ANAEROBIC BIOCONVERSION OF COLLAGEN TO METHANE

by

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ABSTRACT

This thesis reports the use of two anaerobic batch reactors (ABRs) to transform a protein solid waste into a renewable energy source. The "heart" of this type of reactor depends on the microbial community within the reactor, their bioconversion potential, and the operational cost. In this research, we developed two ABRs using highly purified enrichments adapted to the anaerobic collagen digestion process. The first reactor, ABRa, was inoculated with a selected highly purified microbial enrichment with functional roles of collagen degradation, coupled with methane production. The second reactor, ABRb, was inoculated with a mixture of two methanogenic microbial communities; one with the acclimated functional role of collagen degradation, and the other with the role of syntrophic volatile fatty acids (VFAs) degradation. We tested which ABR biotransformed more efficiently the collagen based on methane and VFAs consumption, and by comparing the microbial population by Denaturing Gradient Gel Electrophoresis (DGGE). Our results showed that ABRa biotransformed approximately 12% of the collagen into 57% methane while ABRb biotransformed only 4% of the waste into 20% methane. DGGE analysis showed similarities on the diversity of the bacterial population between and inside both of the ABRs on the first 45 days. However, the bacterial population remains 100% homologous, and no differences were detected on the methanogenic population at 90 days. In conclusion, some anaerobic microbial communities have the potential to transform the collagen into biogas. However, using one adapted consortium to transform the protein into methane is more efficient than combining several trophic microbial groups that were acclimated separately from different environments.

RESUMEN

Esta tesis trata sobre el uso de dos bioreactores anaeróbicos “ABRs” (por sus siglas en inglés) para transformar desperdicios sólidos proteicos en una fuente de energía renovable. El “corazón” de este reactor depende de la comunidad microbiana, su potencial de bioconversión, y el costo operativo del mismo. En esta investigación, se utilizaron enriquecimientos altamente purificados para desarrollar dos bioreactores anaeróbicos. El primer reactor “ABRa’’ se inoculó con una comunidad proveniente de un enriquecimiento capaz de degradar el desperdicio (colágeno) y producir metano. El segundo reactor “ABRb’’ se inoculó utilizando una mezcla de dos comunidades microbianas, una comunidad especializada en la degradación de colágeno, y la otra comunidad especializada en la producción de metano e intermediarios de degradación, los ácidos grasos volátiles y se compararon las comunidades microbianas utilizando Electroforesis de Gradiente Desnaturalizante “DGGE” (por sus siglas en inglés). Nuestros resultados demuestran que ABRa biotransformó aproximadamente 12% del colágeno en 57% metano, mientras que el ABRb biotransformó solo 4% del desperdicio en 20% metano. Los análisis de DGGE indican que hubo similitud en la diversidad bacteriana de los bioreactores durante los primeros 45 días. Sin embargo, la población bacteriana y metanogénica de ambos reactores se mantuvo 100% homóloga al finalizar 90 días. En conclusión, algunas comunidades microbianas tienen el potencial de transformar el colágeno en biogás. Sin embargo, utilizar un consorcio adaptado a transformar la proteína a metano resulta más eficiente que combinar varios niveles tróficos que fueron aclimatados por separado de diferentes ambientes.

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This project is dedicated to the people that always believe in me and in my capacity to do this research, especially to my wife Marie, my parents, Edwin, and Nitza. Especially I dedicate this work to the memory of my always remembered grandfather Alfonso Piñero.

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I also want to thank the Bio Science and Engineering Initiative (BioSEI) for the funding and the resources for the development of this research. Furthermore, I want to thank Mr. Cherry for supplying the industrial waste materials to do this research. Lastly, I would like to acknowledge my family, and thank them for their inspiration, unconditional support, and love.

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1 INTRODUCTION

In natural environments, the anaerobic digestion of organic matter such as proteins, carbohydrates, and lipids involves a complicated process of several metabolic reactions from at least three different functional microbial groups that constitutes as a whole an anaerobic microbial community (30). The first trophic level is occupied by hydrolytic fermentative bacteria (4). These bacteria hydrolyze proteins, carbohydrates, and lipids to sugars, amino acids, and fatty acids. The second trophic level is occupied by the acetogenic and hydrogen producing bacteria (also known as syntrophic bacteria), which transform the fermentation product of the first trophic level into carbon dioxide, hydrogen, and acetate (33). The third trophic level is occupied by the methanogens (assuming that the electron acceptors available are limited to CO_2) which convert acetate, carbon dioxide and hydrogen to methane in a process called methanogenesis (28). However, it is this complex microbial community as a whole that has been utilized in anaerobic bioreactors to produce a clean and renewable energy source like H_2 or CH_4 (13).

1.1 Motivation

In worldwide landfills more than 52% of the solid waste, such as newspapers, food scraps, clothing, furniture, and grass clipping are continually being decompose to methane by anaerobic microbial communities contributing with 7% of the total atmospheric methane concentration (Energy Administration, 1/12/11 website, http://tonto.eia.doe.gov/country/country_energy_data. cfm?fips=RQ). If the constant methane

emissions from landfills are not collected, they will escape to the atmosphere and contribute to global warming, which in turn could induce melting of glacial areas and promoting mass extinction of species not suited to higher temperatures (8). Unfortunately, Puerto Rico does not have a mechanism to monitor or collect the methane emissions from its 32 open landfills. However, being one of the main solid waste producers in the world, we consider that Puerto Rico landfills are a contributing part of the global methane emissions problem (Environmental Protection Agency 1/24//11 website, http://tonto.eia.doe.gov/country/ country_energy_data.cfm?fips=RQ).

Natural alternatives to reduce the methane emissions from landfills, such as the use of biofilters has been documented (11). These biofilters take advantage of methane oxidizing bacteria and has the potential to reduce methane emissions by at least 62% (11). Another solution to this problem is the use of methane collectors. This method requires a set of pipes connected in landfills, allowing the collectors to remove the majority of methane produced and avoiding the biogas escaping to the atmosphere. On the other hand, another possible solution to reduce Puerto Rico's landfill methane emissions is the use of anaerobic bioreactors. These reactors are an efficient alternative to collect methane produced by the digestion of solid wastes such as papers, food scraps, and proteinaceous residuals (10). These digesters are design to harbor microbial communities selected to produce methane in the absence of electron acceptors like nitrate, iron, and sulfate (35). More importantly, when this biogas is collected it can be a useful renewable energy source given its calorific value of 55.530 kJ/kg (26).

Even though these possible solutions exist, in our Island we are not taking action to reduce methane emissions from landfills, neither taking advantage of the biogas calorific properties to possibly reduce the dependence and high cost of importing large amounts of fossil fuels (3.540 millions of dollars every day) (Energy Administration 1/12/11 website, http://www. eia.doe.gov/state/territory_profile_pr.html and (22). Perhaps, modifying Puerto Rico's municipal solid waste management by sorting the biodegradable waste in anaerobic bioreactors, will reduce the atmospheric methane emission, increase the landfills life, and will supply an alternative energy source which is useful to develop a better quality of life (35).

1.2 Literature Review

During the last decade the use of anaerobic bioreactors to produce biogas has proliferated and gained popularity (9). For example, anaerobic batch reactors have been successfully used for the bioconversion of fruit scraps to methane with a conversion up to 95% of the original mass (7). Additionally, these digesters have been used for the treatment of other wastes like, synthetically formulated wastewater (30) and sludge (13). It has been reported, that anaerobic digesters are able to generate methane, CO_2, and ammonia as the main products when using a synthetic waste stream containing the protein, bovine serum albumin (BSA), as the only carbon source (30). Furthermore, the bioconversion of gelatin into methane was possible using a co-culture of *Clostridium collagenovorans* and *Methanosarcina Barkeri* (14). This co-culture was able to biotransform 85% of the polypeptide (gelatin) to methane and reach steady state in 16 days (14).

Steady state condition is observed when a bioreactor is continually showing degrading rates and methane production rates without changes in the functionality of the reactor over time due to the stability of the microbial community (10). However, anaerobic microbial populations in bioreactors have been monitored using molecular approaches like 16S rRNA gene clone libraries (24). Another effective tool to describe the stability of the microbial community within the bioreactor is Denaturing Gradient Gel Electrophoresis (DGGE). This technique has been described as a fingerprinting method due to its ability to separates polymerase chain reaction generated fragments of the same size from different DNA templates based in their sequence and the dominant microbial organisms (32). It is possible to use only one of these molecular techniques to monitor the changes in the microbial population inside the bioreactor to predict reactor stability.

In this research, we were interested in developing an anaerobic reactor that transform an industrial solid waste, collagen, into a renewable energy source using microorganism from natural environments. The "heart" of the digester depends on the bioconversion potential reflected in the amount of methane produced and the stability of the remaining digestate (10). Additionally, it was our interest to make specific enrichments to select for the functional trophic groups that are involved in this solid waste digestion that will include; collagenolytic bacteria, amine oxidizing bacteria, and syntrophic bacteria with their mutualistic partners the hydrogenotrophic methanogens. Collagenolytic bacteria have the functional role within the reactor to hydrolyze the peptide bonds between the three structured polypeptides of collagen in order to produce gelatin (4). The proteolytic bacteria make possible the gelatin degradation; in most cases the same collagenolytic group is responsible for this degradation. These

reactions make possible the hydrolyzation of the peptide bonds producing free amino acids (33% glycine, 11% alanine, 21% of proline w/v) (12). Those, amino acids are transformed to volatile fatty acids (acetate (C2) from glycine, propionate (C3) from alanine, and valerate (C5) from proline with an oxidative deamination by amine oxidizing bacteria (4). Moreover, the formed VFAs (acetate, propionate, and valerate) in turn are transformed to methane by syntrophic bacteria in collaboration with hydrogenotrophic methanogens and also with the help of acetoclastic methanogens.

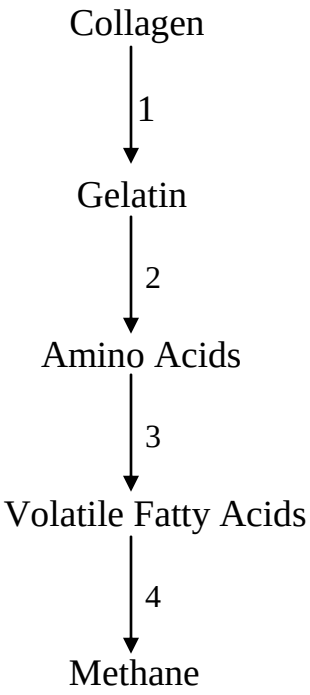

Figure 1. Anaerobic collagen digestion. (1) Hydrolization of three structured polypeptides of collagen by collagenolytic bacteria (2) Hydrolyzation of the gelatin peptide bonds by proteolytic bacteria. (3) Oxidative deamination of the amino acids by amine oxidizing bacteria. (4) Syntrophic oxidation of the volatile fatty acid by syntrophic bacteria, follow by hydrogenotrophic or acetoclastic methanogenesis by methanogens.

1.3 Objective

The main objective of this research was to prove that an industrial solid waste could be transformed into a renewable energy source using an anaerobic batch bioreactor. The specific aims that developed the anaerobic batch bioreactor consisted in the following:

1) Find collagenolytic anaerobic bacteria, and anaerobic syntrophic microorganisms capable of degrading acetate, propionate and valerate from natural environments.
2) Biotransform an industrial solid waste, collagen, into methane using an anaerobic batch reactor.
3) Describe the diversity of the microbial community in the anaerobic batch reactors bioreactors.

2 METHODOLOGY

2.1 Samples

Liquid samples and sediments were taken by submerging a 25mL sterile bottle in anoxic environments. Samples were collected from the sludge of the Mayagüez residual waste water treatment plant. Our second target for sampling was the grease trap from the cafeteria of the University of Puerto Rico - Mayagüez Campus. All the proteinaceous waste residues such as, bones, and cartilage (that contain or are made of collagen) are dispensed into this chamber. The third sample we took was from an oxidation pond from a coffee plantation in Maricao, Puerto Rico. We sampled this oxidation pond to test if collagen degraders can be found in a place that the main component is coffee pulp residue that is not related to collagen (as a negative control). The next sample was taken from the bottom of the Lucchetti water reservoir in Yauco, Puerto Rico. Another two samples were taken from sediments of a grass paddy in Mayagüez and a rice paddy field in Lajas, Puerto Rico. All the bottles were transported at room temperature to our laboratory and placed inside a vinyl anaerobic chamber (Coy Laboratories) with a mixture of anaerobic gases (N_2 and H_2 90:10).

2.2 Microcosm enrichments

In order to set the anaerobic proteolytic enrichments, we prepared in anaerobic tubes 9 ml of autoclaved medium (as same as described in 21). We introduced, the tubes to the anaerobic chamber, open it, and aseptically we added the equivalent to 1% of the industrial solid waste (w/v), collagen. After adding the collagen, the headspace of the enrichments (6 ml) was exchanged with an anaerobic gas (Nitrogen: Carbon dioxide 80:20) in a gassing station using strict anaerobic techniques (33). Finally, we added 1 ml of inoculum (just liquid) from each of the environmental samples, and we documented the visual collagen solubilization in the samples. In addition, we set volatile fatty acids enrichments that consisted of 9 ml of medium with 10mM of acetate, propionate, or valerate. The VFAs enrichments were inoculated by adding 1ml of the sample taken from the rice paddy field. We did exactly the same with the sediments from the water reservoir. Additionally, all the enrichments were incubated at 37ºC in the dark without shaking and processed using the following controls: an autoclaved control that consisted of 9 ml of medium, 1ml of autoclaved sample and 1% of collagen or 10mM of each VFA. An unamended control that consisted of 9 ml of SB medium, 1 ml of sample and no carbon source, and a negative control that consisted of 9 ml of SB medium and 1% of collagen or 10mM of each VFA, but without inoculums. The microbial activity within the enrichments was predicted by the quantification of the VFAs and methane production.

Furthermore, in order to purify the anaerobic collagen degraders, all the enrichments that showed visual solubilization of the solid waste and increase turbidity were transferred

into a serial dilution series with dilutions up to 10^7 (1ml of inoculum, 9 ml of SB medium, and 1% of collagen). Similarly, we transferred all the VFAs enrichments inoculated with the rice paddy field to a serial dilution series (1ml of inocula, 9ml of SB medium, and 10mM of one of the VFAs). All the dilutions series were incubated at 37ºC in the dark without shaking. Phase contract microscopy (Leica DMI3000B) was used to observe and compare the morphotypes in all of the incubations that solubilize the collagen and /or produced methane.

2.3 The effect of temperature and UV light on collagen degradation

We transferred 20% from all of the highest dilution tubes that showed visual collagen solubilization and turbity into a new tube containing SB medium to a final volume of 20 ml. The ratio of collagen was maintained to 1% in all the incubations. In order to search the optimal temperature in which our cultures degrade collagen the fastest, we incubated the bottles at three different temperatures; 37ºC, 25ºC, and room temperature (less than 25ºC) during a month to observe the collagenolytic activity. All the samples that did not achieve collagen degradation at 25ºC or at room temperature after a month of incubation were switched to 37ºC. In addition, we exposed the collagen to UV lights for 5, 10, and 15 minutes to determine the effect on the degradability of the protein (also looking for an alternative to sterilize the material). After exposure, the collagen was introduced into anaerobic tubes containing 9 ml of SB medium and 8% of the highest dilution from the

grease trap enrichment that showed turbidity and visual collagen solubilization as inoculum. We incubated the tubes for a month at 37ºC.

2.4 Microbial biomass for reactors

In order to obtain more microbial biomass to inoculate two ABRs, we transferred 8% from the water reservoir 20 ml bottle incubated at 37ºC, to three 100 ml bottles containing SB medium. We repeated this procedure using the grease trap original enrichments as inoculum. Also, we transferred 5ml of the VFA highest dilutions that showed VFA consumption coupled with methane production from the rice paddy (for acetate, propionate, and valerate) to a 50 ml serum bottles filled with 20 ml of SB medium, and 20mM of VFAs. After a month of incubation at 37ºC, we proceed to transfer it into three 100 ml serum bottles, which contained 10mM of acetate, propionate, or valerate. These bottles were incubated at 37ºC in the dark and without shaking for a month to obtain the maximum growth of the microbial populations.

2.5 Anaerobic batch reactors

We started up two anaerobic batch reactors (ABRa and ABRb) in triplicates. Our ABRs consisted of a sterile 1L schott bottle, with 500 ml of SB medium, 1% of blended collagen and 60 ml of inocula. The homogenization of the collagen was done with a blender (Warning Corporation) inside a vinyl anaerobic chamber (Coy Laboratories) with a mixture

of anaerobic gases (N_2 and H_2 90:10). We added 500 ml of SB medium and 1% of collagen in a blender for three minutes at maximum speed. After adding the collagen, the reactors were sealed and once outside the anaerobic chamber the headspace of the enrichments was exchanged with anaerobic gases (Nitrogen: Carbon dioxide 80:20) in a gassing station using strict anaerobic techniques (33). As inocula for ABRa, we used approximately 10% of the culture (60 ml per each 500 ml in the schott bottle) from three of our 100 ml water reservoir enrichments previously transferred. To inoculate the second reactor, ABRb, we mixed 60 ml from the grease trap enrichment and pre mix (40 ml) from each individual enrichment (rice paddy) on different VFAs, into a sterile 250 ml schott bottle. After mixing, we used 60 ml of the mixture as inoculum for each ABRb 500mL schott bottle.

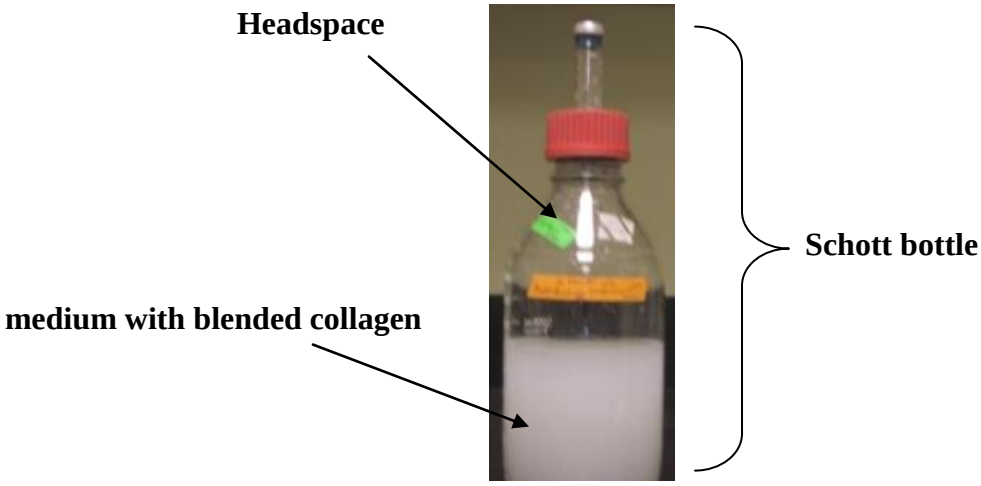

Figure 2. Picture of the anaerobic batch reactor.

2.6 Analytical methods

Methane production was quantified in our experiments using a Shimadzu GC-2010, equipped with an FID detector and a Porapak N column. The GC parameters and conditions of detection were kept as previously described (5). A standard curve was prepared using dilutions of a highly purified and certified gas mixture containing 20% of methane balanced with helium (Linde). Headspace samples (0.2ml) were taken periodically to measure the methane production in all the incubations. The concentration of methane obtained from the headspace samples of the ABRs was converted to grams of methane applying the Ideal gas Law. Additionally, the amount of collagen converted to methane, was calculated using the grams of methane produced in the ABRs. Furthermore, VFA concentrations were analyzed in our experiments using a Shimadzu HPLC equipped with an UV detector at 210nm, and a Rezex Roa- Organic Acid (H+) column following a previously described method (1). Concentrations of VFAs were calculated using a standard curve using high quality chemicals and converted into grams using their molecular weight. In addition, samples of 3 ml were periodically taken from the fluids within the ABRs, to measure the pH using a meter (Metler Toledo).

2.7 DNA extraction and PCR

We used the FastDNA SPIN (MPBio) commercial Kit following the manufacturer instructions to extract DNA from the fluids of the ABRs and the highest serial dilution tube

that showed collagen or VFAs degradation. The only modification in the extraction procedure was as follow: we collected 1.5 ml of each fluid sample in a 2.0 ml microtube instead of 0.5g of soil, then the sample was centrifuged the sample for 5 minutes at 16,168 RCF. Once centrifuged, the supernatant was removed and the pellet was re-suspended in the microtube, the cell suspension was transfered to a lysing tube, and then we followed the instructions of the FastDNA SPIN Kit.

For the amplification of bacterial DNA we used 2 µl of extracted DNA (between 0.5 to 20ng), 12.3 µl of deonized sterile water, 5 µl of 5x buffer or green master flex buffer, 2.5ul of 25mM dNTPs, 0.25 µl of each primers at 10 pmol GM5F (with GC clamp) and DS907R, 2.5 µl of 25 mM $MgCl_2$, and 0.2 µl of Taq polymerase (Fisher Scientific or Promega). Finally, we vortex and spun the final volume (25µl) for 10 seconds prior to cycling. The primers amplify a 550bp fragment of the Bacterial 16S rRNA gene as previously described (24). However, for the methanogenic DNA amplification we used 2 µl of extracted DNA (between 0.5 to 20ng), 7.2 µl of deonized sterile water, 5 µl of 5x buffer or green master flex buffer, 0.625 µl of 10 mM dNTPs, 2 µl of 25 mM $MgCl_2$, 5 µl of each 2 pmol A1106F and A1378R (these primers amplify 350bp fragment of the Arquea 16S rRNA gene as previously described (32), and 0.2 µl of Taq polymerase (Fisher Scientific or Promega). The DNA of *Syntrophus aciditrophicus* was used as the positive control for the bacterial PCR. Also a negative no DNA template was always included. However, the Bacterial DNA samples were amplified using the following program in an Eppendorf thermocycle: denature (94°C/4 min), 30 cycles of denaturing (94°C/1.25min), annealing (53ºC/0.75min) and elongation

(72°C/2min). The final step was an elongation (72ºC/10min) followed by a hold at 4ºC. DNA of *Methanospirillum hungatei*, was used as a positive control for the PCR amplification of the methanogenic population. Also a negative control with no DNA template was always included. The methanogenic DNA samples were amplified using the same thermocycler and following a program consisting of: denature (94°C/5 min), 35 cycles of denature (94°C/0.5min), annealing (55ºC/0.5min) and elongation (72°C/2min). The final step was an elongation (72ºC/3min) followed by a hold at 4ºC. All PCR product sizes were confirmed in a 1% agarose-gel electrophoresis (111v, 75min), using a quick load 100bp DNA ladder (New England Biolabs) for size comparison. Finally, the gels were stained with ethidium bromide, and visualized by UV transillumination using a versadoc Bio-Rad.

2.8 DGGE analysis

PCR products were separated by denaturing gradient gel electrophoresis (DGGE) using a D-code universal mutation detection system (Bio Rad) Bacterial PCR products were separated using DGGE parallel gradient with a build in denaturant gradient gels ranging from 30 to 50% denaturants (urea and formamide; 100% denaturant is 7M and 40% respectively) in a 8% acrylamide, run at 100v, for 12 h at 60°C. Similarly, the methanogenic PCR products were separated using a parallel gradient gels with a build in denaturant gradient from 50 to 70% urea and formamide in a 8% acrylamide, run at 100 v, for 10-12 h at 60°C. However, the voltage was maintained at 50V until the gels reached 60ºC, at which point the voltage was increased to 100V for the remainder of the electrophoresis. The sample volume was

adjusted individually to load approximately the same quantity of DNA, based on the intensity of the PCR product. The gels were stained using ethidium bromide and documented using a versadoc (BioRad) system.

DGGE profiles were compared using similarity comparisons with the Hamming Distance program. Each band showed in the gel lanes was coded for its presence or absence within a lane in a hand calculated matrix following this equation; D_{xy}= 1-2 (N_{XY}/ N_x+N_y). Additionally each lane was compared using a similarity matrix. Trees were constructed using the Hamming Distance values calculated for each comparison (indicating the number of bands similar between lanes) as an input for the NEIGHBOR program (Mega version 5.0; (29).

3 RESULTS

3.1 Microcosm enrichments

We detected collagen solubilization with the following sediments in our enrichments: waste water treatment plant, water reservoir, grease trap, and from sediments of an oxidation pond in a coffee plantation, and a grass paddy. Collagen degraders were not detected in sediments from a rice paddy. However, methane was detected only in the water reservoir enrichment. None of the controls showed collagenolityc activity (autoclaved controls, unamended controls and negative controls). In addition, the rice paddy and the water reservoir dilution sets with acetate, propionate, and valerate, showed complete VFA

degradation and methane production on only each of the 10^{-1} dilutions after 7 days of incubation.

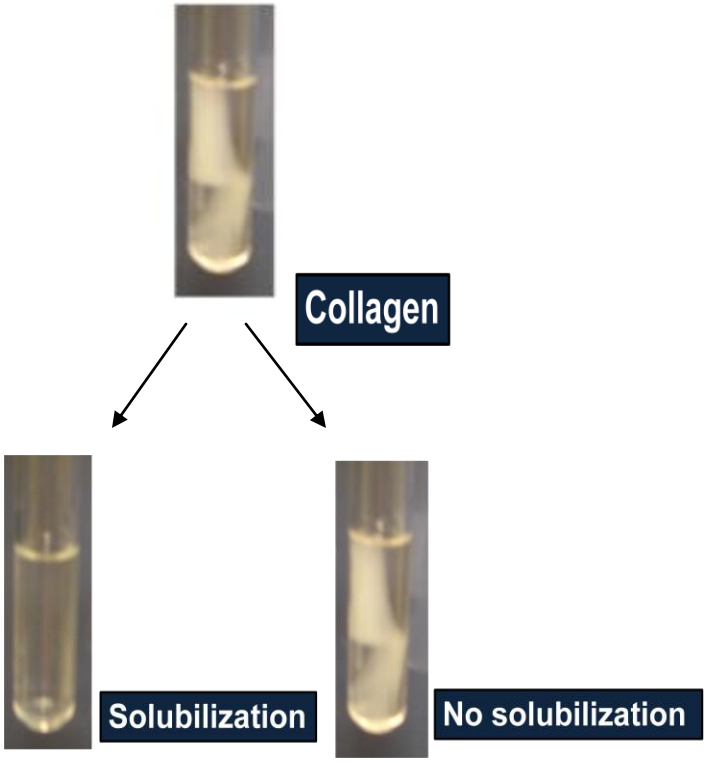

Figure 3. Solubilization of the solid waste collagen.

Table 1. Preliminary collagen enrichments

Environments	Solubilization time (days)	Methane production
Grease trap	3	-
Grass paddy	27	-
Coffee oxidation pond	28	-
Waste water treatment plant	6	-
Rice paddy	-	-
Water reservoir	26	+

*Collagen solubilization was documented by visual observation. Methane production was detected using a Gas Chromatograph.

3.2 Serial dilutions

After incubating the bottles for a month, all of the environmental enrichments degraded the collagen, but at different time intervals with presumed lag phases that ranged from 3 to 28 days (Table 1). The grease trap dilution series presented collagen solubilization up to 10^{-7}, in 10 days, (being the fastest enrichment that showed this capacity). The waste treatment plant dilution series showed solubilization in 13 days and up to dilution 10^{-7}. The grass paddy and the coffee oxidation pond sediments showed collagen solubilization in 30 days but at different population sizes 10^{5} and 10^{2} respectively. In addition, the dilution series with sediments from the water reservoir, showed collagen solubilization up to 10^{-6}, in 30 days too,

but was the only set that showed methane production simultaneously with protein degradation. In addition, phase contract microscopy revealed very similar morphologies with spore-forming rods in all of the highest dilutions from all different environments that degraded the waste.

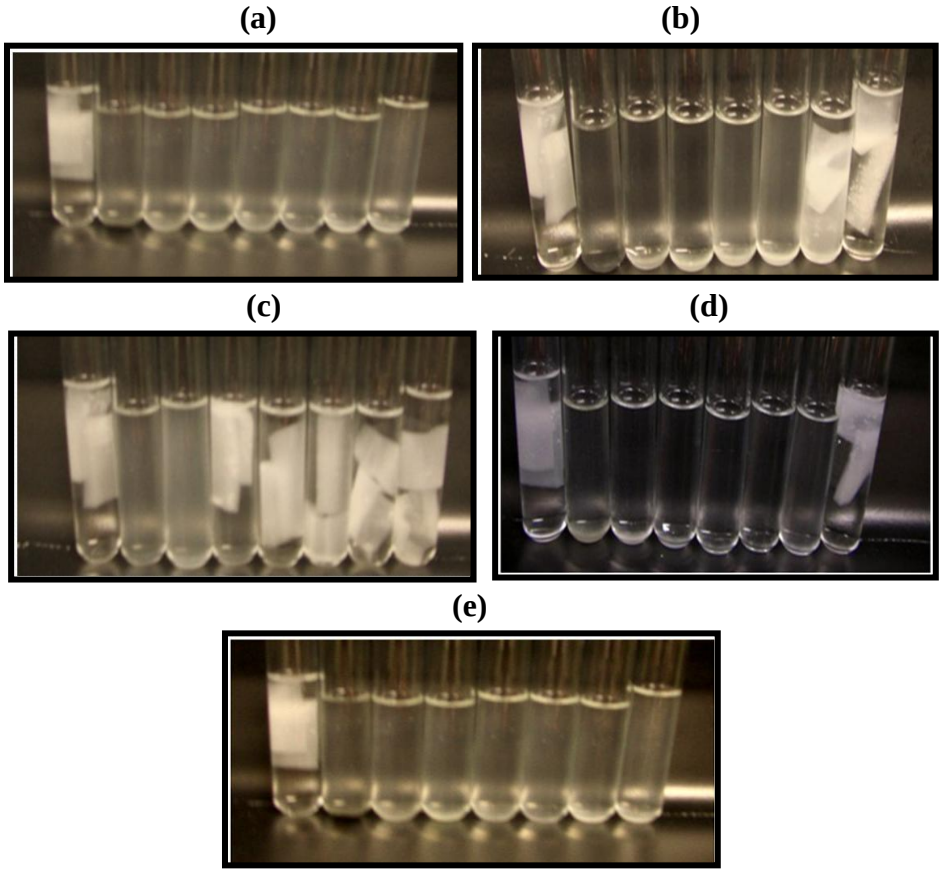

Figure 4. Collagenolytic activity in the serial dilutions. (a) waste water treatment plant, (b) grass paddy, (c) oxidation pond, (d) water reservoir, (e) grease trap. The first tube in all the dilution sets is the negative control (without inoculation).

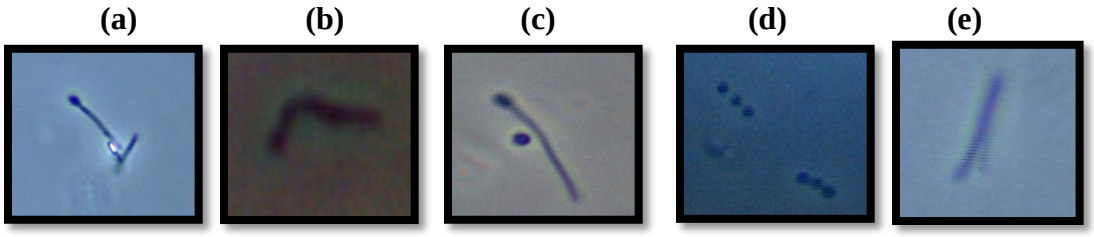

Figure 5. Microscopy of the highest dilutions that showed collagen degradation. (a) water reservoir, (b) oxidation pond, (c) grass paddy, (d) grease trap (e) waste treatment plant.

3.3 The effect of temperature and UV light on collagen degradation

Visual collagen solubilization was detected in all enrichments at 37ºC. Only the grease trap and the water reservoir showed collagen solubilization at 25ºC, and only the grease trap showed collagen solubilization at room temperature. However, when we changed back to 37ºC, the 25ºC and room temperature incubations that did not show collagen degradation, we observed collagen degradation in all of them after 24 hours.

However, when we exposed the collagen at UV light for 5 minutes, we observed that collagen was partially degraded. When we exposed the protein at UV light for 10 minutes, we observed that less collagen was degraded and more remained in the tube. However, no collagen solubilization was observed in the tube containing the collagen exposed to 15 minutes at UV light.

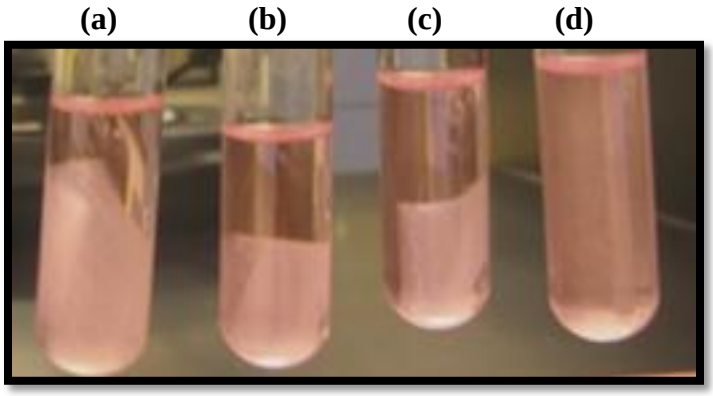

Figure 6. The effect of UV light on the collagen degradation. (a) collagenolytic activity after exposing the collagen at UV light for 15 minutes. (b) collagenolytic activity after exposing the collagen at UV light for 10 minutes. (c) collagenolytic activity after exposing the collagen at UV light for 5 minutes. (d) collagenolytic activity with no UV light collagen exposition.

3.4 Anaerobic batch reactors

ABRa biotransformed 12% of the proteinaceus material into approximately 60% methane (0.6g) (Table 2). However, ABRb bioconverted 4% of the collagen into 20% (0.4g) methane (Table 2). Acetate, propionate, and lactate were the metabolites detected in ABRa (Figure 7). All of these VFAs were completely consumed producing 60% methane during 90 days (Figure 7). Butyrate, lactate, propionate, and acetate were the metabolites detected in ABRb. All the VFAs except for acetate, were consumed in ABRb. However, acetate concentrations remained very high in this reactor throughout the 90 days of operations (Figure 8).

Table 2. Metabolites detected in both ABRs (mean values)

	ABR a Day 1	ABR b Day 1	ABR a Day 45	ABR b Day 45	ABR a Day 90	ABR b D7.790
Methane (g)	0	0	0.4	0	0.6	0.2
Acetate (g)	0	0	1.5	2.9	0.6	2.0
Propionate (g)	0	0	0	1.4	0	0.4
Butyrate (g)	0	0	1.4	0.6	0	0.4
Lactate (g)	0	0	0.1	0.1	0	0
Total mass (g)	0	0	3.4	5.0	1.2	3.0
% of collagen converted to methane	0	0	8.0	0	12	4.0
pH	7.2	7.2	6.8	6.2	6.5	5.8

*Concentrations of methane obtained from the headspace samples of the ABRs were converted to grams applying the Ideal gas Law. Concentrations of VFAs and lactate were converted into grams using their molecular weight. The amount of collagen converted to methane, was calculated using the grams of methane produced in the ABRs.

Table 3. Standard deviations of metabolites showed in table 2.

	ABR a Day 1	ABR b Day 1	ABR a Day 45	ABR b Day 45	ABR a Day 90	ABR b Day 90
Methane (g)	0	0	0.3	0.1	0.2	0.1
Acetate (g)	0	0	0.4	0.8	0.6	1.2
Propionate (g)	0	0	0.1	0.4	0	0.2
Butyrate (g)	0	0	0.4	0.8	0.3	0.5
Lactate (g)	0	0	0.3	0.1	0.2	0.3
Total mass (g)	0	0	0.6	1.4	0.4	1.7
% of collagen converted to methane	0	0	0.3	0.1	0.2	0.1
pH	0	0	0.2	1.2	0.1	1.8

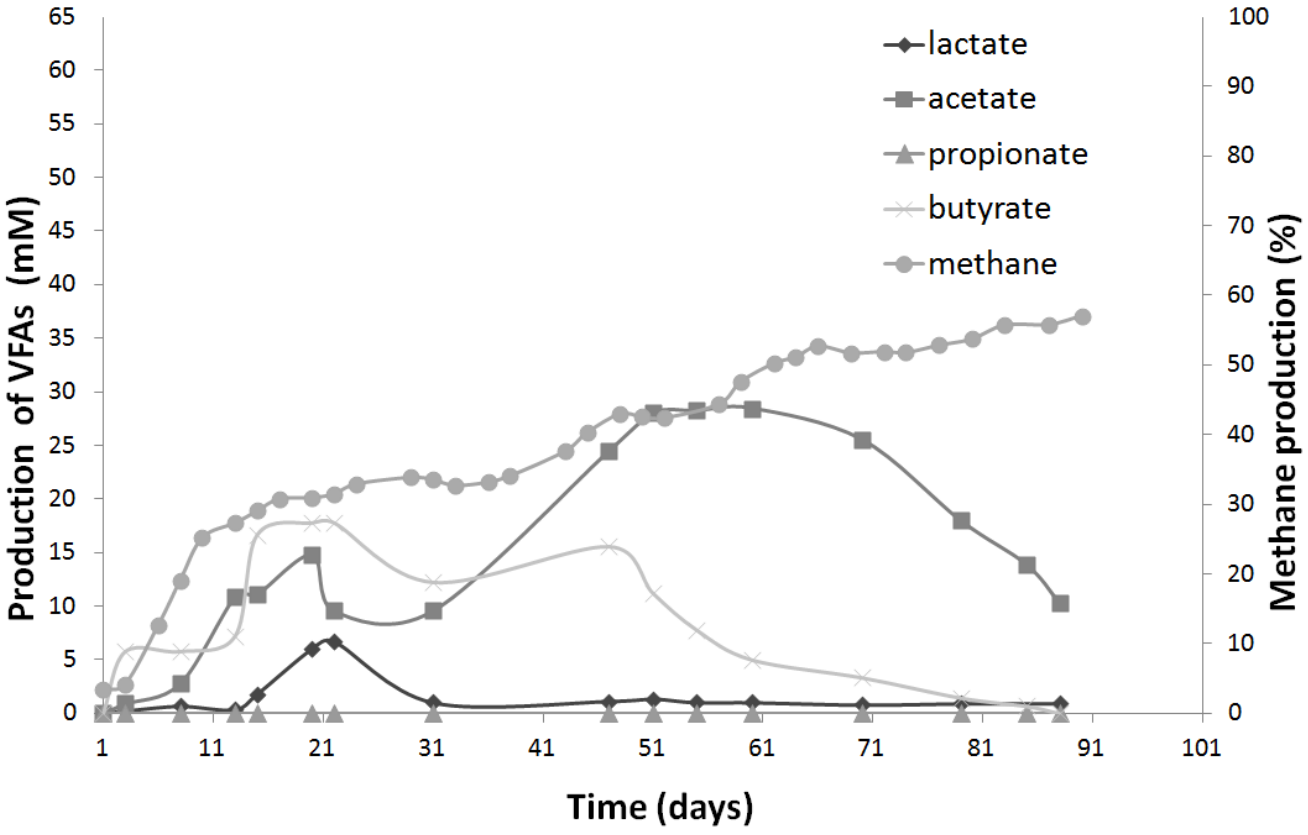

Figure 7. Chemical composition inside ABRa, (mean values).

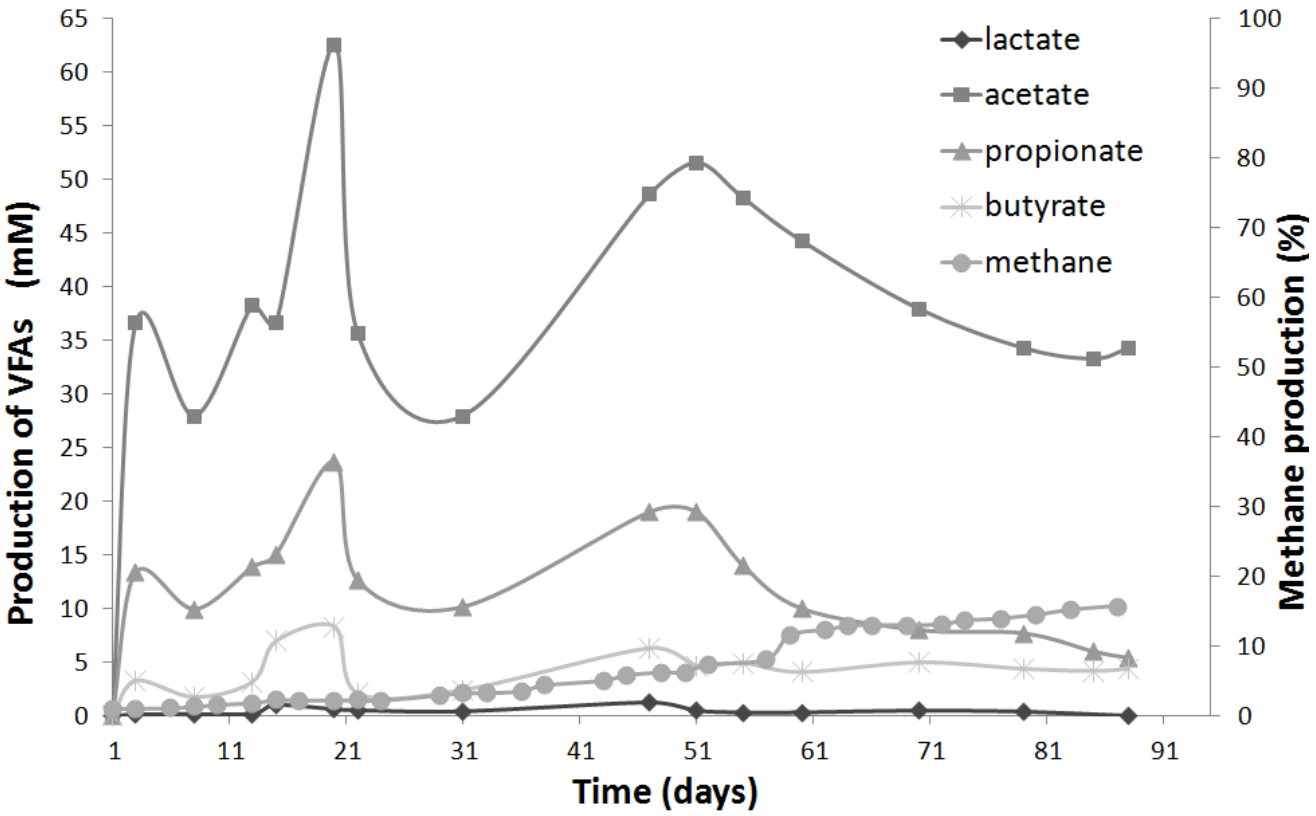

Figure 8. Chemical composition inside ABRb, (mean values).

3.5 PCR and DGGE

Our PCR products amplified with primers GM5F-GC and DS907R showed a band of 550-bp (data not shown). Additionally, PCR products amplified with the 1106F-GC and 1378R primers showed a band of 350bp (data not shown). None of the negative control (PCR products without DNA template) showed any amplification (or PCR fragments of expected size).

DGGE analysis of the enrichments showed high bacterial population diversity, with six to twelve bands (Figure 9). The DGGE-profile of the ABRs bacterial population showed a low diversity, with 2 to 3 bands (Figure 10). However, the DGGE profile of all the VFAs enrichments and ABRs showed homologues diversity of Archea, composed of one band (Figure 11). These bands were 100% similar to the band of *M. hungatei* (our positive control).

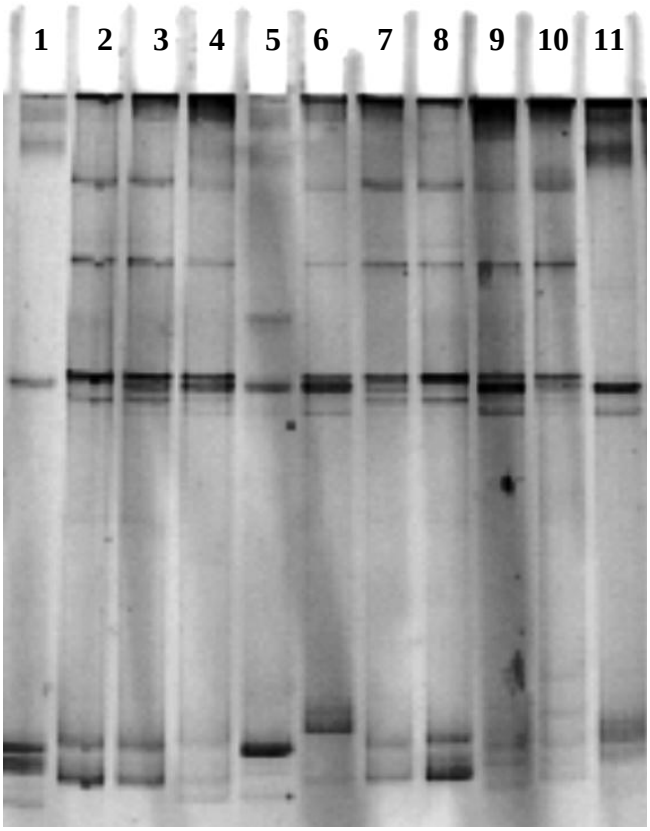

Figure 9. DGGE profile of the bacterial population diversity presented in the microcosm enrichments. (1) water reservoir enriched with collagen. (2) water reservoir enriched with acetate. (3) rice paddy enriched with acetate. (4) grease trap enriched with collagen. (5) ABRb. (6) *Syntrophus aciditrophicus* amplified DNA added to amplified DNA from grass paddy enriched with collagen. (7) grease trap enriched with collagen. (8) waste treatment plant enriched with collagen. (9) grass paddy enriched with collagen. (10) coffee oxidation pond enriched with collagen.(11) ABRa. DGGE was run at 100v, for 12 h at 60°C, with a 30 to 50% in an 8% acrylamide gel.

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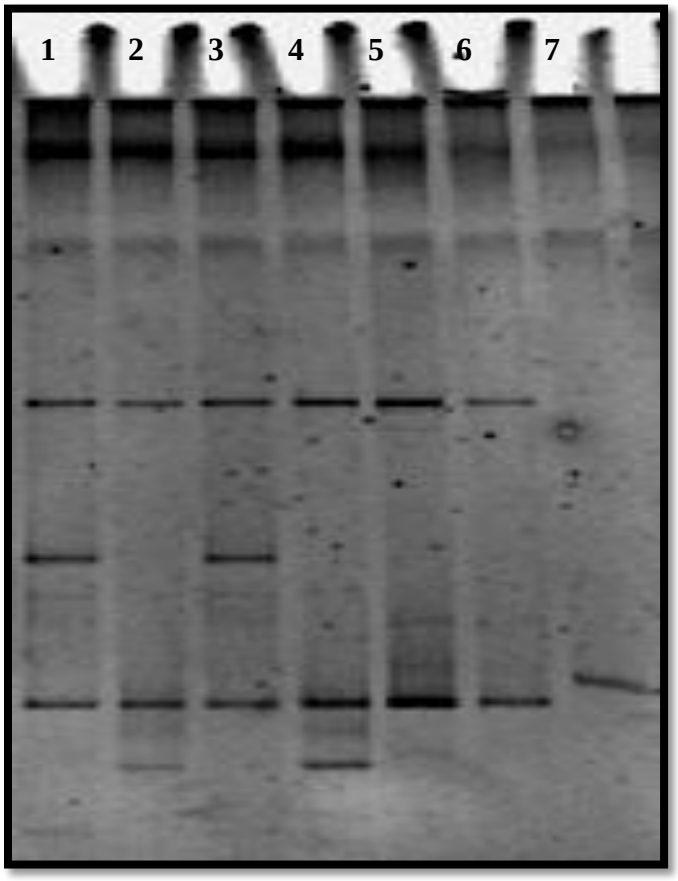

Figure 10. DGGE profiles of the bacterial population present in ABRs. (1) ABRa day 1. (2) ABRb day 1. (3) ABRa day 45. (4) ABRb day 45. (5) ABRa 90. (60) ABRb day 90 days. (7) 16S rRNA gene amplified of *Syntrophus aciditrophicus*. DGGE was run at 100v, for 12 h at 60°C, with a 30 to 50% in an 8% acrylamide gel.

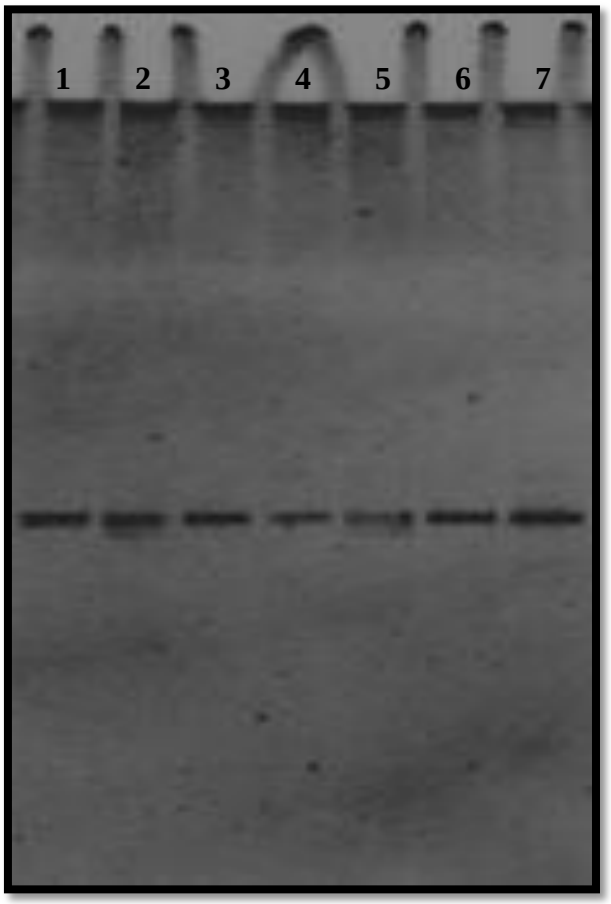

Figure 11. DGGE profiles of the methanogenic population present in ABRs. (1) ABRa day 1. (2) ABRb day 1. (3) ABRa day 45. (4) ABRb day 45. (5) ABRa day 90. (6) ABRb day 90 days. (7) 16S rRNA gene amplified of *Methanospirillum hungatei*. DGGE was run at 100v, for 12 h at 60°C, with a 50 -70% in an 8% acrylamide gel.

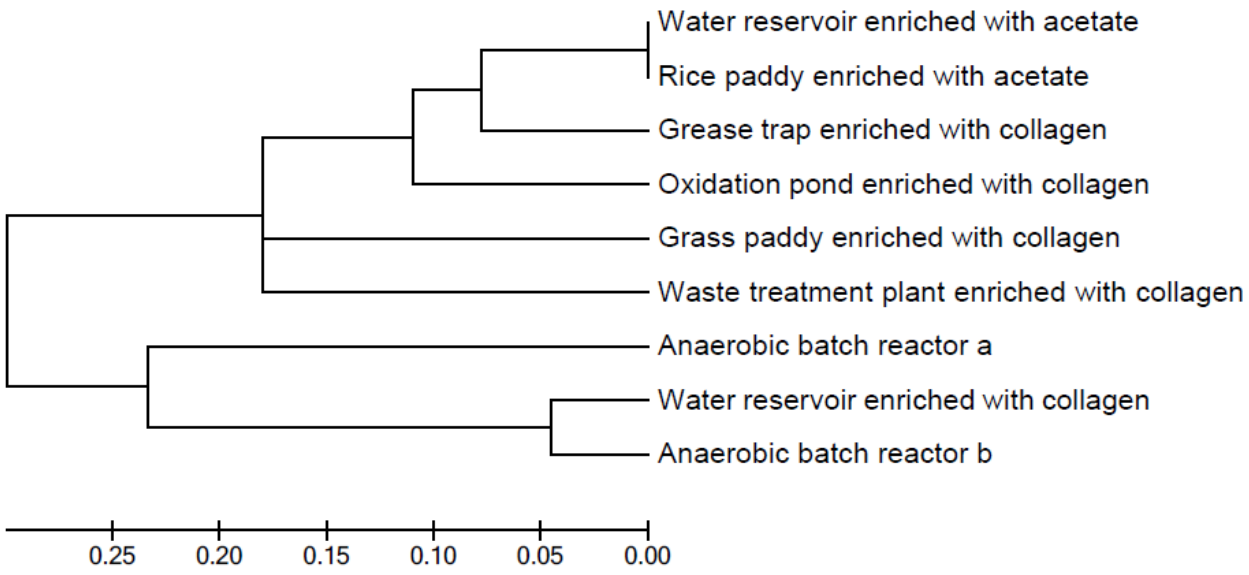

Figure 12. Relationship between the DGGE banding patters showed in Figure 9.

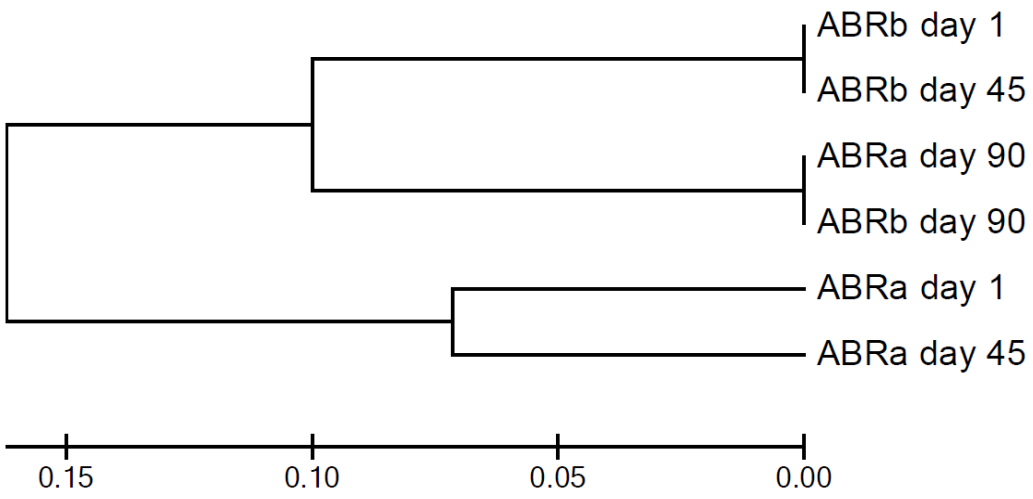

Figure 13. Relationship between the DGGE banding patters showed in Figure 10.

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4 DISCUSSION AND CONCLUSIONS

4.1 Microcosm enrichments

Our first experiments reveal that anaerobic collagenolytic enzyme activity can be detected in almost all of the environments we tested at 37ºC. There is evidence that classify proteases that remain active at temperatures ranging from 20ºC to 37ºC, as mesophilic enzymes. However, the majority of the anaerobic microbes from our samples incubated at 25ºC, and room temperature, only express collagenase activity when they were incubated at 37ºC. This information suggest that our cultures maintain their viability during a month at 25ºC and room temperature without degrading collagen, suggesting that almost all the proteolytic bacteria presented in our samples can survive for a month without apparent degradation of the collagen. Perhaps, our cultures maintain their viability by the formation of an endospore. However, we still do not know if the inability to degrade the collagen at lowers temperatures (25ºC or less) is due to inactivation of the enzyme or lack of translation. One thing is clear, our enrichments seems to degrade collagen at 37ºC.

4.2 Serial dilutions

Moreover, our results indicate that microbial populations obtained from environments exposed to a high protein material like the grease trap and waste water treatment plant contain higher populations (10^7) capable of degrading the collagen (Figure 4). The bacterial populations size obtained from the oxidation pond, the grass paddy, and from the water

reservoir were 10^2, 10^5, and 10^6 respectively. This data suggests that collagenolityc population sizes obtained from environments within a low protein material are lower than the ones obtained from environments exposed to high protein content. It is possible that the grease trap and the waste water treatment plant consortiums showed collagen degradation to higher level, because they were adapted to perform this activity in their original habitats. However, the water reservoir was the only environment that contains the complex anaerobic community that is required to perform anaerobic collagen digestion coupled to methane production. Therefore, we selected this microbial consortium to develop our first bioreactor, and combined the collagen degraders from the grease trap with the methanogenic couples obtained from the enrichments developed with rice paddy sediments on acetate, propionate, and valerate to develop our second bioreactor.

In addition, the data indicates that exposing collagen to UV light affected its degradation and this effect was augmented with time of exposure (Figure 6). Previous studies (19), have reported that the triple helical structure of the collagen is destroyed with UV irradiation. Perhaps, the UV exposure caused a conformational change in the structure of collagen such that the catalytic enzymes could not recognize the damage structure of the protein.

4.3 Anaerobic batch reactors

In the anaerobic batch reactors which we analyzed, (Figure 7 and Figure 8) differences were detected between the ABRs chemical composition. Based on the mass balance analysis (Table 2), the anaerobic batch reactor a biotransformed more efficiently the protein into methane than the anaerobic batch reactor b. This bioreactor produced more than 40% of the total methane in 45 days. Additionally, almost all the VFAs were degraded in this digester throughout the 90 days. Therefore, we consider that this microbial community was adapted to perform the complete collagen digestion process.

However, ABRb chemical composition showed a very low methane production and a high accumulation of VFAs. We attribute this low methane production to the high concentration of VFAs detected from the first days and during the experiment. According with Lee et al (17) the methanogenesis process can be inhibited by the accumulation of metabolic intermediaries like VFAs. Our data suggest that this bioreactor converted all the collagen -via oxidative deamination into acetate, lactate, butyrate and propionate in the first 45 days. It is possible that the syntrophic couples could not adapt to the high rate of VFAs production in this digester.

In addition, previous research has reported (2) that DGGE profiling method can be useful for diagnose the presence and abundance of microorganisms in bioreactors. As we presented here, other research (28) has described that the diversity on the bacterial population is higher than the methanogenic population diversity inside ABR and in anoxic environments. Based

on the identical positioning band patterns presented in Figure 9, it indicates that many of these bands (same organisms, bacteria) are present in all of the samples. Therefore, we constructed a neighbor joining tree to show which consortiums contain the most similar or divergence bacterial population diversity, Figures 12 and 13.

The DGGE profile of ABRs (Figure 10) showed three bacterial bands at the first 45 days. Two of these bands were in this digester until the end at 90 days. Acetate, lactate, and butyrate were generated by the anaerobic degradation of collagen in both of the bioreactors. Acetate was the main intermediary in both of the reactors and also observed in the digestion of gelatin (14). However, the VFAs concentration and its production rate in ABRb were higher than ABRa, suggesting that the oxidative deamination process in ABRb transformed all the collagen into VFAs. Therefore, we attribute the difference in VFAs production to the difference find in the bacterial diversity at the first 45 days showed in the DGGE bacterial gel, Figure 10. However, our neighbor joining tree constructed with the Hamming Distance values (Figure 13) confirmed that the ABRs bacterial population totally converged after 90 days.

Furthermore, DGGE analysis illustrates *M. hungatei* as the only methanogenic archeon obtained from all the rice paddy and water reservoir enrichments on acetate, propionate, and valerate. There is evidence that showed that this Archeon can generate methane from butyrate, lactate, propionate, and valerate oxidation when coupled with a suitable syntrophic bacterium (28, 15, 6, and 34). Following an exhaustive search of the literature no one ever has reported that *M. hungatei* is capable of producing methane as a hydrogenotrophic partner

in a syntrophic acetate oxidation partnership. Therefore, the novel approach we present here, propose that this hydrogenotrophic organism, can produce methane by consuming the H_2 provided from the syntrophic oxidation of acetate.

However, it have been reported that chemotaxis in *M. hungatei* respond to a gradient of acetate with concentrations between 0.1 to 20mM, but no one ever have reported why this attraction occurs (20). Following an exhaustive search of the literature no one has reported how syntrophic bacteria and methanogens find each other to form a syntrophic couple. We hypothesize, that chemotaxis in this Archeon can be the key of how this flagellar methanogen find their syntrophic partner to engage in a syntrophic relationship. Moreover, this can be another explanation for the differences between the batch reactors, besides the similarities on the microbial population. It is possible that in anaerobic batch reactor a, *M. hungatei* find their syntrophic partner as result of chemotaxis. However, due to the high concentration of acetate remaining in anaerobic batch reactor b at 90 days, *M. hungatei* was not attracted and therefore the acetate could not be degraded. We are the first group to show evidence (by DGGE and Gas Chromatography) that illustrates that *M. hungatei* could be the hydrogenotrophic partner in syntrophic acetate oxidation.

In addition, DGGE profile of the ABRs methanogenic population diversity showed only a band patter homologues to the *Methanospirillum hungatei* jf1, appears from time zero, constantly to the end at three months in both of the bioreactors (Figure 11). It appears that the ABRs methanogenic population converged on the same hydrogenotropic methanogen, *M hungatei*. As was previously discussed it is probably that this Archeon was producing

methane from acetate, lactate, and butyrate degradation. Maybe, the absence of other hydrogenotrophic organisms, forced one or more syntrophic bacteria present in the ABRs to select *M. hungatei* as their methanogenic partner, with the exception of acetate in ABRb.

4.4 Future works

If we compare this data with the result obtained from the anaerobic reactors used for the digestion of fruit scraps (7), they showed a conversion of 95% of the original waste. Furthermore, other research (14) showed that the co-culture of *Clostridium collagenovorans* and *Methanosarcina barkeri* was able to biotransform 85% of the polypeptide (gelatin) to methane. However, our consortiums were able to transform between 4-12% respectively of the solid waste into methane. If we convert the percent of methane produced in our ABRa to watts, (seen in the appendices) we found that this bioreactor was able to generate 9.49 Watts. This amount of energy is capable to turning on an 8 W/h lamp for more than one hour. On the other side, after applying the same formulas to ABRb we obtain a generation of only 2.83 Watts. This confirms that the bioconversion potential of ABRa was almost four times more than ABRb. However, if we convert to Watts the VFAs reminding at 90 days in ABRb, it can generate almost 50 Watts, (five times more energy than ABRa). This amount of energy is capable to turn on an 8 W/h lamp for more than five hours.

Therefore, in order to increase the production of methane in ABRa, we have to promote our culture to produce more VFAs, with a production rate that permit the formation of syntrophic couples. It is possible that using *Methanosarcina barkeri*, instead of *M. hungatei,*

as showed in the digestion of gelatin (14) it could produce more methane. To increase methane production in ABRb, we need to separate the collagenolytic bacteria and the VFAs producers from the syntrophic degraders in a baffled reactor. This type of digester, let us to control the metabolic flow between their chambers and avoid methanogenesis inhibition by VFA accumulation (13 and 9).

4.5 Conclusion

In conclusion, studying the functional capability from an adapted microcosm bring crucial information about how this community respond to specific conditions. Anaerobic batch reactor technologies can be a useful tool to digest solid wastes such proteins like collagen. Therefore, studying the functional capacity from an adapted microcosm bring crucial information about how this community respond to specific conditions. This thesis reports, how a single adapted consortium digest a solid waste into biogas. Moreover, the DGGE and GC profile of the ABRs indicates that *M. hungatei* was the only methanogen producing methane by the syntrophic oxidation of acetate, butyrate, and lactate. However, more research is needed to get insight into the factors that regulate and optimized the interaction of anaerobic collagen bacteria and the syntrophic couples in order to attend environmental and eco-social major problems.

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6 Appendices

6.1 Mass balances

In order to calculate the methane mass inside the ABRa we applied the ideal gas law:

Methane % showed by the Gas chromatograph. ABRa = 57% of CH_4

Methane mass in this bioreactor can be estimated applying the following equation:

CH_4 mass in ABRa = 57% of CH_4 / 100 of the total gas mass = 0.57g

We applied the same equation to obtain the methane mass inside ABRb.

ABRb= 16% of CH_4

CH_4 mass in ABRb= 16% of CH_4 / 100 of the total gas mass = 0.16g

*The application of mass balances over our anaerobic batch bioreactors for carbon is defined.

The following general anaerobic digestion reaction scheme was assumed for the collagen utilization:

Collagen → Volatile fatty acids + Carbon dioxide + Methane + Biomass

In our study we quantified the VFAs, and methane production. For this reason we assumed that all the collagen was degraded and transformed into VFAs, methane, and biomass.

Mass balance in ABRa

5g of collagen → 0.62g (acetate) + 0.38g (butyrate) + 0.02g (lactate) + 0.57g (methane)

5g (collagen) = 100% of the initial carbon source

To calculate the % of carbon recovery we applied the following equation:

% of carbon mass recovery = 100 * (1.59g)/ 5g = 31.8 %

To calculate the % of carbon mass missing in the reactor we applied the following equation:

% of carbon mass missing in the ABRa = 100% - 31.8% = 68.2%

To calculate the % of carbon recovered in methane we applied the following equation:

% of carbon mass in methane = 100* 0.57g / 5g = 11.4%

We applied the equations showed previously to obtain the mass balance in the ABRb.

Methane % detected by the Gas chromatograph inside ABRb = 17% of CH_4

CH_4 mass in ABRb= 17% of CH_4 / 100 of the total gas mass = 0.17g

Mass balance in ABRb

5g of collagen → 2.0g (acetate) + 0.38g (propionate) + 0.39g (butyrate) + 0.04g (lactate) + 0.22g (methane)

5g (collagen) = 100% of the initial carbon source

To calculate the % of carbon recovery we applied the following equation:

% of carbon mass recovery = 100* (3.03g)/ 5g = 61 %

To calculate the % of carbon mass missing in the reactor we applied this equation:

% of carbon mass missing in the ABRb = 100% - 61% = 39%

To calculate the % of carbon in methane applied the following equation:

% of carbon mass in methane = 100* 0.16g / 5g = 3.4%

6.2 Energy production

To calculate the amount of energy that can be generated (Watts) W by the methane produced we used the following equations:

For the ABRa:

$$0.57g\ CH_4 \quad X \quad \frac{1kg\ CH_4}{10^3 g\ CH_4} \quad = \quad 0.00057kg\ CH_4$$

To obtain the Kilo Joules (KJ) of methane in ABRa we applied the following equation:

Methane calorific value = 55,530 KJ/Kg

$0.00057kg\ CH_4$ X 55,530 KJ/Kg = 31.6521 KJ

To obtain the Watts generated we used the following equations:

1KJ/h = 0.0003 kiloWatts (KW) 31.6521 KJ X 0.0003 KW = 0.00949 KW

1 KW= 1000 W 0.00949 KW X 10^3W/1KW = 9.49 W

To calculate the amount of energy that can be generated (Watts) W using the methane produced by the ABRb we used the following equations:

$$0.17g\ CH_4 \quad X \quad \frac{1kg\ CH_4}{10^3 g\ CH_4} \quad = \quad 0.00017kg\ CH_4$$

Methane calorific value = 55,530 KJ/Kg

$0.00017kg\ CH_4$ X 55,530 KJ/Kg = 9.4401 KJ

1KJ/h = 0.0003 kiloWatts/h (KW)

9.4401 KJ X 0.0003 KW = 0.00366KW
1 KW= 1000 W
0.00283 KW X 10^3W/1KW = 2.83 W