order to develop a sediment free enrichment a 5mL aliquot was used to inoculate a new anaerobic bottle containing 95mL of medium supplemented with 10mM acetate as previously described (Jackson, 1999); a negative control was also prepared simulating the enrichment but sterilized. The headspace of all the bottles was exchanged with anaerobic gases to N_2:CO_2 (80:20 v/v) at a final pressure of 10psi and incubated at 35 °C in the dark without shaking. The microbial activity was assessed by quantifying methane production and acetate consumption weekly (section 2.2.3).

2.2.2 Lactate enrichment and controls

A 1 mL of the enrichment of mechanical soil sample in 10mM acetate (section 2.2.1) was added to an anaerobic bottle containing 99mL of supplemented medium with 10mM lactate. This enrichment was incubated at 35°C and lactate degradation and methane production were monitored bi-weekly. Therefore, we performed several experimental controls to demonstrate lactate and syntrophic acetate oxidation in our enrichments. The experimental controls were as follows: lactate; acetate; lactate with BESA; lactate with BESA and sulfate; lactate and sulfate; and lactate and acetate at equimolar concentrations. The negative controls consisted of 100mL of supplemented medium with each different conditions mentioned previously but without cells. From the enrichment of mechanic soil sample in lactate, 1 mL was removed to each experimental

control in a bottle of about 100 mL in an atmosphere of N_2:CO_2 (80:20 v/v) and were incubated at 35 °C without shaking. In order to perform a statistical analysis, all enrichments and controls were carried out in triplicates. All experimental controls were monitored every two days for the first 14 days; subsequently these were monitored weekly for 80 days. Also, to describe the morphotypes of the grown cultures we monitored the community as lactate was consumed and as acetate was produced using a Phase contrast microscope (Leica DMI3000B) at 100X magnification and the Micrometric SE Premium Program. Furthermore, we also performed molecular analyses of the 16S rRNA genes of bacteria and archaea of the enrichment in lactate and the controls. Then, we used DGGE to analyze the complexity of the microbial community enriched.

2.2.3 Chemical Analysis

The concentration of volatile fatty acids in the different controls was determined every week with a Shimadzu (LC-2010) High Performance Liquid Chromatograph (HPLC) and the production of methane with a Gas Chromatograph (GC) (Shimadzu GC-2010). The enrichments were sampled anaerobically with a syringe (1 mL) following strict anaerobic and aseptic techniques. One milliliter of culture fluid was placed in a 1.5 ml Eppendorf tube and then preserved frozen at -20°C until used. Before analysis, the samples were thawed at room temperature, mixed with 0.1g of Dowex (Acros Organic; New Jersey, USA) to remove the impurities, centrifuged for 5 minutes at 13,000 rpm (Eppendorf centrifuge 5415D), and 200 µL of the supernatant was injected into the HPLC. The HPLC was equipped with a 20 µL loop; a Rezex ROA-Organic Acid H^+ column (C18 300 by 7.8mm, 10µL) and the parameters were maintained as previously described (Jackson, 1999). Moreover, methane production was

determined by Gas Chromatography (Shimadzu GC-2010) using a stainless steel column (Porapak N 80/100, 10ft x 1/8 in ss, detector FID) as previously described (Bastviken et al., 2004). Samples of 0.2 mL of the headspace were taken every week to measure methane production. We measured spectrophotometrically the concentration of sulfide in controls that contained sulfate, according to Cline (1969) and with Kleinsteuber (2008) modifications. Concentrations of the samples were calculated using standards prepared from anoxic sulfide stock solutions.

2.2.4 DNA extraction

All extractions were done using the FastDNA SPIN Kit for Soil (Qbiogene Inc., Carlsbad, CA). DNA extraction began with a cell suspension, which consisted of centrifuging a total volume of 3mL from an active culture; centrifuging 1.5ml of fluid culture twice in an Eppendorf tube. The pelleted cells were re-suspended in 0.5 mL of supernatant fluid and used in its entirety with the FastDNA SPIN Kit for Soil. The manufacturer's protocol was used with some modifications described below. Cell lysis by bead beading was carried out in a vortex using a Mo-Bio vortex adapter (Mo Bio Inc., Carlsbad, CA) for 5 min at maximum speed and then a centrifugation for 13,000 rpm for 10 minutes (Eppendorf Centrifuge 5415D). The time of mixing the lysate with the DNA Binding Matrix Suspension was for 10 minutes, and we use two washes with SEW-S solution. In the final step, the DNA was eluted in 75 to 100 μL of TE buffer and quantified (260nm of optical density) using a nanodrop ND-1000 instrument (Thermo Scientific, Wilmington, DE).

2.2.5 Molecular analysis

Following the previous degradation analysis, we performed a molecular analysis of the 16S rRNA genes of bacteria and archaea using the following parameters:

A. Archaea 16S rRNA

PCR amplifications of Archaeal 16S rRNA gene were done in a MyCycler Thermal Cycler (Bio-Rad) using the primers 1106F-GC CLAMP, 1378-reverse and the thermal program described previously (Watanabe et al., 2006). The PCR master mix (final volume of 50µL) was composed of 10µL of PCR buffer (5x) (Promega), 1.25µL of dNTP's mix (10 mM) (New England Biolabs), 4µL of $MgCl_2$ (25mM) (Promega), 10µL of each primer (2pmol/µL each), 0.4 µL of Taq DNA polymerase (5U/µL) (Promega), 2µL of template DNA [10ng/ µL] and 22.35 µL of water.

B. Bacterial 16S rRNA

Partial 16S ribosomal DNA (rDNA) sequences were amplified from the enrichments using the primers GM5F-GC CLAMP and DS907-reverse and the thermal program described previously (Muyzer et al., 1993). The PCR master mix reaction (final volume of 50µL) were done in a MyCycler Thermal Cycler (Bio-Rad) used 10µL of PCR buffer (5x) (Promega), 5µL of dNTP's mix (2.5mM) (New England®), 5µL of $MgCl_2$ (25mM) (Promega), 0.25µL of each primer (10pmol/µL each), 1 µL of Bovine Serum Albumina (BSA) (New England®), 0.2 µL of Taq DNA polymerase (5U/µL) (Promega), 2µL of template DNA [10ng] and completed with water. The thermal program consist of (i) 4 min at 94°C; (ii) 30 cycles of 1.25 min at 94°C, 45 sec at 53°C, and 2 min at 72°C; (iii) and a final extension step of 10 min at 72°C.

C. **PCR gel amplifications**

All PCR amplifications were confirmed performing an agarose gel electrophoresis (1.5%) at approximately 115 volts in 1X Tris-acetate-EDTA (TAE Buffer), using a quick load 100bp DNA ladder (New England Biolabs) for size comparison. The gels were stained with ethidium bromide (10mg/mL), and visualized by a Versadoc (Bio-Rad).

2.2.6 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed with the PCR amplifications of a 16S rRNA gene of archaea and bacteria. We use a D-code universal mutation detection system (Bio Rad, Hercules, CA) and the electrophoresis was performed in 8% acrylamide gels (ratio of acrylamide to bis-acrylamide 37.5:1 (J.T. Baker) for 12 h at 100V in a denaturant gradient of urea and formamide (100% denaturant agent was defined as 7M urea and 40% formamide). DGGE of 16S rRNA gene of archaea and bacteria were performed in a gradient of 40 to 60 % (archaea) and 25 to 45 % (bacteria) of urea and formamide respectively. The voltage was maintained at 50V until the gels reached 60°C, at which time it was placed at 100V until the end of the electrophoresis. We performed a cladogram of the DGGE, which represents the classification of organisms and their phylogenetic relationship, based on the comparison between bands. Therefore, a matrix was calculated with the following equation: $Dxy = 1\text{-}2\ (N_{XY} / N_X + N_Y)$. Where, D_{XY} is the distance between two lanes (populations), N_{XY} is the number of bands equal in both lanes, N_X is the number of bands in lane x, N_Y is the number of bands in the lane Y. Then this matrix was used to construct a Neighbor Joining Tree in the program MEGA version 5.0 (Tamura et al. 2011).

2.3 Results

2.3.1 Chemical Analysis

We detected that the acetate production is based on the lactate degradation, but acetate is not consumed until lactate is completely degraded. After lactate is consumed in approximately 7 days, methane production was proportional to lactate degradation and was stabilized until day 49, and then it increases due to acetate degradation. The increase of acetate production at 42 days was probably related to an error in reading (Figure 2.1). Therefore, when we repeated the experiment, we added different controls with sulfate as a possible electron acceptor and BESA as an inhibitor of methanogenesis. Lactate enrichment showed faster lactate degradation than original experiment, however acetate was not produced proportionally although methane production also increased when acetate was degraded (Figure 2.2). We estimate that in the lactate enrichment, approximately 10% of the lactate is converted into methane (Appendix A.1). If we compare these results with the Lactate-acetate enrichment (Figure 2.6), which showed also a 96% of lactate degradation but in this enrichment acetate and methane were produced proportionally (Table 2.1). Furthermore, in the same way, acetate is not degraded until lactate is completely degraded and this increased methane production. In presence of an electron acceptor as sulfate (Figure 2.3), lactate was 98% degraded and methane and acetate were not produced. If we compare lactate degradation with or without sulfate (Figure 2.8) we can observe that lactate was degraded faster when sulfate was present, inhibiting the production of methane. Moreover, lactate enrichment with methan0ogenesis inhibitor showed only 24% of lactate degradation and furthermore 100% of lactate was not converted to acetate because only 28% was to acetate production (Figure 2.5). Thus, if we observe lactate enrichment with BESA and sulfate, only present a 46% of lactate degradation although another electron acceptor was present (Figure 2.4).

Then, if we analyze the enrichment of lactate with a methanogenesis inhibitor but without another electron acceptor, we can observe that only 24% of lactate was degraded (Figure 2.5). We observed that the acetate produced in the different enrichments were not degraded while BESA was in the sample, even when sulfate was present as a possible electron acceptor (Table 2.1). In sulfate enrichments, we could observe that there was an increased production of sulfide in the enrichment that did not have BESA as an inhibitor of methanogenesis (Figure 2.9). However, we observe that although in some enrichment acetate generated by lactate degradation also was degraded, when we change the culture directly to acetate, it was not degraded (Figure 2.7). We expected that acetate degradation occur, but we don't have a possible explanation of why it didn't occur (Figure 2.7).

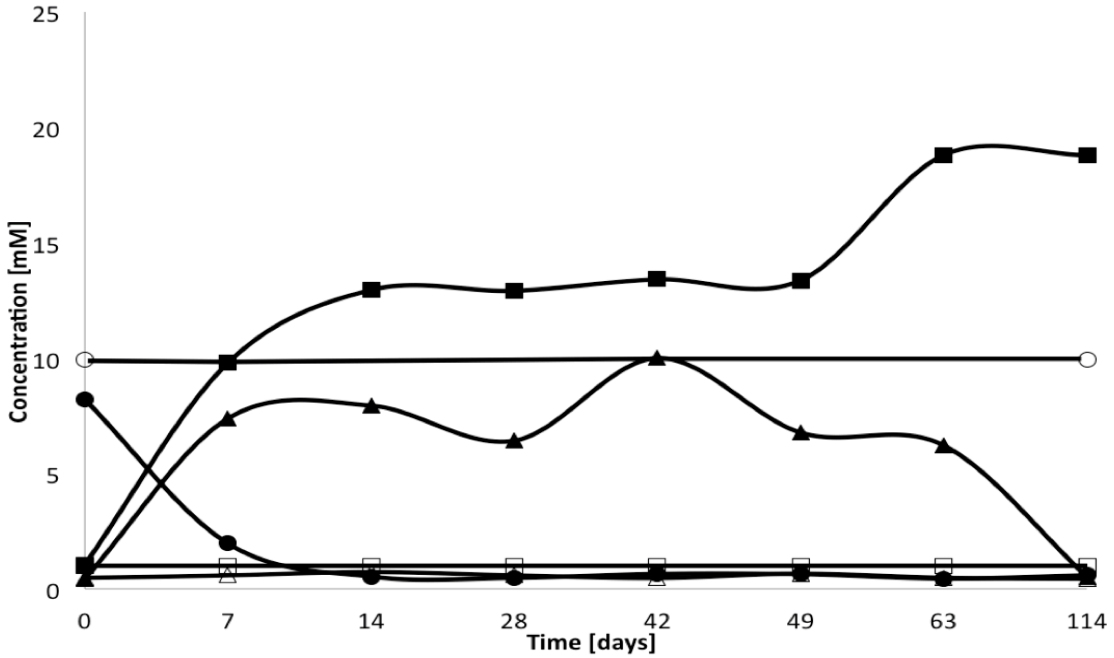

Figure 2.1: Methane production from lactate and acetate degradation in the mechanical soil sample enrichment (First Chemical Analysis). The graphs demonstrate that lactate degradation (●) was in approximately 9 days generating a proportional formation of acetate (▲), which then was also degraded. Methane production (■) is observed when the lactate is degraded, and increased as the acetate produced is degraded. Negative control of lactate (○) was not degraded, and thereby acetate (△) and methane (□) production in the negative control were not present.

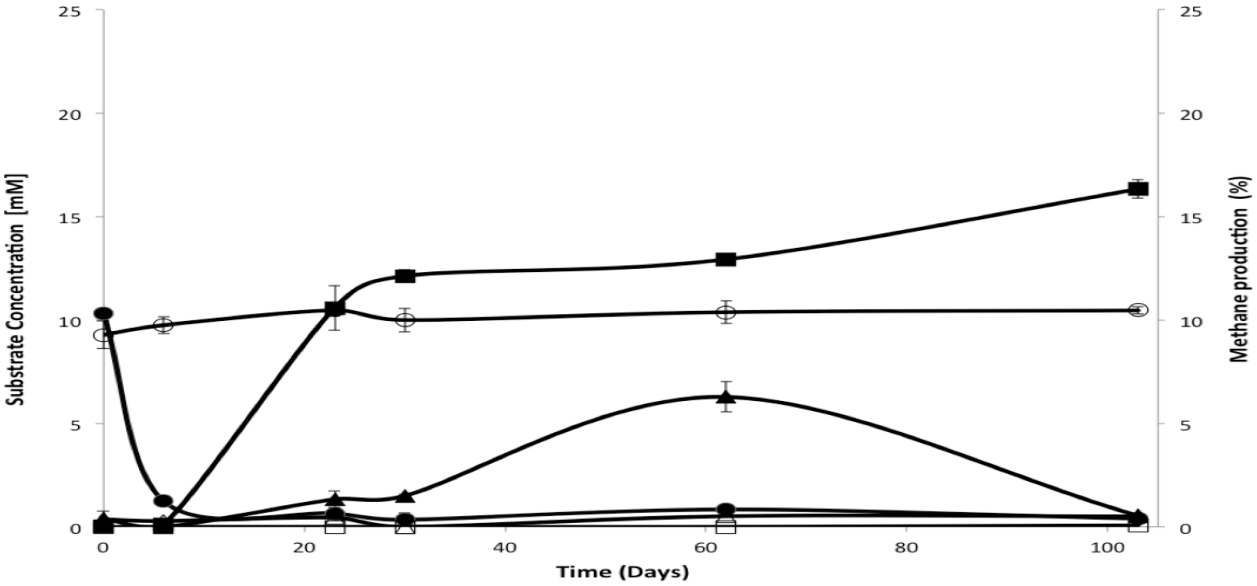

Figure 2.2. Methane production based on the lactate degradation by mechanical soil sample enrichment (Second Chemical Analysis). The graphs demonstrate that lactate (●) was degraded in approximately 6 days and acetate (▲) was produced but not proportionally. Methane (■) production from lactate increased when the acetate was degraded. Negative control of lactate (○) was not degraded, and thereby acetate (△) and methane (□) production in the negative control were not present.

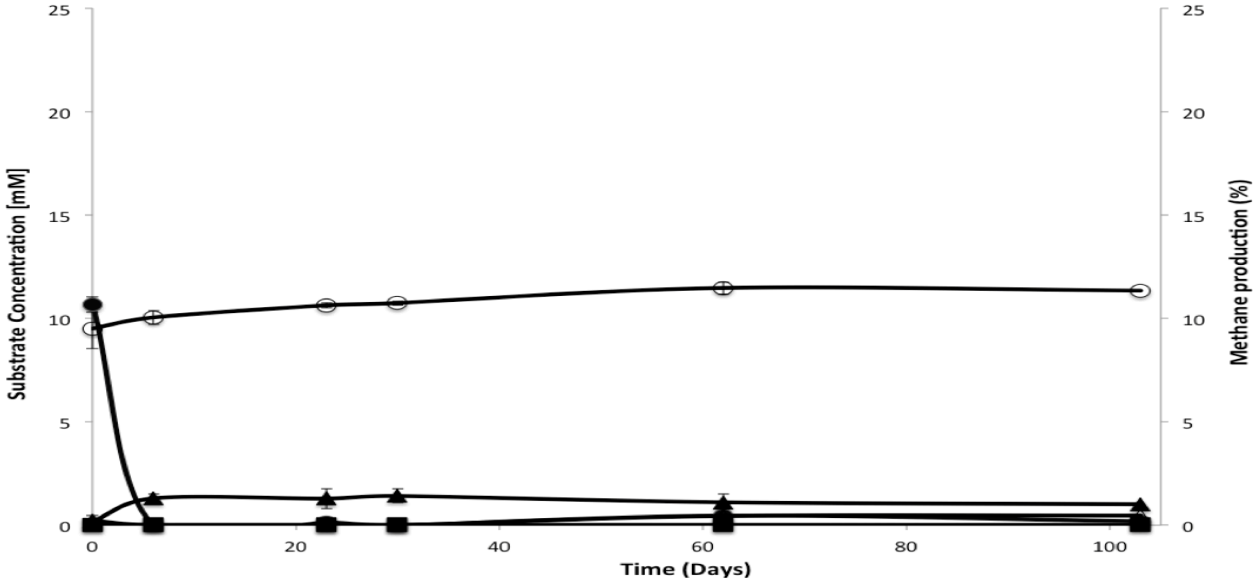

Figure 2.3. Lactate degradation with sulfate as an electron acceptor. The graphs demonstrate that lactate (●) was degraded in approximately 6 days and the acetate (▲) was produced but not proportionally. Methane (■) was not produced. Negative control of lactate (○) was not degraded, and thereby acetate (△) and methane (□) production in the negative control were not present.

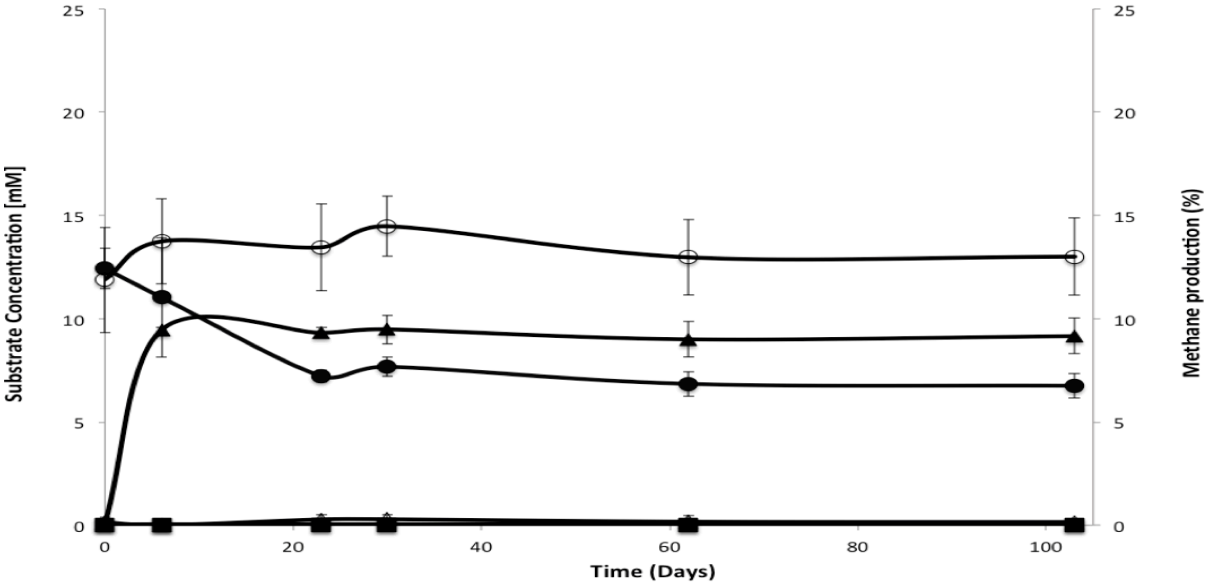

Figure 2.4. Lactate degradation in presence of a methanogenesis inhibitor (BESA) and sulfate as an electron acceptor. The graphs demonstrate that lactate (●) was partly degraded when an electron acceptor and an inhibitor of methanogenesis are present. Acetate (▲) was produced by the partial degradation of lactate, and there was no production of methane (■). Lactate in negative control (○) was not degraded, and thereby acetate (△) and methane (□) production in the negative control were not present.

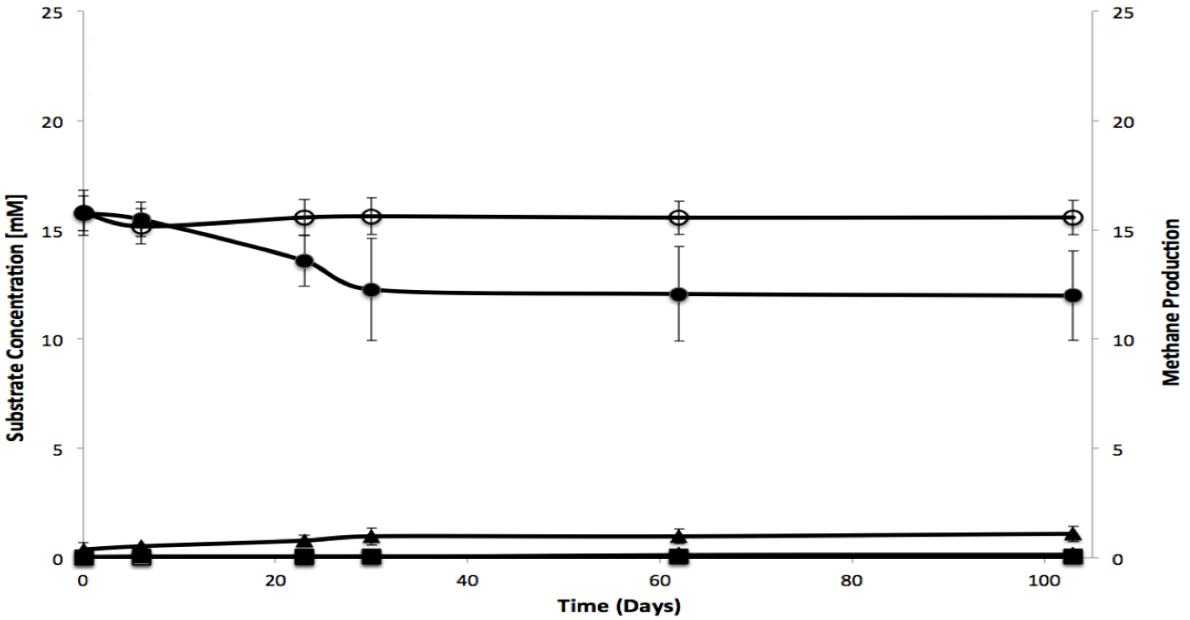

Figure 2.5. Lactate degradation in the presence of an inhibitor of methanogenesis. The graphs demonstrate a partial degradation of lactate (●) and a minimal production of acetate (▲) and there was no methane production (■). Lactate (○) in negative control was not degraded and thus no production of acetate (△) or methane (□).

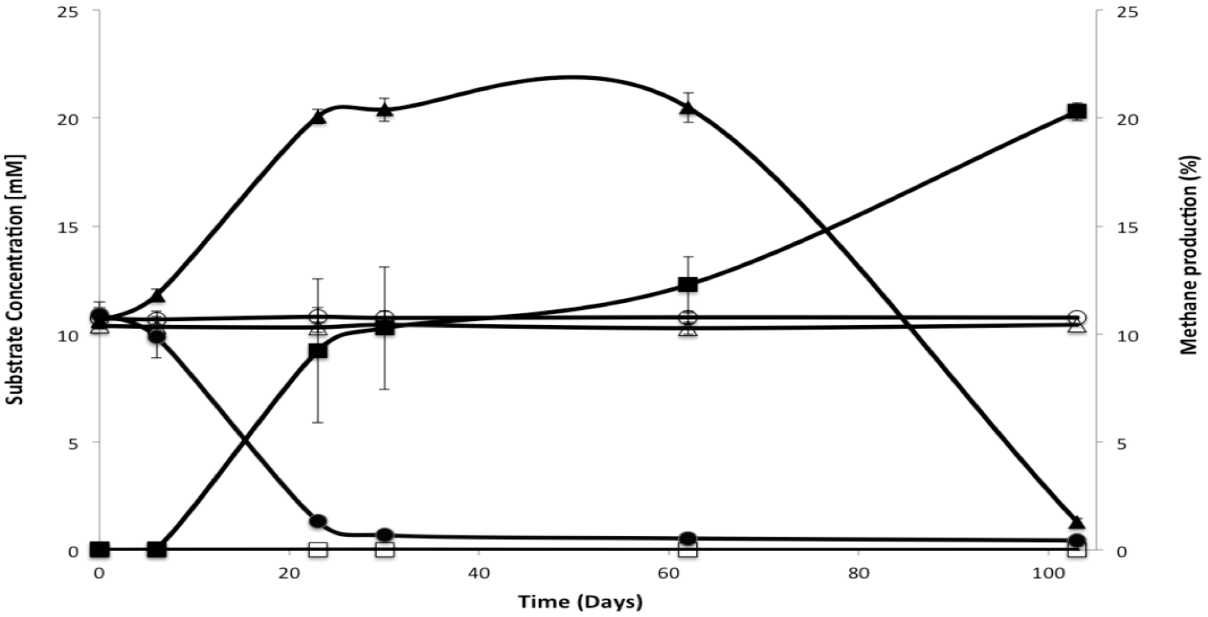

Figure 2.6. Lactate degradation in the presence of another substrate. The graphs demonstrate that when we add the same amount of acetate and lactate in the sample, lactate (●) was degraded, producing an increase in the concentration of acetate (▲) and methane (■). Methane production increased when the acetate was degraded. Furthermore, lactate in (○) negative control was nor degraded and therefore did not produce acetate (△) or methane (□).

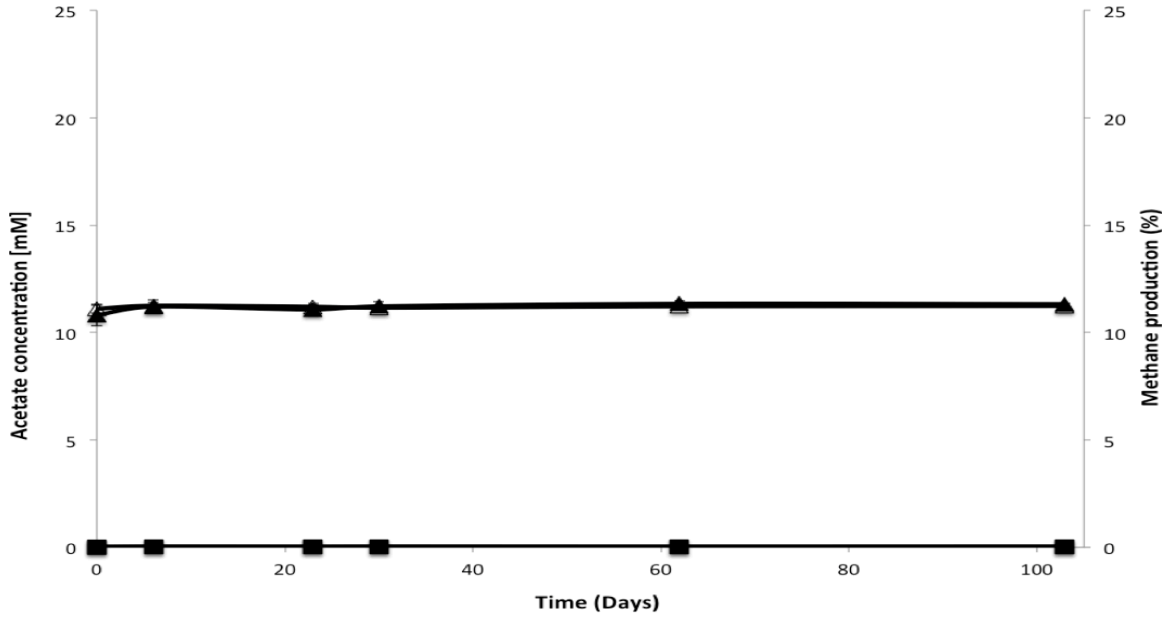

Figure 2.7. Chemical Analysis when exposing the culture to another substrate. The graph demonstrates that when the culture was exposed to acetate (▲), substrate degradation did not occur and therefore there was no production of methane (■). Acetate (△) in negative control was not degraded and thus no production of methane was observed (□).

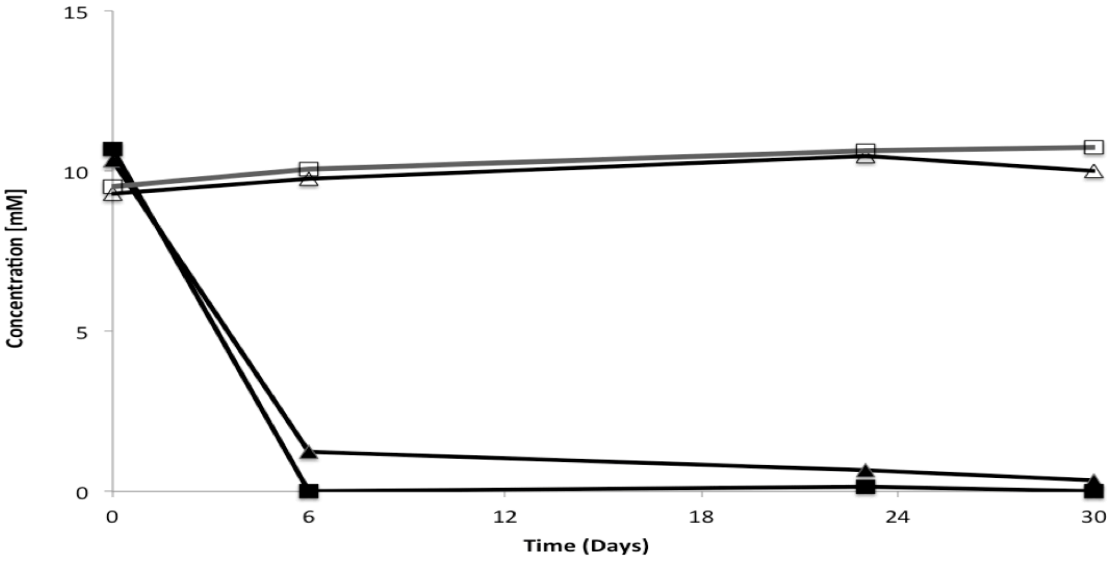

Figure 2.8. Comparative lactate degradation when sulfate was present. The graph demonstrates that when the enrichment has sulfate (■) lactate degradation was faster than when it is not present (▲). Negatives controls with sulfate (□) or without sulfate (△) did not present substrate degradation.

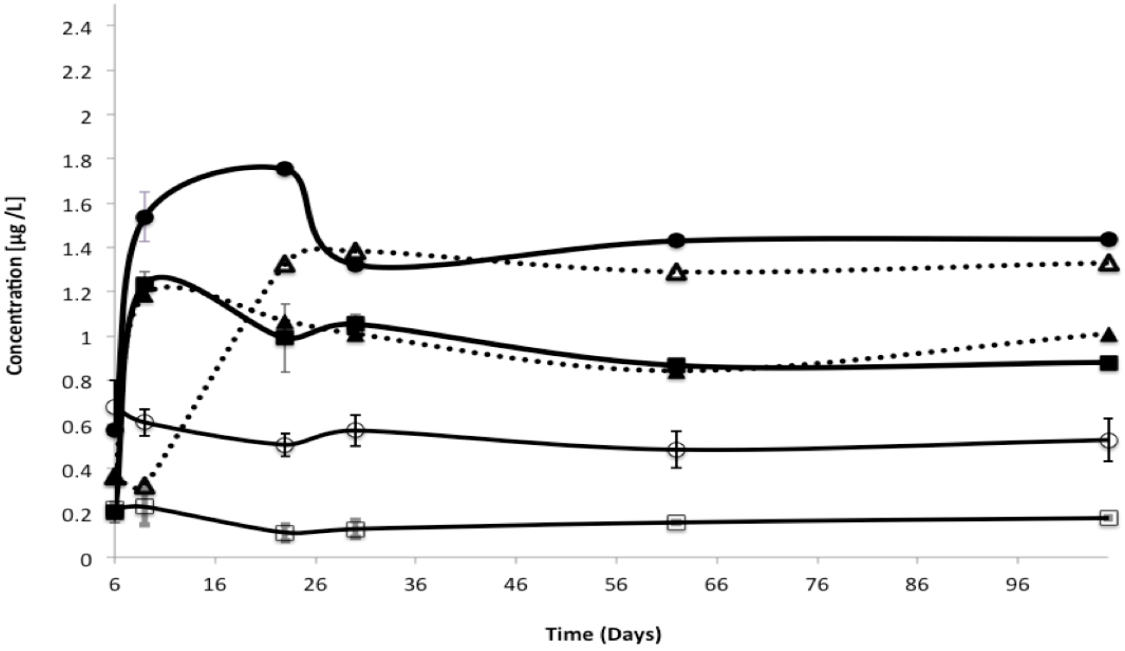

Figure 2.9. Sulfide analysis of the culture exposed to sulfate with and without an inhibitor of methanogenesis. The graph demonstrate that when the enrichment was exposed to lactate with sulfate (●) was obtained more production of sulfide that when was exposed to the same condition but with an inhibitor of methanogenesis (■). A sulfate reducer bacterium, *D. vulgaris*, was exposed to sulfate (△) and sulfate with BESA (▲), obtaining more production of sulfide when BESA was not present. The negative controls with sulfate with BESA (□) and sulfate (○) did not show an increase in the initial concentration.

Table: 2.1: Chemical Analysis of the samples

	Substrate degradation (%)	Observations
Lactate	96% Lactate	Lactate degradation faster. Methane production increase when acetate produced was degraded.
Lactate Sulfate	98% Lactate	No acetate neither methane production
Lactate BESA	24% Lactate	No methane production No complete degradation
Lactate Sulfate BESA	46% Lactate	No methane production Acetate produced was not degraded
Lactate Acetate	96% Lactate 87% Acetate	Lactate was degraded and acetate produced was not degraded until lactate was totally degraded. Methane production increase. Possible Consortium of 3 microorganism
Acetate	0%	No acetoclastic degradation.

2.3.2 Molecular Analysis

To study the microbial community in the sample, we perform a molecular analysis with different genes as the 16S rRNA of bacteria, 16S rRNA of methanogenic archaea, cap protein gene of the *S. aciditrophicus* and 16S rRNA of the *Syntrophus* genus. Therefore, after optimizing the PCR, we were able to obtain amplification of all samples for the 16S rRNA of bacteria (Figure 2.10) and archaea (Figure 2.11). DGGE of the PCR amplifications of the 16S rRNA of bacteria shows that regardless of the different experimental conditions used, the population of bacteria that is present appears to be the same (Figure 2.12). We can observe two bands that may represent two different microorganisms and apparently none of them shows similarity with SB. Moreover, when we observed the DGGE of the PCR amplifications of the 16S rRNA of archaea (Figure 2.13), methanogenic archaea populations appear to be the same in all enrichments, although in

lactate-BESA and acetate enrichments we can observe (a higher number of bands) more bands than others (Figure 2.13, Lanes 4 and 6). Furthermore, DGGE bands of both enrichments were similar to the original culture (Figure 2.13, Lane 7). Also, something that we can observe is that all samples have the control microorganism, JF1. Nevertheless, different enrichments of Mechanic Soil Sample were favored by two different methanogenic archaea and one of them is JF1.

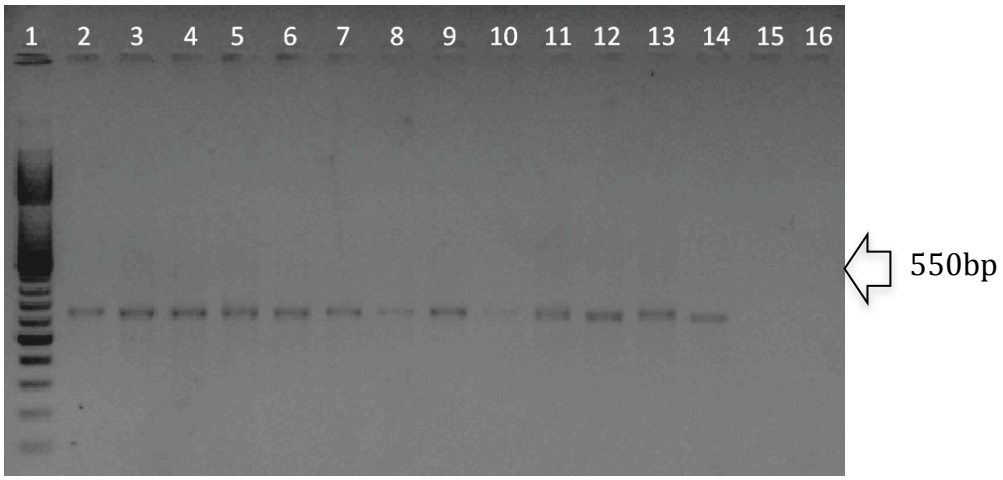

Figure 2.10: Agarose gel electrophoresis demonstrating a representative amplification of the Bacterial 16S rRNA gene of the different enrichments of the Mechanic Soil Sample. The gel was a 1.5% agarose gel, ran for 1.5 hours at 111V. 1. 100bp ladder, 2 and 9. Lactate enrichment, 3 and 11. Lactate-sulfate enrichment, 4 and 12. Lactate-sulfate-BESA enrichment, 5.Lactate-Acetate enrichment, 6. Colony of roll tube in Lactate (A), 7. Lactate enrichment, 8. lactate original culture, 10. Lactate enrichment replica 2, 13 and 14. *S. aciditrophicus,* 15. Negative PCR control and 16. Empty.

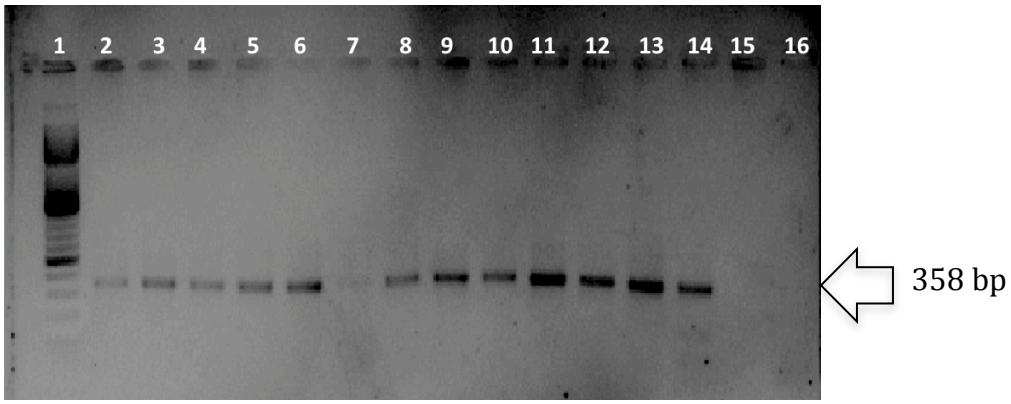

Figure 2.11 Agarose gel electrophoresis demonstrating a representative amplification of the Archaea 16S rRNA gene of the different enrichments of the Mechanic Soil Sample. The gel was a 1.5% agarose gel, ran for 1.5 hours at 111V.1.100bp ladder, 2. Lactate enrichment, 3. Lactate-sulfate enrichment, 4. Lactate-sulfate-BESA enrichment, 5. Lactate-BESA enrichment, 6. Lactate-acetate enrichment, 7. Lactate enrichment (Nested-PCR), 8 and 9. Acetate enrichment, 10 and 11. Lactate original culture, 12 and 13. Lactate enrichment, 14. JF1, 15. Negative PCR control, 16. Empty.

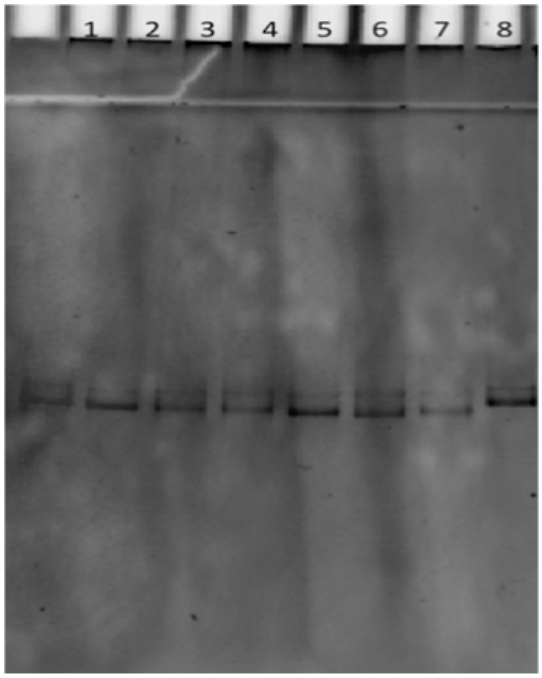

Figure 2.12: Denaturing gradient gel electrophoresis of the bacterial community present in the different enrichments of the Mechanic Soil Sample. The gradient of the gel presented was of 25-45% of bis-acrylamide at 60˚C for 12 hours. 1. Lactate enrichment, 2. Lactate-sulfate enrichment, 3. Lactate-sulfate-BESA enrichment, 4.Lactate-acetate enrichment, 5- Colony of roll tube in lactate, 6. Lactate enrichment, 7. Lactate original culture, 8. SB.

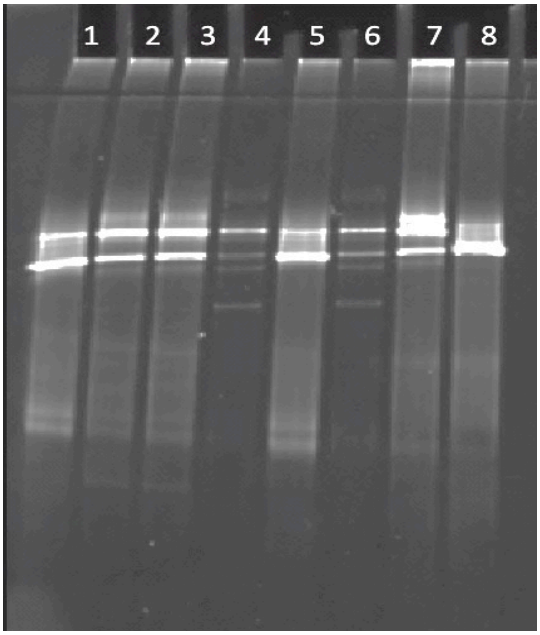

Figure 2.13 Denaturing gradient gel electrophoresis of the methanogenic archaeal community present in the different enrichments of the Mechanic Soil Sample. The gradient of the gel presented was of 40-60% of bis-acrylamide at 60ₒC for 12 hours. 1. Lactate enrichment. 2. Lactate-sulfate enrichment, 3. Lactate-sulfate-BESA enrichment, 4. Lactate-BESA enrichment, 5. Lactate-acetate enrichment, 6. Acetate enrichment, 7. Lactate enrichment (original culture), 8. JF1.

2.4 Discussion and Conclusions

In this research we had aim at demonstrating the syntrophic acetate oxidation by a present bacteria in the environmental soil sample that was taken from the front of a mechanic workshop. To demonstrate the syntrophic acetate degradation, we perform different enrichments with lactate using as controls a methanogenesis inhibitor and sulfate such as a possible electron acceptor. Then, we perform a chemical and molecular analysis to the different enrichments. In the chemical analysis, we observed that when we added BESA, an inhibitor of methanogenesis, lactate was not degraded and no methane production was obtained. Furthermore, we also observed that when we added sulfate, lactate was degraded faster than without sulfate but there was not methane production. However, in the enrichments that had sulfate and BESA, we

observed that the degradation of lactate was partially inhibited. Suggesting that the BESA also inhibits sulfate reduction bacteria. Nevertheless, BESA (2-bromoethanesulfonic acid) is a structural analog of coenzyme M that has been reported as an inhibitor of methanogenesis because inhibits the methyl transfer reaction in methanogens using H_2 and CO_2 (Gunsalus et. al., 1978). BESA has not been reported in the literature with other capacity, therefore we questioned why in the presence of an electron acceptor such as sulfate, lactate degradation was inhibited. Therefore we wonder if exist another function to BESA, beyond being a cofactor of a coenzyme M.

On the other hand, to observe the community of bacteria and archaea in our samples, DGGE analysis were performed using specific primers. DGGE of the 16S rRNA of bacteria shows that although the sample was enriched in different ways, the population of bacteria that is present is the same. Moreover, methanogenic archaea populations appear to be the same in all enrichments, although in lactate-BESA and acetate enrichments we can observe more bands than others. However, these bands are also present in the original culture and if we observe the chemical analysis of these two enrichments, these did not show degradation of the substrate. Nevertheless, the molecular technique used does not allow us to see whether the amplified DNA is of a living cell and if it was fulfilling a role in our enrichment. Moreover, we can also observe two bands present in all enrichments, being one of them in the same position of our control, JF1 suggesting that it is one of the two methanogenic archaea in our culture.

Hence, this technique of DGGE could have implications if we use it to analyze the population because it may be the case are microorganisms that have more than one copy of the 16S rRNA gene and produce more than one band. As an example, the syntrophic bacteria, *Pelotomaculum schinkii* that contains two distinct 16S rRNA gene sequences, with a 96.8%

similarity between them (de Bok et al., 2005). These cases also occur in archaea, were reports indicate one to four copies of the 16S rRNA gene (Arcinas et al., 2004) and one to two copies of the mcrA gene (Luton et al, 2002). These cases could show us two different bands in a DGGE, and therefore can be interpreted as two different microorganisms when in reality they are not. We cannot predict how many bacteria we have in the culture, but we suggest that we need more advanced molecular studies to conclude how many different microorganisms are in the sample and who they are. However, in the case of the population of methanogenic archaea in the culture, we can mention that we have two methanogenic archaea and one of which is our control microorganism, JF1. We suggest that we have a possible consortium of three microorganism composed of one sulphate reducing bacteria and two methanogenic archaea, one of then is JF1. To better molecular study of our community, we turn to Chapter 3 of in this thesis.

Chapter Three

Design and validation of PCR primers targeting the cap protein gene of *S. aciditrophicus*

3.1 Introduction

Currently there are no known mechanisms involved in the formation of a syntrophic relationship. Recent studies suggest that the flagellum mediates the symbiosis (Watanabe et al., 2009). In this research they studied the relationship between syntrophic bacteria (*Pelotomaculum thermopropionicum*) and methanogenic archaea (*Methanothermobacter thermautotrophicus*). They found that these microorganisms formed co-aggregate when grown on a syntrophic substrate and that physical contact between them was essential to degrade it. Watanabe et al. (2005), determined that the microorganisms needed physical contact in order to degrade the substrate provided. If the substrate required that a high quantity of electrons be removed, then the microorganisms performing that task needed to have a closer distance. In the study by discovering why the importance of physical contact to create a syntrophic relationship, they found that the flagellar cap protein, FliD, of *P. thermopropionicum* recognized the membrane of its known partners, a significant step for the formation of a relationship (Watanabe et al., 2009). However, we do not know if this is similar in all syntrophic relationships. An example of this is the case of *S. aciditrophicus*, because this bacterium possesses all the genes for the formation of the flagellum, but its phenotype was described as a non-motile microorganism (Jackson et al, 1999). For this reason, we decided to create PCR primers to amplify the gene of the cap protein of SB, and then use them to find the gene in different environments enriched with various substrates.

3.2 Materials and methods

3.2.1 Primers design for the cap protein of *S. aciditrophicus*

We designed primers for the putative gene of the flagellar cap protein of *S. aciditrophicus* with the purpose of identifying this microorganism in any environmental sample. *S. aciditrophicus* has in its genome two cap proteins, the large one contains 1,035 aminoacids (GenBank: ABC77244) and the other has 220 amino acids (GenBank: ABC77208). Specific primers that amplify both sequences of the cap protein genes were designed. We verify each primer set for possible homodimers, heterodimers and determined their optimal melting temperatures using web-based programs such as DNA Technologies (IDT) and BLAST. We use the In-silico PCR Amplification program to predict the amplification product size. The sequences amplified in In-silico PCR were introduced in Blast to observe the similarity with other sequences from microorganisms in the database. To check the specificity of primers, DNA from *S. aciditrophicus* was used as a positive control in PCR experiments and DNA of the *Desulfatibacillum alketivorans* (AK-01; a gift from Dr. Amy Callaghan from the University of Oklahoma), microorganism with the most similar cap protein in the database. The primer set analyzed for the long sequence of cap protein (37SBcaplg F and 2802SBcaplgR) were used to amplify the positions in the genome of SB from 1,380,560 to 1,383,333 bp, producing a PCR product of 2,766 bp. In the same matter, the primers designed for the short sequence of the cap protein (381SBcapsh F and 645SBcapsh R), were used to amplify the regions from 1,352,824 to 1,353,088 base pairs (bp) within the genome of SB, rendering a PCR product of 265 base pairs. We use these primers for molecular analysis of the population using the qPCR technique. In this technique we use different primer sets for a possible analysis of the community present in the mechanic soil sample enriched with lactate (see chapter 2). In addition, we use these primers of

the cap protein to look for this gene in different environments, and then we performed RFLP analysis to compare the amplified gene with that of the control organism, SB.

3.2.2 DNA extraction

All extractions were done using the FastDNA SPIN Kit for Soil (Qbiogene Inc., Carlsbad, CA). DNA extraction began with a cell suspension, which consists of centrifuging a total volume of 3mL; centrifuging 1.5ml of fluid culture twice in an Eppendorf tube. The pelleted cells were re-suspended in 0.5 mL of supernatant fluid and used in its entirety with the FastDNA SPIN Kit for Soil. The manufacturer's protocol was used with some modifications described below. Cell lysis by bead beading was carried out in a vortex using a Mo-Bio vortex adapter (Mo Bio Inc., Carlsbad, CA) for 5 min at maximum speed and then a centrifugation for 13,000 rpm for 10 minutes (Eppendorf Centrifuge 5415D). The time of mixing the lysate with the DNA Binding Matrix Suspension was for 10 minutes, and we use two washes with SEW-S solution. In the final step, the DNA was eluted in 75 to 100 μL of TE buffer and quantified (260nm of optical density) using a nanodrop ND-1000 instrument (Thermo Scientific, Wilmington, DE).

3.2.3 Molecular analysis

A mechanic soil sample enriched in lactate was selected for molecular analysis to elucidate the possible syntrophic relationships that may occur in the sample. Initially, chemical analysis of the sample was performed to observe the degradation of the substrate and the intermediaries of metabolism, in addition for measuring methane production. Following the previous chemical analysis, we performed a molecular analysis with four different primer sets that would amplify: the 16S rRNA genes of bacteria and archaea individually, cap protein and specific primers to the *Syntrophus* genera. All PCR amplifications were confirmed performing an agarose gel

electrophoresis (1.5%) at approximately 115 volts in 1X Tris-acetate-EDTA (TAE Buffer), using a quick load 100bp DNA ladder (New England Biolabs) for size comparison. The gels were stained with ethidium bromide (10mg/mL), and visualized by a Versadoc (Bio-Rad). The primer sets for both bacterial and archaeal 16S rRNA gene were also used for DGGE analysis. Furthermore, PCR were performed using the following parameters:

A. Archaea 16S rRNA

PCR amplifications of Archaeal 16S rRNA gene were incubated in a MyCycler Thermal Cycler (Bio-Rad) using the primers 1106F-GC CLAMP, 1378-reverse and the thermal program described previously (Watanabe et al., 2006), which amplifies a fragment of 358 bp. The PCR master mix (final volume of 50µL) was composed of 10µL of PCR buffer (5x) (Promega), 1.25µL of dNTP's mix (10 mM) (New England Biolabs), 4µL of $MgCl_2$ (25mM) (Promega), 10µL of each primer (2pmol/µL each), 0.4 µL of Taq DNA polymerase (5U/µL) (Promega), 2µL of template DNA [10ng/ µL] and 22.35 µL of water.

B. Bacterial 16S rRNA gene

Partial 16S ribosomal DNA (rDNA) sequences were amplified from the enrichments using the primers GM5F-GC CLAMP and DS907-reverse and the thermal program described previously (Muyzer, 1993), which amplifies a fragment of 550 bp. The PCR master mix reaction (final volume of 50µL) were incubated in a MyCycler Thermal Cycler (Bio-Rad) used 10µL of PCR buffer (5x) (Promega), 5µL of dNTP's mix (2.5mM) (New England®), 5µL of $MgCl_2$ (25mM) (Promega), 0.25µL of each primer (10pmol/µL each), 1 µL of Bovine Serum Albumina (BSA) (New England®), 0.2 µL of Taq DNA polymerase (5U/µL) (Promega), 2µL of template DNA [10ng] and completed with water. The thermal program consist of (i) 4 min at 94°C; (ii) 30

cycles of 1.25 min at 94°C, 45 sec at 53°C, and 2 min at 72°C; (iii) and a final extension step of 10 min at 72°C.

C. SB16S

The specific primer set of 16S rRNA gene to Syntrophus genera are (SB16Sf [5'CTTGCAAGCCCTCACTTA3'] and SB16Sr [5'CCGTCAAGTACAAAGGCT3'), which amplifies a fragment of 280bp, kindly provided by Dr. Cody Sheik from the University of Oklahoma. The PCR master mix reaction (final volume of 50µL) used 10µL of PCR buffer (5x) (Promega), 5µL of dNTP's mix (2.5mM) (New England®), 4 µL of $MgCl_2$ (25mM) (Promega), 2µL of each primer (10µM/µL each), 0.2 µL of Taq DNA polymerase (5U/µL) (Promega), 2µL of template DNA [10ng] and completed with water. The thermal program consist of (i) 5 min at 94°C; (ii) 30 cycles of 0.5 min at 94°C, 0.5 min at 54°C, and 0.5 min at 72°C; (iii) and a final extension step of 15 min at 72°C.

D. SB cap primers

PCR amplifications were carried out in a MyCycler Thermal Cycler (Bio-Rad) using the primers specifically created (section 2.2.1) to the cap protein of *S. aciditrophicus.* The PCR master mix (final volume of 25µL) was composed of 5µL of PCR buffer (5x) (Promega), 2.5 µL of dNTP's mix (2.5 mM) (New England Biolabs), 2 µL of $MgCl_2$ (25mM) (Promega), 0.625 µL of each primer (2pmol/µL each), 0.2 µL of Taq DNA polymerase (5U/µL) (Promega), and 2µL of template DNA [10ng/ µL] and completed with water. The thermal program consisted of (i) 5 min at 95°C; (ii) 30 cycles of 1 min at 95°C, 1.25 min at 48.8 °C, and 1 min at 72°C; (iii) and a final extension step of 10 min at 72°C.

3.2.4 Restriction Fragment Length Polymorphism Analysis of the cap protein amplification product

To determine if the sequence found in the cap protein of the mechanic soil sample and our positive control (*S. aciditrophicus*) are similar, we analyzed the PCR product of both samples through RFLP. Furthermore, we only used the enzyme RsaI in the RFLP analysis performed to the cap protein gene amplified in different environments. Whereas for the Mechanic Soil Sample, we conducted the RFPL using five endonucleases in separate reactions: Hinf I, Alu I, DdeI, RsaI and EcoRI. The RFLP Digestion reactions (final volume of 30 µL) were prepared using 0.1 µL of 1X enzyme buffer (New England Biolabs®),), 0.5 µL of BSA (New England Biolabs®), 0.1 µL of the enzyme (10u/ µL) (Promega), 20 µL of PCR amplification and completed with water. Then, digestion reactions were incubated in a MyCycler Thermal Cycler (Bio-Rad) for 1 hour at 37 ºC. Reaction mixtures were analyzed in a 2% agarose gel for 1.5 hours at 111V. The gel was stained with ethidium bromide (10mg/mL), and visualized by a Versadoc (Bio-Rad).

3.2.5 Quantitative PCR (qPCR)

The purpose of using this technique with different primer sets is to make an analysis of possible syntrophic relationships using genes of methanogenic archaea (Archaea 16SrRNA), bacteria (Bacterial 16S rRNA), bacteria of the *Syntrophus* genus, and bacteria that have the cap protein of *S. aciditrophicus*. We use "My iQ" from BioRad to do all the qPCR in collaboration with Dr. Mostafa Elshahed from Oklahoma State University. The master mix consisted of 12.5 µL of SYBR Green, 1 µL of each primer set [2 pmol/ µL each], 3 µL of DNA sample [10ng/µL] and 25µL with water. The program consisted of 25 cycles with 2 steps: denaturing and annealing. The temperature parameters were empirically optimized for each following primer set (section

2.2.4). As standards for the functional genes, we used DNA from *S. aciditrophicus* and *Methanospirillum hungatei* (JF1) in dilutions 1:10.

3.2.6 Denaturing Gradient Gel Electrophoresis

DGGE was performed with the PCR amplifications of a 16S rRNA gene of archaea and bacteria. We use a D-code universal mutation detection system (Bio Rad, Hercules, CA) and the electrophoresis was performed in 8% acrylamide gels (ratio of acrylamide to bis-acrylamide 37.5:1 (J.T. Baker) for 12 h at 100V in a denaturant gradient of urea and formamide (100% denaturant agent was defined as 7M urea and 40% formamide). The gradient for the DGGE gels of the 16S rRNA gene of the arquea and bacteria gels were 40 to 60% and 25 to 45% respectively. The voltage was maintained at 50V until the gels reached 60 °C, at which time it was placed at 100V until the end of the electrophoresis. We performed a cladogram of the DGGE, which represents the classification of organisms and their phylogenetic relationship, based on the comparison between bands. Therefore, a matrix was calculated with the following equation: Dxy = 1-2 (N_{XY} / N_X + N_Y). Where, D_{XY} is the distance between two rails (populations), N_{XY} is the number of bands equal in both lanes, N_X is the number of bands in lane x, N_Y is the number of bands in the lane Y. Then this matrix was used to construct a Neighbor Join Tree in the program MEGA version 5.0 (Tamura et al. 2011).

3.2.7 Isolation of the acetate and lactate degrading consortium or couple.

After analyzing the degradation of the substrate, we attempt to isolate the microorganisms. To isolate the microorganisms responsible for the syntrophic acetate oxidation or lactate degradation, we performed anaerobic serial dilutions in the medium described previously (Jackson, 1999) with about 2% agar using the roll tube technique as described by Balch & Wolfe (1976) using a

tube roller (custom made). After sterilizing the medium, 1 mL of the culture were transferred to 9mL of the roll tube. The tubes were placed horizontally in the tube roller and then turn on the instrument and as it turns, ice was placed around the tube to solidify the agar. The roll tube was incubated at 37°C in a vertical position for approximately two to three weeks, monitoring the development of colonies every week. The isolated colonies were removed inside anaerobic glove box using a Pasteur pipette with a modified tip and were transferred to medium previously described. After inoculation, we exchanged the gases in a gassing station that containing N_2/CO_2 (80:20 v/v). The pure cultures were incubated at 37°C and methane production was monitored weekly. Also, these colonies were observed under microscopy and molecular analyses were performed.

3.3 Results

3.3.1 PCR optimization using *S. aciditrophicus* DNA

DNA gradients were performed using SB DNA as a control and both primer sets. Primers of the long sequence of the cap protein, SBcaplargeF37 and SBcaplargeR2802, amplified from 0.017 ng to 87 ng (Figure 3.1). Furthermore, primers for the short sequence amplified from 0.067 ng to 8.7 ng (Figure 3.2). Moreover, in the database, the microorganism with the cap protein more similar to SB was *Desulfatibacillum alketivorans* (AK-01), which we perform a PCR using both set of cap protein primers and DNA of AK-01 and DNA of SB. We demonstrate that these primer set only amplified for SB and although not amplified the known closest cap protein in the database, we know that may exist other microorganism that not exist in the database than may have a similar or equal cap protein of SB.

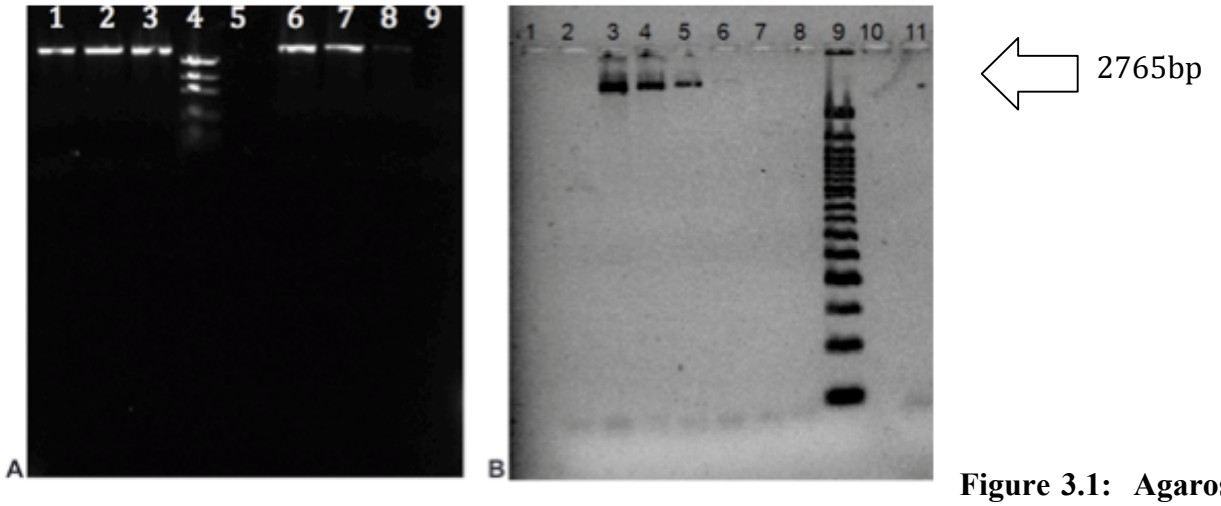

Figure 3.1: Agarose gel electrophoresis of a PCR of the cap protein using a DNA Gradient and the primer set SBcaplargeF37 and SBcaplargeR 2802. DNA gradient was using *S. aciditrophicus* DNA in 1% agarose gel ran at 115V for 1 hour (A) and 1.8% agarose gel (B). Figure A is representative of a PCR DNA Gradient in the following order; 5.8 ng (1), 8.7 ng (2), 17.4 ng (3), Low DNA Mass ladder (4), Empty (5), 43.5 ng (6), 42 ng (7), 87 ng (8), Negative Control (9). Figure B is representative to 1.8% agarose gel of PCR DNA Gradient in the following order (1) Empty, (2) 3 ng, (3) 1.09 ng, (4) 0.067 ng, (5) 0.027 ng, (6) 0.017 ng, (7) 0.0045 ng, (8) 0.00106 ng, (9) 50 bp ladder, (10) Empty, (11) Negative Control.

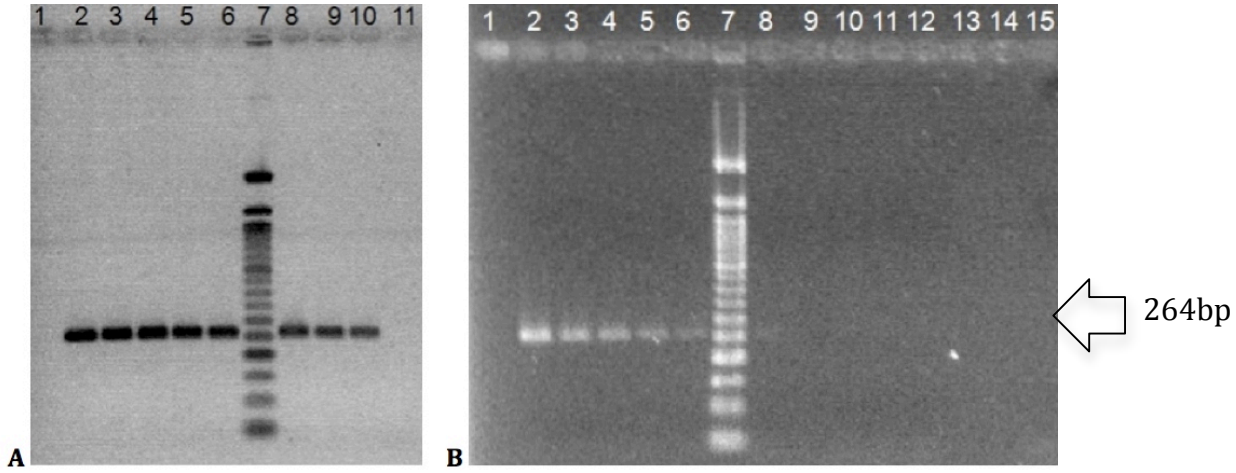

Figure 3.2: Agarose gel electrophoresis of a PCR of the cap protein using a DNA Gradient and the primer set SBcapshortF381 and SBcapshortR645. DNA gradient was using *S. aciditrophicus* DNA in 1.8% agarose gel at 115V for 1 hour (A & B) with a 50 bp ladder. Figure A is in the following orden: 1= Empty, 2= 8.7 ng, 3= 4.35 ng, 4= 2.125 ng, 5= 1.06 ng, 6= 0.53 ng, 7= 50 bp ladder, 8= 0.266 ng, 9= 0.133 ng, 10= 0.067 ng, 11= Negative Control. Figure B is in the following order: 1= Empty, 2= 0.067 ng, 3= 0.01675, 4= 0.0084 ng, 5= 0.0042 ng, 6= 0.0021 ng, 7= 50 bp ladder, 8= 0.00105 ng, 9= 0.0005 ng, 10= 0.00026, 11= 0.00013 ng, 12= 0.00007 ng, 13= Empty, 14= Negative Control.

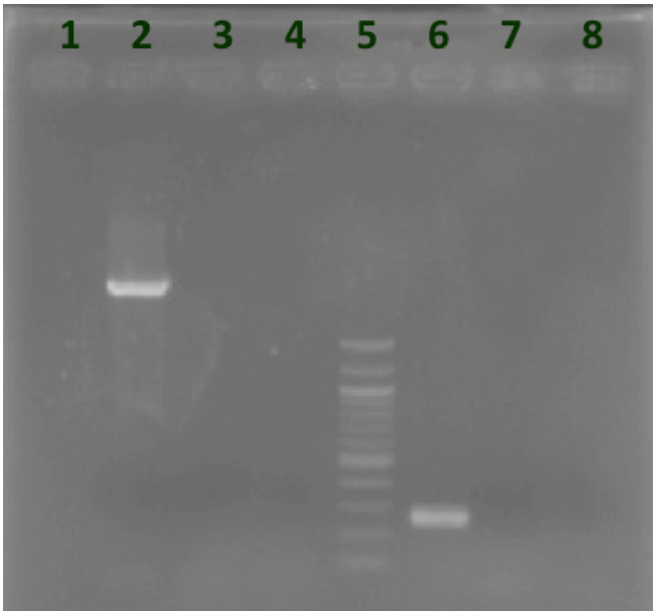

Figure 3.3: Agarose gel of a PCR of the most similar microorganism using the cap protein primers created. The primer set used were SBcaplargeF37/SBcaplargeR2802 (lanes 2 to 4) and SBcapshortF381/SBcapshortR645 (lanes 6 to 8). The gel was run in 1.8% agarose gel at 115V for 1 hour. 1= Empty, 2 and 6= *S. aciditrophicus*, 3 and 7= *Desulfatibacillum alketivorans* (AK-01), 4 and 8= Respective Negative control.

3.3.2 PCR of the cap protein in different environments

To determine if we can use these primers to delimit the distribution of *S. aciditrophicus* in nature, we performed PCR amplifications of this gene from 27 different environments. The DNA of previously enriched sediments from different environments (rice paddy field, mechanic soil sample, water and sediment lake sample, coffee paddy field, dry forest sediment sample, human feces, pond, sludge and petroleum contaminated soil samples) which were used as template for PCR amplifications were previously enriched in different substrates such as propionate, acetate, butyrate, benzoate, lactate, and valerate. We performed PCR amplifications of different environments with two primers set specially designed for the cap protein (SBcaplargeF37/SBcaplargeR 2408 and SBcapshortF381/SBcapshortR645). The amplifications

of primers set SBcaplargeF37 and SBcaplargeR2808 are shown in the Figure 3.4. Furthermore, the amplifications of primers set SBcapshortF381SBcapshortR645 are shown in the Figure 3.5. Results showed amplification of the primers set of the short sequence in 11 environments (Mechanic soil sample enriched in butyrate, lactate, acetate and benzoate; Rice paddy field enriched in butyrate, valerate, propionate and acetate; Coffee paddy field enriched in butyrate; La Plata Lake enriched in benzoate and butyrate; and soil sample of "Bosque Seco de Guánica") Figure 3.5. Moreover, the primers set of the large sequence of the cap protein only amplified in 5 environments (Mechanic soil sample in butyrate and benzoate; Rice paddy field and Coffee paddy field in butyrate; La Plata Lake in Butyrate) (Figures 3.4). To verify that the cap protein gene amplified in these environments is similar to SB, we performed RFLP analysis, which is shown below.

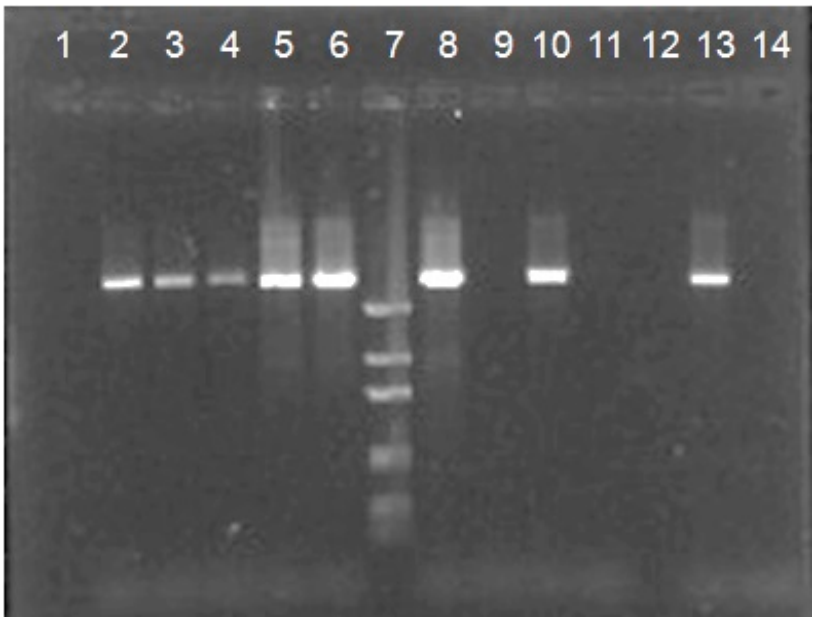

Figure 3.4: Agarose gel electrophoresis of a PCR of the cap protein of different environments using primer set SBcaplargeF37 and SBcaplargeR2802. The PCR was performed using10 ng of DNA and the agarose gel was run in 1.5% agarose gel. 1= Empty, 2= Rice Paddy Field in Butyrate, 3= Coffee Paddy Field in Butyrate, 4= Mechanic Soil Sample in Butyrate, 5 and 6= Mechanic Soil Sample in Benzoate, 7= Low DNA Mass Ladder, 8= La Plata

Lake in Benzoate, 9= La Plata Lake in Butyrate, 10= La Plata Lake in Benzoate, 11= "Bosque Seco de Guánica" in Benzoate, 12= Empty, 13= *S. aciditrophicus*, 14= Negative Control.

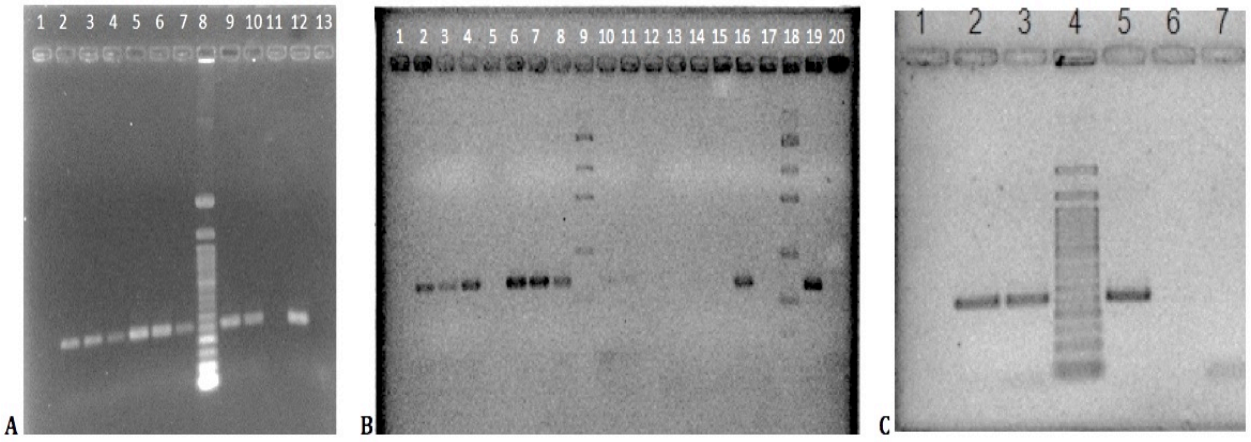

Figure 3.5: Agarose gel electrophoresis of a PCR of the cap protein of different environments using primer set SbcapshortF381 and SbcapshortR645. The PCR was performed using **10ng of DNA** in 1.5% agarose gel. **A:** 1= Empty, 2= Rice Paddy Field in Butyrate, 3= Coffee Paddy Field in Butyrate, 4= Mechanic Soil Sample in Butyrate, 5= Mechanic Soil Sample in Benzoate, 6= La Plata Lake in Benzoate, 7= La Plata Lake in Butyrate, 8= 50 bp ladder, 9= La Plata Lake in Benzoate, 10= "Bosque Seco de Guánica", 11= Empty, 12= *S. aciditrophicus*, 13= Negative Control. **B:** 1= Empty, 2= Rice Paddy Field in Acetate, 3= Rice Paddy Field in Propionate, 4= Rice Paddy Field in Valerate, 5= Empty, 6 and 7= La Plata Lake in Benzoate, 8= La Plata Lake in Butyrate, 9 and 18= 50 bp ladder, 10= Grass in cellulose, 11= Grass, 12= Empty, 13= Guánica Dry Forest, 14= Guánica Dry Forest in propionate, 15= Guánica Dry Forest in acetate, 16= Guánica Dry Forest in Benzoate, 17= Guánica Dry Forest in crotonate, 19= *S. aciditrophicus*, 20= Negative Control. **C:** 1= Empty, 2= Mechanic Soil Sample in lactate, 3= Mechanic Soil Sample in acetate, 4= 50 bp ladder, 5= *S. aciditrophicus*, 6= Empty, 7= Negative Control.

3.3.3 RPLP of the amplification of the cap protein (large sequence)

We performed a RFLP amplification using the primer set for the large sequence of the cap protein. For this technique, we used PCR amplifications of the cap protein gene of the Mechanic Soil Sample enriched in lactate and the positive control, SB. We perform the RFLP analysis with five different enzymes to both samples. RFLP data analysis suggests that the Bacteria in The Mechanic Soil Sample share almost all the restriction sites with the cap protein of SB. Therefore, we suggest that both cap protein are fairly similar (Figure 3.6). Moreover, the RFLP analysis for

the cap protein gene amplified in different environments also presents similar cuts to SB (Figure 3.7). The environment with the cap protein gene more similar to SB is La Plata Lake enriched with Benzoate, while that Mechanic Soil Sample enriched with benzoate is the less similar. Coffee Paddy Field and Rice Paddy Field, both enriched with butyrate, show clear bands but similar to SB.

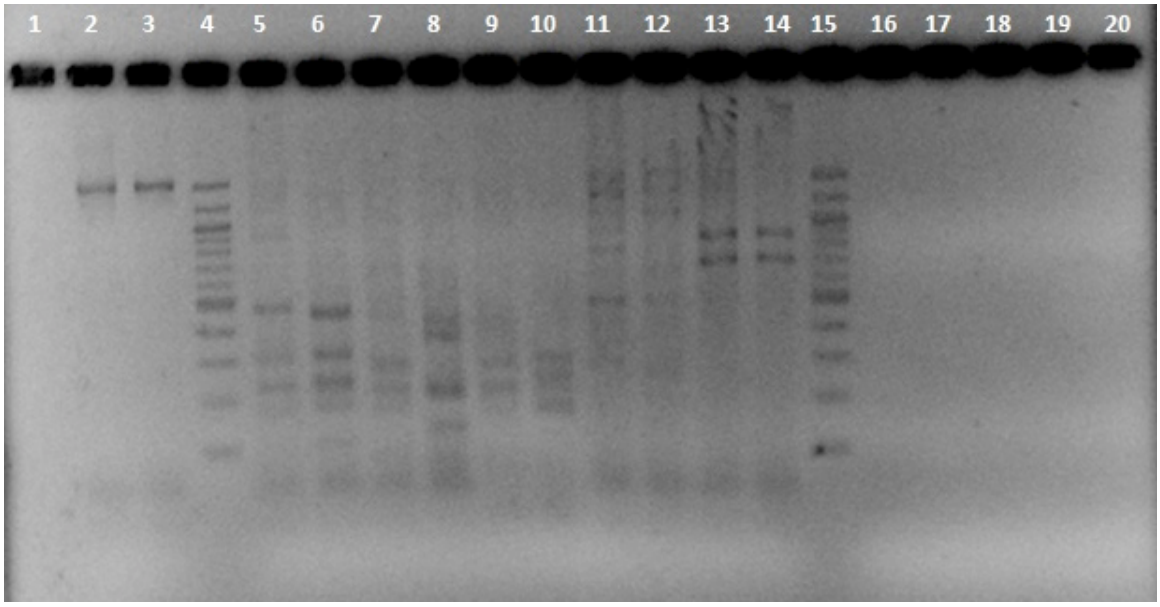

Figure 3.6: Agarose gel electrophoresis of the RFLP of PCR amplification using the primer set of SBcaplargeF37 and SBcaplargeR2802. The gel was a 2% agarose gel, run at 111 V for 1.5 h. Lane 1. Empty, Lane 2 and 3. Uncut PCR amplification of the Mechanic Soil Sample and SB, respectively. Lane 4 and 15. DNA ladder 100bp. Following the order previously described, after the DNA ladder in lane 4, the samples were cut by different restriction enzymes such as Hindf I (lanes 5 and 6), AluI (lanes 7 and 8), DdeI (lanes 9 and 10) and RsaI (lanes 11 and 12), and EcoRI (lanes 13 and 14).

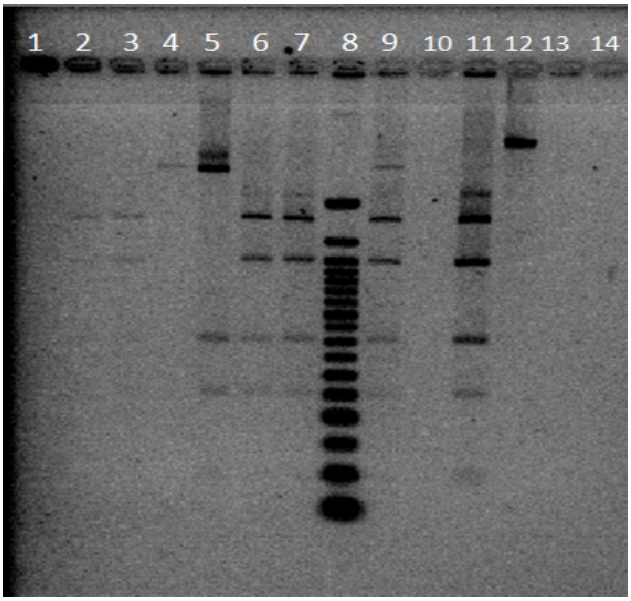

Figure 3.7: Agarose gel electrophoresis of the RFLP of PCR amplification of different environmental samples using the primer set of SBcaplargeF37 and SBcaplargeR2802. Agarose electrophoresis of the RFLP of PCR amplification using the primer set of large The gel was a 2% agarose gel, run at 111 V for 1.5 h. 1= Empty, 2= Rice Paddy Field enriched with butyrate, 3= Coffee Paddy Field enriched with Butyrate, 4= Mechanic Soil Sample enriched with butyrate, 5= Mechanic Soil Sample enriched with benzoate (dilution 10^{-4}), 6 and 9= La Plata Lake enriched with benzoate, 7= La Plata Lake enriched with butyrate, 8= 50 bp loading ladder, 10= Empty, 11= SB, 12= SB (uncut PCR), 13= Negative Control of the Digestion and 14= Empty.

3.3.4 qPCR Analysis of the Mechanic Soil Sample using different primer set

Analyzing the ratios of the copy of the cap protein versus the amount of bacteria in the sample shows that all the bacterium present in the sample has the cap protein (Table 3.1). Of those bacteria that possess the cap protein, which tell us that it is a 100% of the bacterial population, approximately 84% are from de *Syntrophus* genera. However, when we observe the ratio of bacteria, which are from the genus *Syntrophus*, the data tells us that 100% of them belong to this genus. This suggests that all bacteria in the sample are from the *Syntrophus* genera and that some 84% of them have the cap protein. Watanabe el al. (2009) suggests that the flagellum media the symbiosis, considering this, when we observe the ratio of copies of archaea versus

bacteria, this show us that 25% of the population are methanogenic archaea. Therefore, Mechanic Soil Sample it has four archaea per each bacteria in the sample.

Table 3.1: qPCR of the Mechanic Soil sample enriched with lactate using different primer set

Primer sets Comparison	qPCR ratios	%
SB CAP: BACT16S	1.0109	101.1
SB CAP: SB16S	0.8392	83.9
SB CAP: ARQ16S	3.9919	399.2
ARQ16S: SB CAP	0.2505	25.1
ARQ16S: BACT16S	0.2533	25.3
SB16S: BACT16S	1.2046	120.5

3.3.5 PCR of the *Syntrophus* genera

To analyze the data of the qPCR, we performed a PCR with specific primers for the *Syntrophus* genera. Data Analysis of the PCR amplification of this gene indicates that exist a bacteria in the Mechanic Soil Sample that is from the *Syntrophus* genera (Figure 3.8). Furthermore, this gene also amplified in other environmental sample enriched with butyrate, benzoate, lactate, valerate, propionate or acetate. (Figure 3.8).

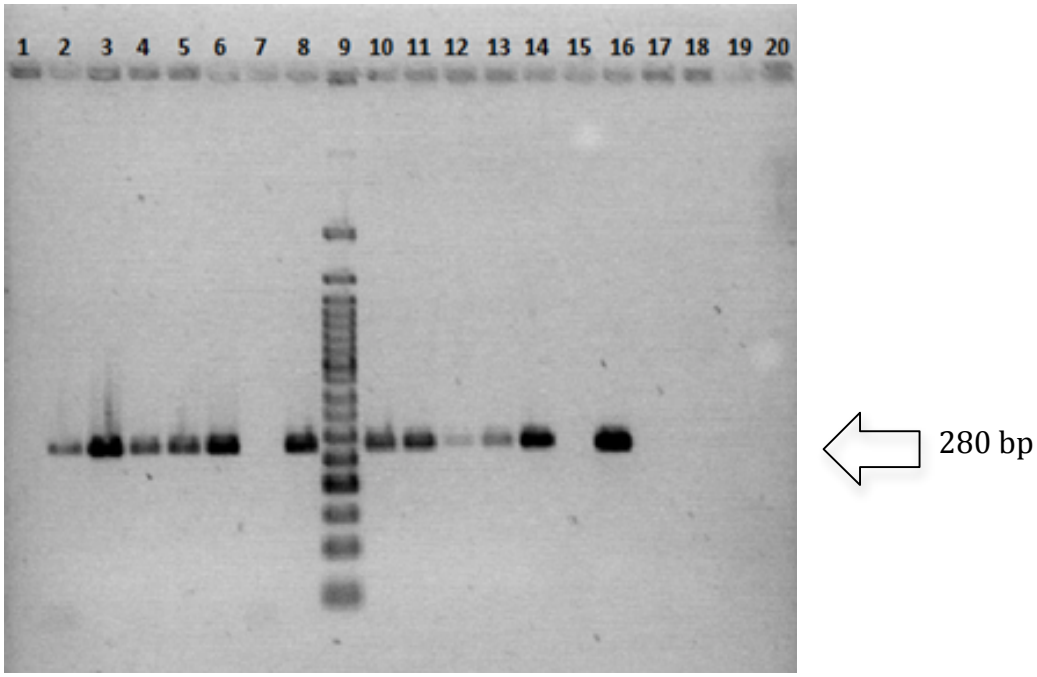

Figure 3.8: Agarose gel electrophoresis of the PCR amplification of the *Syntrophus* genera. The gel was run in 1.8% agarose for 1 h in 120V. 1= Empty, 2= Mechanic Soil Sample enriched with butyrate, 3= Mechanic Soil Sample enriched with benzoate, 4= Mechanic Soil Sample enriched in lactate, 5= Coffee Paddy Field enriched with butyrate, 6 and 8= La Plata Lake enriched with benzoate, 7= La Plata Lake enriched in butyrate, 9= 50 bp ladder, 10= Rice Paddy Field enriched with butyrate, 11= Rice Paddy Field enriched with valerate, 12= Rice Paddy Field enriched with propionate, 13= Rice Paddy Field enriched with acetate, 14= Guánica Dry Forest enriched with benzoate, 15= Empty, 16= SB, 17= Empty, 18= Negative PCR, 19 to 20= Empty.

3.3.6 PCR and DGGE of the 16S rRNA of bacteria and archaea

To observe the community of bacteria and archaea in the sample, we perform a PCR to the DNA of Mechanic Soil Sample enriched with lactate using primers for each gene. We observe the amplification of the sample and its respective controls for both genes of the 16S rRNA of bacteria and archaea gene (Figure 3.9). After amplification of both genes, we performed a DGGE gel in urea-formamide gradient of 25-45% to bacteria and another gel of 40-60% to archaea amplification gene (Figure 3.10). In a DGGE gel of the 16S rRNA of archaea, we observe that in the sample have four bands, which would represent four different methanogenic archaea, being one of these JF1. The band that is presented below in both samples is not being taken into consideration for the analysis because it is present in our positive control (pure culture), and in all our samples managed in the laboratory (Figure 3.10 A). Furthermore, when we analyze the

community of bacteria in the DGGE, we observe that we have four different bands in the sample, two of which are very clear. (Figure 3.10 B). One of these slight bands has the same position as the DGGE band of SB. These results may contradict our qPCR data, unless that in our sample there is another member of the *Syntrophus* genera, which possesses a similar cap protein like SB. It should be noted that this technique of DGGE could separate sequences with difference of only one nitrogenous base.

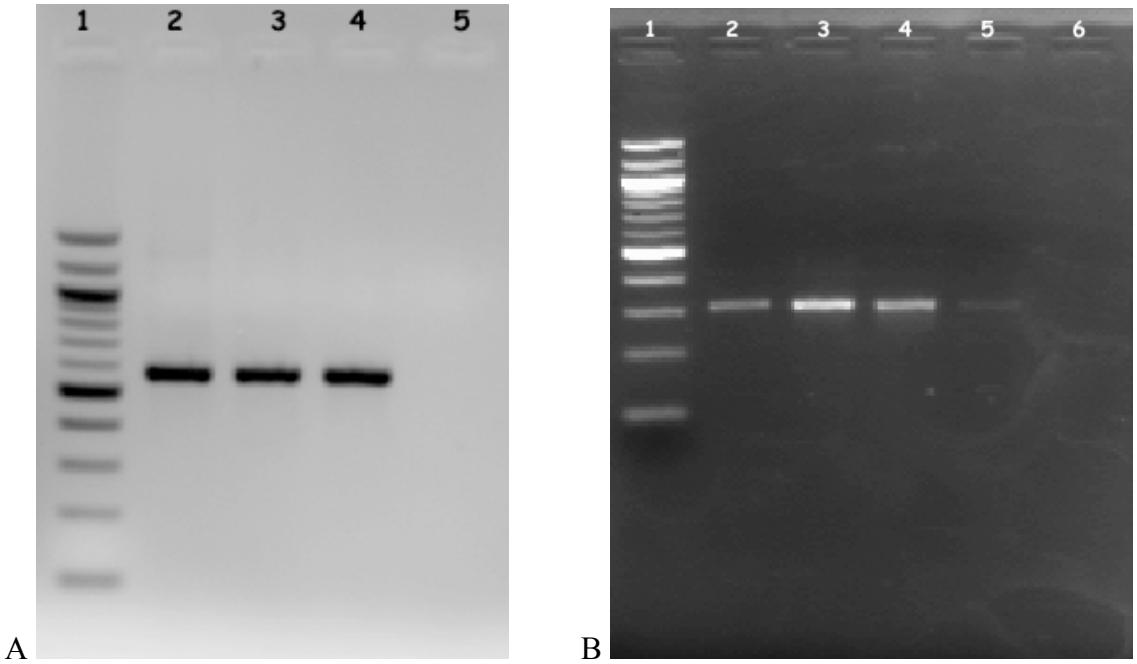

Figure 3.9: Agarose gel electrophoresis of the PCR amplification of the 16S rRNA genes of bacteria (A) and archaea (B). The gel was run in 1.8% agarose for 1 h in 120V. A: 1= 100bp ladder, 2= Mechanic Soil Sample enriched with lactate, 3 and 4= SB, 5= Negative PCR. B: 1= 100 bp ladder, 2= Mechanic Soil Sample enriched in lactate, 3= JF1 (7ng), 4= JF1 (14ng), 5= JF1 (28 ng), 6= Negative PCR.

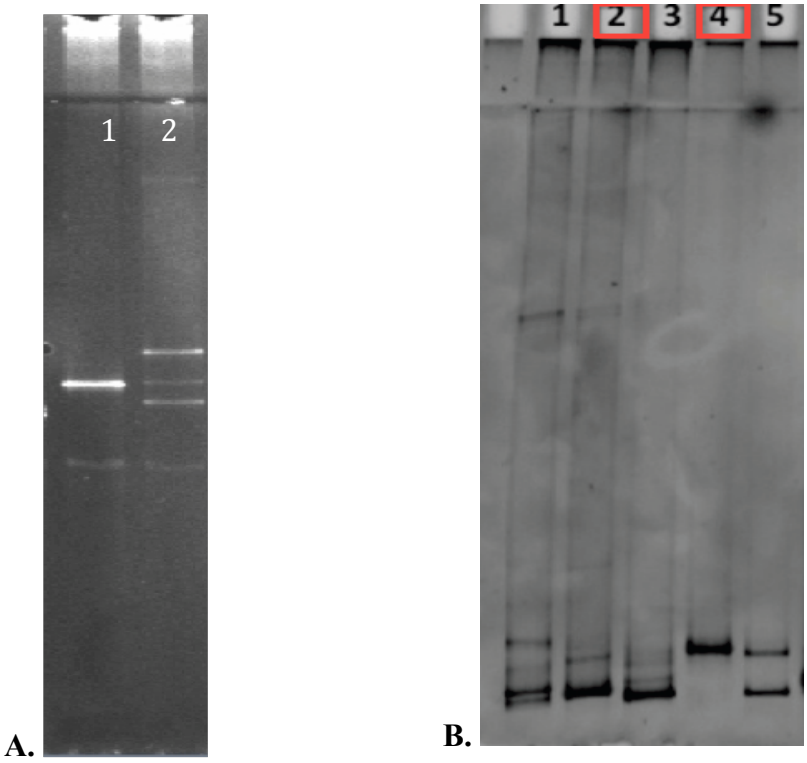

Figure 3.10: DGGE for the 16S rRNA of archaea (A) and bacteria (B) gene amplification. DGGE A is in a gradient of 40-60% of urea and formamide. 1= JF1, 2= Mechanic Soil Sample enriched with lactate. B is a DGGE in a gradient of 25-45% of urea and formamide. 1, 3 and 5= other environmental sample. 2= Mechanic Soil Sample, 4= SB.

3.3.7 Isolation of the acetate and lactate degrader consortium or couple.

For the isolation of the lactate and acetate degraders and the potential partners, we developed serial dilutions in addition to use roll tube technique. Preliminary data of the cultures observed under phase contrast microscope show us that each colony presents two or three different morphologies (Figures 3.11-3.15). In lactate colonies we observed vibrio and coccus (Figures 3.11 and 3.12), as well as aggregates of microorganisms (Figure 3.11:B). Lactate enrichment with BESA as inhibitor of methanogenesis showed morphologies of rod shaped, similar to SB

morphology, and coccus (Figure 3.13). Moreover, enrichment with sulfate showed many vibrios and spirillum morphologies or large vibrio (Figure 3.14). Finally, enrichment of lactate with sulfate and BESA showed small colonies, which presented coccus morphology (Figure 3.15). Therefore, in different enrichments appear to be three different morphologies. For future studies, another student will be responsible for more thoroughly analyzing these cocultures.

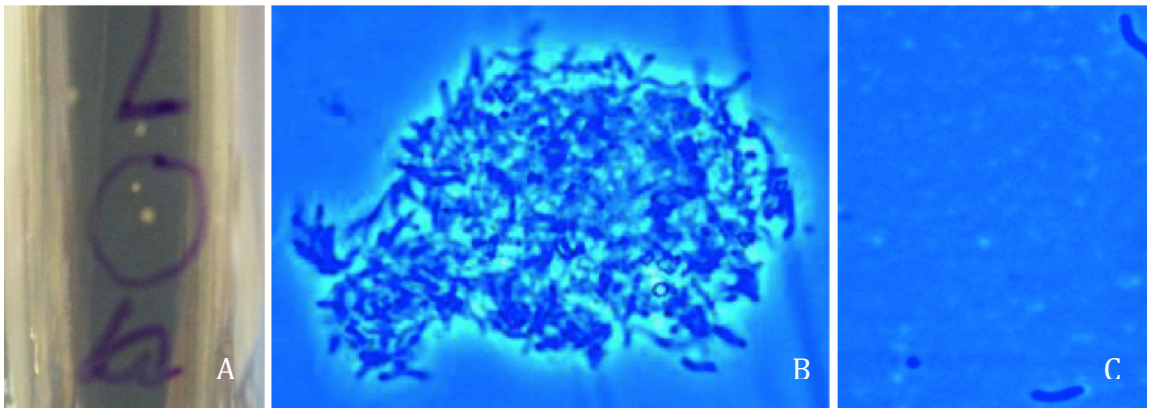

Figure 3.11: Morphologies of the roll tube colony (L1a) of the lactate enrichment. A) Roll tube colony (L1a) in lactate enrichment. B) Aggregates of microorganism in the colony 1a of lactate enrichment. C) Morphology of vibrio and coccus of the 1a of lactate enrichment.

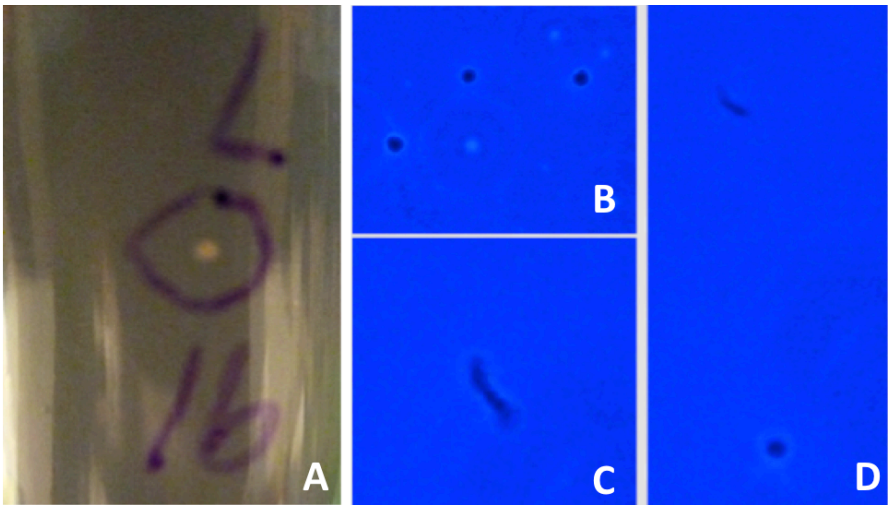

Figure 3.12: Morphologies of the roll tube colony (L1b) of the lactate enrichment. A) Roll tube colony (L1b) in lactate enrichment. Morphology of coccus (B), vibrio (C) and vibrio and coccus (D).

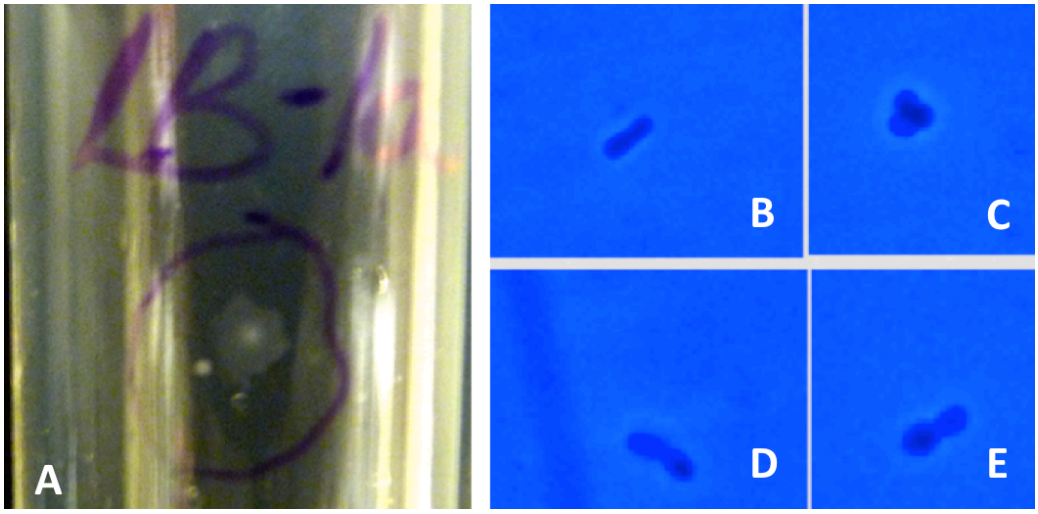

Figure 3.13: Morphologies of the roll tube colony (LB-1a) of the lactate with BESA enrichment. A. Roll tube colony (LB-1a) in lactate with BESA enrichment. Morphologies of rod shaped (B, D and E). Morphology of coccus (C).

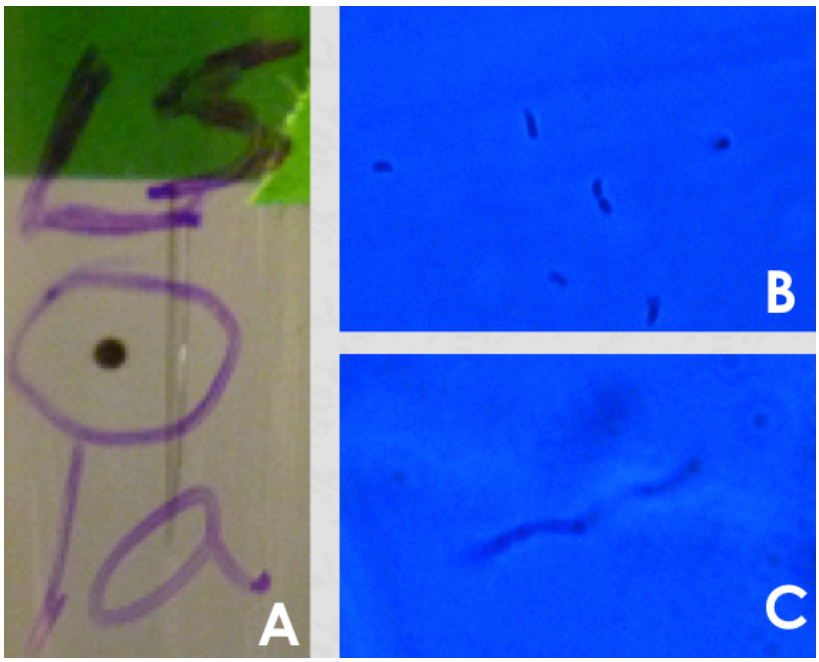

Figure 3.14: Morphologies of the roll tube colony (LS-1a) of the lactate with sulfate enrichment. Roll tube colony (LS-1a) in lactate with sulfate enrichment. A. Roll tube colony (LS-1a) in lactate with sulfate. Morphologies of vibrios (B) and spirillum or large vibrio(C).

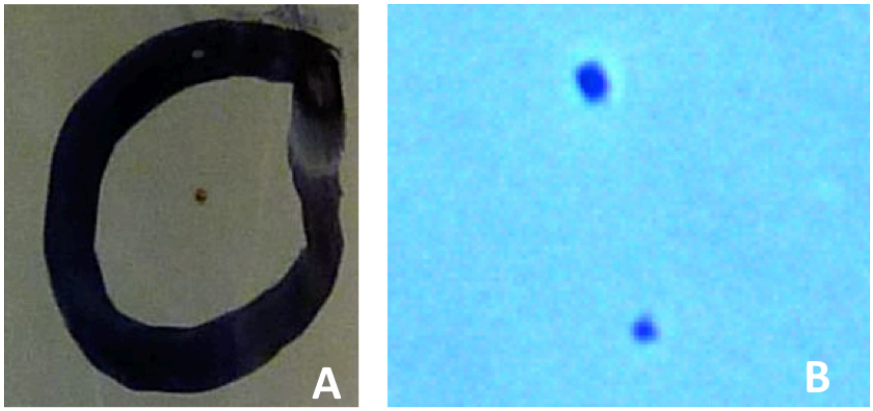

Figure 3.15: Morphologies of the roll tube colony of the enrichment of lactate with sulfate and BESA. Roll tube small colony (A) and morphology of coccus (B).

3.4 Discussions and Conclusions

3.4.1 Molecular analysis with the cap protein primers

We design two primer set to amplify the gene of the cap protein of SB. In the database we found two sequences of the cap protein, one of them of 1,035 aminoacids and another of 220 aminoacids. Hence, we created a primer set to amplify a portion of the large sequence (37SBcaplg F and 2802SBcaplgR) and a portion of the small sequence (381SBcapsh F and 645SBcapsh R) of the cap protein of SB. Then, amplification was performed using a SB DNA to a gradient, which would check the sensibility of each primer set. Primers for the long sequence showed a detection limit of 0.017 ng, while primers for the short sequence amplified until 0.00105 ng. It is suggested that if our microorganism possesses only one copy of this gene, this would mean that the long sequence primers could amplify samples that have as a minimum of 48,000 cells with this gene, while the short sequence primers could amplify up to at least 300 cells with this gene. This shows us that the short sequence primer set is more sensitive in terms of the minimum quantity of the cells with this gene that we need in a sample to be able of

amplifying it. Also, we confirmed this when we amplified the gene in the same natural environments using both primers set. Short sequence primers amplify the cap protein gene in 11 different environments, while for the long sequence primers amplify only 5 of these environments. This shows us that if we used the primers to amplify the short sequence of the cap protein in different environmental samples, if these are more sensitive could know more precisely if the gene is or not present. The gene of the cap protein of SB was present in natural environments such as: Mechanic Soil Sample (enriched in benzoate and lactate), Rice Paddy Field (enriched in valerate, propionate and acetate), Coffee Paddy Field and Coffee Paddy Field enriched in Butyrate, La Plata Lake (enriched in Benzoate and Butyrate), and in Guánica Dry Forest enriched in Benzoate. Therefore, we know that SB can degrade benzoate and fatty acids of 4 to 8 carbons, for which we may assume that it can be present in enriched environments with benzoate, butyrate and valerate. However, we can't tell if SB is active or not in the sample because we don't possess 100% of the sequences of the microorganisms, besides not know if there are others who have the same sequence of the cap protein and still not appear in the database. Another possibility is the existence of microorganism that is not found in the database, which possesses a cap protein gene similar to that SB, or a known microorganism in a database that does not have its cap protein sequenced. However, these environments amplified with the specific primers to the genus *Syntrophus*, which might indicate that is SB or a new microorganism of this genus and therefore does not appear in the database.

3.4.2 Molecular analysis of the Mechanic Soil Sample enriched in lactate

We molecularly analyzed the Mechanic Soil Sample enriched with lactate, using different primer sets. In the molecular analysis of the cap protein using both primers sets, the data shows that we

have a microorganism with the cap protein of SB. When we compared in the RFLP the cap protein amplified with SB, the data suggest that the bacteria in our enrichment share almost all the restriction sites with SB. Moreover, qPCR data shows that we have only one bacterium in the sample that have the cap protein of SB and also belongs to the genus *Syntrophus*. Furthermore, the data indicated that for each bacterium in the sample that has the cap protein, we have four copies of the 16S rRNA of the methanogenic archaea. DGGE analysis of the archaeal 16 rRNA gene show that in the sample we have four bands, which would represent four different methanogenic archaea, being one of these JF1 *Methanospirillum hungatei,* known syntrophic partner of SB. Moreover, DGGE analysis of the bacterial 16 rRNA gene show that in the sample we have four different bands and one of these bands has the same position as the DGGE band of SB. Therefore, we have a known syntrophic couple in our sample, although we do not know whether they are to exercise any role. Hence, we do not know if they are working as partners or are interacting with other microorganism in the culture. This molecular analysis using a qPCR with different primer sets can help us to understand how populations may be correlated to form a syntrophic relationship. However, when we add the DGGE data, we can see that in our sample there are only two-methanogenic archaea and four different bacteria. This may represent that in the sample is found greater amount of bacteria than possible syntrophic partner. DGGE analysis contradicts the qPCR data that showed us that only exist a single bacterium in the sample. Hence, we have an environmental sample that possesses one microorganism with a similar cap protein of SB and it appears to belong of the genus *Syntrophus*. Nevertheless, we need more rigorous testing as sequencing to confirm that the present microorganism in the sample is SB and to see what could be its possible syntrophic partner.

In conclusions, we have a community of microorganisms that can perform lactate oxidations and apparently have the cap protein gene with a specific function that we do not know. The primer set performed specifically for the cap protein gene of SB not amplified to the more similar to the SB cap protein in the database. However, we don't have certainty that the amplified cap protein in our samples belong to the other microorganism that does not appear in the database, which we can suggest that is a new microorganism, an old microorganism that has acquired new genes or an old microorganism that possess both functions. Moreover, our data suggest that BESA not only inhibits the methanogens in the sample but also inhibiting microorganisms in our sample to degrade lactate when sulfate is present. We suggest further studies to verify that BESA has this new capacity, because it has not been shown previously.

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Appendices

Mass balances

To calculate the methane mass-produced for each enrichment, we took into consideration the enrichments of lactate and lactate-acetate because it's were the only ones in produce methane and therefore these present substrate degradation. Then we convert the concentrations of substrates and methane to grams using the followings parameters:

CH_4 mass of lactate enrichment =

Final methane: 15.9 % of CH_4/ 100 of the total gas mass= 0.159 g

CH_4 mass of lactate-acetate enrichment =

Final methane: 20.3 % of CH_4/ 100 of the total gas mass= 0.203 g

Lactate enrichment:

* Started with:
10.32 mM of lactate = 10.32mM (1mol/1000μM) (112.05)
= 1.1556 g of lactate

0.4 mM of acetate = 0.4 mM (1mol/1000μM) (82.03)
= 0.0328 g of acetate

*Enrichment finished with:
0.4 mM of lactate = 0.4 mM (1mol/1000μM) (112.05)
= 0.0448 g of lactate

0.54 mM of acetate = 0.54 mM (1mol/1000μM) (82.03)
= 0.0442 g of acetate

Lactate-acetate enrichment started with:

* Enrichment started with:
10.9 mM of lactate = 10.9mM (1mol/1000μM) (112.05)
= 1.221 g of lactate

10.58 mM of acetate = 10.9mM (1mol/1000μM) (82.03)
= 0.867 g of acetate

*Enrichment finished with:

0.43 mM of lactate = 0.43 mM (1mol/1000μM) (112.05)
= 0.049 g of lactate

1.28 mM of acetate = 1.28 mM (1mol/1000μM) (82.03)
= 0.105 g of acetate

To the substrate degradation we use the following equations:

$2CH_3CHOCOO^- + H_2O \rightarrow 2CH_3COO^- + CH_4 + H^+ + HCO_3^-$
$2CH_3COO- + 2H_2O \rightarrow 2HCO_3- + 2CH_4$

$2CH_3CHOCOO^- - 3H_2O \rightarrow 3CH_4{}^+ + 3HCO_3 + H^+$

Appendix A.1: Mass balance of the enrichments Lactate and Lactate-Acetate

Enrichment	Lactate (g)		Acetate (g)		Methane (g)		
	Initial	Final	Initial	Final	Estimated	Real	Difference
Lactate	1.1556	0.0448	0.03	0.442	1.7634	0.159	1.6044
Lactate-acetate	1.221	0.049	0.867	0.105	2.6985	0.203	2.4955

Lost methane = (Estimated-Real)/Estimated

Lactate enrichment = 90.9 %

Lactate-Acetate enrichment = 92.4%

Appendix A.2: Gibbs free energy of de reactions involving in enrichment of lactate and its controls.

All calculations were performed at mM concentration and using the Gibbs free energies of formation tables (Tauer et al., 1997), and we use the following equation:

$\Delta G^0 rx = \Sigma_n \Delta G^0 f$ (products) - $\Sigma_n \Delta G^0 f$ (reagents)

Lactate enrichment

$10\ CH_3CH_2OHCOO^- + 10\ H_2O \rightleftarrows 10\ CH_3COO^- + 10\ HCO_3^- + 10H^+ + 20H_2$

$20\ H_2 + 5HCO_3^- + 5H^+ \rightleftarrows 5\ CH_4 + 15\ H_2O$

$10\ CH_3COO^- + 10\ H_2O \rightleftarrows 10\ CH_4 + 10\ HCO_3^-$

$10\ CH_3CH_2OHCOO^-_{(aq)} - 15\ H_2O_{(liq)} \rightleftarrows 15\ CH_{4(g)} + 15\ HCO_3^-_{(aq)} + 5H^+_{(aq)}$

ΔG^0_{rx} = [(15*-50.75 kJ/mol) + (15*-586.85 kJ/mol)+(5*0 kJ/mol)] –
[(10*-517.81 kJ/mol) + (15*-237.18kJ/mol)]
= -828.23 kJ/mol

Enrichment of Lactate with sulfate -

$10\ CH_3CH_2OHCOO^-_{(aq)} + 10\ SO_4^{-2}_{(aq)} \rightleftarrows 30\ CO_{2\,(g)} + 10\ SH^- + 10\ H_2O_{(liq)} + 15\ H_{2\,(g)}$

ΔG^0_{rx} = [(30* -394.36 kJ/mol)+(10*12.05 kJ/mol)+(10*-237.18 kJ/mol)+(15*0 kJ/mol)] –
[(10*-237.18 kJ/mol)+(10*-744.63 kJ/mol)]
= -1657.75 kJ/mol

Enrichment of Lactate with sulfate and BESA

$12\ CH_3CH_2OHCOO^-_{(aq)} + 2\ SO_4^{-2}_{(aq)} + BESA \rightleftarrows 7\ CH_3CH_2OHCOO^-_{(aq)} + 5\ CH_3COO^-_{(aq)} + 2HS^-_{(aq)} + 5\ CO_{2(g)} + 3\ H_2O_{(liq)} + 7\ H^+_{(aq)}$

ΔG^0_{rx} = [(7*-517.81 kJ/mol) + (5*-369.41 kJ/mol) + (2*12.05 kJ/mol) + (5*-394.36kJ/mol)
+ (3*-237.178 kJ/mol)+ (7*0 kJ/mol)] - [(12*-517.81 kJ/mol)+(2*-744.63)]
= -427.969 kJ/mol

Enrichment of Lactate with BESA

$15\ CH_3CH_2OHCOO^-_{(aq)} + 3\ H_2O_{(liq)} + BESA \rightleftarrows 12\ CH_3CH_2OHCOO^-_{(aq)} + 3\ CH_3COO^-_{(aq)} + 3\ CO_{2(g)} + 7\ H_{2(g)} + H^+_{(aq)}$

ΔG^0_{rx} = [(12*-517.81 kJ/mol) + (3*-369.41 kJ/mol) + (3*-394.36 kJ/mol) +
(7*0 kJ/mol)+(1*0 kJ/mol)] – [(15*-517.8 kJ/mol) + (3*-237.18 kJ/mol)]
= -26.343 kJ/mol

Enrichment of the same concentration of Lactate and Acetate

$10CH_3CH_2OHCOO^- + 10CH_3COO^- + 10H_2O \rightleftarrows 20CH_3COO^- + 10HCO_3^- + 10H^+ + 20H_2$
$20\ H_2 + 5\ HCO_3^- + 5\ H^+ \rightleftarrows 5\ CH_4 + 15\ H_2O$

$10CH_3CH_2OHCOO^- + 10CH_3COO^- + 5H_2O \rightleftarrows 20CH_3COO^- + 5CH_4 + 5HCO_3^- + 5H^+$
$20\ CH_3COO^- + 20\ H_2O \rightleftarrows 20\ CH_4 + 20\ HCO_3^-$

$10CH_3CH_2OHCOO^-_{(aq)} + 10CH_3COO^-_{(aq)} - 25H_2O_{(liq)} \rightleftarrows 20CH_{4(g)} + 25HCO_3^-_{(aq)} + 5H^+_{(aq)}$

ΔG^0_{rx} = [(25*-50.75) + (25*-586.85 kJ/mol) + (5*0 kJ/mol)] – [(10*-517.81 kJ/mol) + (10*-369.41 kJ/mol)+ (25*-237.178 kJ/mol)]
= -1138.35 kJ/mol