Screening Azorean geothermal hot spring aerobic microbial communities for solubilization and viscosity reduction of sewage sludge

Tese de Doutoramento

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Doutoramento em

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SUMÁRIO

Os Açores são ricos em manifestações secundárias vulcânicas terrestres onde podemos encontrar uma grande diversidade microbiana, quase inexplorada em aplicações biotecnológicas. Neste trabalho, construímos três consórcios bacterianos termófilos aeróbios para pré-tratamento de resíduos, de forma aumentar a produção de biogás. Os consórcios foram construídos a partir de microrganismos da biomassa em decomposição proveniente das fontes termais das Furnas. O processo de enriquecimento iniciado a partir do mesmo inóculo levou ao desenvolvimento de três consórcios distintos, Azotm36, Azotdp36 e Azotf36 enriquecidos em lamas, misturas de gramíneas e penas, respetivamente. A eficiência de hidrólise foi monitorizada seguindo se ensaios para determinar o Potencial Bioquímico de Metano (BMP) de resíduos pré-tratados. A amplificação da região hipervariável V3/V4 do gene 16s rRNA foi amplificada para caracterização filogenética dos consórcios usando piro sequenciação. Os generos predominantes nos consórcios foram, *Ureibacillus* (52%), *Thermobacillus* (41%) e *Brevibacillus* (99%) para, Azotm36, Azotdp36 e Azotf36, respetivamente. A eficiência mais alta de pré-tratamento foi observada no consórcio Azotf36 com 97% de decomposição das penas, aumentando 95% a produção de metano, BMP (59 LCH_4/Kg SV adicionado) relativamente a penas não tratadas. O pré-tratamento do consórcio Azotdp36 alcançou 61% de solubilização das misturas de gramíneas aumentando a produção de metano em 77% (168 LCH_4/Kg SV adicionados) relativamente a gramíneas não tratadas. O pré-tratamento mais baixo foi obtido no consórcio Azotm36 com 51% de solubilização das lamas produzindo mais 20% de metano (200 LCH_4/Kg SV adicionado) relativamente a lamas não tratadas. Estes resultados, mostram o valor encontrado nas comunidades bacterianas das fontes hidrotermais para aplicações em biotecnologia.

SUMMARY

The Azores are rich in terrestrial volcanic secondary events where we can find a great microbial diversity, almost unexplored in biotechnology application. In this work, we constructed three aerobic thermophilic bacterial consortia for wastes pretreatment to enhance biogas production. The consortia were constructed from microorganisms found in decomposition biomass from Furnas hot springs. Enrichment process that started from the same inoculum led to the development of three distinct consortia Azotm36, Azotdp36 and Azotf36 enriched in sewage sludge, grass mixtures and feathers, respectively. Hydrolysis efficiency was monitored followed by assays of to determine Biochemical Methane Potential (BMP) of pretreated wastes. 16S rRNA gene hypervariable regions V3/V4 were amplified for phylogenetic characterization of consortia using 454 pyrosequencing. The predominant genera in the consortia were *Ureibacillus* (52%), *Thermobacillus* (41%) and *Brevibacillus* (99%) for, Azotm36, Azotdp36 and Azotf36, respectively. The highest pretreatment efficiency was observed in Azotf36 consortia with 97% of feather decomposition producing more 95% of methane, BMP (59 LCH_4/Kg VS added) than untreated feathers. The Azotdp36 pretreatment achieved 61% of grass mixtures solubilization producing more 77% of methane (168 LCH_4/Kg VS added) than untreated grass. Lowest pretreatment results were achieved in Azotm36 with 51% of sewage sludge solubilization producing more 20% of methane in pretreated sludge (200 LCH_4/Kg VS added) than untreated sewage sludge. These results, show the value found in hydrothermal hot springs bacterial communities for applications in biotechnology.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

ABC	Aerobic Bacterial Consortium
AD	Anaerobic Digestion
ATBCB	Azorean Thermophilic Bacteria Collection Biobank
BMP	Biochemical Methane Potential
C/N	Carbon to nitrogen
COD	Chemical oxygen demand
DMSS	Dairy Milk Sewage Sludge
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
ND	Not determined
NDt	Not detected
OTUs	Operational Taxonomic Units
rpm	Revolutions per minute
RS	Reducing sugars
SSS	Slaughterhouse Sewage Sludge
STP	Standard temperature and pressure conditions
TKN	Total kjeldahl Nitrogen
TS	Total solids
UASB	Upflow anaerobic sludge blanket reactor

V/V	volume/volume
VFA	Volatile fatty acids
VS	Volatile solids
W/V	Weigh/volume
WWTP	waste water treatment plant

1. | INTRODUCTION, RESEARCH AIM

1.1. | BIOMASS DEGRADATION BY MICROBIAL COMMUNITIES

The growing global energy requirements together with the limited availability of fossil fuels, unstable energy prices, and environmental problems require the use of renewable energies. The currently used feedstocks for anaerobic digestion (AD) are brief, and therefore, it is important to explore new substrates for utilization in AD to reserve the growing needs. The abundance and availability of sewage sludge and lignocellulosic biomasses worldwide as well as their high protein and carbohydrate content make these materials an attractive feedstock for energy production. Currently, the utilization of activated sewage sludge and lignocellulosic residues as feedstock for methane production is not disseminated due to the difficulty decomposing and access their contained energy (Horváth et al., 2016).

Convenient alternative approaches to screen essential microbial groups that maintain a desired function of a mixed population are desired. Such approaches can be employed to allow the selection and enrichment of known functional consortia with user defined attributes for specific functions like waste degradation. Different microbial strains coexisting in a microflora can have diverse physiological attributes. They may complement or inhibit mutually in a mixed population that thrives under various environmental constraints and conditions. In nature, degradation of biomass is achieved by the interaction of diverse microbial communities working in a symbiotical way or by competing for resources (Weimer et al., 1991; Wang et al., 2010). In a complex biological degradation system, individual microbial groups existing in a syntrophic relationship, in which each bacterial group with distinct roles and functions, would constitute a significant link in the chain of bioconversion. Different strains coexisting in a system may compete or inhibit mutually. It appears that maintaining functional strains with user defined attributes in desired quantities and activities in a continuous flow reactor is extremely difficult. To infer a complete understanding of these interactions is generally not an easy task. Strain isolation using conventional methods and screening practices can cultivate in vitro a limited portion of the desired microbes (Tajima et al., 1999).

It is also possible that some key functional strains not always can be cultured and studied by the present conventional isolation methods. Thus, important information for understanding the mechanisms of a specific metabolic pathway are ignored or unknown. The function of a consortium is of primary interest for practical applications. Artificial microbial selection is the deliberate and systematic course of action or approach by which adaptive traits become universal to the populations because of competitive reproduction. When microbial traits have a heritable phenotype, artificial selective pressure can be realized by any aspect of the environment, including competition with peers of the same or other traits. Thus, artificial selection approach can be directional, resulting in a adaptive strain propagation, and it can be the driving force of microbial evolution (Williams & Lenton., 2007). Artificial selection is widely employed through microbial genetics such as cloning that allow the survival of bacterial strains with user defined phenotypes for specific functions. (Lee et al., 2013; Adav et al., 2009).

The biodegradation of biomass using complex microbial communities has been proposed as a highly efficient approach for biotechnological applications. Although microbial decomposition of wastes has been studied extensively, most of these studies used pure cultures of microorganisms (Zhao et al., 2014).

When comparing with pure cultures, specific features of fermentation with microbial consortia can bring the possibility of utilizing less costly or blended substrates like whey, molasses, lignocellulose, and sewage sludge. Additionally the synergies of various enzymatic systems and combination of metabolic pathways of different microorganisms will end in efficient utilization, contributing to product purification and reducing the costs. Working with a thermophilic microbial consortium does not have any sterile requirement, decreasing the sterilization costs (Street & Bey, 2014). Also, the consortia at thermophilic temperatures can lead to the reduction of pathogenic bacteria in wastes, accomplished by the heat on pathogenic microbes. Therefore, biotechnology centred on microbial consortia, could be an added value option relative to the traditional biotechnology based on pure cultures to produce chemical substances and by-products with additional value in industrial biotechnology (Sabra et al., 2010). Mixed culture of microbial consortium also normally show greater efficiency and productivity from the substrate. This is related to the interactions among cells in microbial consortium, in which metabolites produced from one strain might affect the metabolism and growth of another strain. The stability of microbial consortium during fermentation is normally an essential problem in industrial process.

Other advantages working with bacterial consortia are associated to quorum sensing, that is characterized by communication of information relying on bacterial density, leading to the recognition of coordinated behaviors through responsive gene expression. The microbial cells can release some specific signal molecules and detect the change of their concentrations spontaneously, thus coordinating behaviors on the establishment of a sufficient quorum (Schertzer et al., 2009).

Mutualism is also an important factor that refers to benefit two or more species to one another when living together, but both of their lives will be affected or even die when separated. There are numerous examples of mutualisms in the fermentation processes with microbial consortia. For instance, the connection between Archaea and bacteria is mutualism during the production of methane in AD process (Stolyar et al., 2007). The mutualism is a major problem when studying these microbial communities by trying to use isolation techniques, because as soon two mutualism strains are separated they end up losing viability. The competition for limited natural resources within a microbial community is known as the selective force that promotes biosynthesis of antimicrobial compounds. Recently, it was shown that these antimicrobial molecules produced in nature are not primarily used as weapons for competition but as tools of communication that may control the homeostasis of microbial communities (Hibbing et al., 2010).

The interaction among cells in microbial consortium plays a main role to the stability of bacterial pretreatment, studies have reported that bacterial consortia have a good cost benefit in wastes pretreatment for AD (Jiang et al., 2017; Nakamichi et al., 2010).

1.2. | ANAEROBIC DIGESTION

Several reactions in the anaerobic process by different groups of bacteria, convert insoluble and complex organic compounds to simple compounds, as they pass through an anaerobic food chain. Methane fermentation is always the last step in the anaerobic food chain. In such processes, insoluble and complex organic compounds are degraded to methane, carbon dioxide and minerals. During the degradation of organic compounds, some of the carbon dioxide is released which is reduced to methane form. All the compounds must be degraded to simple organic and inorganic compounds for methane-forming bacteria to be able use. For example, formates, methanol, methylamine, acetate, inorganic hydrogen gas and carbon dioxide are formed (Khanal, 2008). Methane is the simplest organic compound at the end of the anaerobic food chain. To have success in the food chain, methane-forming bacteria are the most influent on this final step (Bruni, 2010).

Methane forming bacteria cannot use organic compounds like butyrate and propionate directly as substrates if they are not first converted to acetate. During the anaerobic processes, syntrophic relationships occur between bacteria. At least two kinds of bacteria are involved in the relationships and the action of one organism is dependent on the activity of another. An example of this is the syntrophic relationship between hydrogen producing bacteria and hydrogen consuming bacteria. Acetate is the most commonly used substrate by methane-forming bacteria which may be degraded in the absence of sulphate. In the presence of sulphate, acetate cannot split into methane and carbon dioxide (Deublein, 2008). The process is the accomplishment of four groups of microorganisms combined actions: primary fermenting bacteria, secondary fermenting bacteria and two types of Archaea. The anaerobic decomposition of organic materials will finally turn into biogas (methane and carbon dioxide), typically divided into three steps. First hydrolysis, substrate is hydrolysed to small units by primary fermenting bacteria. Then acidogenesis and acetogenesis, the formed soluble oligomers and monomers are converted into acetic acid, hydrogen and carbon dioxide by primary fermenting bacteria and secondary fermenting bacteria. In the last step methanogenesis, acetic acid, hydrogen and carbon dioxide are converted into biogas by the Archaea (Deublein, 2008). For the optimal work of the decomposition process, the dependence of these three steps should work equally well and deliver to the next step the substrate required. For example, if hydrolysis is inhibited, the substrate to the second and third step will be limited and there is a reduction in methane production (Gerardi, 2003).

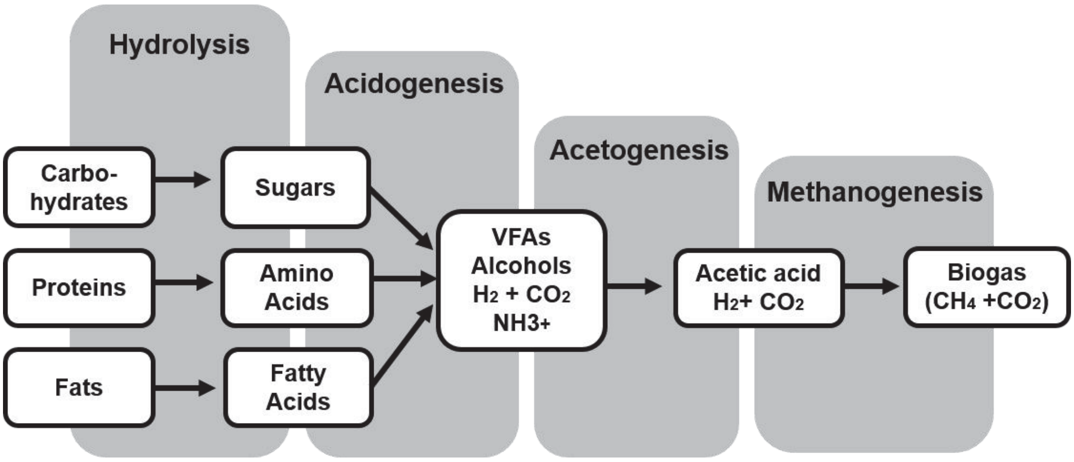


Figure 1. Anaerobic Digestion process adapted from Ugwuanyi (2005)

1.2.1. | Hydrolysis

Organic waste can be converted into biogas and energy saving investment costs being recuperated in a short period of time. The process of AD begins with the hydrolysis, which is the limiting step of AD (Braun, 2003).

In this step, complex organic polymers are hydrolysed into smaller unites such as sugars, long-chain fatty acids and amino acids. This is taken out by different groups of obligate or facultative fermentative bacteria through excreting extracellular enzymes (Kaseng et al., 1992). The proteolytic bacteria produce proteases that catalyse the hydrolysis of proteins into amino acids; the cellulolytic and xylanolytic bacteria produce cellulases and xylanases that degrade cellulose and xylan to glucose and xylose, respectively; and the lipolytic bacteria produce lipases that break down lipids to glycerol and long chain fatty acids (Figure 1).

1.2.2. | Acidogenesis

The dissolved sugars, long chain fatty acids and amino acids produced by hydrolysis are utilized in this step both by fermentative bacteria or by anaerobic oxidizers (Gujer & Zehnder, 1983), forming acetate and more short chain fatty acids, alcohols, hydrogen and carbon dioxide (Figure 1). Acidogenesis is a strong and often the fastest step in the whole AD process. When protons are used as electron acceptor with hydrogen production, the oxidation of substrate by fermentative bacteria provides the largest amount of energy. In a well operated anaerobic reactor, about 70-80% of the hydrolysis products will be converted directly to methanogenic substrates like, hydrogen, carbon dioxide and acetate, with the remaining 20-30% transformed into other intermediate products, such as volatile fatty acids (VFAs) and alcohols (Ahring, 2003). The intermediate products produced in acidogenesis step are unable to be utilized by the methanogens, and must be even more degraded in the acetogenesis step by acetogens.

1.2.3. | Acetogenesis

Since the intermediate products must be further oxidized to acetate, H_2 and CO_2 prior to they are used by methanogens, the acetogenesis step is vital for the successful production of biogas. In contrast to the fermentative bacteria, the acetogens are obligately symbiotic bacteria (Westermann, 1996). Under standard conditions, these oxidation processes are endothermic, energy demanding and can't grow when the H_2 pressure is high. Only when H_2 pressure is lower than a specific level, the oxidation of propionate and butyrate can be achieved. Also in contrast to the fermentative bacteria, acetogens cannot switch their metabolic pathway but decrease of H^+ to H_2. Therefore, the proceeding of acetogenesis is relying on the presence of hydrogen utilizing methanogens to remove H_2.

1.2.4. | Methanogenesis

The final step of the AD is named methanogenesis where the CH_4 production occurs. This process is carried out by methanogenic Archaea, which metabolize the end products of the past reactions, mainly H_2, CO_2, and acetate, to form CH_4 (Figure 1). Methanogens belong to Archaea, a unique group of microorganisms, phylogenetically different from the main group of prokaryotic microorganisms. It is projected that, under stabilized conditions, about 70% of methane is produced by the acetate utilizing methanogens and 30% by the hydrogen consuming methanogens (Klass, 1984). Methanogenesis is regarded as the main force of the whole anaerobic degradation. In contrast to some of the acetogens, methanogenesis is an energy generating process under standard conditions. Only if the presence of the hydrogen using methanogens keeping the partial pressure low, could the acetogens complete a catabolic oxidation which would not be energy yielding if the hydrogen consuming bacteria had not been present. The main products of the anaerobic process are carbon dioxide and methane, and small quantities of nitrogen, hydrogen, ammonia and hydrogen sulphide and the mixture of these gases products is biogas (McInerney et al., 1980).

1.2.5. | Process parameters

Inside all the biological processes, keeping the consistency of the living conditions is important. A change in temperature, substrates or substrate concentration could result in a shutdown of biogas production. The microbial metabolism process depends on many parameters. Multiple parameters should be taken into consideration and be monitored for an optimum fermenting process. (McInerney et al., 1980).

The optimal pH range can be divided into two sets, 5.5 to 6.5 for acidogens and 7.8 to 8.2 for methanogens. It is important to adjust the pH value in the optimal range considering anaerobic performance is affected by as light pH changes. For the combined cultures pH ranges from 6.8 to 7.4 will be the ideal. In the low pH environment, the activity of methanogens will be reduced, resulting in the accumulation of acetic acid and hydrogen. At higher partial pressure of hydrogen, propionic acid-degrading bacteria can be inhibited which causes the build-up of VFA, which reduces the production of acetic acid making the pH drop further and eventually, the biogas process fails. (Khanal, 2008).

Temperature is one of the more important factors influencing the anaerobic process especially in methane production. Compared to the operating temperature, the variation in temperature has far more influence the methane forming bacteria. Moreover, it affects not only the methane forming bacteria but also volatile acid forming bacteria (Gerardi, 2003). Maintaining the optimal digester temperature is the most important problem during anaerobic process. The C/N ratio of the substrate should be within the range of 16:1 to 25:1. Because not much biomass is developed with the anaerobic process, the need for nutrients is very low. Just as too low C/N ratio causes an increase in ammonia production and an inhibition of methane production, too high a C/N ratio causes negative influence in protein formation and a decline in the energy and structural metabolism of the microorganisms. It is necessary to keep a balanced composition of C/N ratio.

The concentration of the inhibitors, the composition of the substrate and the adaptation of the bacteria to the inhibitor are all issues that influence the determination of the inhibition process. Commonly inhibitors include oxygen, sulphur compounds, organic acids, nitrate, ammonium and ammonia or heavy metals (Khanal, 2008).

1.2.6. | Advantages of anaerobic digestion

This process of waste management and of energy production has many environmental advantages and offers significant advantages over other types of waste treatment, like less biomass sludge is created in comparison to aerobic treatment technologies (Ward et al., 2008). Another known advantage for the treatment of high organic concentration waste waters, effective in treating wet wastes of less than 40% dry matter is one the possibilities of nutrient recycling and reduction of waste volumes. The slurry produced is an improved fertilizer in terms of its availability to plants, the effective pathogen removal (Sahlstrom, 2003) and minimal door emissions. However, problems such as low CH_4 yield and process fluctuations are often encountered in AD, preventing this technique from being an extensively applied treatment method (Bolzonella et al., 2006).

1.2.7. | Constrains of anaerobic digestion

The major reasons why some feedstocks are not ideal for biogas production are they cannot be digested by microorganisms. Digestion by microorganisms is very difficult to achieve, digestion could be achieved but in a very slow way, and the presence of inhibitors in the feedstock or the production of inhibitory compounds during microbial degradation may inhibited such digestion. The objective of the pretreatment is to facilitate the digestion process by removing these barriers and to make the organic content of the substrate easily accessible by the microbial community (Taherzadeh & Karimi, 2008).

During the first step of AD (the hydrolysis), the hydrolytic bacteria convert the insoluble complex organic matter into monomers and soluble oligomers like fatty acids, amino acids, and sugars. The enzymes involved in this process are cellulases, hemicellulases, lipases, amylases, and proteases (Taherzadeh & Karimi, 2008). Therefore, in AD all types of substrates can be hydrolysed, however, the rate of the hydrolysis step is highly dependent on the characteristics of a given substrate. Hydrolysis can proceed relatively fast if the necessary enzymes are produced by microorganisms and suitable surface area for physical contact between the enzymes and the substrate is provided. However, substrates with more recalcitrant structure, like cellulose, need longer period to be degraded, and the degradation is usually not complete. The hydrolysis step is often considered as the rate-limiting step when utilizing these types of substrates (Deublein & Steinhauser, 2011). Therefore, an initial pretreatment step, which converts raw materials to a form that is easily accessed to AD microbial communities for further degradation is needed (Zhang, 2008). The ideal pretreatment to be employed for processing these feedstocks should be cost-effective, increase feedstock accessibility to microorganisms, should not use or produce substances that inhibit biogas production, should not demand high energy, and should not generate by-products that are harmful to the environment. (Horváth et al., 2016; Patinvoh et al., 2016).

1.3. | PHYSICAL AND CHEMICAL PRETREATMENTS

Pretreatment techniques such as mechanical thermal, chemical, biological, and their combinations are utilized to make the organic material become a lot more accessible to anaerobic microorganisms. This process improves the overall digestion efficiency and the degree of digestion, lowering retention time and increasing methane production rates (Ariunbaatar et al., 2014).

Mechanical pre-treatment favours solubilisation of particulate matters in liquid state which increases the yield of the biomass. In general, in mechanical pre-treatment the most frequently techniques used are ultrasonic pre-treatment, grinding and high pressure homogenization. With these methods, the aim is to enhance the degradability of organic materials by disrupting and lysing the bacterial cells. (Baredar et al., 2016).

Milling is among the physical pretreatments that was proven to be effective by shearing, increasing the surface area, and reducing the degree of polymerization, thus improving the hydrolysis yield by 5–25%. Degree of such improvement depends on type of biomass, as well as the duration and type of milling. Overall, it has been repeatedly shown that smaller particle sizes result in higher yields (Lennartsson et al., 2011; Teghammar et al., 2012). That is why the physical pretreatment is often carried out in combination with other pretreatment methods. However, in some cases, the chemical agent used for the pretreatment can act as a potential inhibitor for the microbial community involved in the AD (Kabir et al., 2013).

Grinding, one dominant pretreatment technique is the wet milling, which is more of a grinding method. Wet milling utilizes small beads to break cell walls, the size of the beads used are therefore critical for maximal sludge disintegration. Of several milling products, the ball mill using smaller diameter balls has the best performance. During grinding sludge is pressed through a conical or cylindrical space with shear-stresses high enough to break the bacterial cell walls. (Wang et al., 1999).

Ultrasonication is an excellent and promising method to improve the biodegradability of the sludge. This method has several benefits such as efficient sludge disintegration (>95%), improvement in biodegradability and bio solids quality, increase in methane production, reduction in retention time, sludge reduction and energy recovery. This method enhances the sludge digestibility by causing disruption to the chemical, physical and biological properties of the sludge. Ultrasonic treatment accelerates the hydrolysis reactions by disrupting cell walls. The bacterial cells are separated by pressure waves and cavitations produced by ultrasonic generator leading to extraction of intracellular organic elements. The disruption of sludge particles obtained from ultrasonication treatment will boost subsequent acidogenesis, acetogenesis and methanogenesis reactions leads to an enhanced methane production and reduction of sludge volume (Pilli et al., 2011). Wang et al. (1999) showed that the methane generation was increased by 69% with corresponding ultrasonic pre-treatment of 40 minutes.

Gasification of indigestible feedstock is used during gasification the feedstock is gasified by exposure to high temperatures (1200 °C) and an oxidizing agent. Steam, oxygen, and air are mainly utilized as oxidizing streams.

The produced syngas is mainly composed of H_2 and CO_2, which can thereafter be fermented for biogas production. In addition, at the end of the gasification, a residual ash, in the form of slang, is left in the gasifier. The composition of the syngas and the ash is affected greatly by the feedstock composition and the process temperature. For example, feedstocks with high amount of carbon generate syngas with high CO content. Furthermore, at lower operating temperatures below 1000 °C, the gas produced contains higher amounts of impurities. The remaining ashes, from thermochemical processes, have been mainly used as building material in road construction. However, ashes consist of toxic components such as heavy metals, which pose an environmental threat. Therefore, alternative uses have been considered. For example, it has been stated that the addition of ash enhanced the biogas production and benefit the alkalinity, and pH of anaerobic digesters One of the main advantages of gasification as a pretreatment process is that it can handle a wide variety of heterogeneous feedstocks. In addition, gasification has a high conversion rate, and both syngas and the remaining ash can be used for biogas production (Richter et al., 2013).

Thermal pretreatment is well identified for a long time that can improve the degradability of organic wastes. While the lipids and the carbohydrates of the wastes are easily degradable, the proteins are protected by cell walls from hydrolysis. Heat supplied during thermal pre-treatment breaks the chemical bonds of the cell wall, thus makes the protein content easily accessible for biological digestion. Maximum bio digestibility, in percentage, means the maximum percentage of substrate chemical oxygen demand (COD) that can be converted to methane (El-Mashad et al., 2004). Effect of thermal pre-treatment is studied in a wide range of temperatures ranging from 60 to 270 ºC. In practice, the optimum temperature for the pre-treatment process is 160 to180 ºC and treatment time between 30 to 60 minutes. Pressure associated with it may vary from 600 to 2500 kPa. Various researches on thermal pre-treatment have been performed to verify this conclusion. Bougrier et al. (2006) compared the thermal pre-treatment (170 ºC during 30 minutes) performance of waste activated sludge obtained from urban waste water plants with untreated sludge. The outcomes suggested that there was positive effect on solubilization rates and methanation when thermal pre-treatment was performed Their experiments revealed that the effect on digestibility of activated sludge was high at 175 ºC. The AD of the thermally pre-treated biomass at this temperature resulted in an increased methane production of 60-70% and higher temperatures driven to decrease in methane production.

Chemical pretreatment is an effective and cost-effective method to hydrolyse the cell walls and thus increase solubility of the organic matter contained within the cells. According to different principles, chemical treatment methods can be categorized into acid and alkaline oxidation. The most regular oxidative methods are ozonation and peroxidation. (Baredar et al., 2016).

Ozonation is a powerful cell-lytic agent, which can damage the microorganisms present in activated sludge and oxidize the organic substances released from the cells (Chu et al., 2009) Among the techniques to disintegrate sludge, ozonation of sludge is one of the effectual way and yields the utmost degree of disintegration. Ozonation process changes the characteristics of the sludge considerably. The sludge biodegradation is influenced by the amount of ozone. Several researchers have investigated the impact of ozone amount on sludge biodegradation. Ozonation treatment has two counter effects: degradation of molecules and cell structures which is responsible for the increase in biogas production and oxidation of organic molecules that is responsible for the decreases in biogas production (Penaud et al.,1999). The biogas yield increased by 80% with ozone treatment of 0.1 g O_3/g COD; the effect was not evident at higher concentration of ozone (Yeom et al., 2002).

Peroxidation techniques, including the well-known Fenton peroxidation and novel reactions of peroxymonosulphate and dimethyl dioxirane, can accomplish a transformation of available COD into soluble BOD improving the biogas production. Fenton pretreatment disintegrates extracellular polymeric substances and ruptures cell walls, releasing intracellular water. Hence increasing the soluble COD and BOD in the sludge water. Low pH value required is the major disadvantage of this method. Kim et al. (2003) studied biogas generation by treating sludge with Fenton peroxidation, techniques on laboratory scale. The outcomes showed a maximum increase of 75% with Fenton, while the biogas production was twice by peroxymonosulphate and by dimethyl dioxirane methods (Zupančič & Gril, 2012).

Thermochemical pretreatment together with thermal treatment is called thermochemical treatment. There is no agreement on the performance of this method. Kim et al. (2003), showed that the order of effectiveness in sludge solubilization was NaOH>KOH>$Mg(OH)_2$ and $Ca(OH)_2$, whereas the pre-treatment with KOH was more efficient than using NaOH and observed no effect on the biodegradability (Penaud et al., 1999). Haug et al., (1978) determined a decrease in biodegradability of 60%. Tanaka et al. (1997) showed that thermochemical pretreatment considerably raised the biodegradability up to 230%. It’s noted that thermochemical method gives the best result compared with thermal, chemical, ultrasonic methods under same conditions. They tested the COD removal rates and biogas production under two conditions (thermal treatment at 170º C and thermochemical at 130 ºC for 30 min). They found that the COD removal rates were considerably increased compared with the untreated raw sludge, being 71% and 60% of raw sludge COD, respectively, when the biogas generations were increased by 54% and 74%.

1.4. | BIOLOGICAL PRETREATMENTS

Biological pretreatment includes two processes aerobic and anaerobic. These are made in raw wastes applying microorganisms as a biological pretreatment prior to AD (Rouches at al., 2016). The objective of biological pretreatment is to enhance the hydrolysis process in an additional stage prior to the main digestion procedure. The enzymes produced by the microorganisms in the pretreatments, can catalyse biological reactions. Almost all the enzymes we know active in proteins fit in six basic classes: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. Enzymatic lysis is taken by a enzyme catalyzed reaction produced by the breakdown of the compounds of the cell wall (Patinvoh et al., 2016).

In the past years, the bioprocessing of wastes has been suggested as an effective and economical method of producing value added products, however, it is still under investigation whether this could be accomplished more efficiently with a single organism or community of organisms. Many natural mixtures of microbes in a symbiotic consortium or engineered symbiosis within a consortium have been demonstrated to improve process efficiency and stability relative to monoculture in pretreatments of wastes. (Zuroff& Curtis, 2012).

Several examples of these biological pretreatments in wastes to achieve the desired sub products, shown that a microbial consortium is a promising alternative to deconstruct wastes rather than microbial mono cultures (Gilmore & O’Malley, 2016).

1.4.1. | Sewage sludge pretreatments

Sewage sludge is constituted by organic materials in which proteins represents 50% of this waste. These proteins are difficult to hydrolyse and pretreatments have been tested to achieve efficient solubilization. The use of thermophilic bacterial consortia with proteolytic and hydrolytic activity can increase sewage sludge biodegradability and solubilization and consequently this could be reflected in the increase of the methane production rate, and in the reduction of digestion time. Also it could be of economic benefit through the increase of methane production efficiency (Zheng et al., 2017). The increased availability of enzymes is believed to stimulate a more efficient release of organic matter from the sludge (Wawrzynczyk et al., 2008). Several authors have shown the benefit of enzyme addition or pretreatment on the conditioning of waste water solids (primary sewage sludges) and enhancement of the degree of dewaterability of anaerobically digested biosolids (Roman et al., 2006). Kim & Sim (2004) improved sludge pretreatment in their study by controlling the amount of enzyme and ozone. Dursun et al. (2006) stated that there was a considerable increase in cake solid content of anaerobically digested sludge (27% as opposed to 18% without enzyme pretreatment) using an enzyme dose at 20 mg/l. Also, other authors investigated enzyme hydrolysis as a moderate and effective mean of extracting extracellular polymers from mixed culture activated sludge flocs. Alpha-amylase, cellulase and proteinase were used in one of these studies (Burgess et al., 2008).

Exoenzymes, like lipases, glucosidases and proteases can be originate from one of three key sources, namely the sewage influent, the activated sludge via cell autolysis, or as enzymes that are secreted by the bacteria. Moreover, Exoenzymes are either cell surface bound (ectoenzymes), in free form (Exoenzymes) in water or adsorbed within the extracellular polymeric substances (EPS) of the sludge matrix (Cadoret et al., 2002). Extracellular enzymes in anaerobic digested sludge can be divided in two classes: ‘cell-free enzyme’ dispersed in the bulk liquid and ‘cell bound enzyme’ associated with the microbial cell surface. The degree of contact between the microbial cells and their substrates is thus of crucial importance. Using fluorescent in situ hybridization, Higuchi et al. (2005) shown that cell-bound alpha-amylase is mainly responsible for the hydrolysis of digested sludge. Proteolytic activity in activated sludge flocs were mainly found in the EPS, while the glycolytic activity was associated with the organic colloidal fraction of the waste water.

Whiteley et al. (2002) showed that protease and phosphatase enzyme activities were essentially associated with the organic particulate matter of the primary sewage sludge. Also, concentration of hydrolases, and the contact that exists between these enzymes and their substrates, were very important in modelling complex particulate substrates during AD (Jain et al.,1992). The activity of these hydrolases declined during both aerobic and AD. Under aerobic conditions a rapid loss of glucosidase activity over the first ten days was associated with a resultant accumulation of polysaccharide material (Novak et al., 2003). In fact, enzyme addition or pretreatments benefit the digestion of waste water solids and enhance the degree of dewaterability of anaerobically digested biosolids. Roman et al. (2006) have also used lipases in the enzymatic treatment of dairy waste water. In contrast the restaurant wastes, which have a uniquely high lipid content, general aerobic and anaerobic waste water treatments are anticipated to contain a major organic fraction especially rich in protein and carbohydrate contents. Proteases and glycosidases are therefore believed to play a crucial role in the degradation of waste water sludges (Burgess et al., 2008).

Sewage sludge heat pretreatments at 60 ºC is a relatively simple process that can establish relationship between the efficiency of sludge reduction and biological reaction of the sludge matrix. Yan et al. (2008) has reported that protease secreting bacteria appeared shortly after heat treatment, with an immediate increase in protease activity in the sludge supernatant after 1 h heat treatment. This protease activity was believed to be due to proteases released from the microbial cells via lysis.

Several authors reported the use of single bacteria enhancing sludge digestion rate significantly, but the use of bacterial consortia in pretreatments of sewage sludge are rare to find. Li et al. (2009) shown that the use of a Brevibacillus could enhance un-sterilized sludge degradation by 11.86% and sterilized by 30%, relative to the control. The results also showed that the inoculation of Brevibacillus sp. KH3 could promote the degradation of EPS and the collapse of cells and inhibit the growth for certain kinds of microorganisms. Other studies used an aerobic thermophilic consortium for simultaneous sludge solubilization, pathogen control and metal leaching. The process comprises a first stage at thermophilic temperatures (60 °C) and a second stage at mild-thermophilic temperatures (45 °C) (Narayanan &Sreekrishnan, 2009). Using a stable aerobic microbial consortium, established by successive sub cultivation Cai et al. (2015) achieved a pretreatment capable of shortened digestion time of sewage sludge by 50% and increased biogas production by 45% compared to raw sludge in the anaerobic process.

1.4.2. | Lignocellulosic pretreatments

Due to the recalcitrant nature and strong elasticity, feedstock rich in lignocellulosic materials can result in a low biogas yield. Therefore, these wastes are pretreated prior to the biogas production. The main purpose of pretreatment for lignocellulosic wastes is to break the lignin region that protects the cellulose and hemicellulose, to make the feedstock more accessible for digestion. Pretreatment also helps to decrease the crystallinity of cellulose and to increase the porosity.

Several pretreatments methods have been suggested for enhancing biogas production from lignocellulosic biomass, which can be classified as, physical, physicochemical, chemical, and biological pretreatments (Hendriks & Zeeman, 2009).

Dilute acid pretreatment at controlled conditions can solubilize almost all the hemicellulose from a biomass and thereby increase the accessibility of microorganisms to cellulose. This method breaks the intra molecular bonds between the lignin, hemicellulose, and cellulose in the cell wall and hydrolyses the hemicellulose (Taherzadeh & Jeihanipour, 2012). However, at a high temperature and acid concentration, undesirable dehydration occurs resulting in the hemicellulose and cellulose being degraded into different types of inhibitory compounds, such as furfural.

Liquid hot water pretreatment at temperatures of 200–230°C, has also been reported as an effective method for the hydrolysis of hemicellulose (Johnson & Elander, 2008). A higher yield of soluble sugars is possible with this method compared to the un-catalyzed steam explosion method. However, the liquid hot water pretreatment method liberates the sugars in the oligomeric form.

Alkaline pretreatment is a suitable method for solubilizing the lignin; it can be carried out at different concentrations of sodium hydroxide, or ammonia. This method causes swelling of the fibers, which results in an increased accessible surface area, reduced degree of polymerization, and decrystallization of the cellulose. Alkaline treatment breaks the intra molecular bonds between the lignin, hemicellulose, and cellulose and disrupts the lignin structure. As a result, the biomass digestibility is increased, although there is a loss of cellulose and hemicellulose during this process (Adney et al., 2009)

As these last pretreatments are examples of pretreatments that can bring a decrease of the quality and quantity of the resulting subs products of value for AD as alternative biological pretreatments using bacterial consortia have been receiving more attention by researchers in the past years. Several reports using mesophilic or thermophilic consortia were shown to be cost effective and delivered enhancing of biogas production. As reported by Yan et al. (2012) a mesophilic microbial consortium pretreatment could achieve 49 % of rice straw hydrolysis within 7 days at 30 ºC. It could also convert lignocellulose into volatile products, and enhanced the biogas yield and methane content, improving biodegradability. Yuan et al. (2012) demonstrated that a thermophilic consortium could pre-treat municipal lignocellulosic wastes at 50 ºC enhancing biogas and methane yields. The pretreatments also produced more volatile organic products comparing with un treated waste and the maximum biogas and methane yields occurred at the time when hydrolysate sCOD reached its maximum.

1.4.3. | Feathers pretreatments

Problems with poultry waste and landfilled poultry waste like feathers might cause problems of land, water and air pollution. Feather waste is a complex structure due to the protein keratin which comprises approximately 90% of the entire feather, and have a high risk of contamination with pathogens. Feathers are composed of 90–95% of proteins and 5–10% of lipids (Onifade et al., 1998). The main protein element is keratin, a highly specific fibrous protein with mechanical strength and defensive abilities. Furthermore, keratin is also the main component of hair, wool, nails, horn, and hoofs. Keratin is distinguished from the other structural proteins by its relatively high cysteine content, which enables it to form disulphide bonds, that serve as structural components, thereby stabilizing the molecule (Hill et al., 2010). Along the high content of cysteine, feathers generally have high concentrations of serine, proline, and acidic amino acids and are deficient in some essential amino acids, like methionine and histidine. The secondary structure of feather keratin comprises 41% α-helix and 38% β-sheet configurations, and 21% disordered regions.

The pretreatment methods used can be categorized into four main categories, that includes physical, thermal, chemical and biological treatments. Physical and thermal treatments run at a high temperature or a high pressure, and in some cases diluted acid or alkali is added as well. The drawbacks of these technologies are high running costs and that certain amino acids will be destroyed (Barone et al., 2006). Hence, recent research focuses on the biological pretreatment methods, which have been reported as successful for keratin degradation. Microorganisms like bacteria and fungi produce enzymes that can break down the keratin and make it soluble. The keratin rich feedstock is inoculated with appropriate bacteria or fungi cultures and incubated for days depending on the activity of the microorganism employed and the characteristics of the substrate. Several microorganisms such as Bacillus sp. (Fellahi et al., 2014; Forgács et al., 2013) and Aspergillus sp. (Mazotto et al., 2013) have been investigated and stated as being effective for hydrolyzing keratin.

Keratin is a protein that can be found in example hair, wool, scales and feathers. It is an insoluble fibrous structural protein. Keratin has several important roles in nature due to its structure; there are two main types of keratin, α and β-keratin. The α-keratin alpha-helixes are dominant over β-keratin beta-sheets. Alpha-keratins are found in mammals and this is a softer kind of keratin, while β -keratins are more common in birds and reptiles and is a hard keratin that adds rigidity to form feathers for example. Most of the different variants of keratins are mechanically strong and unreactive (Whitford, 2013; Matikevičienė & Grigiškis, 2015; Brandelli & Riffel, 2010).

Pathogens from the poultry waste could spread to animal feed, but if pretreated with biological treatment, using enzyme keratinase in the case of feather degradation, the enzymes will also degrade prions. The biological pretreatment is cheaper method than for instance chemical treatment, even though this is a time-consuming method. Also, handlings with corrosive chemicals are decreased if working with enzymes instead. The resulting hydrolysate will be high in nutrition level and therefore suitable for animal feed or for biogas production. Degradation of feathers has earlier been performed by alkaline hydrolysis and steam pressure cooking; this nevertheless may destroy valuable amino acids as methionine, lysine and histidine and is also considered as high-priced method. The resulting hydrolysate is low in value but suitable for biogas production purpose (Abbasi & Abbasi, 2010). Enzymes have been found and isolated in keratin rich sources as poultry waste for instance. The most common bacteria that produce keratinase are deriving from the genera *Streptomycetes* and *Bacillus*. The most common catalytic type is the serine protease. Keratinase characteristics can vary from each other depending on parameters such as producer, source of isolate, properties of the biochemical and biophysical sort, like for instance pH and temperature. Generally, they have optimal activity; at neutral to alkaline pH and at temperature range between 40-60 ºC, however alkalophilic and thermophilic conditions have been also shown to be optimal for some enzymes. For enzymes produced by different strains of *Bacillus licheniformis* the optimal pH range was found between 7.5 - 10. Some bacteria that produce keratinase are *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus cereus*.

The mechanism of how keratinase is acting during hydrolysis is not fully elucidated. But it seems that reduction of cysteine bridges may have an influence on keratin degradation. Keratinases show higher activity on soluble proteins like casein than on keratin. It has also been proved that in most cases native keratin is difficult to be hydrolysed by purified keratinolytic enzymes due to high amount of disulphide bonds presented in its structure. So, it is not only the release of extracellular keratinolytic proteases that control the hydrolysis of keratin, but the reduction of disulphide bonds, called sulfitolysis, plays an essential role as well. Other utilization areas for keratinase applications have a variety of utilizations for this enzyme other than hydrolysis prior to biogas production. Mentioning only a few of them is for instance as in detergent applications and in the woolen textile industry. Furthermore, some novel applications are in enhancement of drug delivery in some tissues and also in the hydrolysis of prion proteins (Brandelli & Riffel, 2010).

1.5. | MOLECULAR CHARACTERIZATION OF BACTERIAL CONSORTIA

The most promising method for the determination of population dynamics is the molecular biological one based on the analysis and differentiation of microbial DNA, such as sequencing and metagenomics (Röske et al., 2014). A massive information can be derived from even very simple microbial communities (Spiegelman et al., 2005). Metabolic networks and stoichiometric models can provide not only to predict metabolic fluxes and growth phenotypes of single organism, but also to capture growth parameters and composition of simple bacterial community (Sabra et al., 2015). Detailed research about microbiome found on microbial consortium has not only profound theoretical significance, but also more substantial application potential, and can be of more benefit for humanity (Jiang et al., 2017).

For instance, to acquire insight into the structure of consortia communities and access to yet uncultivated microorganisms, many recent studies have taken a cultivation independent method by gene pyrosequencing methodologies sequencing 16S rRNA genes or metagenomes from thermophilic communities.

Two studies focusing on terrestrial compost systems degrading lignocellulosic substrates at 60 ºC presented that enriched communities dominated by *Paenibacill*i, *Rhodothermus*, and *Thermus*, showed that changes in the feedstock led to community level responses. Another study of a switchgrass-degrading bioreactor with temperature cycled up to 54 ºC documented enrichment of a variety of *Firmicutes* and a few phylotypes in the *Chloroflexi*, *Proteobacteria*, and *Actinobacteria* (Allgaier et al., 2010) Even with the potential for high-temperature communities to serve as sources of novel cellulases, no such studies have explored the composition and structure of cellulolytic microbial communities at higher temperatures. It is well recognized that microbial communities in 75 ºC habitats are distinct from those at lower temperatures, even at the phylum level and therefore the potential for applied and basic scientific discovery resulting from the investigation of cellulolytic communities in high-temperature environments such as terrestrial hot springs is significant (Spear et al., 2005). 16S rRNA gene pyrosequencing methodologies were then employed to characterize the microbial communities that colonized the enrichments and to compare them to those in corresponding sediment samples to examine the effect of lignocellulosic enrichment (Peacock et al., 2013).

Regarding to their physicochemical conditions, terrestrial hydrothermal springs are highly diverse habitats, providing an extended range of ecological niches. These niches possess extreme conditions, such as high temperature, low or high pH, and the presence of toxic ions. Their extreme features are expected to lead to limited biodiversity, making hydrothermal habitats best model systems to study principles of community structure and function. Prokaryotic diversity in hydrothermal ecosystems has become extensively studied, estimates on community composition, however, have been performed mostly based on clone libraries with the known bias of cloning based technologies and reasonably low sample size considered (Swingley et al., 2012). With next generation sequencing methods, the analysis of larger 16S rRNA gene data sets has become feasible, making it possible to increase the extent of sequence-based biodiversity studies, leading to more reliability. Analysis of large sets of short rRNA gene fragments comprising variable regions has been applied to infer community composition and biodiversity estimates in terrestrial and marine habitats (Nacke et al., 2011; Roesch et al., 2007; Will et al., 2010; Harmsen et al., 1997). However, the analysis is usually based on PCR-amplified DNA fragments, which still suffers from potential primer bias. Direct sequencing of metagenomic DNA can prevent this bias, increasing, however, the amount of data available for analysis since non 16S rRNA gene sequences are also generated (Inskeep et al., 2010). Also, phylogenetic analysis of the metagenome, with fluorescence in situ hybridization (FISH), which allows direct quantification of specific microbial lineages in a sample, directly are possible. Brock and Brock (1967) shown a detailed description of the hot springs of Furnas Valley pointing out the thermal and chemical complexity of the Furnas springs and a major difference regarding other terrestrial hydrothermal areas: in Furnas, the largest spring is at the highest elevation and is alkaline, whereas some of the lower springs are smaller and more acidic. This is completely opposite to the situation found in Yellowstone National Park and Iceland (Allen & Day, 1935; Barth, 1950). Here the higher springs are small and acidic and the lower ones large and alkaline. The hot springs of Furnas Valley have been a valuable source for thermophilic microorganisms and thermostable enzymes with useful features for biotechnological applications (Albuquerque et al., 2008;

França et al., 2006; Friedrich & Antranikian, 1996). However, only little data are available on overall diversity (Hamamura et al., 2012).

In a recent study by Sahm et al., (2013) the microbial community of two hot springs of Furnas Valley was characterized based on two different rRNA gene-based methods. The combination of different primer dependent and independent methods revealed major differences, focusing the importance of combining different methodological approaches. A large dataset of short rRNA gene sequences and metagenome analysis revealed that the habitats were dominated by three to four genera, both for the bacterial and the Archaea population. Archaea diversity was particularly limited in the acidic spring (pH 3, 51 ºC), in which *Thermoplasma* represented almost 90%. However, overall abundance of Archaea in this acidic environment was low as revealed by FISH, while the hot slightly alkaline spring (pH 8, 92 ºC) had almost even abundances of Archaea and Bacteria. Unlike other hydrothermal habitats, the high temperature slightly alkaline low sulphate spring was dominated by heterotrophic bacteria, probably due to a high content of dissolved organic carbon, suggesting that not all hydrothermal habitats are dominated by chemolithoautotrophs and that a wide range of chemical characteristics needs to be considered when analyzing hot springs. The results shown enrichment of heterotrophic, polymer-degrading genera in the Furnas springs which make them particularly promising for the search of unique thermostable enzymes for application in biotechnology and biorefinery of the second generation.

1.6. | RESEARCH AIM

The objective was to find bacterial consortia that could solubilize sewage sludge to enhance biogas production, the results led us to select two more wastes substrates with high value for biogas enhancement. The thermophilic consortia were used in a two-stage digestion system. The first stage consisted on a thermophilic aerobic bacterial pretreatment using a selected consortium to improve Slaughterhouse sewage sludge (SSS), grass mixtures or feather hydrolysis and the second stage was the AD of pretreated wastes.

2. | MATERIAL AND METHODS

2.1. | WASTES PHYSICAL AND CHEMICAL CHARACTERIZATION

2.1.1. | Wastes sources

The wastes used in the present work were collected from the waste water treatment plants (WWTP) from two local industrial producers (Figure 2). Sewage Sludge and feathers were collected from a slaughterhouse and a dairy milk industry of São Miguel Island. Grass mixtures were collected from local university garden and air dry before use.

Figure 2. Glass containers with sewage sludge for sterilization and storage at 4 ºC until use.

2.1.2. | Physical analysis Total Solids, Volatile Solids

Total and volatile solids were determined according to the method described in Standard Methods, in triplicate (APHA et al., 1999). For the determination of Total Solids (TS), a sample of waste in triplicate was dried at 105 ºC for until constant weight was achieved. The content of volatile solids (VS) was determined after sample was calcined at 550° C, until constant weight was achieved. Total solids and VS of wastes substrates were determined before and after pretreatments.

2.1.3. | Chemical Oxygen Demand

2.1.3.1. | Substrates COD

To determine COD balance during pretreatments chemical oxygen demand of wastes was determined before, during and at the end of pretreatments using a closed reflux, titrimetric method according to (APHA et al., 1999). The COD from substrate (COD substrate) and COD of substrate residue (COD substrate residue) during pretreatment were analysed using potassium dichromate as an oxidant, using 25 mg of wastes. For grass mixtures, wastes samples were previously milled into a 1 mm powder (Pommier et al., 2010).

2.1.3.2. | Soluble COD and pH

The soluble chemical oxygen demand (sCOD) of hydrolysate, and COD of medium (COD medium) were analysed following centrifugation at 8000g for 10 minutes, and sampling of supernatant only. During the pretreatment, the hydrolysate pH was recorded at day 0, 1, 2, 3, 4, 5, 6 and 7. The analysis of soluble COD (centrifuged for 10 minutes at 15 000 rpm) and total, was carried out by closed reflux with titration method. The method is based on the oxidation of organic matter present in the sample with a set amount and excess of potassium dichromate in acidic medium. After digestion during 2 hours at 150°C and cooling, the excess potassium dichromate is titrated with iron and ammonium sulphate, using an indicator of Ferroin to detect the turning point (indicated by the passage of a yellow-greenish to brownish-orange). The concentration of organic matter, is expressed in mg O_2/L.

2.2. | ENRICHMENT OF AEROBIC THERMOPHILIC CONSORTIUM

2.2.1. | Inoculum sampling

Sediments, mud, biofilm, water, feathers, decaying small branches and leaves were collected inside and near hydrothermal springs samples at Furnas, São Miguel, Azores, in March of 2013. All necessary permits were obtained for the described field studies from the regional government of the Azores (Licença Nº 26/2013/DRA). A description of the different sampling sites is provided in

Table 1. Several samples were collected at hot springs in Furnas Lagoon (sampling site a) (Figure. 1a) and in Furnas Valley solfataric field with acidic springs (Figure. 1b). The sampled biomass was supplemented with water from the respective hydrothermal spring. Sediments and mud were characterized by brownish, ochre, grey, black or green appearance. The biofilms encountered along the effluent stream of one hydrothermal vent exhibited black, white, light-grey, green, orange, yellowish or brownish colors (Figure. 3b).

Figure 3. Enrichment inoculum collecting sites a) Furnas lagoon hot springs. b) Furnas Valley solfataric field with acidic springs.

Table 1. Sample sites hydrothermal springs physical and chemical characteristics

Site	Temp. (ºC)	sample pH	Biotope
A	90	6	Caldeira dos Vimes (water/decaying branches)
	77	9	Small hydrothermal spring at solfataric field near caldeira dos Vimes (water/mud/decaying branches)
	52	5	Small hydrothermal spring at solfataric field near caldeira dos Vimes (water/mud/feathers)
	90	5.3	Caldeira da Lagoa das Furnas (water /mud)
	80	8	Small hydrothermal spring at solfataric field near caldeira da lagoa das furnas (water/mud/feathers)
B	98	7.5	Caldeira do Esguicho (water/mud)
	60	4	Small hydrothermal spring at solfataric field near Caldeira do Esguicho (mud and decaying leaves)
	72	3	Small spring in Solfataric field near Caldeira do Esquicho (water/soil)
	65	2	Small spring in Solfataric field near Caldeira do Esquicho (water/soil)
	60	7	Small hydrothermal spring near Caldeira do Asmodeu (water/soil)
	94	8.5	Caldeira Grande (water/mud)

Samples collected sites with a wide variety of physicochemical characteristics. In situ temperatures ranged from 45 to 94 ºC, whereas pH values varied between 2.5 and 9 (Table 1).

To carry out the collection a steel spatula and bottles of 500 ml sterile with lid was used or transport of samples to the laboratory (Figure 4).

Figure 4. 500 ml glass container autoclaved used for the collection of samples from sediments of hydrothermal springs

For collecting samples from hydrothermal vents with difficult access a rod extendible pole with a container on the edge was used (Figure 5 b). Samples between 100 to 300 g of mud were collected, and the temperature was registered using a thermometer installed at the end of the pole (Figure 5 a) and b)).

Figure 5. a) temperature measurement in situ using thermometer sensor adapted to a 3-meter extensible pole b) Example of sampling in hard to reach boilers using the extensible pole.

For collection of water samples sterilized glass bottles of 1 liter were used. For each sample, the sampling site temperature and pH were registered. The samples were placed in a thermal box to prevent the decrease of temperature and transported to the lab for inoculum preparation. These samples were used later in the lab to inoculate enrichment bioreactors with wastes for the selection of consortia with hydrolytic capacity in the digestion of organic wastes.

2.2.2. | Enrichment process

For the enrichment process a minimal medium AEMAC (Azorean enrichment medium for aerobic consortia) adapted from (Sahm et al., 2013) was used (Table 2), and pH adjusted to the desired value using NaOH or HCl.

In table 3 are described the characteristics of the medium used, substrate type, concentration, and treatments carried out during the preparation of media.

Table 2. Composition and characteristics of the culture medium AEMAC used.

Reagents	Concentration
NaCl	3 g/l
KCl	1 g/l
$MgS0_4$x7 H_2O	0,1 g/l
$(NH_4)_2 S0_4$	1,5 g/l
Trace elements DSM141	1 ml/l
Vitamin solution DSM141	1 ml/l
Yeast extract	0,01% W/V
Carbon source	1% W/V

Table 3. Culture medium, waste substrate and concentration used at three different pH

Culture medium	Wastes substrates	Wastes concentration	pH
AEMAC	DMSS	10 g/l TS	3/7/9
H_2O	DMSS	10 g/l TS	3/7/9
AEMAC	SSS	10 g/l TS	3/7/9
H2O	SSS	10 g/l TS	3/7/9
AEMAC	Olive Oil	1% v/v	3/7/9
AEMAC	Grass mixtures	1% w/v	3/7/9
AEMAC	Chitin (galleria mellonella skin)	0,2% w/v	3/7/9
AEMAC	Chicken Feathers	0,2% w/v	3/7/9
AEMAC	Skim milk	1% w/v	3/7/9
AEMAC	Starch	1% w/v	3/7/9
H_20	Nutrient agar	1% w/v	3/7/9

Before enrichment tests was necessary to eliminate bacteria and spores present in sludge. Thus, ensuring that bacteria grown in sludge originated in the samples collected in the hydrothermal springs. We started with the tyndallization on the sewage sludge to remove all vegetative microbial communities and temperature resistant spores from the waste. This ensured that the only microorganisms growing in the sludge during enrichment will have origin in the inoculum from hydrothermal springs. The sludge and feathers were transported to the laboratory in hermetically sealed bags and the tyndallization process was done immediately. The sewage sludge was introduced in one liter glass containers with cover (Figure 1) and submitted to tyndallization. After tyndallization the wastes were stored at 4 ºC until use.

Feathers and chitin were washed prior to tyndallization process. Also, according to the type of waste and to maintain the constitution structure of the substrates different tyndallization temperatures were used. Three sterilization 15 minutes 121 ºC, two incubation times of sludge at 60 ºC between each autoclaving, the first incubation with a duration of 2 hours and the second with 24 hours. This method allows the spores of fungi or bacteria germinate between the two autoclave sterilization, dying in the next heating sterilization.

Table 4. Temperatures of tyndallization of the different wastes selected

Wastes Substrates	Tyndallization temperatures
DMSS	121 ºC
DMSS	121 ºC
SSS	121 ºC
SSS	121 ºC
Grass mixtures	100 ºC
Chitin (galleria mellonella skin)	100 ºC
Chicken Feathers	100 ºC

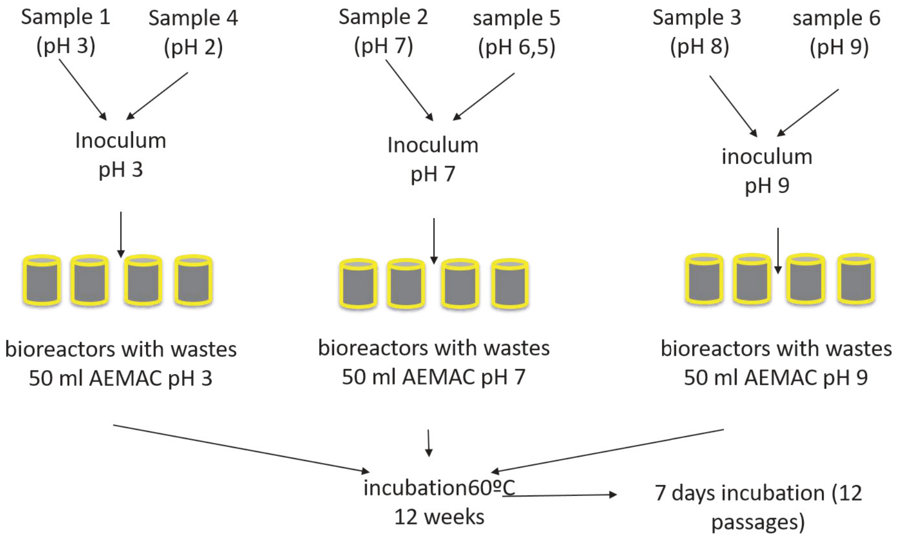


Figure 6. Schematics of inoculum distribution

To proceed to the beginning of the enrichment culture media were prepared according to the table 2. Each bioreactor was prepared at three pH (pH 3,7 and 9) and with their respective controls. 100 ml bottles with a working volume of 50 ml were used for the enrichment of samples from boilers (figure 6). The bottles were kept at a temperature of 60 °C for 7 days with horizontal agitation (figure 7). After a 7-day enrichment, a 5 ml of sample was taken from each bioreactor and transferred into a new 50 ml of minimal AEMAC medium with wastes. This procedure was repeated several times in fresh medium and sub cultured several times to obtain a microbial community capable of degrading wastes.

After the second subculture was gradually being withdrawn to the yeast extract, so that the growth of consortia and bacteria depend on nutrient substrates in exclusively added. This procedure was repeated until they have performed a minimum of 6 passages allowing to select and the adapt of the consortia to the different substrates provided. We monitored bacterial growth using a contrast phase microscope (Leica, Cambridge).

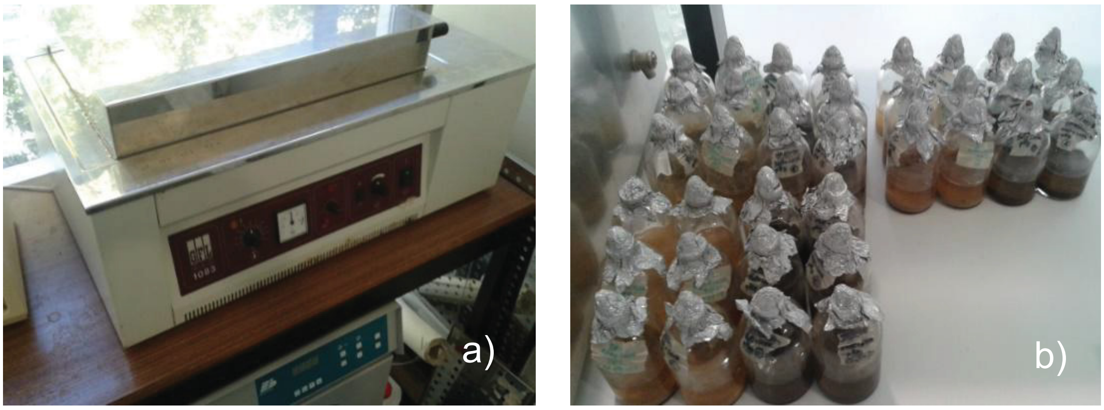


Figure 7. a) bath used for incubation of bottles of enrichment b) Example of the bioreactors for enrichment of the consortia to substrates

2.2.3. | Enzymatic activity

2.2.3.1. | Agar diffusion technique

Enzymatic activities from the consortia hydrolysates were monitored during enrichment process with four week intervals (Table B in annex) using agar diffusion technique for the presence of Proteases Cellulases, Xylanases and Amylases enzymes according to the specific substrate. 1% w/v of substrates were added to AEMAC medium with 1.5% agar. A sample of 200 µl from the consortia hydrolysate was collected and centrifuge at 10 000 r.p.m 15 minutes to remove bacteria and wastes residues. Holes with 50 mm diameter were punctured in the agar plates and the 200 µl of centrifuge hydrolysate was added in two times allowing hydrolysate to diffuse into the agar. After the plates were kept at room temperature for 3 hours allowing further diffusion of the hydrolysate. After diffusion, the plates were incubated at 60 ºC being revealed after 24 hours. Plates with CMC and Xylan were flooded with an aqueous solution of 1% Congo red in water followed by 1 N NaCl with intermittent shaking at 25 rpm for 15 minutes. After 1 N HCl, was added and changed the dye color (blue to violet). the enzymatic activity was inhibited with 1 N NaOH, which changed the dye color (brown to red). Activity was revealed by the diameter in millimetres of the clear zone around the holes on the agar plate. Plates with starch were revealed after plates incubation by flooding agar plates with Gram's iodine (2.0 g KI and 1.0 g iodine in 300 ml distilled water) for 3-5 minutes (Kasana et al., 2008).

During pretreatments, the hydrolysates were also screened for enzymes using the same agar diffusion technique, additionally enzymatic screening was used with APIzym chromogenic substrates for the enzymes shown in the results chapter. Enzymatic screening was also done after the isolation of bacteria from the selected consortia, the isolates were screened by growing them on the agar plates with the substrates. Isolates that presented growth and a clearing zone around the colonies were considered as positive for the specific substrate enzymes.

2.2.3.2. | APIzym assay

Each API-ZYM gallery was inoculated with two drops of adequate centrifuged hydrolysate from the consortia and incubated for 4 h at 60 ºC. The galleries were then activated by adding one drop of ZYM A and ZYM B reagents and after 5 minutes, the revealed color developed in each enzymatic reaction, were visually assigned by means of the color chart supplied with the kit system.Qualitative results were registered and presented as positive or negative for the presence of the enzymes.

2.3. | CONSTRUCTION OF THE BIOBANK

To build the Bank of thermophilic aerobic consortia and isolated bacteria that produced thermostable enzymes, techniques for preservation and maintenance for thermophilic bacteria were done according to the techniques used in the collections of the Leibniz Institute institute-DSMZ German collection of microorganisms and cell cultures. (Spring, 2006). Consortia and isolated bacteria were conserved in fresh culture medium supplemented 20% of glycerol at -80 ºC (Figure 8). At the end of each passage, part of consortia cultures were centrifuge at 13 000 r.p.m. and culture medium supplemented with 20% of glycerol was added to the bacteria and wastes substrates. Also, a preservation of the consortia was made at room temperature without any supplements at the end of each enrichment.

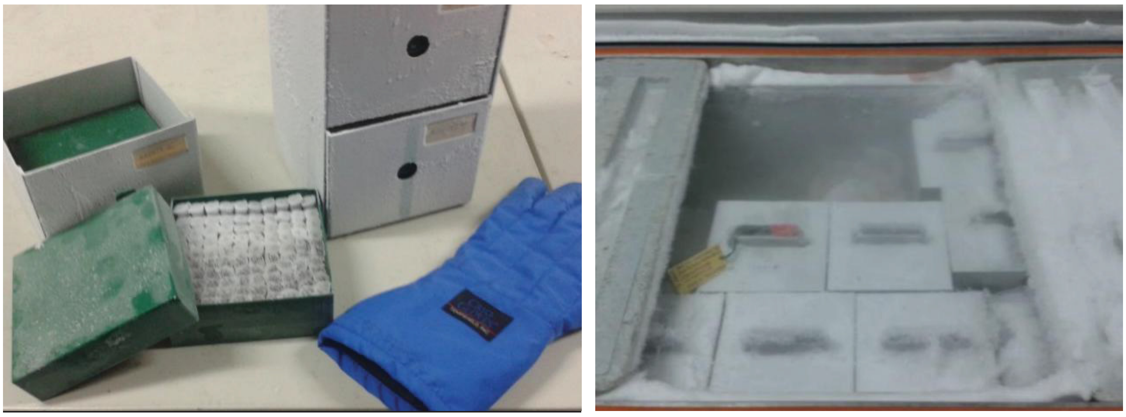


Figure 8. Preservation of consortia and isolated bacteria at -80 ºC in 20% of glycerol.

2.4. | CONSORTIA HYDROLYSIS EFFICIENCY DURING PRETREATMENTS

2.4.1. | Dry weight

Final substrate weights were determined using the hydrolysate (including both the fermentation broth and the residual waste) following centrifugation at 13 000 r.p.m., for 10 minutes. The resulting precipitate was water rinse to remove bacterial biomass, although in the more porous wastes bacterial biomass was not completely removed which could underestimate dry weight values. After washing, residues were filtered throughout a filter paper and oven dried at 105 ºC until constant weight was achieved.

2.4.2. | Percentage of solubilization

Percentage of solubilization was based on the values of COD balance tables using COD of substrate and sCOD at two time of pretreatment, first at the selected day of pretreatment stop time, and the second at the end of the pretreatments. The percentage of solubilization was calculate according to the formula A in table E in annex.

2.4.3. | Soluble protein

The hydrolysates were recovered and the pH stabilized to 7 with Tris-HCl 1 M pH 5.75 buffer (Naimov et al., 2008). To determine protein concertation hydrolysates were centrifuge at 10 000 r.p.m. for 15 minutes, and dilutions were prepared. Protein concentration was determined using Pierce reagent (Pierce BCA Protein assay kit, Cat. 23225).

2.4.4. | Reducing sugars

For the quantification of reducing sugars the 3,5-dinitrosalicylic acid (DNS) protocol according to Miller (1959), was used. DNS solution was prepared by dissolving 0.25 g of DNS acid and 75 g of sodium potassium tartrate in 50 ml of 2 M sodium hydroxide solution. Then, the final volume was brought up to 250 ml using distilled water. Prepared solutions were stored in the dark. Sterilized polystyrene 96-well plates were used to carry out the DNS reactions. Series dilution of fructose as standard was prepared. Then, 40 µl of a 10-minute centrifuge at 13 000 r.p.m. hydrolysates were added to the wells. Then 160 µl of DNS solution was added into each well. The micro well plate, with its top covered to minimize evaporative loss, was wrapped with aluminum foil to prevent melting from the direct contact with the dry oven. 30 minutes in the 100 ºC dry oven. Finally, the test plates were analysed with a microplate reader to determine the optical density of each sample at 540 nm and directly proportional to the amount of reducing sugar.

2.4.5. | Amino acids

For the determination of free amino acids in the hydrolysates of feathers at the end of pretreatment the hydrolysate was centrifuge at 13 000 r.p.m. and supernatant filtered through a 0.2 cellulose filter to remove bacteria and any waste residues. The filtered supernatant was concentrated in a Amicon Ultra 10K (Milipore). Concentrated supernatant containing the amino acids were lyophilized before amino acids identification by HPLC. The identification and quantification of free amino acids present in the supernatant was made as a service by subcontracting SGS, Portugal. S.A.

2.4.6. | VFAs

The volatile fatty acids concentrations for the three tested volatile acids (acetic acid, propionic acid and butyric acid) were performed by HPLC using a Waters system, composed by a Waters 600S controller with a Waters 626 pump, a Mandel scientific 530 column heater and an absorbance detector waters 486 that was equipped with an Aminex HPX-87H column (300 mm × 7.8 mm). The separation of volatile fatty acids was based in ion moderated partition chromatography technique, with ionic exclusion, hydrophobicity and molecular size exclusion. Samples were analysed at 40°C, with 0.01N H_2SO_4 as the mobile phase at a flow rate of 0.80 ml/min. Data analyses were performed using a detection wavelength of 210 nm. The retention times were used for qualitative criteria, and the peak areas as quantitative criteria. Calibration was done using external standards of the respective components. Liquid samples for organic acid analyses were taken on days 0, 1, 2, 3, 4, 5 and 6, by removing 1 ml of the liquid. The samples were submitted to protein precipitation by using 20% (v/v) of TCA, and maintained at 4 ºC for 30 minutes. Samples were centrifuged for 10 minutes at 13 000 r.p.m, filtered through a 0.45 µm polytetrafluoroethylene filter, and injected (10 µl) directly into the HPLC system.

2.5. | BIOCHEMICAL METHANE POTENTIAL ASSAYS

2.5.1. | Anaerobic digestion batch assays

The BMP assays were adapted from Angelidaki et al. (2009). Total solids (TS) and volatile solids (VS) contents of the various pretreated and unpretreated substrates was accessed at the end of the pretreatment period. For each pretreated and unpretreated waste, the tests were carried out in 115 ml duplicate serum bottles capped with rubber septum sleeve stoppers under anaerobic conditions. Periodically pressure inside the sealed bioreactors was monitored as the methane content that was analysed by gas chromatography every 7 days during the 42 days of anaerobic digestion.

2.5.1.1. | Inoculum

The anaerobic inoculum was collected from an up flow anaerobic sludge blanket reactor collected from the local central of biogas digesters at Agraçor piggery industry. The central works with a co-digestion of different wastes from food industries, slaughterhouse sludge and dairy milk industries residues. The inoculum as a source of methanogens in pig manure and at the point of collection the central had been stopped for one week although the inoculum was active and still produced biogas for one more week as the pressure inside the recipient was monitored during the “degassed” period. The total solids of the inoculum were 8 g TS/L and the volatile solids 4 g VS/L.

Necessary nutrients/micronutrient/vitamins are required for optimal performance of anaerobic microorganisms. Nutrient medium containing macro and micronutrients, buffers, vitamins etc. 10% v/v of the anaerobic basic medium was added to the anaerobic bioreactors with the inoculum and substrate. Anaerobic Basic Medium was prepared from the following stock solutions, (chemicals given next are concentrations in g/l, in distilled water). Stock solution A: NH_4Cl, 100; NaCl, 10; $MgCl_2$ $6H_2O$, 10; $CaCl_2$ $2H_2O$, 5. Stock solution B: K_2HPO_4 $3H_2O$, 200. Stock solution C: resazurin 0.5. Stock solution D: trace-metal and selenite solution: $FeCl_2$ $4H_2O$, 2; H_3BO_3, 0.05; $ZnCl_2$, 0.05; $CuCl_2$ $2H_2O$, 0.038; MnCl2 $4H_2O$, 0.05; (NH_4) $6Mo_7O_{24}$ H_2O, 0.05; $AlCl_3$, 0.05; CoCl2 $6H_2O$, 0.05; NiCl2 $6H_2O$, 0.092; ethylenediaminetetraacetate, 0.5; concentrated HCl, 1 ml; Na_2SeO_3 $5H_2O$, 0.1.Stock solution E: vitamin mixture (components are given in mg/l): Biotin, 2; folic acid, 2; pyridoxine acid, 10; ridoflavin, 5; thiamine hydrochloride, 5; cyanocobalamine, 0.1; nicotinic acid, 5; P-aminobenzoic acid, 5; lipoic acid, 5; DL-pantothenic acid.

To 974 ml of distilled water, the following stock solutions were added (stock solution A), 10 ml; (stock solution B), 2 ml; (stock solution C), 1 ml; (stock solution D), 1 ml and (stock solution E), 1 ml. The mixture was gassed with CO_2. Cysteine hydrochloride, 0.5 g and $NaHCO_3$, 2.6 g, were added and the medium was dispensed to serum vials. Before inoculation the vials were reduced with Na_2S $9H_2O$ to a final concentration of 0.025% (Angelidaki & Sanders 2004).

2.5.1.2. | Experimental reactors

The samples collected from the pretreated bioreactors were incubated in 115 ml batch vials at 37 ºC, with a stirring speed of 150 rpm under strict anaerobic conditions. The methane production was regularly measured by gas chromatography. Mixtures of pretreated wastes from the concentrations of Azotm36 2.5% TS, Azotdp36 5% TS, Azotf36 2.5% TS were added to the anaerobic digestion bioreactors. using a ratio of 70% v/v inoculum / 20 % v/v pretreated waste and 10% v/v of anaerobic basic medium. Corresponding to the following values of VS; Azotm36 (pretreated sludge 0.17 g VS added, unpretreated 0.18 g VS added), Azotdp36 (pretreated grass mixtures 0.48 g VS added, unpretreated grass mixtures 0.7 g VS added) and in Azotf36 (pretreated feathers 0.85 g VS added, unpretreated feathers 0.87g VS added). A total of 10 ml of each pretreated solubilized waste and unpretreated waste with the anaerobic methanogenic inoculum and anaerobic basic medium was loaded into 115 ml glass bottles which were then sealed with a rubber septum and screw cap as shown in figure 9. The working volume was 50 ml and the head space of 65 ml, the resulting headspace of the sealed bottles was flushed with pure carbon dioxide gas for 5 minutes at room temperature. Finally, the assay bottles were loaded into an incubator at 37 ºC, during 42 days of anaerobic digestion. The maximum methane yield was calculated per kg VS of substrate added in each vial. The assay is performed in closed vessels (115 ml) depending on the homogeneity of the substrate the more homogeneous the substrate the smaller the volume of the vessel is required (Figure 9). Thereafter, all the reactors were placed in an incubator at 37 ºC for a period of 42 days. All the reactors were manually agitated once a day.

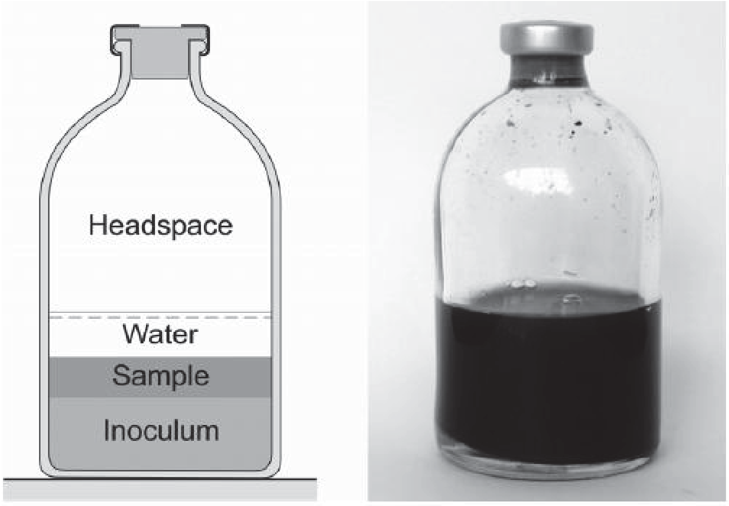


Figure 9. Example of an assay vessel for anaerobic biodegradability test.
Adapted from Angelidaki et al., (2009)

2.5.2. | Biogas volumes

The manometric pressure was measured daily in the headspace of the reactors through the septum with a needle connected to a digital pressure manometer (Digitron, England – type 2025P, up to 2000 mbar). The data collected from the pressure were used to calculate the biogas volumes trough the AD assays. Biogas production was monitored for every bottle throughout the duration of each experiment. Bottles were maintained in the incubator during measuring biogas volume were done immediately with a needle pressure transducer and calculations were made to convert pressure readings into biogas volumes at standard temperature and pressure. Biogas volume adjusted to STP was calculate using the mbar pressure results using the equation B in table E in annex. This equation was used to convert pressure readings into biogas volumes at standard temperature and pressure. Where volume of dry gas at standard temperature and pressure, in ml pressure of gas phase at time of reading, in mbar volume of head space in bottle; 65 ml standard temperature; 273 K, standard pressure; 1013 mbar temperature of gas phase at time of reading.

2.5.3. | Methane content

The biogas samples were collected from the bioreactors through the septum with an adapted needle connected to a tube and to the pre-vacuum Giggenbach bottles filled with NaOH 4 N (Giggenbach, 1975; Giggenbach, 1989). Analysis of the gas samples were done in the laboratory of gas geochemistry of the Research Institute for Volcanology and Risk Assessment of the University of the Azores following the methodologies described by Caliro et al. (2015) and Moreno et al., (2016). The gases in the headspace of the bottle (CH_4, N_2, O_2, Ar, He and H_2) were analysed by gas chromatography on a Perkin Elmer Clarus 580 gas chromatograph equipped with two thermal conductivity detectors (TDC) and two columns: a PLOT column (MSieve, 5 Å; 30 m x 0.53 mm x 50 µm; He, as the carrier gas) and a Packed column (2 m x 1/8” SS Molecular Sieve 13 x 60/80 PE 8000; Ar as the carrier gas). CH_4, N_2, O_2, Ar were measured on the first channel with He as carrier gas using a flux of 10 ml/min. He and H_2 were analysed in the second channel using Ar as carrier gas with a flux of 20 ml/min. The temperature of the detectors in both channels is

150 ºC, and in the oven, is 25 ºC. CO_2 was analysed by potentiometric titration with an automatic titrator from Radiometer Copenhagen, model VIT90 Video Titrator using HCl 0.1 N as titrant and H_2S by colorimetric titration with mercury acetate 0.001 M as titrant, using dithizone for end point detection. For the determination of methane content a simple percentage where calculated by the mean of duplicate bioreactors moles of CH_4 comparing to the other gases obtained from the CG results. For the determination of BMP results by bioreactor, the mean of cumulative CH4-COD equivalents (mg COD-CH_4) was calculated based on the mmoles of CH_4, according to the formula C in table E in annex. These values were used to calculate cumulative BMP that was expressed in L CH_4/ Kg VS added. The values of VS at the end of pretreatments were used for each waste. The formula used for cumulative BMP calculation is formula D in table E in annex. These formulas and standard equivalents calculations where based on previous determination made by Neves (2009).

2.6. | BACTERIAL COMMUNITY PHYLOGENETIC ANALYSIS

2.6.1. | Total genomic DNA extraction

For the bacterial community consortia characterization, the technique of 454 pyrosequencing was used contracting as service in the Next Gen sequencing unit (GENOINSEQ) at Biocant Park, Cantanhede, Portugal. For the preparation of genomic DNA from the consortia we first proceeded to the genomic DNA extraction and checked the qualitative and quantitative validation of gDNA. The DNA was then amplified with Universal bacterial primers (16s_8F: 5-AGA GTT TGA TCC TGG CTC AG -3, 16S_1492R: 5-CGG TTA CCT TGT TAC GAC TT-3) according to (Tuner et al., 1999) for the 16S SSU rRNA gene sequence to confirm the gene amplification before pyrosequencing. The DNA extraction was done from three independent replicates of each consortia. Total community gDNA was isolated applying the PowerMax^{TM} Soil DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, USA) for isolation of genomic DNA from all three consortia. The kit is prepared to remove PCR inhibiting compounds, including humic substances, and the brown color often associated with soil DNA. The kit is ideal for environmental samples that contain high humic substance content, including compost, sediment and manure that was the case of the wastes in which we enriched the consortia. Microorganisms were lysed by a combination of SDS and further disruption agents, and mechanical force using beads.

Approximately 2 g of the consortia culture centrifuge pellet and waste residues were collected when the sCOD reached the highest values during pretreatments and used for DNA extraction. The DNA was eluted in 2 ml sterile water and quantified by using a spectrophotometer at a wave length of 280 nm. To confirm the integrity of gDNA a 12 µl of total gDNA was added to the wells of an 0,8% (w/v) agarose gel and electrophoresis run at 100 V 30 minutes (Figure A and B in annex). The following reaction mixtures were used for amplification: 2.5 µl 10x reaction buffer, 2 mM MgCl2, 0.2 mM dNTPs, 0.2 µM each primer, 0.75 U Taq DNA polymerase (Biotaq™ DNA Polymerase, Bioline Ltd, U.K), 5 µl

DNA and water to a final volume of 25 µl. The PCR conditions consisted of an initial denaturation step at 95 ºC for 3 minutes followed by 30 cycles of amplification at 95 ºC for 30 s, the annealing temperature 50 ºC and was done for 30 s, the polymerization at 72 ºC for 1 minute and a final extension step at 72 ºC for 7 minutes. Before gDNA was send to pyrosequencing the confirmation for the amplification of 16S rRNA gene was checked in a 1% (w/v) agarose gel and electrophoresis run at 100 V 30 minutes (Figure C in annex).

2.6.2. | 16S rRNA gene analysis of microbial communities

2.6.2.1. | 454 pyrosequencing PCR

Samples were prepared for 454 pyrosequencing by PCR amplification of the V3V4 hypervariable region with fusion primers containing the Roche-454 A and B Titanium sequencing adapters, an eight-base barcode sequence, the forward primer 5'– ACTCCTACGGGAGGCAG-3' and the reverse primer 5'– TACNVRRGTHTCTAATYC -3' (Wang & Qian, 2009). PCR reactions were performed in 40 µL reactions with Advantage Taq (Clontech) using 0.2 µM of each primer, 0.2 mM dNTPs, 1X polymerase mix and 6% DMSO. The PCR conditions were 94 ºC for 5 minutes followed by 30 cycles of 94 ºC for 30 s, 44 ºC for 45 s and 68 ºC for 60 s and a final elongation step at 68 ºC for 10 minutes The amplicons were purified with Agencourt AMPure XP and quantified by fluorimetry with PicoGreen (Invitrogen, CA, USA), pooled at equimolar concentrations and sequenced in the A direction with GS 454 FLX Titanium chemistry, according to manufacturer's instructions (Roche, 454 Life Sciences, Brandford, CT, USA) at Biocant (Cantanhede, Portugal).

2.6.2.2. | 16S rRNA gene microbial community taxonomy

The raw pyrosequencing reads were processed using Metabiodiverse, an automatic pipeline implemented at Biocant. In a first step, sequencing reads were assigned to the appropriate samples based on the respective barcode. Reads were then, quality filtered to minimize the effects of random sequencing errors by elimination of sequence reads with <150 bp and that contained more than two undetermined nucleotides. Sequences in which the reverse primer was reached were additionally cut. Finally, sequences with more than 50% of low complexity regions, determined by DustMasker (Sogin et al., 2006) and chimera sequences, identified by UChime (Edgar, 2011), were discarded. The sequences were grouped by USearch (Edgar, 2010) according to a phylogenetic distance of 3%, creating the Operational Taxonomic Units (OTUs). Richness of population (rarefaction curves) and the diversity indices (Chao1) were calculated using the Mothur package (Schloss et al., 2009). The taxonomy of each OTU was identified through a BLAST search against the Ribosomal Database Project II (RDP) database (Cole et al., 2008). The best hits were selected and subjected to further quality control. All sequences with an alignment of less than 40% as well as those with an Evalue greater than $1e^{-50}$ were rejected. Additionally, a bootstrap test was applied to the OTUs to identify the least common taxonomy level. Only the sequences with a bootstrap greater than 70% after 100 replicates, as obtained by seqBoot from Phylip package (Felsenstein, 1989), were kept. The taxonomic assignment of the OTUs was completed with the attribution of the NCBI taxonomy identification number, which allowed the complete taxonomy construction of all identified organisms. Finally, for each taxon identified in the sample, the total number of sequences was summed up, providing the abundance of all identified organisms, for population statistics analysis.

2.7. | ISOLATION AND MOLECULAR IDENTIFICATION OF HYDROLYTIC ACTIVE COLONIES

2.7.1. | Bacterial isolation

For Bacteria isolation from the consortia, specific agar plates for enzyme detection (section 2.2.4) were used by spreading 200 µl of consortia culture in the plates. Colonies with activity and nearby colonies were isolated in agar plates. After isolation, colonies that maintained growth were screened for enzymatic activity by growing directly in enzyme agar plates during 24 to 48 hours the colonies presenting the highest activities were selected for molecular characterization.

2.7.2. | Isolates molecular characterization

Genomic DNA was extracted according to previously described methods (JuarezPerez et al., 1997). DNA extraction was done by freeze-thawing thermic shock some plated colonies with 24-hour incubation of isolates were re-suspended in H2O aliquot of 1000 µl in Eppendorf tubes and freeze at -80 °C until ice formation. The samples were thaw by a thermic shock at 90°C in a boiling water-bath for 5 minutes. Cell debris was removed by centrifugation for 5 minutes at 13 000 r.p.m. and the supernatant was used directly for PCR. The following reaction mixtures were used for amplification: 2.5 µl 10x reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM each primer, 0.75 U Taq DNA polymerase (Biotaq™ DNA Polymerase, Bioline Ltd, U.K), 5 µl DNA and water to a final volume of 25 µl. The PCR conditions consisted of an initial denaturation step at 95 ºC for 3 minutes followed by 30 cycles of amplification at 95 ºC for 30 s, the annealing temperature 50 ºC and was done for 30 s, the polymerization at 72 ºC for 1 minute and a final extension step at 72 ºC for 7 minutes. Subsequently, the PCR products were purified by employing the GeneJET PCR Purification Kit (Thermo Scientific) and quantified spectrophotometrically. Purified PCR products were sequenced (Stabvida, Portugal) and the identity of the consensus sequences was checked using BLAST (Altschul et al., 1990).

2.8. | STATISTICAL ANALYSIS

Data was submitted to single factor analysis of variances (ANOVA) was used to determine if significant differences existed between results obtained under different experimental procedures. Statistical significance was established at a $p < 0.05$ level analysis were done with software SPSS version 20.0 (IBM corp. 2011).

3. | RESULTS

3.1. | CONSTRUTION OF A BIOBANK WITH AEROBIC THERMOPHILIC CONSORTIA (ATBCB)

3.1.1. | Enrichment

During the enrichment process and once a week the data relative to the bacterial growth in liquid and solid medium was registered (Figure 10 and Table 5). For the enrichment process, different substrates (DMS, SSS, grass mixtures and feathers) of interest related to biogas production were used.

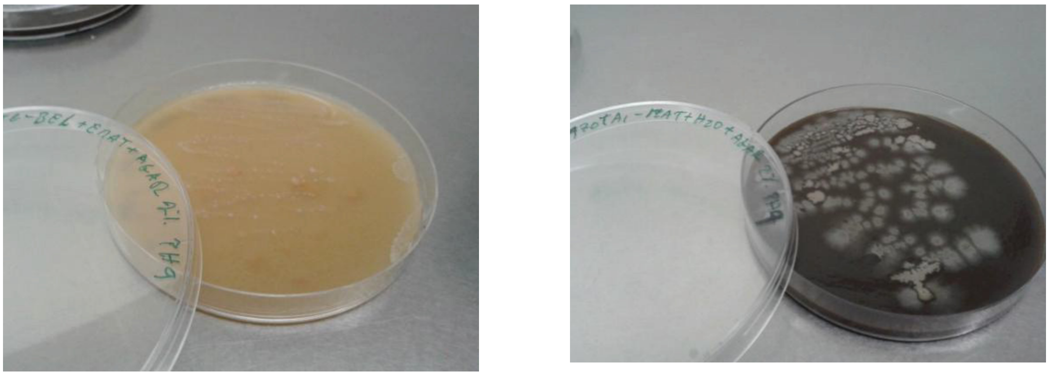


Figure 10. Examples of confirmation of growth in solid medium with sewage sludge substrate.

Each substrate was manipulated to perform the enrichment of bacterial samples at pH 3, 7 and 9, resulting in a total of 33 bioreactors. The enrichment process was done along twelve weeks, with weekly successive passages to a fresh medium. Observation of bacteria in liquid medium and solid medium were registered. Also, enzymatic activity was screened in interval periods of 4 weeks.

We considered that at the end of each passage we end up with a unique consortium. The twelve successive passages were done for the establishment of a stable consortium, therefore, at the end of each week we stored part of the consortium at -80 ºC in 20% of glycerol and at room temperature before inoculating in a fresh medium for the next passage.

3.1.1.1. | Bacterial growth monitorization

During the twelve week of enrichment the bacterial growth of each consortia was monitored weekly. Observation under the microscope of the bioreactors with liquid medium and wastes revealed that most of the consortia presented growth in bioreactors with pH 9 and 7, although bioreactors with pH 3 the bacterial growth was lower or absent (Table 5 and Table A in annex). The consortia (Azotb1 to Azotb34, Azotb2 to Azot35, Azotb3 to Azotb36, Azotm1 to Azotm34, Azotm2 to Azotm35 and Azotm3 to Azotm36) enriched in bioreactors with sewage sludge (DMSS and SSS), presented growth in all pH from the beginning to the end of enrichment. In sewage sludge bioreactors, the decomposition effects were more difficult to determine visually, so we proceeded to the enrichment, and at the end of the twelve weeks the decomposition rate of the consortia was done by sludge residue dry weight.

The consortia enriched in grass mixtures also presented growth along the enrichment period in all pH tested. Although bioreactors at pH 7 and 3 (consortia Azotdp1 to Azotdp34 and Azotdp2 to Azotdp35) presented growth, grass mixtures decomposition was more visible after the second week, in consortia Azotdp6 to Azotdp36 at pH 9.

The consortia enriched with feathers presented different growth. In bioreactors with pH 3 (Azotf1 to Azotf34) no bacterial growth was detected. In bioreactors with pH 7 bacterial growth was observed only in the first 5 weeks of enrichment (consortia Azotf2 to Azotf14). The consortia Azotf3 to Azotf36 from pH 9, were the only that presented growth and feathers decomposition after the second week of enrichment.

The bacterial growth in the other substrates (Table 5) was observed in all pH until they were stored, the enrichments for these substrates were stopped earlier as they had availability of nutrients and were not difficult to hydrolysate, nonetheless they were a good source of bacteria and did not require a long period of enrichment. An exception was the consortia enriched in chitin (consortia Azotc1 to Azotc7, Azotc2 to Azotc8 and Azotc3 to Azotc9) that did not grow in all the pH tested after the third week of enrichment.

At the end of the enrichment the main selected wastes DMSS, SSS, grass mixtures and feathers presented at least one consortia with growth, allowing us to assess the decomposition rate of each one at the end of the enrichment process.

The results of bacterial growth in solid medium (Table 5 and table A in annex) showed that when plating a consortium that presented growth in liquid enrichment medium not always bacterial could grow in the solid medium. This data reveals that it can be difficult to isolate bacteria from these consortia using the traditional methods.

Table 5. Bacterial growth during the process of enrichment and construction of consortia (full results table A in annex).

Consortium	pH	Liquid medium	Substrate	Growth in Liquid medium	Growth in solid medium
Week 1					
Azotb1	3	AEMAC	DMSS	+	+
Azotb2	7	AEMAC	DMSS	+	+
Azotb3	9	AEMAC	DMSS	+	+
AzotbH1	3	H_2O	DMSS	+	+
AzotbH2	7	H_2O	DMSS	+	+
AzotbH3	9	H_2O	DMSS	+	+
Azotm1	3	AEMAC	SSS	+	+
Azotm2	7	AEMAC	SSS	+	+
Azotm3	9	AEMAC	SSS	+	+
AzotmH1	3	H_2O	SSS	+	+
AzotmH2	7	H_2O	SSS	+	+
AzotmH3	9	H_2O	SSS	+	+
Azoto1	3	AEMAC	Oilve oil	+	-
Azoto2	7	AEMAC	Oilve oil	+	+
Azoto3	9	AEMAC	Oilve oil	+	+
Azotdp1	3	AEMAC	Grass mixtures	+	-
Azotdp2	7	AEMAC	Grass mixtures	+	+
Azotdp3	9	AEMAC	Grass mixtures	+	+
Azotf1	3	AEMAC	Chicken Feathers	-	-
Azotf2	7	AEMAC	Chicken Feathers	+	+
Azotf3	9	AEMAC	Chicken Feathers	+	+
Azotc1	3	AEMAC	Chitin	+	-
Azotc2	7	AEMAC	Chitin	+	-
Azotc3	9	AEMAC	Chitin	+	+
Azots1	3	AEMAC	starch	+	+
Azots2	7	AEMAC	starch	+	+
Azots3	9	AEMAC	starch	+	+
Azotp1	3	AEMAC	Skim Milk	+	+
Azotp2	7	AEMAC	Skim Milk	+	+
Azotp3	9	AEMAC	Skim Milk	+	+
Azotn1	3	Nutrient broth	Nutrient broth	+	+
Azotn2	7	Nutrient broth	Nutrient broth	+	+
Azotn3	9	Nutrient broth	Nutrient broth	+	+

3.1.1.2. | Enzymes

For the detection of supernatant enzymes from the consortia, filtered supernatant were used in agar plates using the agar diffusion technique (Figure 11) as described in material and methods section.

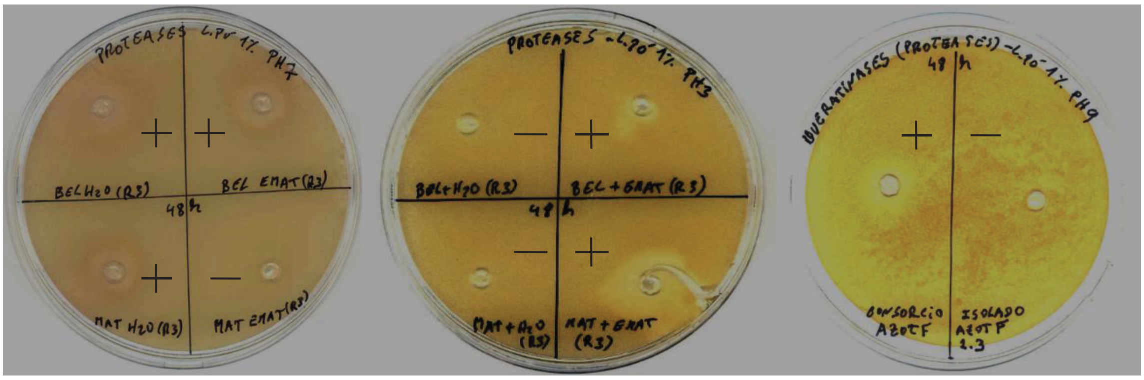


Figure 11. Examples of proteases detection using supernatant of the consortia, incubation temperature 60 ºC during 48 hours.

We optimize and perform enzymatic detection of thermostable enzymes in the supernatants that were monitored at the end of 4 consecutive passages. The results obtained where recorded for positive or negative presence of proteases, lipases, cellulase, xylanase and amylases enzymes.

In what concerns the consortia enriched in DMSS and SSS, proteases were detected in enrichment bioreactors at the pH 9, 7, 3 up until the end of enrichment (Table 6 and Table B, C and D in annex). The consortia enriched in grass mixtures bioreactors at pH 9 produced cellulases and xylanases. The consortia enriched at pH 7 produced only xylanases during enrichment process. For the consortium enriched at pH 3 cellulases or xylanases were not detected at any time of enrichment process (Table 6 and Table B, C and D in annex).

In consortia enriched in feathers substrate that presented growth bioreactors at pH 7 and 9, proteases were present in the first four weeks of enrichment, after ward bioreactors in pH 7 lose bacterial growth and only consortium enriched at pH 9 presented growth and the proteases up until the end of enrichment assay (Table 6 and Table B, C and D in annex). For the consortia enriched in olive oil in pH 7 and 9, (except for pH 3), presented growth and protease activity up until the end of enrichment. The consortia enriched in skim milk, starch and nutrient broth in all pH, presented growth and protease activity during the 4 weeks of enrichment. The consortia enriched in chitin substrate presented growth during the first two weeks for pH 7 and 9, in pH 3 no growth was register from the beginning. After the second week bacteria lost growth and no enzymes were assayed for this consortium.

Table 6. Registration sheets for consortium enzymatic activities during the process of enrichment and construction of consortia for bioreactors of pH 9 (full results Table B, C and D in annex)

Consortium	Liquid medium	Substrate	Enzymes (pH 9)			
			protease	cellulase	xylanas	amylase
Week 4						
Azotb12	AEMAC	DMSS	+	ND	ND	-
AzotbH12	H_2O	DMSS	+	ND	ND	-
Azotm12	AEMAC	SSS	+	ND	ND	-
AzotmH12	H_2O	SSS	+	ND	ND	-
Azoto12	AEMAC	Olive oil	+	ND	ND	-
Azotdp12	AEMAC	Grass	+	+	+	-
Azotf12	AEMAC	Feathers	+	ND	ND	ND
Azotc12	AEMAC	Chitin	ND	ND	ND	ND
Azots12	AEMAC	starch	+	ND	ND	+
Azotp12	AEMAC	Skim milk	+	ND	ND	-
Azotn12	H_20	Nutrient broth	+	ND	ND	+

This information was used also as a criterium for the selection of the best hydrolyzing consortia in the degradation of sewage sludge feathers and grass mixtures that were used in the current work.

To ensure that our bacterial consortium was from the bacteria seeded form the inoculum of the hot springs, tyndallization was done to all the substrates in the enrichment bioreactors. In figure 12 we can see an example of two enrichment bioreactors after passage three, number one is the control not presenting bacterial growth and number two is the inoculated with hot spring bacteria. During this enrichment process the consortia that didn’t lose growth in liquid medium during the enrichment process of twelve weeks, maintained the enzymatic activity for the tested enzyme

Figure 12. Example of enrichment by the end of third passage. 1) control-medium AEMAC + SSS at pH 3. 2) Azotm3- AEMAC + SSS at pH 3 with sample inoculum of hot springs.

3.1.2. | The Biobank

To build the Biobank with thermophilic consortia and bacteria we used for procedures in accordance to biobank collections of the Leibniz institute-DSMZ German collection of microorganisms and cell cultures (Spring, 2006). The BioBank, AZOREAN THERMOPHILIC BACTERIA COLLECTION BIOBANK (ATBCB) located at Department of biology and belongs to the Center of Biotechnology of Azores (CBA). This BioBank is composed with a total of 250 consortia and 70 isolated bacteria.

3.1.3. | Aim of Biobank Construction

The physical BioBank ATBCB was created to ensure the storage and preservation of consortia and bacteria discovered during the current work. The BioBank is composed with a total of 250 consortia and 70 isolated bacteria that are characterized for their capability of producing thermostable enzymes for hydrolysis of different kinds of wastes. The activities and future potential applications in the pre-treatment of waste or other biotechnological applications is the main aim of the construction of this biobank. Although, in the current work we used the bacterial consortia for the pretreatment of wastes to enhance biogas production, these organisms and their activities may have several other applications that do not fit within the scope of this project being an asset for future research and knowledge of microbial diversity in Azores.

3.1.4. | Remarks

For the first-time bacteria from hot springs of Furnas were used to perform enriched consortia in twelve substrates. We enriched consortia that could grow and release enzymes that enable waste biotransformation in DMS, SSS, grass mixtures and feathers.

In substrates from DMS and SSS bacterial growth was present during the all enrichment process, also proteases were present during the enrichment. At the end of the process, the six consortia (Azotb34, Azotb35, Azotb36, Azotm34, Azotm35 and Azotm36), that maintained growth were test for their efficiency of sludge decomposition and one was selected for further assays. All the other 66 consortia that present growth during the process were stored. The sludge substrate was one of the substrates that presented bacterial growth in all the three-pH revealing that bacteria can extract nutrient from this waste, allowing them to maintain growth through all the twelve weeks of enrichment.

Concerning the grass mixtures substrates, growth was present in all consortia enriched at the three substrates during all the enrichment process but only in the consortium Azotdp36 at pH 9, cellulase and xylanase activities were maintained up until the end of the process. The three consortia Azotdp 34, Azotdp35 and Azotdp 36 where was also selected for further evaluation grass mixtures decomposition.

For the consortia enriched in feathers substrate, only Azotf36 enriched at pH 9 presented growth, proteases activity and feather decomposition up until the end of enrichment assay. This consortium was used for further studies on feather decomposition and hydrolysis efficiency.

The biobank is constituted with the 10 consortia selected to be used in this work and the 240 consortia that maintained growth and were not used in the present work were stored in the ATBCB. The 70 isolates resulted from the isolation of bacteria from the consortium Azotm36, Azotdp36, and Azotf36, to characterize the bacteria and the production of thermostable enzymes in these three consortia.

The construction of the ATBCB Biobank was built to insure the preservation of used consortia and bacteria used in the present work. It also as a great value as several other bacteria and consortia presented thermostable enzymes being the Biobank a deposit of bacterial consortia and isolates that may be used in future research projects.

3.2. | ENHANCING ANAEROBIC DIGESTION OF SEWAGE SLUDGE USING A THERMOPHILIC MICROBIAL CONSORTIUM PRETREATMENT

As we know, large amounts of waste activated sludge, containing organic and mineral components, are produced by municipal and industrial waste water treatment plants. The sewage sludge hydrolysis and solubilization is an important step for the improvement of the AD. Solubilization methods like mechanical, physical, chemical and biological have been described and tested in both laboratory and full scale experiments (Odegaard, 2004). In the hyperthermy (40–60 ºC), thermophilic aerobic process we can find hot-stable enzymes produced by the thermophilic bacteria in sewage sludge that help to dissolve the insoluble organic substances. Since over 50% of organic materials inside sludge is protein and the protein hydrolysis is determined as the limiting step for sludge digestion. Increasing the Proteolytic enzyme activity and enhancing protein hydrolysis can promote the sewage sludge AD ability effectively (Bomio et al., 1989).

The enhancement of AD by either endogenous enzymes or added industrial enzymes has been demonstrated. Application of enzymes has been shown to improve sludge reduction as well as biogas production (Wawrzynczyk et al., 2003). These wastes can be pretreated and used as a feedstock for biogas production providing nutrients for anaerobic microorganisms. In this work, we investigated the efficiency of an aerobic thermophilic bacterial consortium

(ATBC) for the hydrolysis and solubilization of sewage sludge to obtain soluble nutrients, thus facilitating digestibility and enhance methane production.

3.2.1. | Selection of a bacterial consortium for sewage sludge hydrolysis

Before selection we compared six consortia enriched in sewage sludge substrates from two different sources, a diary milk industry and a slaughterhouse. The total dry weight loss, and presence of enzymatic activity was used as a criteria selection.

The highest dry weight loss was achieved with the consortia enriched in sewage sludge at pH 9 in both substrates. Consortium Azotm36 enriched in slaughterhouse sludge substrate showed dry weight loss of 30.35% which was 1.8 times higher than consortium (Azotb36) with 16,67%. Enrichments made in pH 3 returned the lowest dry weight loss in both substrates and the consortia enriched in pH 7 achieved 17.86% and 5.65% of solubilization in dairy and slaughterhouse sludge substrates respectively (Figure 13). Statistically no differences were find between the consortia enriched in pH 3. Although the consortia enriched at pH 7 were statistically different form each other. The selection of our consortium, was based on its hydrolysis efficiency when compared with the other consortia at enriched at different pH. Also, there was a statistically difference with $p < 0.05$ (DF=17.5 F= 52.49, sig=0) when comparing consortium Azotm36 with the other consortia using a mean computation of dry weight loss with a Tukey multiple range test.

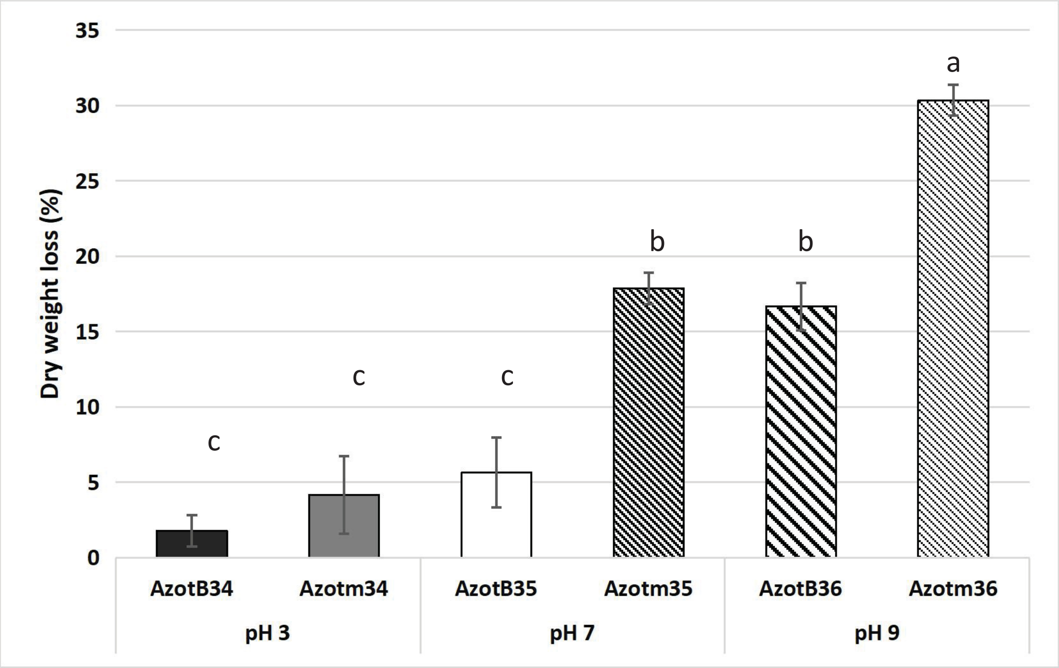


Figure 13. Dry weight loss percentage of consortia enriched in sewage sludge from dairy milk industry and slaughterhouse at 3 different pH. Values represented are subtraction to controls. The mean values represented by the same letters indicate non-significant differences in dry weight loss between the consortia P≥0.05 (Tukey multiple range test)

The stability of the consortium Azotm36 was demonstrated by its ability to maintain hydrolysis after tolerating several rounds of subculture in AEMAC medium with slaughterhouse sewage sludge substrate for more than 2 years and to maintain activity after being stored at room temperature or frozen at -80 ºC in 20% glycerol for periods of more than 6 months.

Other factor of selection was the presence of enzymes of interest in the supernatant. During enrichment process the detection of proteases, were accessed in agar diffusion technique, using the filtered supernatant obtained (Figure 14). The enzymatic assay was performed in all consortium enriched at the three-different pH 3, 7 and 9.

Figure 14. Proteases enzymes in the supernatant detected using agar diffusion technique

At the end of enrichment, we accessed the presence of two other enzymes lipases and amylases in the consortia enriched in DMS and SSS. The consortia enriched at pH 7 presented amylases and proteases lacking the present of lipases. The consortia enriched at pH 9 in both substrates presented all 3 tested enzymes in the supernatant being the Azotm36 consortium the one with the highest activities for the three enzymes (Table 7).

Table 7. Results of supernatant activity detection with agar diffusion technique, substrates used, starch, skim milk and tributyrin.

Substrate	Consortium	pH	Amylases	Proteases	Lipases
D.M.S	AzotB34	3	NDt	+	NDt
	AzotB35	7	+	+	-
	AzotB36	9	++	++	++
S.S.S	Azotm34	3	NDt	+	NDt
	Azotm35	7	+	++	-
	Azotm36	9	+++	+++	++

3.2.2. | Effect of consortium on sewage sludge during pretreatment

3.2.2.1. | Weight loss

At the end of pretreatment time (144 hours) we accessed the effect of consortium Azotm36 in the dry weight loss at three different concentrations 1% TS, 2.5% and 5% TS of slaughterhouse sewage substrates. The dry weight loss at concentrations of 1% TS and 5% TS presented the lower sludge weight losses when comparing with the concentration of 2.5% TS (Table 8). The effect of temperature (60 ºC) and agitation on sewage sludge dry weight loss was verified in the controls reactors. At the end of pretreatment reactors with the controls without the consortium Azotm36 were as follows; 5.4%, 10.23% and 14.81% for dry weight loss for concentrations of 1%, 2.5% and 5% respectively. In general, a temperature at 60 ºC and an agitation of 150 r.p.m., was higher at we increase the concentrations of sewage sludge substrate. In table 8 is presented the effect of the Azotm36 consortium on the percentage of dry weight loss at the end of 144 hours of pretreatment at three different sludge concentrations. To evaluated only the effect of our consortium in the sludge we subtracted the dry weight loss values of consortium with the respective controls. The consortium Azotm36 was most efficient at a concentration of sludge of 2.5% of TS achieving 34.7% of sludge dry weight loss. The efficiency of hydrolysis at 1% was 30.15% and at 5% of TS dropped to 9.89%. There was a statistically difference with $p< 0.05$ (DF=8 F= 58.66, sig=0). The lower hydrolysis in 5% concentration cloud be associated with the higher substrate content reducing available oxygen or the higher concentrations of toxic inhibitory bacterial growth factors present in sewage sludge.

Table 8. Dry weight loss of Azotm36 in three SSS concentrations, mean values presented are subtracted to the controls. The mean values and standard errors and standard errors represented by the same letters indicate non-significant differences in dry weight loss between the waste concentrations P≥0.05 (Tukey multiple range test)

Substrate concentration	Percentage of Weight loss
1% TS	30.15 ± 2 (a)
2.5% TS	34.66 ± 2 (a)
5% TS	9.88 ± 1 (b)

3.2.2.2. | sCOD and COD balance

To determine when to stop pretreatment and to minimize consumption by our consortium of soluble organic materials, soluble COD, COD loss and the rate of COD loss (Loss ratio) were monitored during pretreatment (Table 9).

The Soluble COD had similar fluctuation during pretreatment in all the three sewage sludge concentrations, soluble COD increased in supernatant up until reach 48 hours of pretreatment afterwards it started a progressive decline to the end of pretreatment. The highest soluble COD value where found after 48 hours at the concentration of 2.5% of TS of sludge with a concentration of 70 680 mg /L. The values for the concentrations of 1% TS and 5% TS where lower reaching the highest values of 30 400 mg/L and 59 600 mg/L, respectively. At the end of pretreatment, the values of Soluble COD add decreased mostly being consumed by the bacteria (Figure 15).

Before pretreatment start, the initial COD in the system was calculated as the sum of COD substrate and COD medium, which was equal to the sum of sCOD in hydrolysate, COD substrate residue and COD loss during pretreatment. This calculation allowed us to know the total COD consumed (COD loss) by our bacteria present in the consortium during the pretreatment (Table 9).

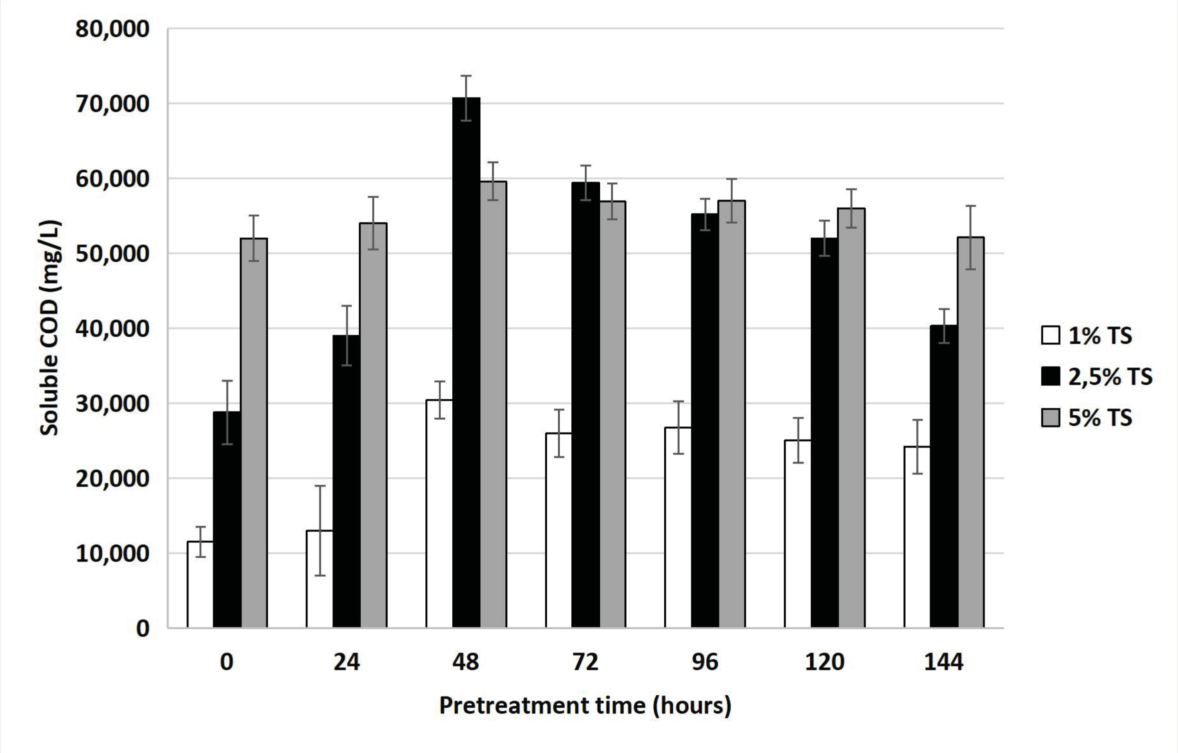


Figure 15. Soluble COD present in supernatant during pretreatment time in three sewage sludge concentrations inoculated with our consortium Azotm36

During the analysis of COD loss, it was found that longer pretreatment times, lead to higher organic materials loss. Indicating us that we should stop the pretreatment in early stages. This would minimize the consumption of organic materials during our consortium culture incubation and make them available for methane production.

At the end of pretreatment, the total COD loss were as follows; the concentration of 1% TS showed 29.9% of COD loss (24 200 mg/L), for 2.5% of TS an 34.2% COD loss (40 300 mg/L), and for 5% TS a COD loss of 9.6% (52 098 mg/L) (Table 8). The lower percentages of COD loss in 5% TS concentration associated when compared with 2.5% TS concentration, could be related to a lower hydrolysis efficiency of our consortia associated at high concentrations of substrate, that would lower the available oxygen in medium needed for our consortia efficiently hydrolyse.

During the first 48 hours, the COD loss was less accentuated in all 3 concentrations, simultaneous the soluble COD reached its peak table 8 This data indicates that in the first 48 hours, soluble organic materials were produced by hydrolytic microorganisms at a higher rate they were consumed by fermentative microorganisms of Azotm36. After the 48 hours, the COD loss raised until stabilize in the last two days of pretreatment, during this time these fermentative microorganisms were also consuming soluble materials but at higher rate than the production by hydrolytic ones.

Taking in consideration this data we selected to stop pretreatment based on two conditions, the first one was a higher soluble COD point and the second it should have a lower COD loss ratio. Meeting this two conditions, we selected the concentration of 2.5% TS at 48 hours of pretreatment as it reached the highest values of soluble COD 70 680 mg/L when compared to 1% TS and 5% TS, and had one of the lowest COD loss ratio of 22.52%, at this point.

Table 9.COD balance during pretreatment with Azotm36

	Before pretreatment			During pretreatment			
Substrate concentration (% TS)	COD substrate (mg/L)	COD medium (mg/L)	Pretreatment time (hours)	sCOD hydrolysate (mg/L)	COD substrate residue (mg/L)	COD loss system (mg/L)	Loss ratio system (%)
1	52348	11490	0	11490	52348	0	0
			24	13000	45848	4990	7.8
			48	30400	28148	5290	8.3
			72	25960	25948	11930	18.7
			96	26760	24448	12630	19.8
			120	25010	23348	15480	24.2
			144	24200	20577	19061	29.9
2.5	131036	28764	0	28764	131036	0	0
			24	39000	119536	1264	0.8
			48	70680	86636	2484	1.6
			72	59400	77636	22764	14.2
			96	55200	73536	31064	19.4
			120	52000	70086	37714	23.6
			144	40300	64835	54665	34.2
5	252396	52000	0	52000	252396	0	0
			24	54000	247196	3200	1.1
			48	59600	238696	6100	2.0
			72	56900	235196	12300	4.0
			96	57000	231196	16200	5.3
			120	56000	229546	18850	6.2
			144	52094	223000	29301	9.6

3.2.2.3. | Percentage of solubilization

The percentage of solubilisation in the pre-treatment, was calculated as the percentage of the initial COD added to the vials that was solubilized during the consortium pretreatment. We calculated the percentage of solubilization at 48 hours and at the end of pretreatment. For the calculations, we took in consideration the soluble COD that was lost by bacteria consumption based on the values of table 8.

Total solubilized COD at 48hours was 73 164 mg/L taking in consideration the already lost COD consumed by our bacteria of 2484 mg/L the pretreatment had a solublization percentage of 34% (Table 10). At the end of pretreatment, we achieved 51% of COD solubilization on slaughterhouse sewage sludge with our consortium Azotm36.

Table 10. Results of percentage e of solubilisation at 48 hours and at the end of pretreatment for the concentration of 2.5% TS.

	COD (mg/L) At 48 hours	**COD (mg/L) At the end of pretreatment**
sCOD−FinalPT	73 164	94 965
sCOD−inicialPT	28 764	28 764
CODtotal−inicialPT	131 036	131 036
PSprt%	34%	51%

3.2.2.4. | Soluble protein

During the pretreatment time the soluble protein was monitored. The effect of temperature and agitation on the sewage sludge soluble protein was considerable and during the pretreatment at each concentration. The soluble protein in the supernatant during the pretreatment time with the Azotm36 consortium were as follows; at 1% of TS concentration started at 2000 µg/ml of soluble protein reached a maximum 3890 µg/ml at 48 hours and progressively declined to the end of pretreatment. This shown that the pretreatment increased the soluble protein by 21.60% in the concentration of 1% TS when compared with the controls. The soluble protein for the pretreated 2.5% TS concentration initiated a concentration of with 3050 µg/ml of protein reaching a maximum of 5770 µg/ml soluble protein at 48 hours. These values when compared with the 2.5% TS controls represented an increase of 24% in soluble protein present in the supernatant. The soluble protein for the 5% TS concentration initiated with 4435 µg/ml of soluble protein reaching its peak at 72 hours of pretreatment with a concentration of 4990 µg/ml. when compared to the controls these values represented only an increase of 2% for the 5% TS substrate concentration. In figure 16 is presented the soluble protein release during Azotm36 consortium solublization of the sludge.

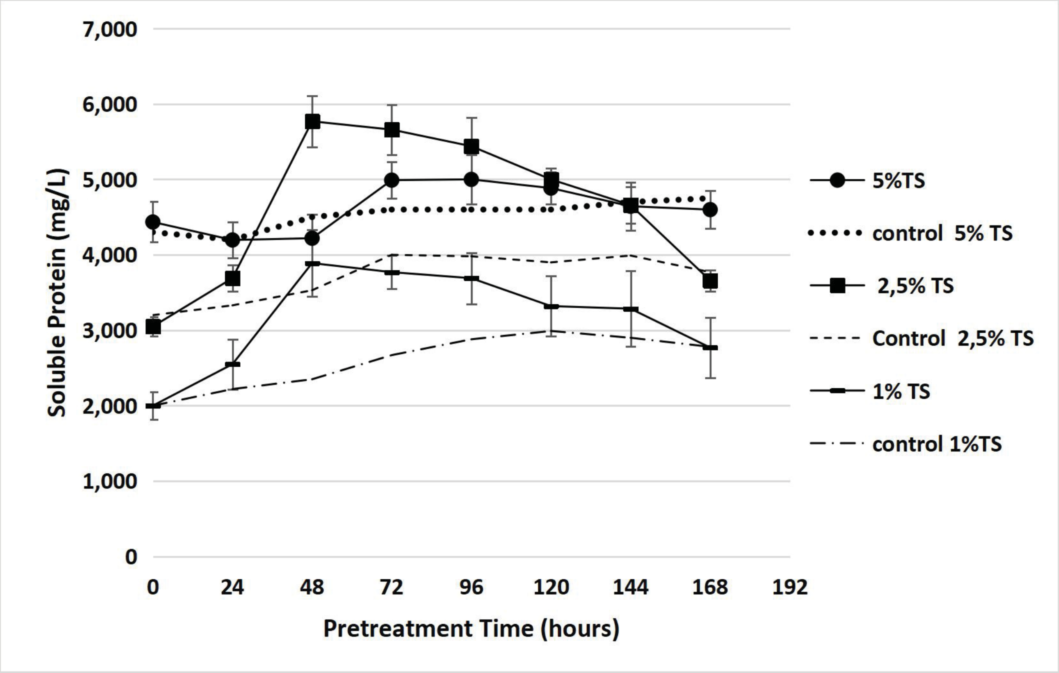


Figure 16. Effect of Azotm36 consortium pretreatment on soluble protein (mg/L).

3.2.2.5. | Enzymes in supernatant of consortia Azotm36

For the detection of enzymes in the hydrolysate supernatant, two tests were used an agar diffusion technique and a API ZYM test kit. The hydrolysate supernatant with 48 hours of pretreatment was tested for the presence of 21 different enzymes. The test results revealed that our consortium is capable of producing proteases and lipases as it could create clear zones in agar plates, 20 mm diameter in skim milk agar plates and 20 mm diameter for tributyrin agar plates.

Additionally, enzymatic activities were obtained in the results for of API ZYM test (Table 11). Our Azotm36 consortium was positive for the enzymes;

Alkaline phosphatase, Acid phosphatase, Phosphohydrolase, Esterase C_4, Esterase, Lipase C_8, Lipase C_{14}, Trypsin, N-acetyl-β-glucosaminidase, α-glucosidase α-mannosidase, α-fucosidase. The results for the not present enzymes were; Leucine amino-peptidase, Valine amino-peptidase, Cystine amino-peptidase, Chymotrypsin, α-galactosidase, β-glucosidase, β-galactosidase, β-glucoronidase. The chromogenic substrates differentiate between hydrolysis of C_4 esters (esterase activity) C_8 ester (ester lipase activity) and C_{14} ester (lipase activity). Usually hydrolysis of tributyrin correlates with the presence of lipases able to hydrolyse glycerides with C_4 fatty acids. Lipolytic thermophiles are found among different phyla of aerobic and anaerobic bacteria.

Table 11. Results of the enzymatic assays in agar plates and API ZYM test kit.

Enzyme assayed for;	**Substrate**	**Results**
Protease	Skim milk agar	+ (15 mm)
Lipase	Tributyrin agar	+(10 mm)
Amylase	Starch agar	+(10 mm)
Alkaline phosphatase	2 naphthyl-phosphate	+
Acid phosphatase	2 naphthyl-phosphate	+
Phosphohydrolase	Naphthyl AS-BI-phosphate	+
Esterase C_4	2 naphthyl-butyrate	+
Esterase Lipase C_8	2 naphthyl-caprylate	+
Lipase C_{14}	2 naphthyl-myristate	+
Leucine amino-	L-leucyl-2-naphthylamide	NDt
Valine amino-peptidase	L-valyl-2-naphthylamide	NDt
Cystine amino-	L-cystyl-2-naphthylamide	NDt
Trypsin	N-benzol-DL-arginine-2-naphthylamide	+
Chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamine	NDt
α-galactosidase	6-Br-2-naphthyl-α-D-galactopyranoside	NDt
β-glucosidase	6-Bromo-2-naphthol-α-D-galactopyranoside	NDt
N-acetyl-β-glucosaminidase	1 naphthyl-N-acetyl-β-D-glucosaminide	+
α-glucosidase	2 naphthyl-2-D-glucopyranoside	+
β-galactosidase	2 naphthyl-β-D-galactopyranoside	NDt
β-glucoronidase	Naphthyl-AS-BI-β-D-glucuronide	NDt
α-mannosidase	6-bromo-2-naphthyl-2-D-mannopyranoside	+
α-fucosidase	2 naphthyl-α-L-fucopyranoside	+

3.2.2.6. | *pH and volatile organic products changes*

The pH of Azotm36 hydrolysates for the three substrate concentrations of 1.0%, 2.5% and 5.0%, declined up until day 2 (Figure. 17). The lowest pH values occurred at 48h for the three substrate concentrations 1.0%. 2.5% and 5.0% of TS. The lowest pH value of the Azotm36 hydrolysates were 7.2, 7.6 and 8 for the concentrations of 1, 2.5% and 5%, respectively. The pH after 48h gradually increased in the three substrate concentrations reaching 8.6 in the 1% of TS and 8.6 and 8.5 in concentration 2.5% and 5% of TS respectively. When substrate concentration was 5% the pH hit the lowest values of 7.2. The decrease of pH values is directly correlated with the acidification of the medium related to the hydrolysis and VFA production.

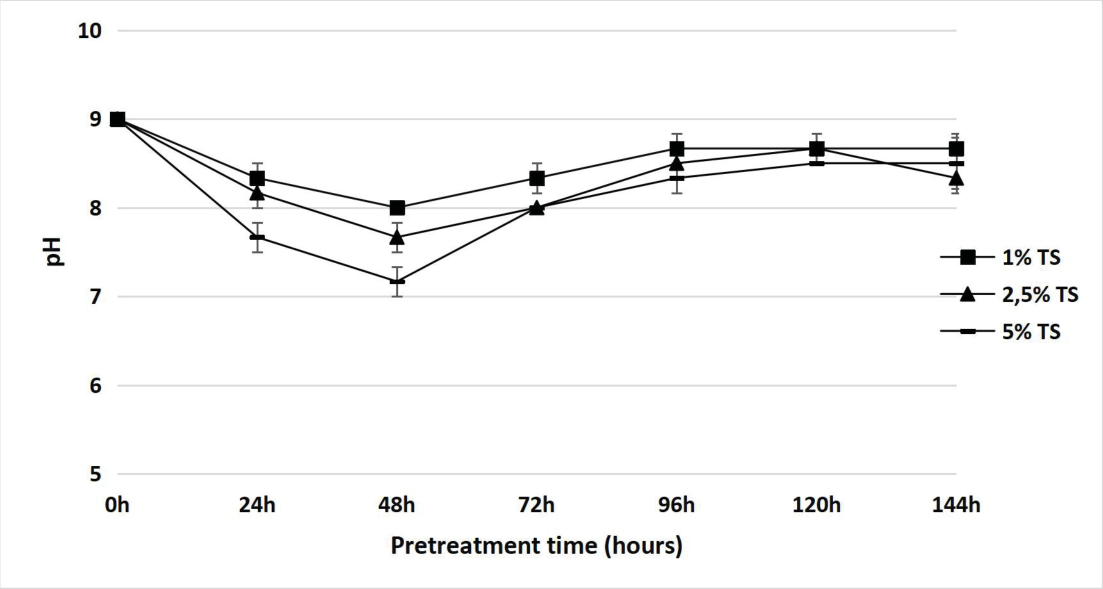


Figure 17. Monitorization of pH value during pretreatment

The monitoring of VFA during pretreatment was made with the previous selected 2.5% TS concentration of sewage sludge substrate, as it was the concentration tested in anaerobic assays.

The concentration of VFAs was registered during 120 hours and a general raise was registered in the first days of pretreatment in the reactors with the consortium Azotm36. At the beginning of pretreatment (0 hours), the concentrations of the three VFAs, in the bioreactors with the control and with our consortium Azotm36 were considered the same and as follows; Acetic acid (0.40 g/L), Propionic acid (0.24 g/L) and Butyric acid (0.47 g/L).

It was clear that the raise of VFAs and the initial high hydrolysis of the sewage sludge contributed for the decrease of the pH (Figure 18). Although after the 48 hours the pH started to raise, this could be in consequence of VFAs general decrease after 48 hours of pretreatment. Analyzing the production of acetic acid in bioreactors with the consortium Azotm36, we saw an increase in the first 48 hours reaching a maximum of 2.16 g/L. After ward acetic acid concentrations decrease till the end of the pretreatment, the concentration of acetic acid in the controls varied between 0.40 and 0.81 g/L during the pretreatments.

The concentration of Propionic acid had an increase during the first 48 hours, declining in the following 2 days and increasing in the last day. The concentrations of propionic acid varied between 0.24 and a maximum of 2.26

g/L, that was reached at 48 hours. The concentration of propionic acid in the controls were between 0.24 and 0.85 g/L, having similar variation as the concentration of propionic acid in the Azotm36 reactors. The other VFA analysed was butyric acid, it started with 0.47 g/L, reaching the maximum at 48hours with a concentration of 3.79 g/L. After 48 hours of pretreatment the concentrations of butyric acid decreased drastically and varied between 0.23 and 0.85 g/L to the end of pretreatments. Butyric acid was detected in the controls at concentrations values between 0.47 and 1.25 g/L stabilizing after 72 hours.

Beside the VFA concentrations was not the first option for the selection of pretreatment stop time, the maximum values for these three VFAs analysed were registered at 48 hours with 8.21 g/L, coinciding with the pretreatment stop time.

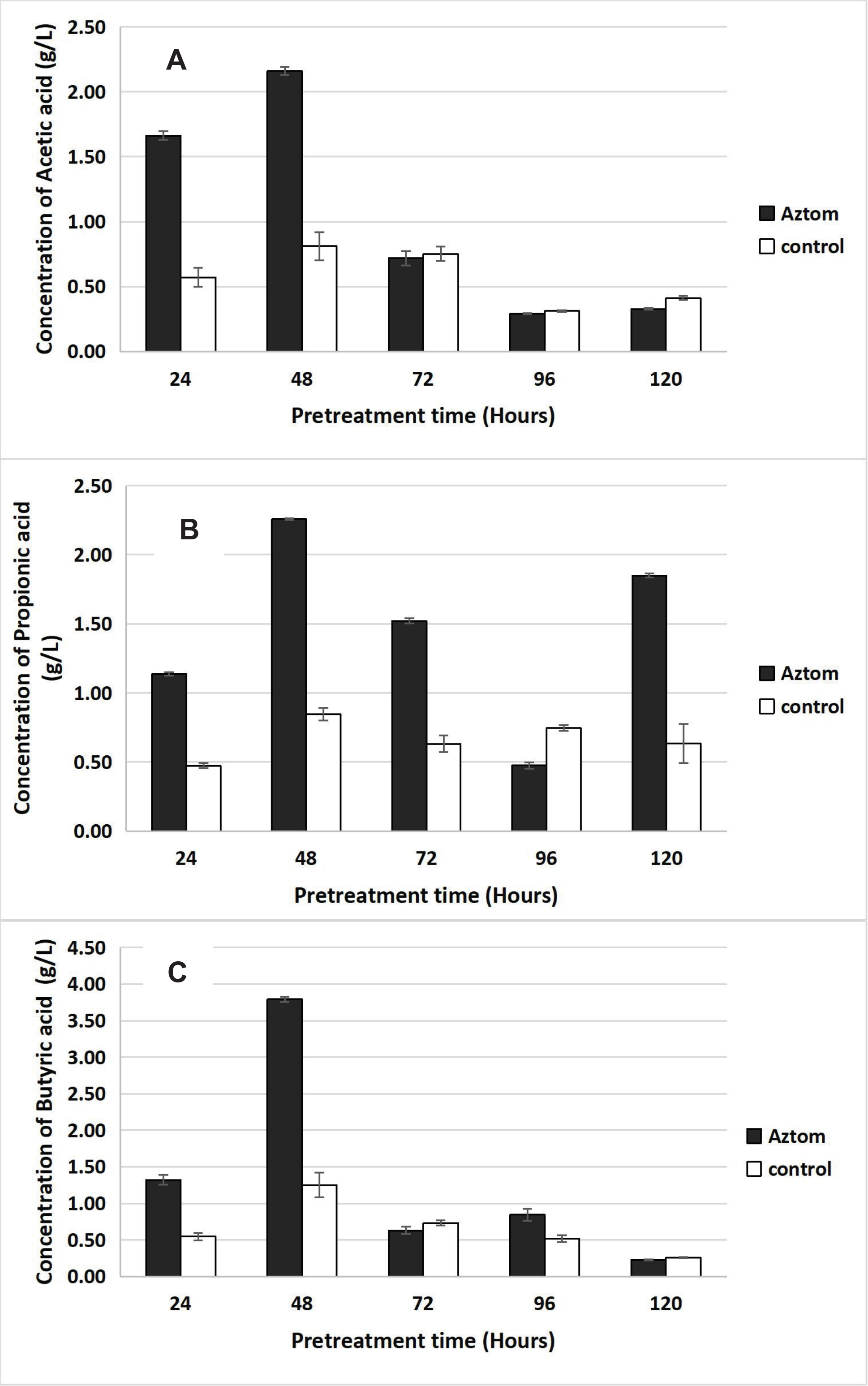


Figure 18. Concentration of VFAs during pretreatments. a) acetic acid, b) propionic acid, c) butyric acid

3.2.3. | Biogas production

3.2.3.1. | Biogas volumes

The total accumulated biogas yields for pretreated sewage sludge at 2.5% TS concentration was not different from the untreated sewage sludge at the end of AD. At the end of the AD bioreactors with pretreated sewage sludge resulted in the production of a total accumulated 1229 ml of biogas. The pretreated sewage sludge produced only 5.3% more biogas than untreated sewage sludge. The volume of biogas production in bioreactors with unpretreated sewage sludge were 1167 ml of total accumulated biogas (Figure 19.) This result shows that Azotm36 pretreatment did not enhance the biogas yields of sewage sludge.

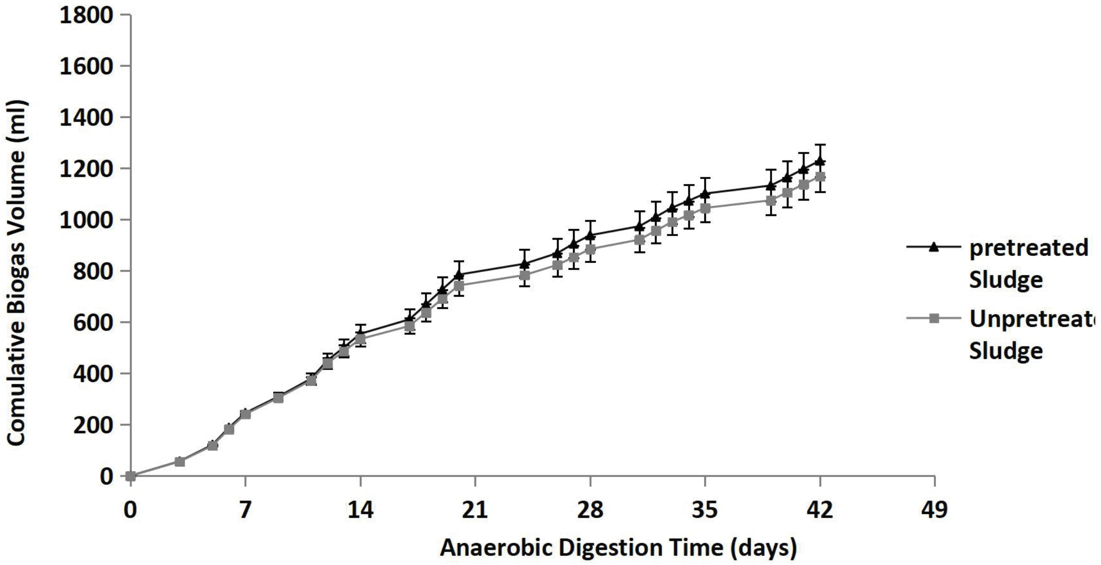


Figure 19. Total accumulated biogas volume during AD of pretreated and unpretreated sewage sludge

The daily accumulated biogas production in figure 20, revealed the days in which more biogas was produced. Analyzing the results, they show the same results as total cumulative biogas production and no differences where noticed in biogas volumes between pretreated and unpretreated sewage sludge anaerobic bioreactors.

These results indicate that our consortium pretreatment is not suitable for the enhancement of total biogas production at the concentrations and conditions tested, although, the production of methane could still be achieved.

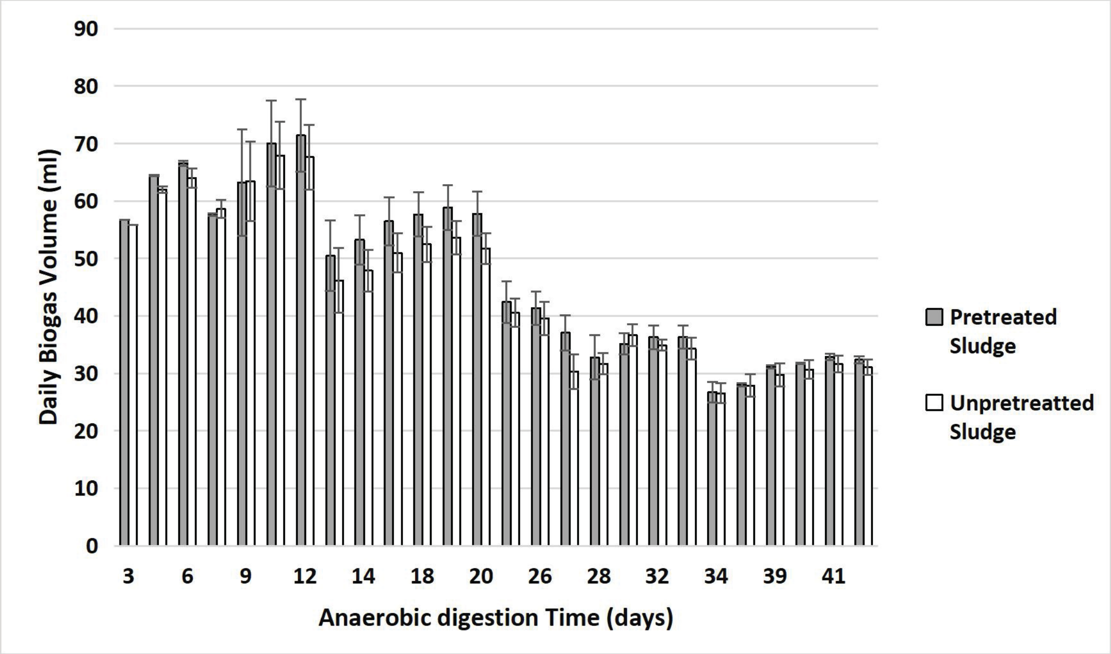


Figure 20. Daily Biogas volume in ml of pretreated and unpretreated sewage sludge

3.2.3.2. | Methane production

The methane content of pretreated sewage sludge was higher than the unpretreated sewage sludge between day 21 and day 28 representing 54% of the total methane produced in bioreactors with Azotm36 pretreated Sludge

(Figure 21). These results were not in frame with the total biogas production that did not shown differences in biogas production in that time interval.

At the end of the AD (day 42) the total methane content in bioreactors with pretreated sewage sludge was 16% higher than in the controls without sewage sludge pretreatments. This result reveals that beside the total biogas production did not varied, we could achieve an enhancement of methane content with the pretreatment of sewage sludge with Azotm36 consortium.

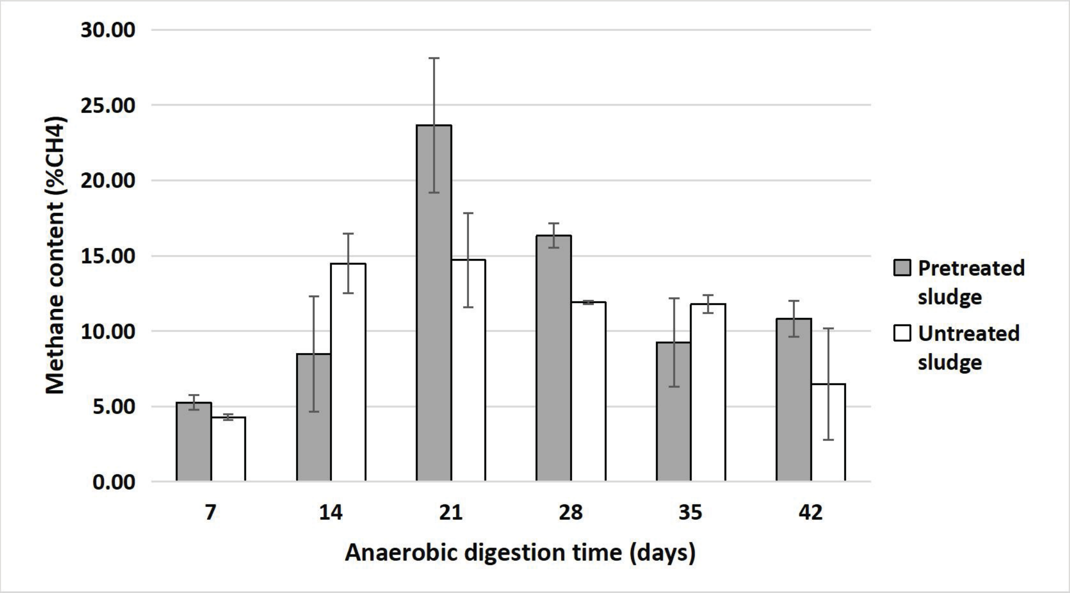


Figure 21. Methane content % in anaerobic bioreactors with and without sewage sludge pretreatment with consortia Azotm36.

The maximum biochemical methane potential was 200.98 LCH_4/Kg VS added, for the pretreated sludge with the consortium Azotm36. This pretreatment resulted in an increase of 20% more biogas production per kilo of volatile solids of sewage sludge added. The untreated sludge biochemical methane production was 167.16 LCH_4/Kg VS added (Figure 22). These values of methane were obtained when the sCOD reached the highest values at 48 hours of pretreatment with a concentration of 2.5% TS of sewage sludge.

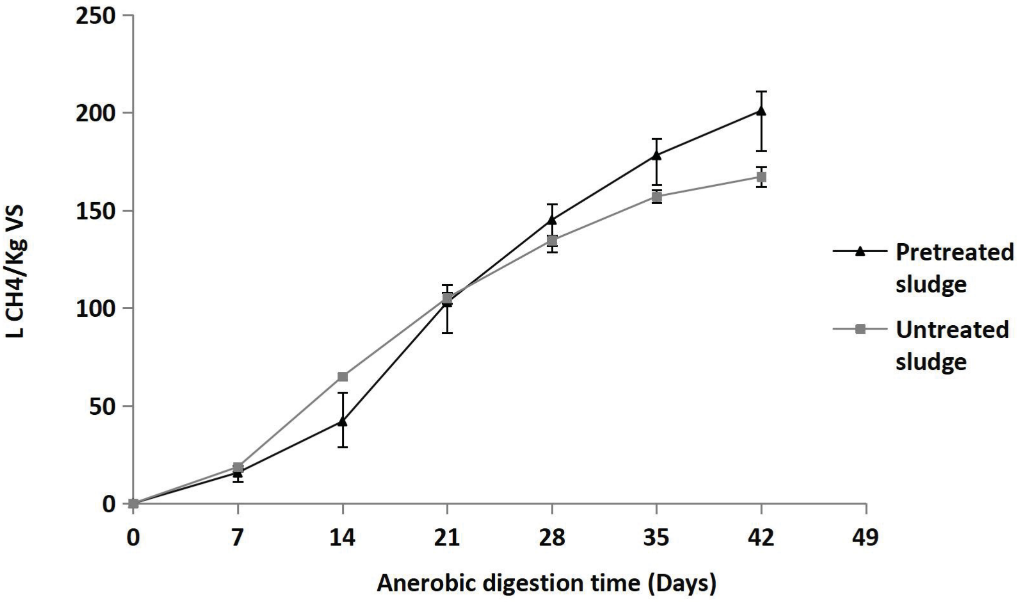


Figure 22. Biochemical Methane Potential curves for Azotm36 pretreated and unpretreated sewage sludge.

3.2.4. | Phylogenetic analysis of bacterial community

3.2.4.1. | Bacterial diversity and richness in Azotm36 consortium

Small-subunit (SSU) rRNA gene sequences from pyrosequencing and obtained OTUs was used to characterize the bacterial diversity and richness and estimated relative abundance of the microbial consortium Azotm36. The adequacy of the sample size for the determination of diversity within the 16S rDNA library was evaluated by rarefaction analysis. Expect as shown in figure 23, the calculated rarefaction curve was partially saturated at 3% divergence which indicates that the analysis covered the bacterial diversity present in the consortium.

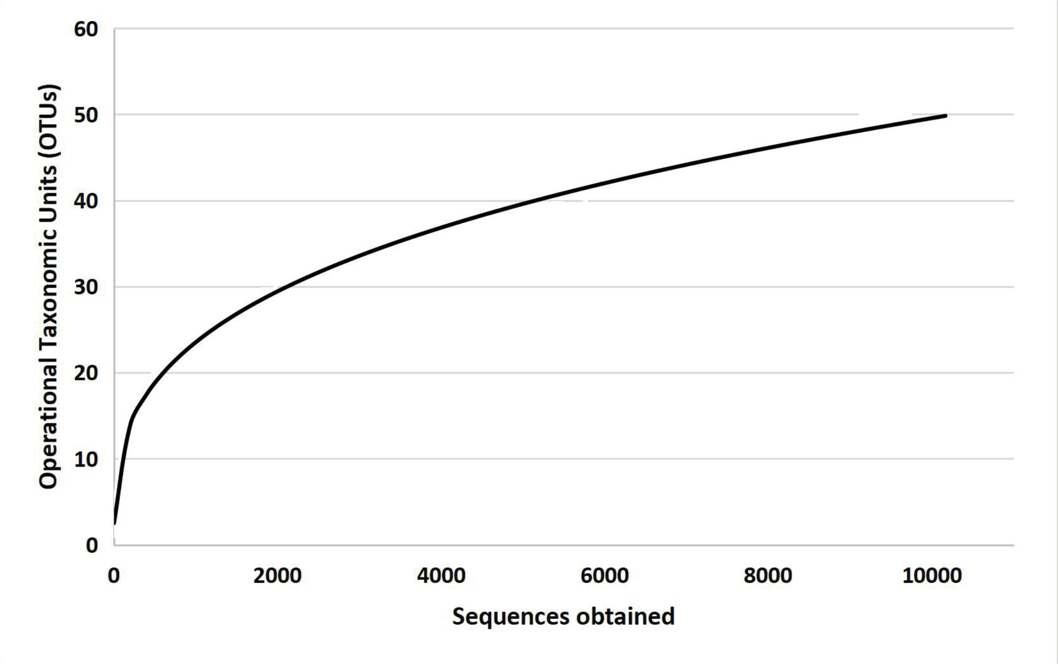


Figure 23. Rarefaction curves from 16S rRNA gene sequences of microbial consortia Azotm36. Sequences were clustered at a genetic distance of 3%. OTU values were calculated by the mean of replicates. Curve was based on a non-linear regression power model

Using Chao1 richness estimator, the maximum number of OTUs for the bacterial community expected at 3% genetic distance was 35.67, 47 and 78.33 for replicates D, E and F respectively (Table 12). The obtained OTUs in replicate was 34 covering 95.33% from the predicted chaos richness estimator of 35.67 OTUs. In replicate B, we obtained 35 OTUs, covering 74.47% from the predicted chaos results of 47. In the replicate C, we obtained 48 OTUs covering 61.28% from expected chaos result of 78.33 OTUs.

Table 12. Diversity indices Chaos results of the 3 replicates from Azotm36

Consortium	Sample	Gene regio	Chao expect	Chao obtained	Coverage %	Reads	OTUs
Azotm36	D	V3	35.67	34	95.33	7787	34
Azotm36	E	V3	47.00	35	74.47	5468	35
Azotm36	F	V3	78.33	48	61.28	10 159	48

3.2.4.2. Composition of the bacterial community in Azotm36 consortium

The 23 414 sequences were analysed in this study after trimming reads sequences of either short length or low quality and removing the chimeras, and selecting the reads without fail blast. The distribution of valid sequences in the replicates were as follows; replicate A with 7787 sequences, replicate B with 5 468 and replicate C with 10 159 sequences. The sequences were used to calculate relative abundance at phylum and genus level of the microbial community from Azotm36.

The relative abundance of populations in Azotm36 consortium were 99.84% belonging to the phylum *Firmicutes* and some bacteria belonging to the phylum *Proteobacteria* with a representation of 0.16% (Figure 24).

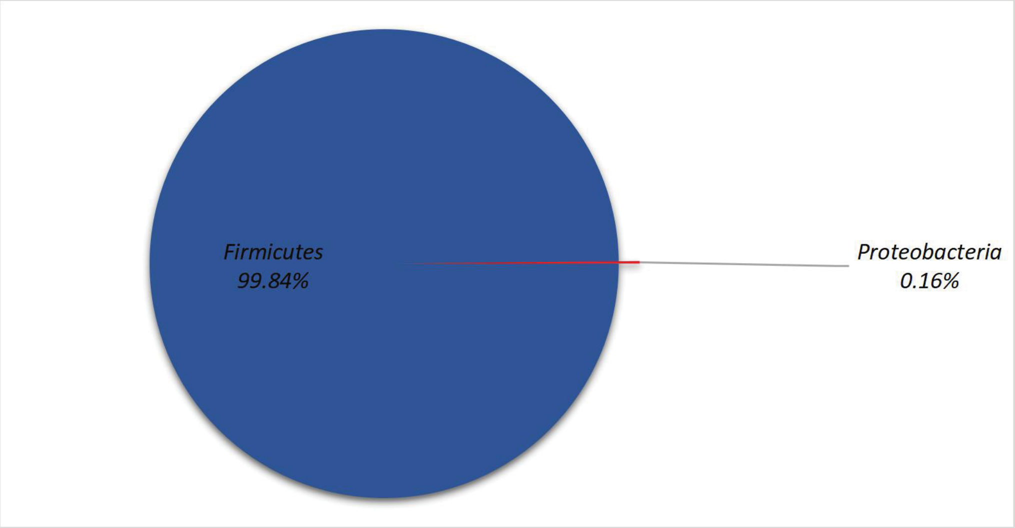


Figure 24. Percentage of mean relative abundance of sequences obtained in Azotm36 bacterial consortia at phylum level

Analyzing the 3 replicates the relative abundance was evenly distributed, for the phylum *Firmicutes* with 99.7% of relative abundance in the three replicates, the phylum *Proteobacteria* had a relative abundance of below 0.21% (Figure 25). The relative bacterial abundance bacterial populations in Aztom36

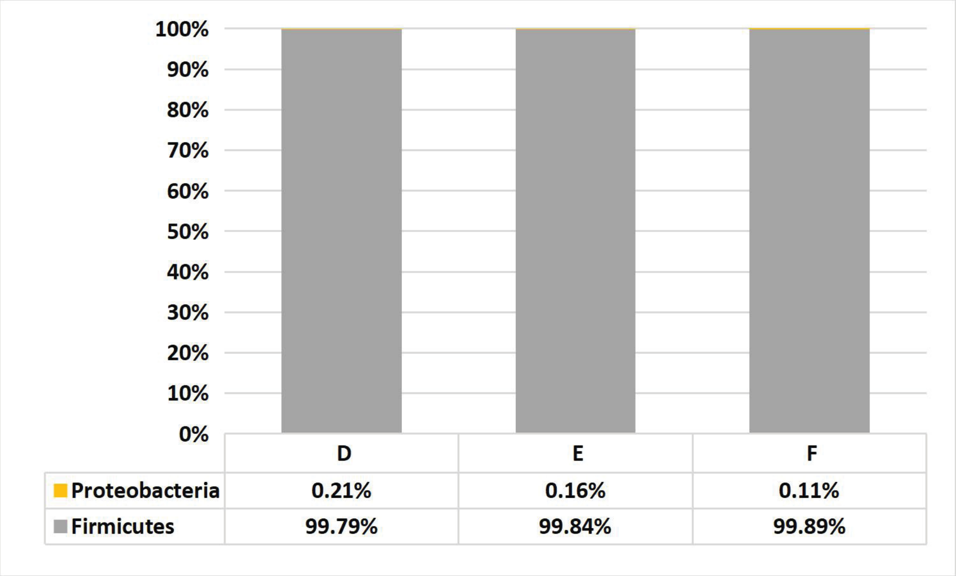


Figure 25. Percentage of relative abundance of sequences obtained in the three replicates of Azotm36 bacterial consortia at phylum level

at genus level were mainly from *Ureibacillus* genus representing 52.19% of the consortium, followed by *Symbiombacterium* (27.84%), *Geobacillus* (2.86%) and *Thermobacillus* with 2.62% of relative abundance. The other six genus represented in the consortium had a representation below 1% and by decreasing order were; *Bacillus* (0.40%), *Aeribacillus* (0.32%), Paenibacillus (0.16%), *Hyphomicrobium* (0.15%), *Thermoaerobacter* (0.05%) and *Brevibacillus* with 0.02%. Relative to the unclassified genus 13.40% of the sequences were not able to be identified with any genus. (Figure 26).

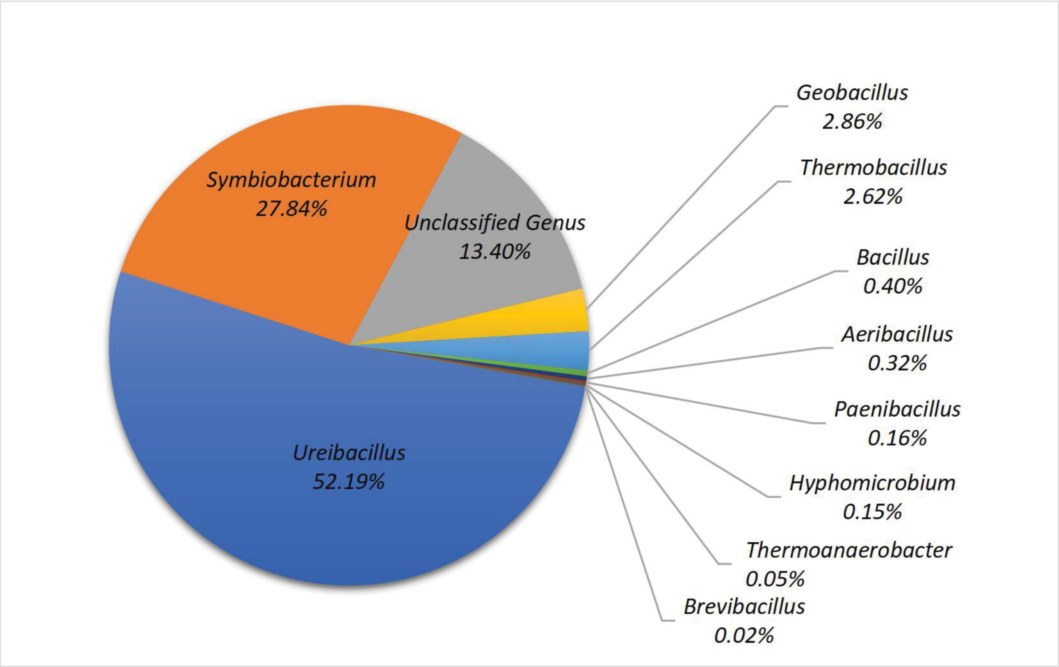


Figure 26. Mean of relative abundance of the total sequences obtained in Azotm36 bacterial consortia at genus level

Analyzing bacterial populations in Aztom36 at species level, we could identify 12 species (12 different OTUs) from a total 117 OTUs sequences (Table 13). All the twelve OTUs were identified as species that belong to the genus

Ureibacillus from family *Planococcaceae*, phylum *Firmicutes*, that was the most abundant genera in our consortium Azotm36. In this *Ureibacillus* genus there were 20 OTUs sequences and 12 of these OTUs blast were identified as related to the species; of *Ureibacillus thermosphaericus* strain ID 51173. A total of 15 OTUs sequences we analysed at species level in genera that had a representation in our consortia below 1% and it was not possible to identified These OTUs at species level (Table 14).

Table 13. Bacterial genus and identified species with high relative representation (above 1%) in consortium Azotm36. Number of classified and unclassified OTUS at species level. The relative abundance percentage was based on the mean on sequences obtained

Phylum>Class>Order>Family>Genus>Species	**Genus rel. abundance**	**Identified species**	**Total OTUs**
Firmicutes>Bacilli>Bacillales>Planococcaceae			
Genus *Ureibacillus*	52.19%	12	20
Ureibacillus_thermosphaericus strain ID 51173		12	
Clostridia>Clostridiales>Clostridiales Family XVIII. Incertae_Sedis			
Genus Symbiobacterium	27.84%	0	22
Firmicutes>Bacilli>Bacillales>Bacillaceae			
Genus Geobacillus	2.86%	0	7
Firmicutes>Bacilli>Bacillales>Paenibacillaceae			
Genus Thermobacillus	2.62%	0	7
Genus below 1% "less represented"	1.10%	0	15
Unidentified Genus	13.40%	0	46
Total	100%	12	117

Table 14. Bacterial Genus and identified species with relative abundance below 1% in consortium Azotm36 Genus. Number of classified and Unidentified OTUS at species level. The relative abundance percentage was based on the mean on sequences obtained.

Phylum>Class>Order>Family>Genus>Species	**Genus rel. abundance**	**Identified species**	**Total OTUs**
Firmicutes>Bacilli>Bacillales>Bacillaceae			
Genus Aeribacillus	0.32%	0	2
Genus Bacillus	0.40%	0	3
Firmicutes>Bacilli>Bacillales>Paenibacillaceae			
Genus Brevibacillus	0.02%	0	1
Genus Paenibacillus	0.16%	0	4
Firmicutes>Clostridia>Thermoanaerobacterales>Thermoanaerobacteraceae			
Genus Thermoanaerobacter	0.05%	0	2
Proteobacteria>Alphaproteobacteria>Rhizobiales>Hyphomicrobiaceae			
Genus Hyphomicrobium	0.15%	0	3
Total	1.10%	0	15

3.2.4.3. | Isolation and molecular identification of hydrolytic active colonies

To identify the hydrolytic bacteria in our consortium we used skim milk and tributyrin agar plates to select the active isolates for proteases and lipases. We isolated 20 colonies with distinct morphological shapes from the consortium Azotm36, from these colonies six isolates presented at least one of the two activities tested. Only three of the isolated bacteria revealed lipase activity on tributyrin substrate and all the six isolates presented protease activity. From these six bacteria, we selected four that presented the highest clearing zone on plates and proceeded its molecular identification. The most closely related bacteria based on BLAST matches to 16S rRNA sequences and their enzymatic capability for production of proteases and lipases are presented in table 15. In the isolate 1 we could detect only the presence of proteases. 16 sDNA analyses pointed 99% similarity with *Aeribacillus pallidus* strain DSM 3670 followed by a

96% of similarity with a *Anoxybacillus vitaminiphilus* strain 3nP4. The isolate 4 was the most active isolate, presenting high protease and lipase activities when compared to the other isolates. The blast of isolate 4 show a similarity of 99% with *Aeribacillus pallidus* strain DSM 3670 followed by a similarity of 96% with *Anoxybacillus calidus* strain C161ab. The enzymatic activities of isolate 6, were visible in both substrates for proteases and lipases activities. The blast results revealed that isolate 6 was very close (99% of similarity) to *Aeribacillus pallidus* strain DSM 3670 and to (95% of similarity) *Anoxybacillus caldiproteolyticus* strain R-35652. These strains blast results revealed bacteria strains that were identified in our consortia at genus level using 454, pyrosequencing method.

Table 15. Identification and enzymatic characterization of the isolated bacteria.

Isolate	Clearing zone diameter (mm)		Best match in GenBank database (accession number)	Similarity (%)
	Skim milk	Tributyrin		
Isolate 1	15	NDt	*Aeribacillus pallidus* strain DSM 3670 (NR_026515)	99
			Anoxybacillus vitaminiphilus strain 3nP4 (NR_108379)	96
			Bacillus alveayuensis strain TM1 (NR_043013)	95
			[*Flavobacterium*] *thermophilum* strain G-21 (NR_104891)	95
Isolate 4	20	20	*Aeribacillus pallidus* strain DSM 3670 (NR_026515)	99
			Anoxybacillus calidus strain C161ab (NR_125532)	96
			Anoxybacillus caldiproteolyticus strain SF03 (NR_115200)	95
			Geobacillus subterraneus strain 34 (NR_025109)	95
Isolate 6	20	10	*Aeribacillus pallidus* strain DSM 3670 (NR_026515)	99
			Anoxybacillus caldiproteolyticus strain R-35652 (NR_116989)	95
			Anoxybacillus tepidamans strain GS5-97 (NR_025819)	95
			Anoxybacillus kamchatkensis strain JW/VK-KG4 (NR_041915)	95

3.2.5. | Remarks

The selection of a stable consortium that could efficiently hydrolysate DMS or SSS was attempted in the present work. During the enrichment process all six consortia enriched at pH3, pH 7 and pH 9 presented growth throughout the twelve weeks and protease activity was detected in all the consortia.

At the end of twelve weeks of enrichment process amylase and lipase activity was tested, and two consortia AzotB9 and Azotm36 presented the highest activities for the three enzymes. Statistically differences were evident when comparing the dry weight loss of consortium Azotm36 enriched in SSS with all the other consortia enriched in other substrates and pH. Azotm36 was the most efficient consortium achieving 30.35% of solubilization of the SSS substrate at pH9 at the end of 7 days, and was selected for further studies.

The monitorization of the consumption of soluble organic material (sCOD) was performed in order selecting the optimal pretreatment stop time. The highest value for sCOD (70 680 mg/L) was obtained at 48 hours of pretreatment in 2.5% TS of substrate concentration. At this point the COD loss ratio percentage (1.6%) was lower compared with other substrate concentrations, meaning that less nutrients were consumed by the consortium Azotm36, leaving more COD available to methanogenic bacteria. Based on these data the pretreatment was stopped at 48 hours and pretreated sludge was transferred to anaerobic bioreactors.

The consortium Azotm36 produced proteases, lipases and amylases, capable of hydrolyse sewage sludge and obtaining soluble nutrients. Proteases were essential to achieve the increase of soluble protein during pretreatment; the highest concentrations were 5770 mg/L registered at 48 hours in the concentration of 2.5% TS.

The maximum values for VFAs were registered at 48 hours with 8.21 g/L, coinciding with the pretreatment stop time. The maximum concentrations of acetic acid and butyric acid in our pretreatment reactors were 2.16 and 3.79 g/L respectively, and propionic acid reached 2.26 g/L at 48 hours which is above limits for inhibition of methanogens.

The volumes of total biogas yield in pretreated sludge substrates were higher than in the unpretreated, producing only more 5.23% of total accumulated biogas at the end of the 42 days. This result shows that although Azotdm9 pretreatment did not enhanced the biogas yields, content of methane in bioreactors with pretreated sludge mixtures were 16% higher than in the controls without sewage sludge pretreatments. The maximum biochemical methane potential of pretreated sludge, was enhanced relatively to the unpretreated sludge. This pretreatment resulted in a 20% raise of biochemical methane potential (200.98 LCH_4/Kg VS added) for pretreated SSS, at the end of the 42 days of AD.

The major phylum in the Azotm36 is *Firmicutes* (99.84%). At genus level the consortium was mainly composed by *Ureibacillus* (52.19%), *Symbiobacterium* (27.84%), *Geobacillus* (2.86%) and *Thermobacillus* (2.62%). Other phyla represent less than 1% in the Azotm36 consortium. Only in the major represented genus *Ureibacillus*, the pyrosequencing results identified twelve strains closely related to the species of *Ureibacillus thermosphaericus*.

All together, these results show that the bacteria community present in the Azotm36 consortium were efficient for the solubilization and pretreatments of SSS, although no differences were seen in the increased total biogas production, the biochemical methane production was enhanced by 20% in pretreated sludge.

All together, these results show that the bacteria community present in the Azotm36 consortium were efficient for the solubilization and pretreatments of SSS, and although no differences were seen in the increased total biogas production, the content of methane in pretreated sludge and the biochemical methane potential was also enhanced in pretreated sludge

3.3. | ENHANCING ANAEROBIC DIGESTION OF LIGNOCELLULOSE BIOMASS USING A THERMOPHILIC MICROBIAL CONSORTIUM PRETREATMENT

The production of biofuels from biomass became a necessity and novel pre-treatments are mandatory to be discovered. Thereby the study of cellulose hydrolyzing bacterial communities is a step in achieving a sustainable future in biofuels development.

Finding microbial pretreatments to unlock wastes high energy values to produce biogas is one of the objectives in the present work. The stabilization of an aerobic thermophilic bacterial consortia with the ability to adapt and hydrolyse different cellulose-rich wastes is proposed. The aim of this work was the construction of a bacterial consortium to attempt the enhance of methane production by using a two-stage digestion system. The first stage consisted on a thermophilic aerobic bacterial pretreatment using a selected consortium to improve grass mixtures hydrolysis and the second stage was the AD of pretreated grass mixtures wastes.

3.3.1. | Selection of a bacterial consortium for lignocellulosic mixtures

After the enrichment process for 3 months, a thermophilic microbial consortium was selected for further studies. Microbial consortium Azotdp36 enriched at pH 9 was selected as it could break down all the filter paper strip and for its efficient lignocellulosic biomass hydrolysis (Figure 27).

Figure 27. Substrate of lignocellulosic biomass (grass mixtures) filtered after 7 days of incubation at 60 ºC. Inoculated with consortium Azotdp36 (right) and the negative control (left)

The three microbial consortia enriched at three different pH in 1% TS of substrate during 7 days revealed that the most effective consortium was enriched at pH 9 and could hydrolyse 50% of the lignocellulosic biomass mix. Consortia enriched at pH 3 and pH 7 solubilized 10% and 27.8% respectively (Table 16). The efficiency of hydrolysis of the selected consortium Azotdp36 was accessed in other high content lignin materials. The consortium was more efficient in low lignin content biomass mixtures like grass mixtures, and is hydrolysis efficiency lowered to 5% in high lignin biomass materials like and wood shavings. There was a statistically significant difference in the dry weight loss between the consortium Azotdp36 pH 9 and the other two consortia Azotdp35 at pH 7 and Azotdp36 at pH 9 determined by one-way ANOVA (DF= 8, 2) (F=23,166) (sig=0.002).

Table 16. Percentage of dry weight loss caused three-microbial consortium enriched at 3 different pH values. Azotdp34 at pH 3, Azotdp35 at pH 7, Azotdp36 at pH 9. Values represented are a subtraction to control. The mean values and standard errors represented by the same letters indicate non-significant differences in dry weight loss between the consortia if P≥0,05 (Tukey multiple range test)

Consortium	Percentage of Weight loss
Azotdp34	10.13 ± 4.79 (b)
Azotdp35	27.86 ± 5.53 (b)
Azotdp36	50.06 ± 1.46 (a)

The stability of the consortium Azotdp36 was demonstrated by its ability to maintain hydrolysis after tolerating several rounds of subculture in medium with cellulosic material for more than 2 years and to maintain activity after being stored at room temperature or frozen at -80 ºC in 20% glycerol for periods of more than 6 months.

3.3.2. | Effect of the consortium on grass mixtures during pretreatment

3.3.2.1. | Weight loss

For the three substrate contents of 1.0%, 2.5% and 5.0% of Total solids, the weight loss of lignocellulosic mixtures dry matter rapidly increase in the first 24 hours 37%, 29% and 10% respectively. After day 1 final weight losses increased gradually till day 5 (Figure 28). There was a statistically significant difference between groups as determined by one-way ANOVA DF= (62, 2) (F=3.610) (sig=0.033).

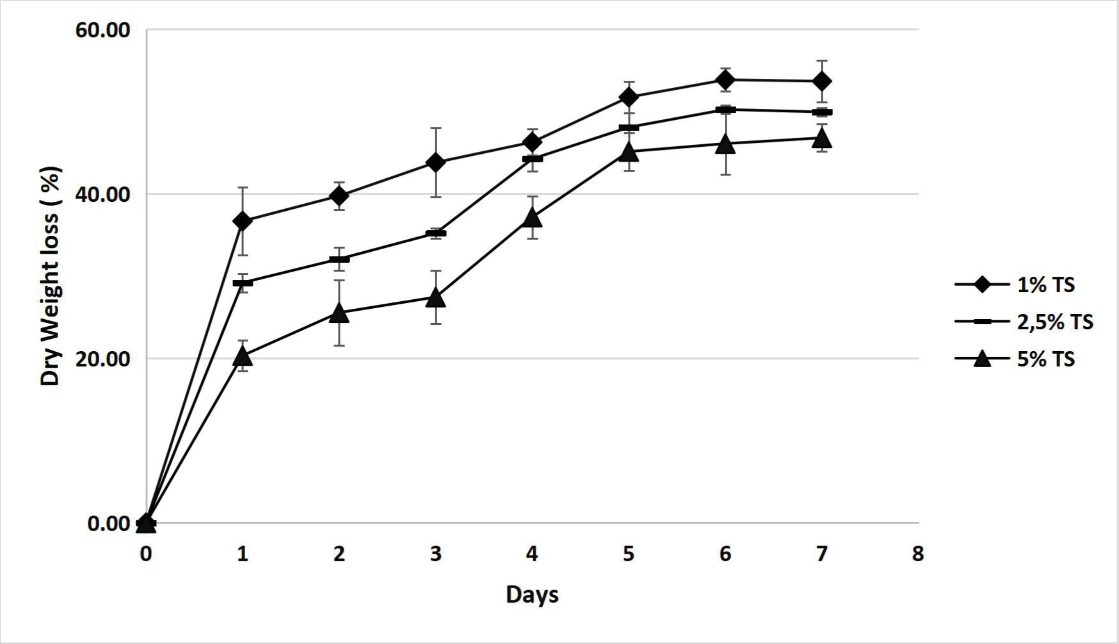


Figure 28. Dry weight loss percentage during pretreatment. Values presented are corrected with the respective controls. The means values represented by the same letters indicate statistically non-significant differences in dry weight loss substrate concentrations if P≥0,05(Tukey multiple range test)

This indicated that the total dry matter of lignocellulosic mixtures was degraded mostly in the first 120 hours. After day 5, the substrate mixtures were degraded more slowly till the end of pretreatment achieving 54%, 50% and 47% for the concentrations of 1%, 2.5 and 5% respectively.

3.3.2.2. | sCOD and COD balance

High sCOD in the hydrolysates indicated that large amounts of soluble substrates are available for AD. Conversion of lignocellulose to soluble products was regarded as the rate-limiting step during the anaerobic So from this perspective, the optimal pretreatment time should equal the time at which the sCOD, or volatile organic product concentration reaches a maximum. This requires the monitoring the consumption of soluble organic material so it could be minimized during pretreatment by microbial consortium.

Similar tendencies in the sCOD concentrations were observed at the 1.0% and 2.5% and 5% concentrations (Figure 29).

The sCOD concentrations increased in the first day rapidly with lower substrate concentrations of 1.0% and 2.5% with more pronounced increase of sCOD. There was a decrease of sCOD after from day 1 to 3 for all the substrate concentrations, this demonstrated that there are both hydrolytic microbes and fermentative microbes in Azotdp36. After day 3, the hydrolytic microbes were hydrolyzing the substrate faster than the fermentative were consuming and the sCOD progressively raised being the highest concentration of 29 400 mg/L achieved in 5% subtract concertation. After day 5 the fermentative microbes took place and consumed the soluble nutrients lowering the sCOD in all 3 concentration. It was noticed that as in high concentrations of substrate contents, higher the sCOD concentrations reached. Although these results did not reflect the total consumed nutrients by the bacteria so we could select the most efficient time in terms of nutrient availability with less consumed by the bacteria consortium.

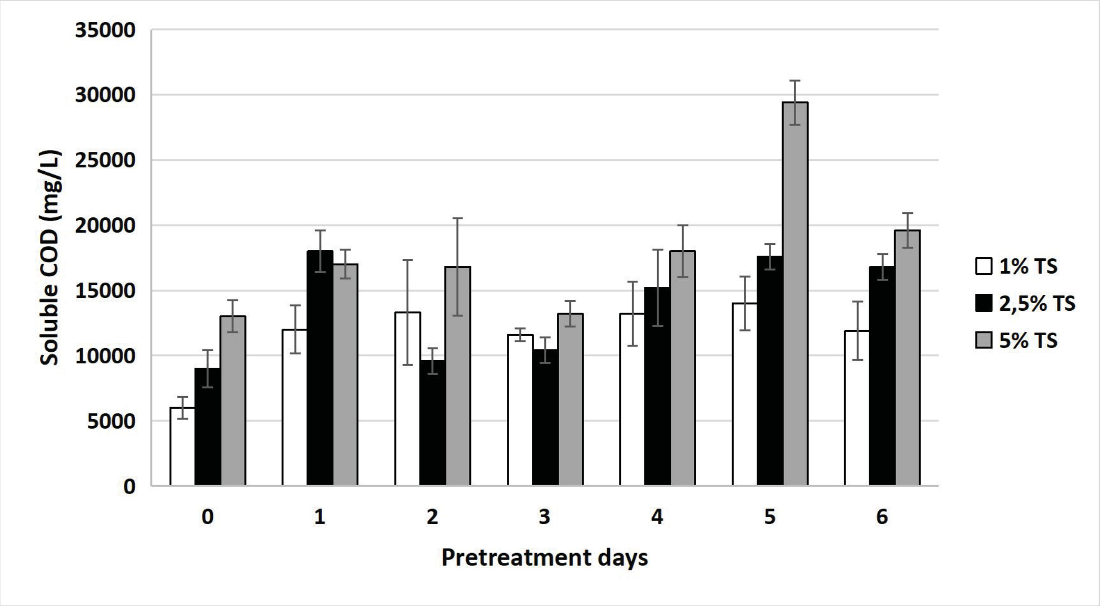


Figure 29. Soluble COD during the 6 days of pretreatment days

As pretreatment time was extended in higher substrate concentrations more organic material was lost. At the end of pretreatment, the COD loss varied between the three substrate concentrations; 1% (21 566 mg O2/L), 2.5% (40 336 mg/L) and at 5% (70 526 mg/L) (Table 17). However, when analyzing loss ratio, it was found that at higher substrate concentrations, lower loss ratio occurred, Meaning less nutrients in the available system; 1% (55.7%), 2.5% (52.1%) and at 5% (50.6%). This could be related with lower oxygen levels in the higher substrates concentrations affecting the degradation ability of aerobic consortium microbes that could be inhibited at lower oxygen levels.

To choose when to stop pretreatment it was taken in consideration the highest hydrolysate concentrations, the rate of loss ratio and sCOD hydrolysate ratio. This allowed us to prevent fermentative microbes from Azotdp36 to consume the available organic materials valuable for methane production. The combination of lowest loss ratio with the highest sCOD ratio allowed us to select when our hydrolytic microbes where more efficient and fermentative microbes consumed less nutrients during pretreatment (so organic materials remain available for methane production). Pretreatment was stopped at day 5 with a concentration of substrate of 5% TS, as it shown the highest sCOD ratio of 2.3 and the lowest loss ratio of 36.2% when compared to the other times and substrate concentrations. The lignocellulosic substrate concentration of 5% at day 5 of pretreatment was used for AD assays.

Table 17. COD balance during pretreatments with Azotdp36

	Before pretreatment			During pretreatment			
Substrate concentration (% TS)	**COD substrate (mg/L)**	**COD medium (mg/L)**	**Pretreatment time (Hours)**	**sCOD hydrolysate (mg/L)**	**COD substrate residue (mg/L)**	**COD loss (mg/L)**	**Loss ratio (%)**
1	32355	6000	0	6000	32355	0	0
			24	12000	24375	2336	6
			48	13000	18975	6736	17.4
			72	11600	14975	12136	31.3
			96	13200	9745	15766	40.7
			120	14000	6345	18366	47.4
			144	11900	5245	21566	55.7
2.5	68000	9000	0	9000	68000	0	0
			24	18000	58200	1156	1.5
			48	9600	50220	17536	22.7
			72	10400	43330	23626	30.5
			96	15200	35880	26276	34.0
			120	17600	27980	31776	41.1
			144	16800	20220	40336	52.1
5	126000	13000	0	13000	126000	0	0
			24	17000	115700	6656	4.8
			48	16000	106450	16906	12.1
			72	13200	97150	29006	20.8
			96	18000	87480	33876	24.3
			120	29400	59480	50476	36.2
			144	19600	49230	70526	50.6

3.3.2.3. | *Percentage of solubilization*

The percentage of solubilization in the pretreatment, was calculated as the percentage of the initial COD added to the vials that was solubilized during the consortium pretreatment. Percentage of solubilization was calculated at 120 hours and at the end of pretreatment. For the calculations, we took in consideration the soluble COD that was lost by bacteria consumption based on the values of table 18.

Total solubilized COD at 120 hours was 79 876 mg/L taking in consideration the already lost COD consumed by our bacteria of 50 476 mg/L, the pretreatment had a solublization percentage of 53%. At the end of pretreatment, 61% of COD solubilization on grass mixtures was achieved with our consortium Azotdp36.

Table 18. Data of percentage of solubilisation at 120 hours and at the end of pretreatment for the concentration of 5% TS.

	COD (mg/L) (At 120 hours)	**COD (mg/L) (At the end of pretreatment)**
sCOD−FinalPT	79 876	90 126
sCOD−inicialPT	13 000	13 000
CODtotal−inicialPT	126 000	126 000
PSprt%	53%	61%

3.3.2.4. | *Enzymes in supernatant of consortia Azotdp36*

Enzymatic hydrolysis of cellulose is a fundamental step in the carbon cycle and in the industrial bioconversion of biomass to biofuels. In nature, cellulose hydrolysis is often catalyzed by enzymes from complex microbial communities, nevertheless these studies are limited to a few isolates. For the detection of enzymes in the hydrolysate supernatant of Azotdp36, two tests were used, An agar diffusion technique and a API ZYM test kit (Table 19). The hydrolysate supernatant with 120 hours of pretreatment was tested in 22 different substrates. The tests in with agar diffusion technique revealed that our consortium is capable of producing cellulases, xylanases and lipases as it could create clear zones in agar plates, 10 mm diameter in cellulose substrate and 20 mm diameter for xylan and tributyrin. Additionally, enzymatic activities were obtained using an API ZYM test. Our Azotdp36 consortium was positive for the enzymes; Alkaline phosphatase, Phosphohydrolase, Esterase C_4, Esterase, Lipase C_8, Lipase C_{14}, Leucine amino-peptidase, Cystine amino-peptidase, Trypsin, β-glucosidase, N-acetyl-β-glucosaminidase and β-galactosidase. The results in the following substrates were negative; Acid phosphatase, α-mannosidase, α-fucosidase, Valine amino-peptidase, Chymotrypsin, α-galactosidase, α-glucosidase, β-glucoronidase. The chromogenic substrates differentiate between hydrolysis of C_4 esters (esterase activity) C_8 ester (ester lipase activity) and C_{14} ester (lipase activity). Usually hydrolysis of tributyrin correlates with the presence of lipases able to hydrolyse glycerides with C_4 fatty acids.

Table 19. Results of the enzymatic assays in agar plates and API ZYM test kit. For Azotdp36

Enzyme assayed for;	**Substrate**	**Results**
Cellulase	Carboxymethyl cellulose	+ (10 mm)
Xylanase	Xylan beechwood	+ (20 mm)
Lipase	Tributyrin agar	+ (20 mm)
Alkaline phosphatase	2 naphthyl-phosphate	+
Acid phosphatase	2 naphthyl-phosphate	-
Phosphohydrolase	Naphthyl AS-BI-phosphate	+
Esterase C_4	2 naphthyl-butyrate	+
Esterase Lipase C_8	2 naphthyl-caprylate	+
Lipase C_{14}	2 naphthyl-myristate	+
Leucine amino-peptidase	L-leucyl-2-naphthylamide	+
Valine amino-peptidase	L-valyl-2-naphthylamide	-
Cystine amino-peptidase	L-cystyl-2-naphthylamide	+
Trypsin	N-benzol-DL-arginine-2-naphthylamide	+
Chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamine	-
α-galactosidase	6-Br-2-naphthyl-α-D-Galactopyranoside	-
β-glucosidase	6-Bromo-2-naphthol-α-D-Galactopyranoside	+
N-acetyl-β-glucosaminidase	Naphthyl-N-acetyl-β-D-glucosaminide	+
α-glucosidase	2 naphthyl-2-D-glucopyranoside	-
β-galactosidase	2 naphthyl-β-D-galactopyranoside	+
β-glucoronidase	Naphthyl-AS-BI-β-D-glucuronide	-
α-mannosidase	6-bromo-2-naphthyl-2-D-mannopyranoside	-
α-fucosidase	2 naphthyl-α-L-fucopyranoside	-

3.3.2.5. | Reducing sugars

During the pretreatment time, soluble reducing sugars were analysed in hydrolysate of selected pretreatment concentration of 2.5% of TS (Figure 30). During this period, cellulose end hemicellulose was converted to simple sugars the highest values of reducing sugars were observed between 48 hours and 96 hours reaching a peak of 1.23 g/L of total reducing sugars. With these results values, a maximum of 45.6 g of reducing sugars per kilo of raw grass could be produced with our consortium Azotdp36 at 48 hours of pretreatment.

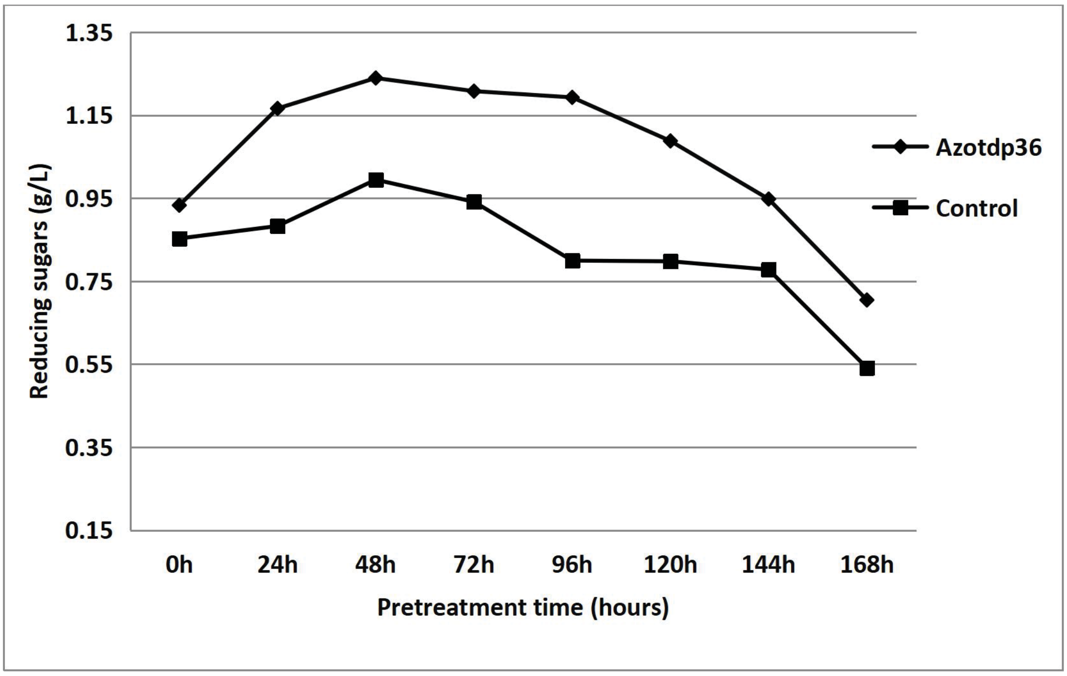


Figure 30. Reducing sugars present in hydrolysate supernatant of Azotdp36

3.3.2.6. | pH and volatile organic products changes

The pH of Azotdp36 hydrolysates for the three substrate concentrations of 1.0%, 2.5% and 5.0%, declined until day 3. The lowest pH values occurred on day 2 for the three substrate concentrations 1.0%, 2.5% and 5.0%. The lowest pH values of the Azotdp36 hydrolysates were 7, 6.6 and 6 for the 1% 2.5% and 5%, respectively. The pH after day 2 gradually increased in the three subtract concentrations reaching the 8.8 in the 1% of TS and 8.4 and 7.6 in concentration 2.5% and 5% of TS respectively. When substrate concentration was 2.5%, the pH hit the lowest values of 6.6, this low value is directly correlated with the acidification of the medium related to the hydrolysis and VFA production. (Figure 31).

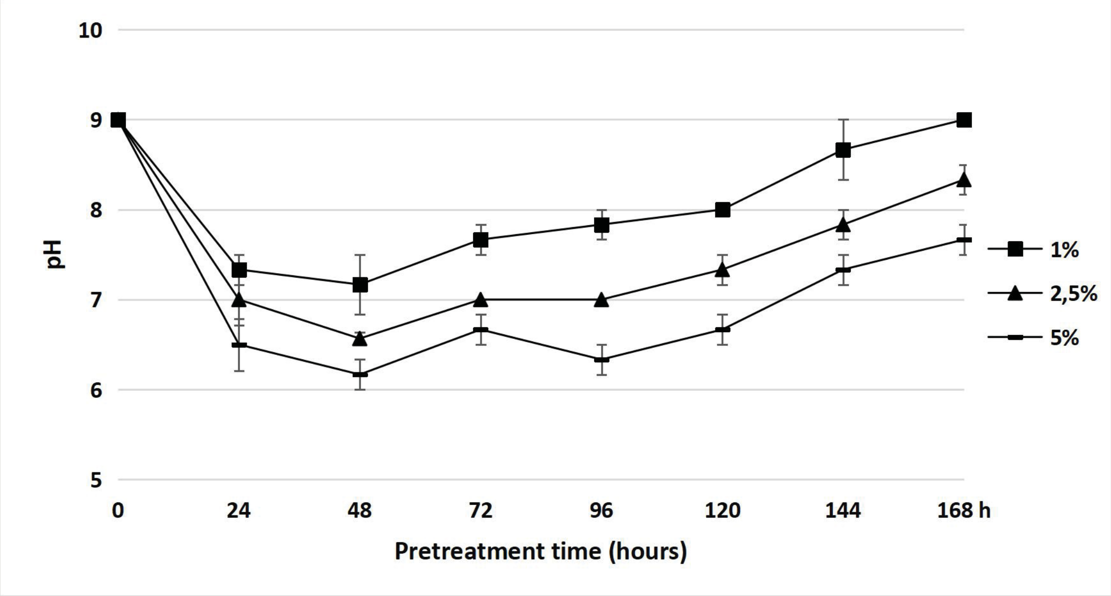


Figure 31. pH variation during pretreatment from the hydrolysate at different substrate concentrations.

The monitoring of VFA during pretreatment was made with the previous selected 5% TS concentration of grass mixtures substrate, as it was the concentration tested in anaerobic assays (Figure 32).

The concentration of VFAs was registered during 120 hours and a general raise was registered in the first days of pretreatment in the reactors with the consortium Azotdp36. At the beginning of pretreatment (0 hours), the concentrations of the three VFAs, in the bioreactors with the control and with our consortium Azotdp36 were considered the same and as follows; Acetic acid (0.22 g/L), Propionic acid (0.21 g/L) and Butyric acid (0.18 g/L).

The high values of VFAs registered at 48hours of pretreatment contributed for the decrease of the pH. Although after the 48 hours the pH started to gradually raise, this could be in consequence of the decrease of some volatile acids, propionic and butyric after 48 hours of pretreatment. Analyzing the production of acetic acid in bioreactors with the consortium Azotdp36, it increased gradually reaching the maximum concentrations between 72 and 120 hours, with a peak at 96 hours of 0.60 g/L. the concentrations of acetic acid in the unpretreated grass (controls) were must lower and varied between 0.22 and a maximum of 0.30 g/L. The concentration of Propionic acid had an increase during the first 48 hours, declining in the following days. The concentrations of propionic acid varied between 0.21 and a maximum of 1.35 g/L, that was reached at 48 hours. The concentration of propionic acid in the controls were between 0.21 and 0.47 g/L, having similar variation as the concentration of propionic acid in the Azotdp36 reactors except at 48 hours. The other VFA analysed was butyric acid, it started with 0.18 g/L, reaching the maximum at 48hours with a concentration of 1.10 g/L. After 48 hours of pretreatment the concentrations of butyric acid decreased drastically after 72 hours it could not be detected in the bioreactors with pretreatment and in controls. Detection of butyric acid in the controls were between 0.18 g/L and 0.63 g/L.

The VFA concentrations was not our first option for the selection of pretreatment stop time, the maximum values for these three VFAs analysed were registered at 48 hours with 2.72 g/L. The values of total three VFA was 1.10 g/L at the time chosen to stop pretreatment (120 hours).

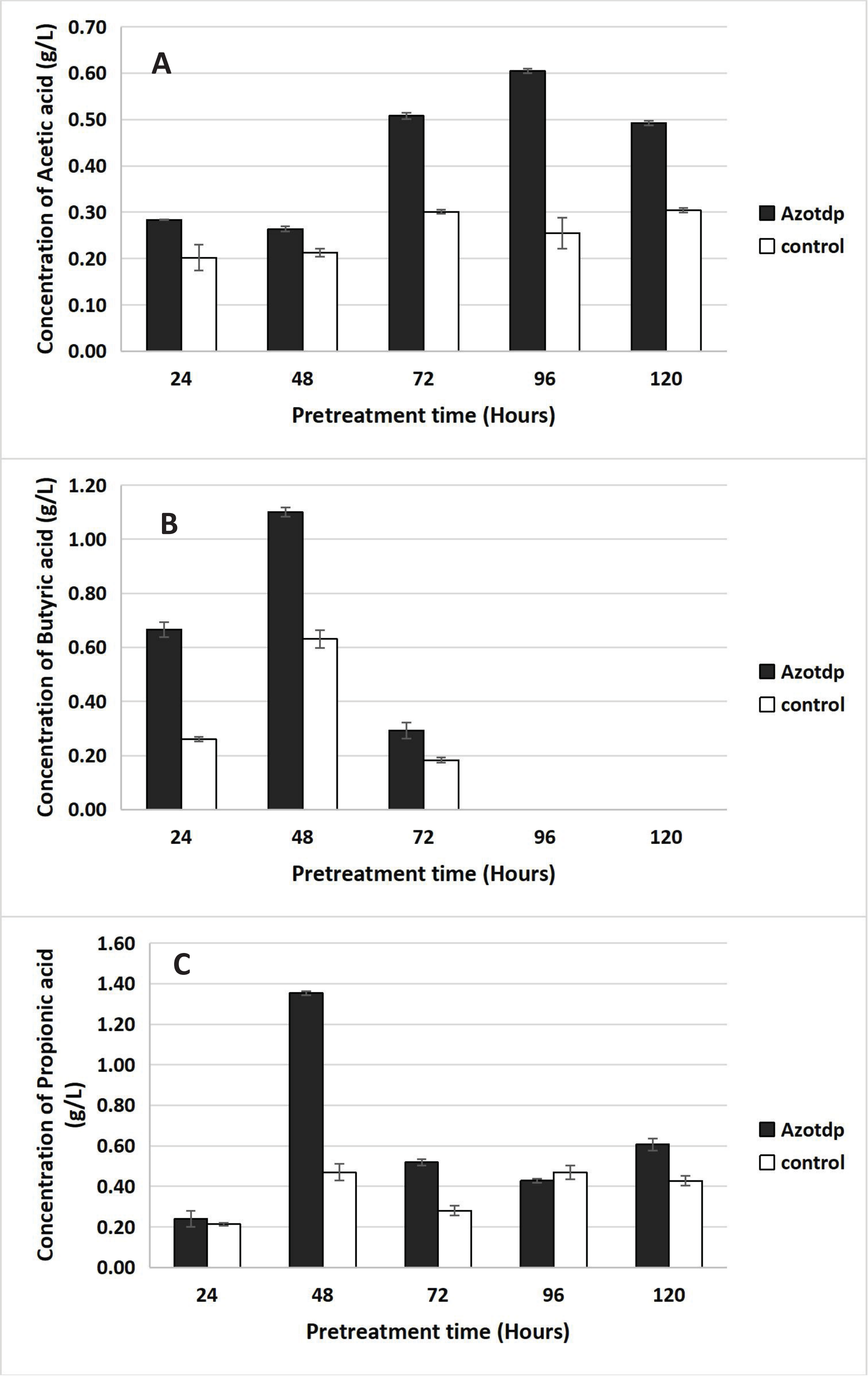


Figure 32. Quantitative analysis of major VFA by GC during pretreatment. (A) acetic acid; (B) propionic acid; (D) butyric acid.

3.3.3. | Biogas production

3.3.3.1. | Biogas volumes

The total accumulated biogas yields for pretreated grass of 5% TS concentration was different than the untreated grass at the end of AD. At the end of the AD bioreactors with pretreated grass resulted in the production of a total accumulated 1 626 ml of biogas per bioreactor. The volume of biogas production in bioreactors with unpretreated grass mixtures were 1375 ml of total accumulated biogas (Figure 33). This result shows that Azotdp36 pretreatment enhanced the biogas yields of grass mixtures.

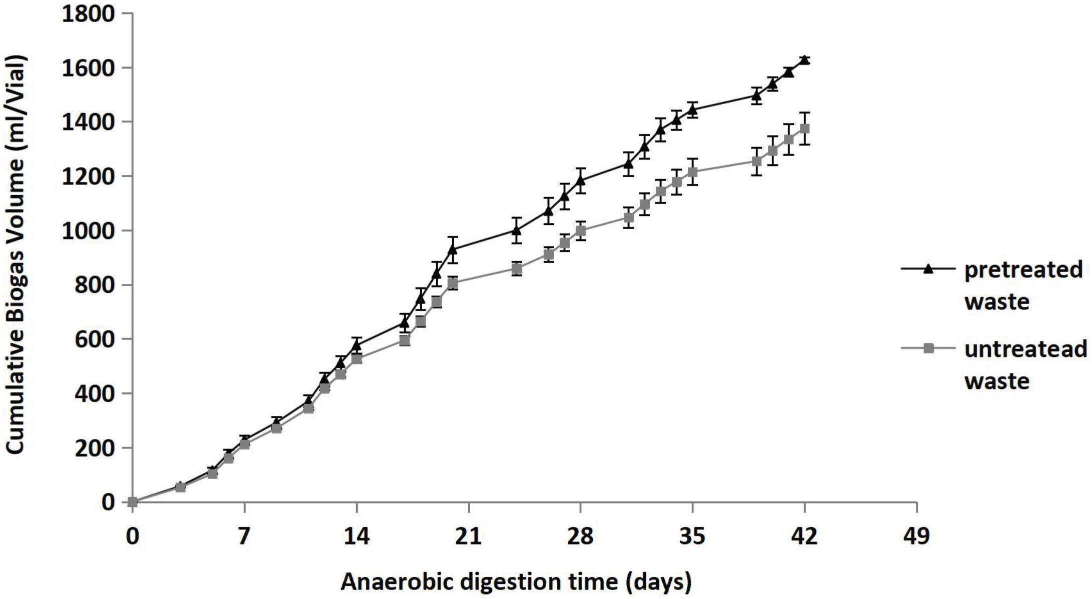


Figure 33. Total accumulated biogas volume during AD of pretreated and unpretreated grass.

The daily accumulated biogas production represented in figure 34, revealed the days in which more biogas was produced in pretreated bioreactors.

Analyzing the results, they show differences in total cumulative. biogas volumes between pretreated and unpretreated anaerobic bioreactors. Between the day 12 and the day 33, AD bioreactors with Azotdp36 consortium of pretreated grass produced higher volumes than the bioreactors with unpretreated grass. After day 33 up until the end of pretreatment no differences were clear between the pretreated and unpretreated anaerobic bioreactors.

These data indicate that our Azotdp36 consortium pretreatment enhances the total biogas production.

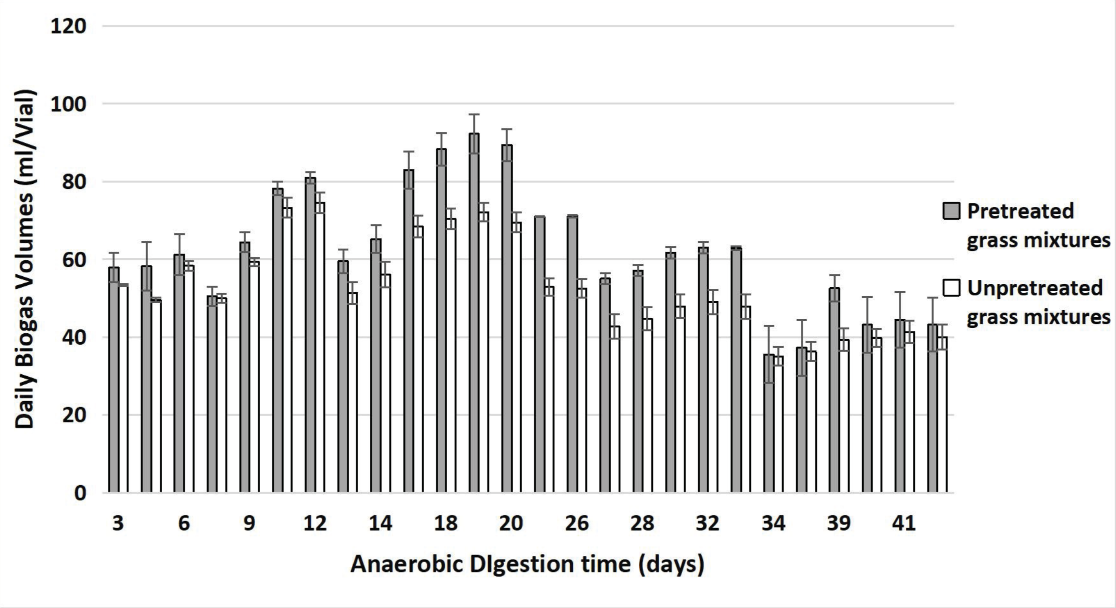


Figure 34. Daily Biogas volume in ml of pretreated and unpretreated sewage grass mixtures

3.3.3.2. | Methane production

The methane content of pretreated grass mixtures bioreactors was higher than the unpretreated, specifically after day 21 up until the end of pretreatment

(Figure 35). These results were in frame with the higher volumes of total biogas production during that time.

At the end of the AD (day 42) the total methane content in bioreactors with pretreated grass mixtures were 21.31% higher than in the controls without grass pretreatment. This result reveals that the pretreatment with Azotdp36 consortium influence total biogas production and enhance methane content in grass mixtures.

The values for methane content in pretreated bioreactors were between 8.55% and 39.12%. The maximum methane yields occurred at day 21 of pretreatment. In addition, the methane production rate of the treated Azotdp36 was faster than that of the untreated sample (Figure 35).

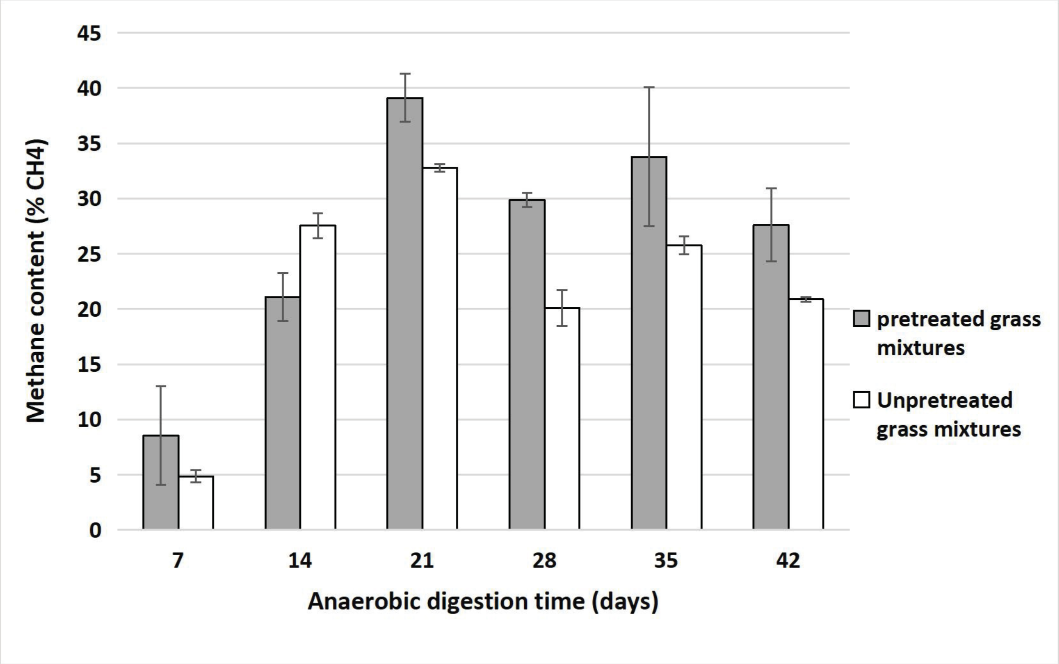


Figure 35. Methane content % in anaerobic bioreactors with and without grass pretreatment with consortia Azotdp36

The maximum biochemical methane potential was 168.36 LCH$_4$/Kg VS added, for the pretreated grass with the consortium Azotdp36. The untreated grass mixtures biochemical methane production was 94.93 LCH$_4$/Kg VS added, (Figure 36). These values of methane were obtained when the sCOD reached the highest values at 120 hours of pretreatment with a concentration of 5% TS of grass substrate. This allow us to conclude that the pretreatment resulted in an increase of 77.35% of biogas production per kilo of volatile solids of grass added.

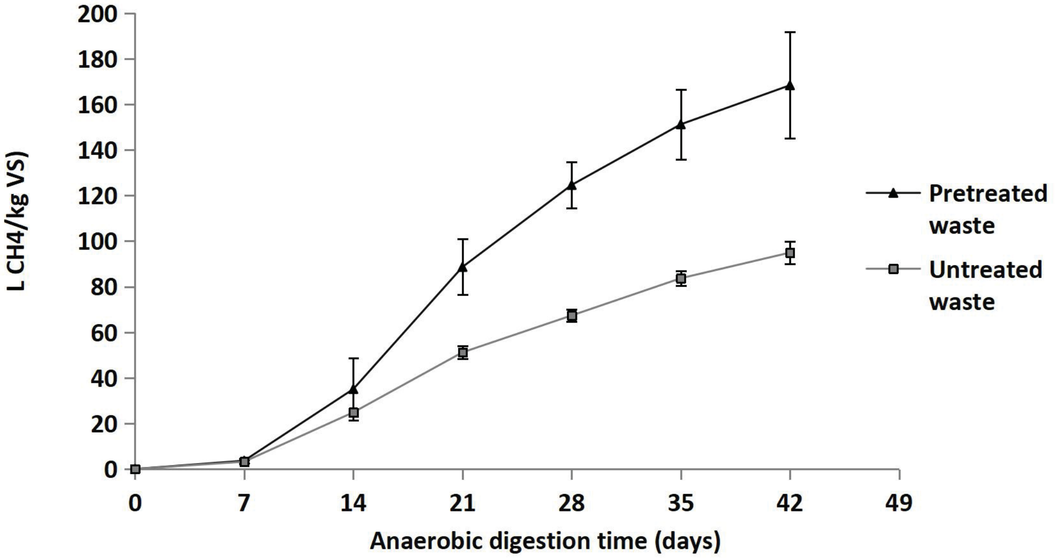


Figure 36. Biochemical methane potential curves for Azotdp36 pretreated and unpretreated grass.

3.3.4. | Phylogenetic analysis of bacterial community

3.3.4.1. | Bacterial diversity and richness in Azotdp36 consortium

Small-subunit (SSU) rRNA gene sequences from pyrosequencing and obtained OTUs was used to characterize the bacterial diversity and richness and estimated relative abundance of the microbial consortium Azotdp36. The adequacy of the sample size for the determination of diversity within the 16S rDNA library was evaluated by rarefaction analysis. Expect as shown in figure 37, the calculated rarefaction curve was partially saturated at 3% divergence which indicates that the analysis covered the bacterial diversity present in the consortium.

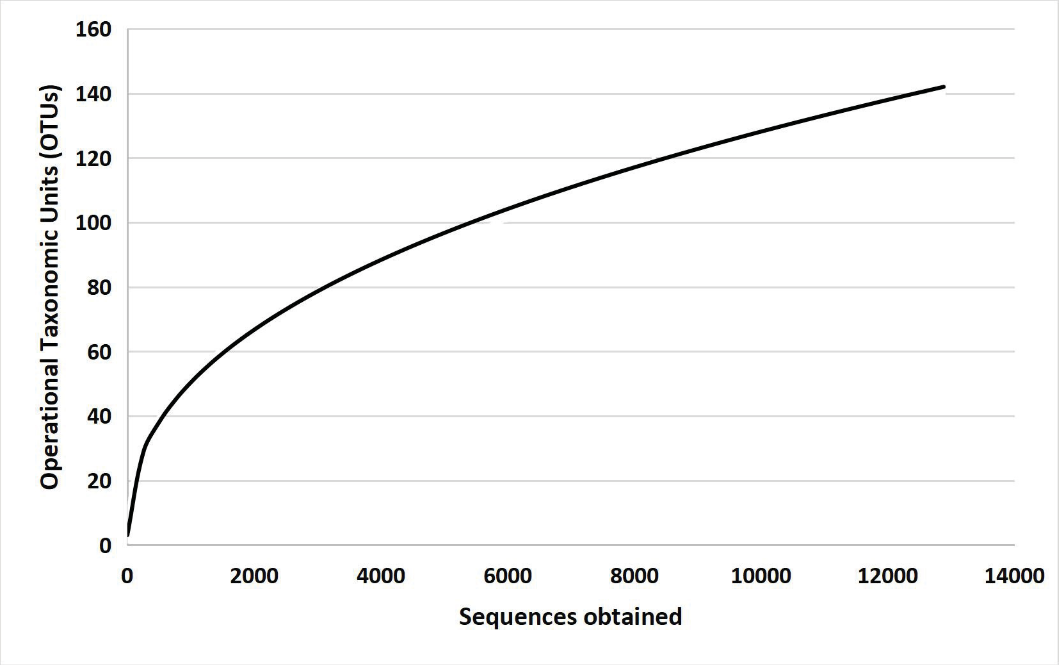


Figure 37. Rarefaction curves from 16S rRNA gene sequences of microbial consortia Azotdp36. Sequences were clustered at a genetic distance of 3%. OTU values were calculated by the mean of replicates. Curve was based on a non-linear regression power model given

Using Chao1 richness estimator, the maximum number of OTUs for the bacterial community expected at 3% genetic distance was 118. 20, 138.50 and 138.55 for replicates A, B and C, respectively (Table 20). The obtained OTUs in replicate A was 93 covering 78.68% of the predicted chaos richness estimator of 118.20. In replicate B, it was obtained 127 OTUs, being the closest one to the predicted chaos results of 139 OTUs, with 91.70% of coverage. In the replicate C, it was obtained 99 OTUs covering 71.46% of expected chaos result of 138.55 OTUs.

Table 20. Diversity indices Chaos results of the 3 replicates from Azotdp36

Consortium	Sample	Gene region	Chao expect	Chao obtained	Coverage %	Reads	OTUs
Azotdp36	A	V3	118.20	93	78.68	7422	93
Azotdp36	B	V3	138.50	127	91.70	12867	127
Azotdp36	C	V3	138.55	99	71.46	8285	99

3.3.4.2. | Composition of the bacterial community in Azotdp36 consortium

A total of 28 573 sequences were analysed in this study after trimming reads sequences of either short length or low quality, removing chimeras, and selecting the reads without fail blast. The distribution of valid sequences in the replicates were as follows; replicate A with 7 422 sequences, replicate B with 12 867 and replicate C with 8 284 sequences. The sequences were used to calculate relative abundance percentage at phylum and genus levels for the microbial community.

The relative bacterial abundance populations in Aztodp36 were mainly from the phylum *Firmicutes* representing 81.21% of the consortium, followed by *Actinobacteria* (6.25%), *Proteobacteria* (2.82%), and 9.72% of the consortium at phylum level were not classified (Figure 38).

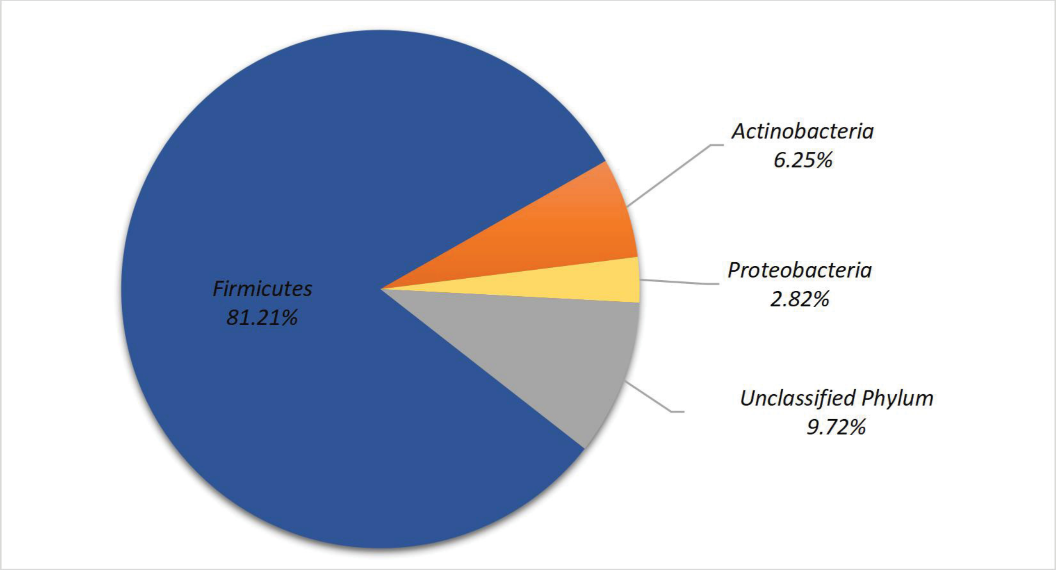
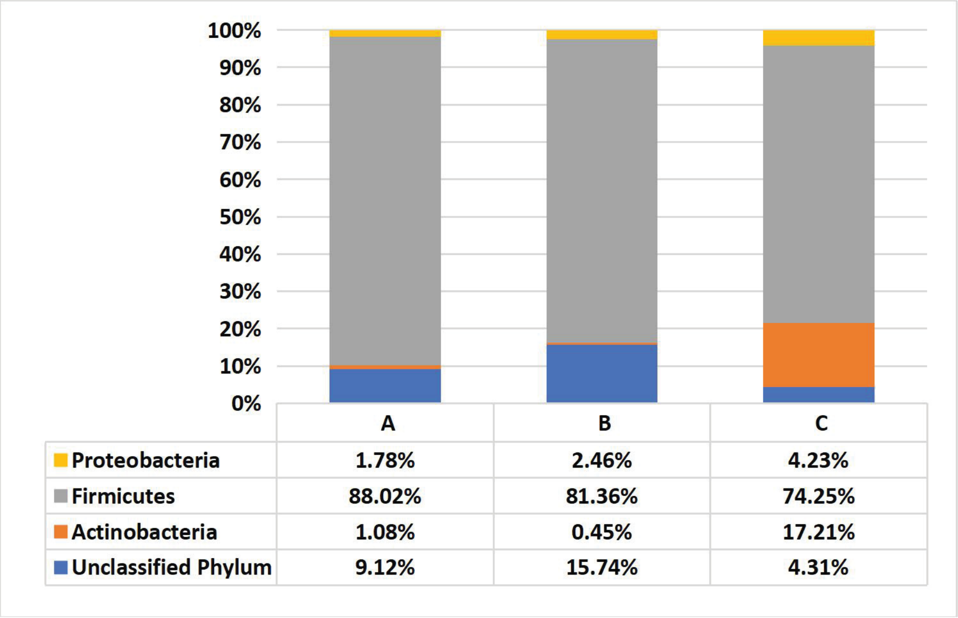


Figure 38. Relative abundance percentage based on the mean of the total sequences obtained in Azotdp36 bacterial consortia at phylum level

Analyzing the 3 replicates, the most represented phylum *Firmicutes* relative abundance was evenly distributed between 74.25 and 88.02%. For the second most represented phylum *Actinobacteria* the values in replicates varies between 1.08% and 17.21%. In less represented phylum *Proteobacteria,* the values were evenly distributed in the three replicates between 1.78% and 4.23% (Figure 39).



	A	B	C
🟨 Proteobacteria	1.78%	2.46%	4.23%
⬜ Firmicutes	88.02%	81.36%	74.25%
🟧 Actinobacteria	1.08%	0.45%	17.21%
🟦 Unclassified Phylum	9.12%	15.74%	4.31%

Figure 39. Relative abundance percentage based on sequences obtained in the three replicates of Azotdp36 bacterial consortia at phylum level

The relative bacterial abundance in Aztodp36 at genus level were mainly from *Thermobacillus* representing 41.18% of the consortium, followed by *Symbiombacterium* (5.89%), *Brevibacillus* (4.48%), *Hyphomicrobium* (2.60%) and *Geobacillus* (2.48%). The other 13 genera in the consortium had a representation below 1% that are by decreasing order; *Ureibacillus* (0.85%) *Bacillus* (0.49%), *Thermobispora* (0.38%), *Paenibacillus* (0.29%), *Caldalkalibacillus* (0.10%), *Aeribacillus* (0.09%), *Devosia* (0.08%), *Thermoaerobacter* (0.07%), *Mesorhizobium* (0.01%), *Planifilum* (0.01%), *Rhizobium* (0.01%), *Thermobifida* (0.01%) and *Cohnella* (0.004%). Relative to the unclassified genus 40.97% of the sequences assigned with any genus. (Figure 40).

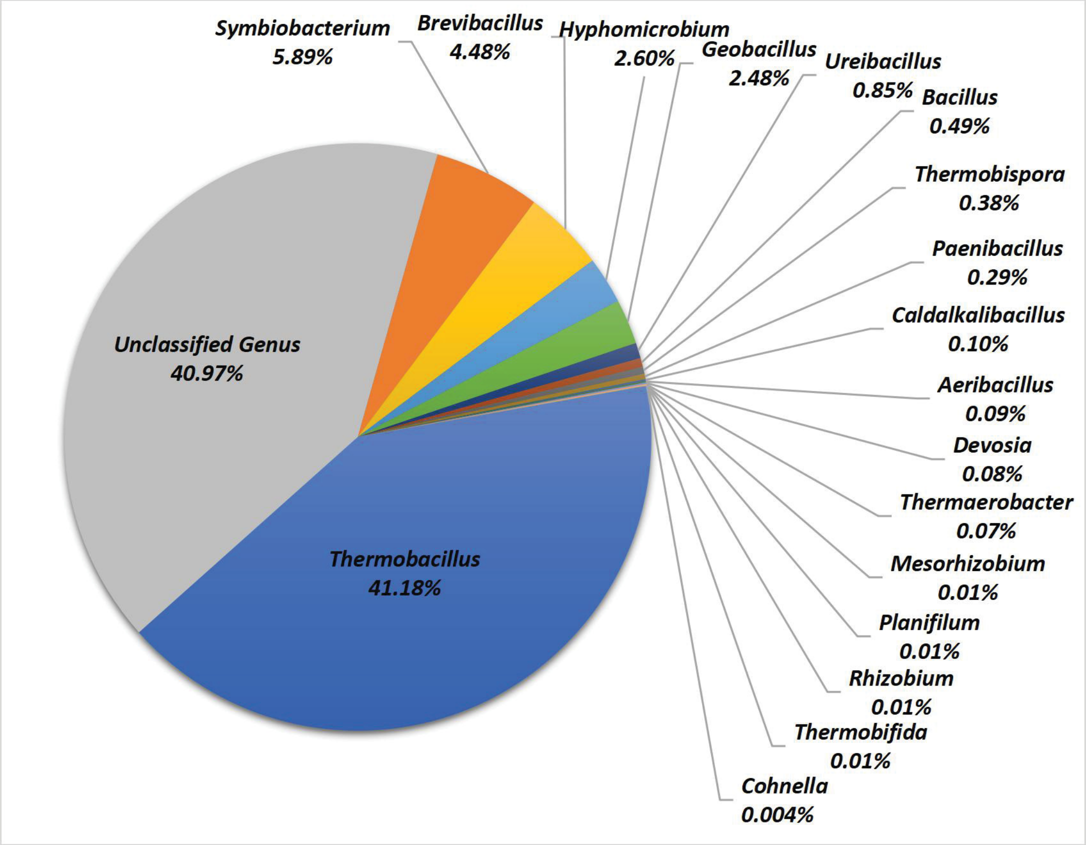


Figure 40. Mean of relative abundance of the total sequences obtained in Azotdp36 bacterial consortia at genus level

Analyzing bacterial populations in Aztodp36 at species level (Table 21), 20 different OTUs from a total 318 OTUs sequences were identified as species. Three of them were assigned as species of e genus *Geobacillus* blasting with *Geobacillus debilis* strain ID 301148, *Geobacillus thermodenitrificans* strain ID 1422 and *Geobacillus stearothermophilus* strain ID 33940, respectively.

Table 21. Bacterial genus and identified species with high relative representation (above 1%) in consortium Azotdp36. Number of classified and unclassified OTUS at species level. The relative abundance percentage was based on the mean on sequences obtained.

Phylum>Class>Order>Family>Genus>*Species*	**Genus relative abundance**	**Identified species**	**Total OTUs**
Firmicutes>Bacilli>Bacillales>Paenibacillaceae			
Genus Thermobacillus	41.18%	0	46
Genus Brevibacillus	4.48%	0	17
Clostridia>Clostridiales>Clostridiales Family XVIII. Incertae Sedis			
Genus Symbiobacterium	5.89%	0	26
Proteobacteria>Alphaproteobacteria>Rhizobiales>Hyphomicrobiaceae			
Genus Hyphomicrobium	2.60%	0	3
Firmicutes>Bacilli>Bacillales>Bacillaceae			
Genus Geobacillus	2.48%	3	16
Geobacillus debilis ID 301148		1	
Geobacillus thermodenitrificans ID 1422		1	
Geobacillus stearothermophilus ID 33940		1	
Genus below 1% "less represented"	2.40%	17	52
Others unclassified Genus	40.97%	0	158
Total	100%	20	318

A total of 52 OTUs sequences analysed at species level in genera, had a representation in the consortia below 1% (Table 22). It was possible to Identify 17 of these OTUs at species level, one belonging to the genus *Thermoaerobacter* from family *Clostridiales Family XVII Incertae Sedis*, phylum *Firmicutes*. The other 16 OTUs were identified at species level in genus *Thermobispora* from the family *Pseudonocardiaceae*, phylum *Actinobacteria*. The specie identified in genus *Thermoaerobacter* was assigned to *T. subterraneus* ID 175696 and the other 16 species from genus *Thermobispora* were assigned to *Thermobispora bispora* ID 2006. A total of 35 OTUs were not identified at species level.

Table 22. Bacterial Genus and identified species with relative abundance below 1% in consortium Azotdp36 Genus. Number of classified and unclassified OTUS at species level. The relative abundance percentage was based on the mean on sequences obtained.

Phylum>Class>Order>Family>Genus>Species	**Genus relative abundance**	**Identified species**	**Total OTUs**
Firmicutes>Bacilli>Bacillales>Bacillaceae			
Genus Aeribacillus	0.09%	0	3
Genus Bacillus	0.49%	0	7
Genus Caldalkalibacillus	0.10%	0	4
Firmicutes>Bacilli>Bacillales>Paenibacillaceae			
Genus Cohnella	0.004%	0	1
Genus Paenibacillus	0.29%	0	4
Proteobacteria>Alphaproteobacteria>Rhizobiales>Hyphomicrobiaceae			
Genus Devosia	0.08%	0	6
Proteobacteria>Alphaproteobacteria>Rhizobiales>Phyllobacteriaceae			
Genus Mesorhizobium	0.01%	0	1
Firmicutes>Bacilli>Bacillales>Thermoactinomycetaceae			
Genus Planifilum	0.01%	0	1
Proteobacteria>Alphaproteobacteria>Rhizobiales>Rhizobiaceae			
Genus Rhizobium	0.01%	0	2
Firmicutes>Clostridia>Clostridiales>Clostridiales_Family_XVII.Incertae_Sedis			
Genus Thermaerobacter	0.07%	1	2
Thermaerobacter subterraneus ID 175696		1	
Actinobacteria>Actinobacteria_class>Actinomycetales>Nocardiopsaceae			
Genus Thermobifida	0.01%	0	1
Actinobacteria>Actinobacteria_class>Actinomycetales>Pseudonocardiaceae			
Genus Thermobispora	0.38%	16	16
Thermobispora bispora ID 2006		16	
Firmicutes>Bacilli>Bacillales>Planococcaceae			
Genus Ureibacillus	0.85%	0	4
Total	2.40%	17	52

3.3.4.3. | Isolation and molecular identification of hydrolytic active colonies

To identify the hydrolytic bacteria in our consortium, CMC and xylan agar plates were used to select isolates with cellulase and xylanase activity. forty colonies with distinct morphologies from the consortium Azotdp36 were isolated. From these colonies, eight were enzyme active and presented at least one of the two activities tested (Table 23). Only six of the isolated bacteria revealed cellulase activity on CMC substrate and all the eight isolates presented xylanase activity. From these eight bacteria, four were selected because they presented the highest clearing zone on plates and were identified based in 16S.

All the four hydrolytic isolates identified, belong to the phylum *Firmicutes* the most predominant phylum in the consortium. The blast results showed that the isolate 7, belong to the second most represented genus in the consortium, the genus *Brevibacillus* from the family *Paenibacillaceae*. In this isolate it was possible to detect the presence of cellulases and xylanases, the blast results of 16 sDNA sequence pointed two high similarity 99% with the species of *Brevibacillus aydinogluensis* strain PDF25 and to the *Brevibacillus thermoruber* strain BT2. The isolates 2, 5 and 8 belong to the genus *Bacillus* from the family *Bacillaceae*, that was one of the genera with a representation below 1% in consortium Azotdp36. The isolate 2 and 5 had similar enzymatic activities, with Isolate 8 being the most active in the production of xylanase and with isolated 2 lacking for the presence of cellulases. The blast results revealed that both isolates 2 and 5 were very close (99% of similarity) to the *Bacillus licheniformis* strain DSM 13 and to *Bacillus sonorensis* strain NBRC 101234. The isolate 8 was set closely (99% similarity) to two different species, *Bacillus licheniformis* strain ATCC 14580 and *Bacillus sonorensis* strain NRRL B-23154. These strains blast results revealed bacteria strains that were identified in our consortia at genus level using 454, pyrosequencing method.

Table 23. Enzymatic characterization and molecular identification and of the isolated bacteria.

Isolate	Clearing zone diameter (mm)		Best match in GenBank database (accession number)	Similarity (%)
	CMC	Xylan		
Isolate 2	NDt	20	*Bacillus licheniformis* strain DSM 13 (NR_118996)	99
			Bacillus sonorensis strain NBRC 101234 (NR_113993)	99
			[*Brevibacterium*] *hal*otolerans strain DSM 8802 (NR_115063)	98
			Bacillus methylotrophicus strain CBMB205 (NR_116240)	97
Isolate 5	12	10	*Bacillus licheniformis* strain DSM 13 (NR_118996)	99
			Bacillus sonorensis strain NBRC 101234 (NR_113993)	99
			Bacillus aerius strain 24K (NR_042338)	99
			Bacillus atrophaeus strain NBRC 15539 (NR_112723)	98
Isolate 7	10	10	*Brevibacillus aydinogluensis* strain PDF25 (NR_117986)	99
			Brevibacillus thermoruber strain BT2 (NR_026514)	99
			Brevibacillus levickii strain LMG 22481 (NR_114928)	97
			Brevibacillus borstelensis strain DSM 6347 (NR_040984)	97
Isolate 8	5	40	*Bacillus licheniformis* strain ATCC 14580 (NR_074923)	99
			Bacillus sonorensis strain NRRL B-23154 (NR_025130)	99
			Bacillus amyloliquefaciens strain BCRC 11601 (NR_116022)	98
			Bacillus subtilis subsp. inaquosorum strain BGSC 3A28 (NR_104873)	98

3.3.5. | Remarks

The selection of a stable consortium that could efficiently hydrolysate grass mixtures was achieved in the present work. Although all the three initial consortia enriched at pH 3, 7 and 9 presented growth during the twelve weeks of enrichment process, only the consortia at pH 7 and 9 presented enzymatic activities for cellulases and xylanases. These results were reflected in the efficiency of grass mixtures hydrolysis, as the most efficient consortium was Azotdp36 at pH 9 that hydrolyse 50% of total dry weight of the grass mixtures at the end of 7 days.

The highest value for sCOD (29 400 mg/L) was obtained during pretreatment at 120 hours in 5% TS of substrate concentration. At this point the COD loss ratio percentage (36.2%) was lower compared with other substrate concentrations, meaning that less nutrients were consumed by the consortium Azotdp36, leaving more COD available to methanogenic bacteria.

The consortium Azotdp36 produced cellulases, xylanases and lipases, capable of hydrolyse cellulose and hemicellulose, the main constituents of plant cell walls. These enzymes were essential to obtain reducing sugars that achieved the highest concentrations of 1.32 g/L between 96 and 120 hours of pretreatment.

The maximum concentrations of acetic acid and butyric acid in our pretreatment reactors were 0.60 and 1.10 g/L respectively, and propionic acid reached a maximum inhibitory methanogens levels of 1.35 g/L at 48 hours.

The volumes of total biogas yield with pretreated substrates were higher than in the unpretreated grass, producing more 18.25% of total accumulated biogas at the end of the 42 days. Also, the methane content of pretreated grass mixtures bioreactors was higher than the unpretreated, this was noticed especially after day 21 up until the end of pretreatment. Production of methane in bioreactors with pretreated grass mixtures were 21.31% higher than in the controls without grass pretreatment. The maximum biochemical methane potential of pretreated grass, at the end of the 42 days of AD was enhanced relatively to the unpretreated grass. This pretreatment resulted in a raise of 77.35% of biochemical methane potential (168.36 $LCH_{4/}Kg$ VS added) for pretreated grass mixtures, at the end of the 42 days of AD.

The relative bacterial abundance populations in Aztodp36 were mainly from the phylum *Firmicutes* representing 81.21% of the consortium. At genus level the consortium was mainly composed by *Thermobacillus* (41.18%), *Symbiobacterium* (5.89%), *Brevibacillus* (4.48%), *Hyphomicrobium* (2.60%) and *Geobacillus* (2.48%), having the other genus a representation below 1% in the Azotdp36 consortium. In the one of the major genus *Geobacillus*, the pyrosequencing analysis identified three OTUs closely related to the species of *Geobacillus debilis*, *Geobacillus thermodenitrificans*. and *Geobacillus* *stearothermophilus*. Other strains were also identified in genus lower representation, in *Thermaerobacter* genus one OTU was closely related to the species of *Thermaerobacter subterraneus*. and in *Thermobispora* genus a strain was closely related to the species of *Thermobispora bispora*.

All together, these results show that the bacteria community present in the Azotdp36 consortium was efficient for the solubilization and pretreatment of grass mixtures, and proved that not only could increase total biogas production, but also methane production rate was faster in pretreated grass. The biochemical methane production was also enhanced by 77.35% in pretreated grass mixtures.

3.4. | ENHANCING ANAEROBIC DIGESTION OF FEATHERS USING A THERMOPHILIC MICROBIAL CONSORTIUM PRETREATMENT

Chicken feathers represent approximately 6% of the entire weight of the chicken, resulting in an immense size of waste. There are different utilizations for feather waste, such as incineration for energy production and animal feed production, after suitable pretreatment. However today most of feather wastes still end up disposed in landfills.

Feathers contain high amount valuable building blocks, amino acids, as they consist of 90% of a protein, keratin. However, feathers should be treated enzymatically rather than mechanically or chemically, which may destroy amino acids and consumes energy (Brandelli et al., 2010). In this work, we investigated the efficiency of an aerobic thermophilic bacterial consortia for the hydrolysis of feathers to obtain keratin soluble protein, amino acids and VFA, thus facilitating digestibility and enhancing methane production.

3.4.1. | Selection of a bacterial consortium for feathers hydrolysis

After the enrichment process for three months (passage twelve), a thermophilic microbial consortium enriched on a feather substrate was selected for further studies. Consortium Azotf36 enriched at pH 9 was selected as it was capable of effectively hydrolyse the chicken feathers (Figure 41). We could see at the end of passage seven, that our consortium was not fully stabilized, achieving an incomplete hydrolysis at 72 hours of incubation, as shown in figure 41 c).

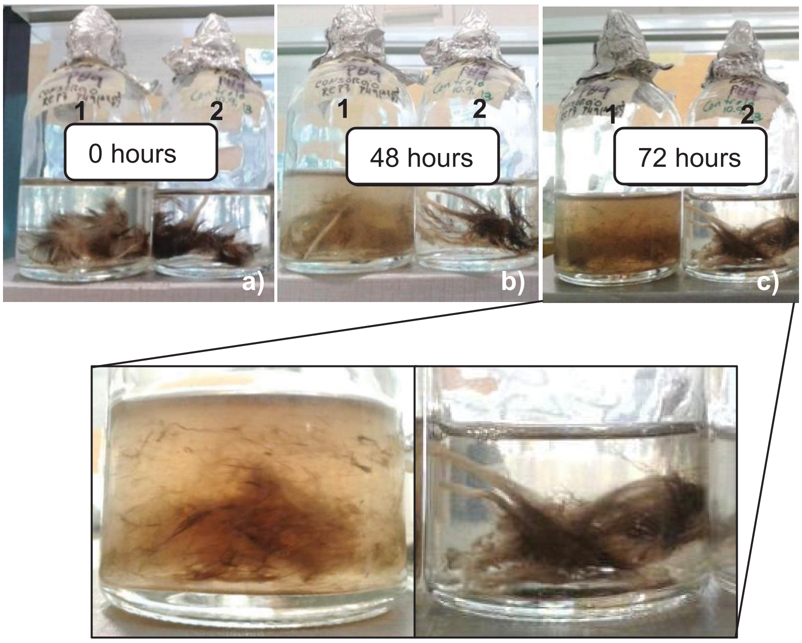


Figure 41. Incomplete hydrolysis of feathers by consortium Azotf36 at the end of passage 7. 1) enrichment reactor with consortium Azotf36, 2) control without bacteria

During the subsequent passages the consortia became more time efficient, achieving complete feather hydrolysis after 48 hours and maintaining this hydrolysis time through the following passages (Figure 42). This indicate us that the enrichment process was complete and after twelve weeks we stopped the enrichment process.

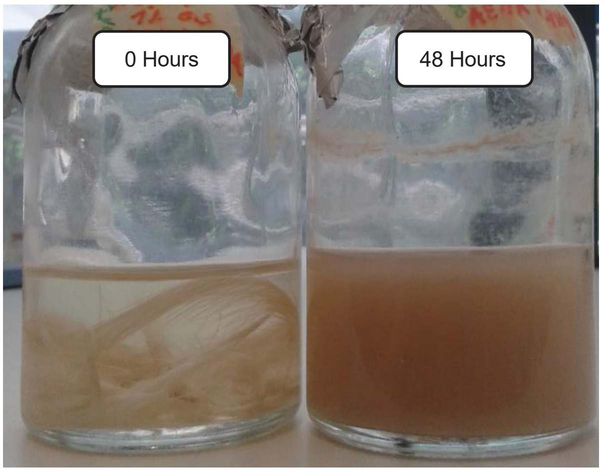


Figure 42. Hydrolysis of feathers after by consortium Azotf36 at the end of 48 hours enrichment passage 12.

The stability of the consortium Azotf36 was demonstrated by its ability to maintain hydrolysis after tolerating several rounds of subculture with feathers substrate for more than 2 years and to keep activity after being stored at room temperature or frozen at -80 ºC in 20% glycerol for periods of more than 6 months.

3.4.2. | Effect of consortium on feathers during pretreatment

3.4.2.1. | Weight loss

At the end of pretreatment time (144 hours) we accessed the effect of consortium Azotf36 in the dry weight loss at three different concentrations of feather substrates 1%, 2.5% and 5% TS. The dry weight loss at concentrations of 1% and 5% TS presented lower feather weight losses when comparing with the concentration of 2.5% TS (Table 24). The effect of temperature (60 ºC) and agitation on feathers dry weight loss was verified in the controls reactors. At the end of pretreatment reactors with the controls without the consortium Azotf36 were as follows; 1.03%, 2.6% and 4.83% of dry weight loss for concentrations of 1%, 2.5% and 5% respectively. In general, a temperature at 60 ºC and an agitation of 150 r.p.m., did not had great influence in feather hydrolysis independent of the feather concentrations. In figure 45 is presented the effect of the Azotf36 consortium on the dry weight loss at the end of 144 hours of pretreatment at three different feathers concentration. To evaluate only the effect of our consortium in the feathers alone we subtracted the dry weight loss values of consortium with the respective controls reactors with same concentration.

Table 24. Dry weight loss % of feathers at three concentrations of TS at the end of 144 hours of pretreatment. Values presented are corrected with the respective controls. The mean values and standard errors represented by the same letters indicate non-significant differences in dry weight loss between the waste concentrations P≥0.05 (Tukey multiple range test)

Substrate concentration	Percentage of Weight loss
1 % TS	76.08 ± 3 (a)
2.5 % TS	93.94± 2 (b)
5 % TS	72.20 ± 5 (a)

The consortium Azotf36 was the most efficient at a concentration of feathers of 2.5% of TS achieving 94% of feathers dry weight loss, which that was statistically different (p≤ 0.05, DF=8 F=14.57, sig=0.005) from the other two concentrations. The efficiency of hydrolysis at 1% and 5% of TS dropped to 76% and 72% respectively.

3.4.2.2. | sCOD and COD balance

To determine when to stop the pretreatment and to minimize consumption by our consortium of soluble organic materials, soluble COD, COD loss and the rate of COD loss (loss ratio) were monitored.

The sCOD had similar fluctuation during pretreatment in all the three feathers concentrations (Table 25). At 48 hours of pretreatment there was a peek of the sCOD in hydrolysates following by a progressive decrease to the end of the process. The highest soluble COD value were found after 48 hours at the concentration of 2.5% of TS of feathers with 10 300 mg /L. The values for the concentrations of 1% TS and 5% TS where lower reaching the highest values of 3 244 mg/L and 7 600 mg/L, respectively. At the end of pretreatment, the values of soluble COD add decreased mostly being consumed by the bacteria (Figure 43).

Before pretreatment start, the initial COD in the system was calculated as the sum of COD substrate and COD medium, which was equal to the sum of sCOD in hydrolysate, COD substrate residue and COD loss during pretreatment. This calculation allowed us to know the total COD consumed or COD loss by our bacteria in the consortium during the pretreatment (Table 22).

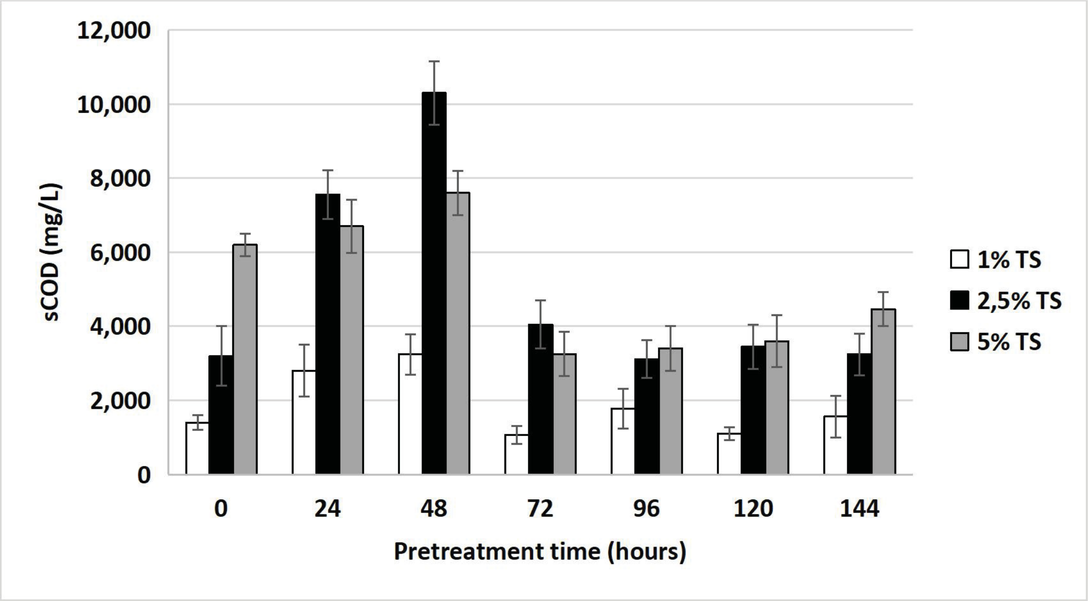


Figure 43. Soluble COD during pretreatment

In the analysis of COD loss, it was found that longer pretreatment times, lead to higher organic materials loss, thus Indicating that we should stop the pretreatment in earlier stages. This would minimize the consumption of organic materials during consortium culture incubation and make them available for methane production.

At the end of pretreatment, the total COD loss were as follows, the concentration of 1% TS showed 72% of COD loss (12 240 mg/L), at 2.5% of TS an 89.02% COD loss (33 206 mg/L), and at 5% TS a COD loss of 69.67% (36 088 mg/L). The lower percentages of COD loss in 5% TS concentration associated with lower values of soluble COD when compared with 2.5% TS concentration, could be related to a lower hydrolysis efficiency of our consortia associated at high concentrations of substrate, that would lower the available oxygen in medium needed for our consortia efficiently hydrolyse.

During the first 48 hours, the COD loss was less accentuated and the soluble COD reached its peak in all 3 concentrations (Table 22). This data indicates that in the first 48 hours, soluble organic materials were produced by hydrolytic microorganisms at a higher rate they were consumed by fermentative microorganisms of Azotf36. After the 48 hours, the COD loss raised until stabilize in the last two days of pretreatment, during this time these fermentative microorganisms were also consuming soluble materials but at higher rate than the production by hydrolytic ones.

Taking in consideration this data we selected to stop pretreatment based on two conditions the first was a highest soluble COD point and the second the lowest COD loss ratio. We selected the concentration of 2.5% TS at 48 hours of pretreatment as it reached the highest values of soluble COD 10 300 mg/L when compared to 1% TS and 5% TS, and one of the lowest COD loss ratio of 22.52%, at this point. Although at 48 hours the COD loss ratio was superior in 1% than 2.5% TS we selected the pretreated feather from the 2.5% TS concentration as it had 3 more times of soluble COD available methanogenic bacteria.

Table 25. COD balance during pretreatment with Azotf36

	Before pretreatment			During pretreatment			
Substrate concentration (% TS)	**COD substrate (mg/L)**	**COD medium (mg/L)**	**Pretreatment time (Hours)**	**sCOD hydrolysate (mg/L)**	**COD substrate residue (mg/L)**	**COD loss system (mg/L)**	**Loss ratio system (%)**
1	15600	1400	0	1400	15600	0	0.00
			24	2800	13100	1100	6.47
			48	3244	10700	3056	17.97
			72	1067	8100	7833	46.08
			96	1778	5800	9422	55.42
			120	1100	4200	11700	68.82
			144	1560	3200	12240	72.00
2.5	34100	3200	0	3200	34100	0	0.00
			24	7556	26500	3244	8.70
			48	10300	18600	8400	22.52
			72	4050	9600	23650	63.40
			96	3111	5500	28689	76.91
			120	3450	2050	31800	85.25
			144	3244	850	33206	89.02
5	45600	6200	0	6200	45600	0	0.00
			24	6700	38400	6700	12.93
			48	7600	28900	15300	29.54
			72	3255	24400	24145	46.61
			96	3400	20400	28000	54.05
			120	3600	15750	32450	62.64
			144	4463	11250	36088	69.67

3.4.2.3. | Pretreatment solubilization

The percentage of solubilisation in the pretreatment, was calculated as the percentage of the initial COD added to the vials that was solubilized during the pretreatment. We calculated the percentage of solubilization at 48 hours and at the end of pretreatment. For the calculations, we took in consideration the soluble COD that was lost due to bacteria consumption based on the values of table 26.

Total solubilized COD at 48 hours was 18 700 mg/L taking in consideration the already lost COD consumed by our bacteria the pretreatment had a solublization of 45%, taking in account that 8 400 mg/L of COD was already consumed by the Azotf36 consortium. At the end of pretreatment, we achieved 97% of COD solubilization using the Azotf36.

Table 26. Results of percentage of solubilization at 48 hours and at the end of pretreatment for the concentration of 2.5 % TS.

	(COD mg/L) At 48 hours	**(COD mg/L) At the end of pretreatment**
sCOD−FinalPT	18 700	36 450
sCOD−inicialPT	3200	3200
CODtotal−inicialPT	34 100	34 100
PSprt%	45%	97%

3.4.2.4. | Enzymes in supernatant of Azotf36 consortium

For the detection of enzymes in the hydrolysate supernatant, two tests were used, an agar diffusion technique and a API ZYM test kit (Table 27). The hydrolysate supernatant with 48 hours of pretreatment was tested for the presence of 21 different enzymes the test results revealed that our consortium is capable of producing proteases and lipases as it could create clear zones in agar plates, 20 mm diameter in skim milk agar plates and 10 mm diameter for tributyrin agar plates.

Additionally, enzymatic activities were obtained in the results for of API ZYM test. Our Azotf36 consortium was positive for the enzymes; Alkaline phosphatase, Acid phosphatase, Phosphohydrolase; Esterase C_4, Esterase, Lipase C_8, Leucine amino-peptidase and β-glucosidase. And negative for the enzymes; Lipase C_{14}, Valine amino-peptidase, Cystine amino-peptidase, Trypsin, Chymotrypsin, α-galactosidase, N-acetyl-glucosaminidase, α-glucosidase, β-galactosidase, β-glucoronidase, α-mannosidase, α-fucosidase. The chromogenic substrates differentiate between hydrolysis of C_4 esters (esterase activity) C_8 ester (ester lipase activity) and C_{14} ester (lipase activity). Usually hydrolysis of tributyrin correlates with the presence of lipases able to hydrolyse glycerides with C_4 fatty acids. Lipolytic thermophiles are found among different phyla of aerobic and anaerobic bacteria. The consortium was also tested for the degradation of keratin from hair, but the azotf36 consortia was not able to decompose keratin fibers from hair.

Table 27. Results of the enzymatic assays in agar plates and API ZYM test kit.

Enzyme assayed for;	Substrate	Results
Protease	Skim milk agar	+ (20mm)
Lipase	Tributyrin agar	+(10mm)
Alkaline phosphatase	2 naphthyl-phosphate	+
Acid phosphatase	2 naphthyl-phosphate	+
Phosphohydrolase	Naphthyl AS-BI-phosphate	+
Esterase C_4	2 naphthyl-butyrate	+
Esterase Lipase C_8	2 naphthyl-caprylate	+
Lipase C_{14}	2 naphthyl-myristate	NDt
Leucine amino-	L-leucyl-2-naphthylamide	+
Valine amino-peptidase	L-valyl-2-naphthylamide	NDt
Cystine amino-	L-cystyl-2-naphthylamide	NDt
Trypsin	N-benzol-DL-arginine-2-	NDt
Chymotrypsin	N-glutaryl-phenylalanine-2-naphthylamine	NDt
α-galactosidase	6-Br-2-naphthyl-α-D-Galactopyranoside	NDt
β-glucosidase	6-Bromo-2-naphthol-α-D-Galactopyranoside	+
N-acetyl-β-glucosaminidase	1 naphthyl-N-acetyl-β-D-glucosaminide	NDt
α-glucosidase	2 naphthyl-2-D-glucopyranoside	NDt
β-galactosidase	2 naphthyl-β-D-galactopyranoside	NDt
β-glucoronidase	Naphthyl-AS-BI-β-D-glucuronide	NDt
α-mannosidase	6-bromo-2-naphthyl-2-D-mannopyranoside	NDt
α-fucosidase	2 naphthyl-α-L-fucopyranoside	NDt

3.4.2.5. | Soluble protein and free amino acids

During the pretreatment time the soluble protein was monitored and the concentration and identification of amino acids at the end of pretreatment was analysed. The effect of temperature and agitation on the feathers soluble protein was relatively low and constant during the pretreatment at each concentration. The soluble protein in the supernatant during the pretreatment time without bacterial consortia (controls) were as follows; at 1% of TS concentration was 85.83 ± 19.36 µg/ml of soluble protein. The mean of soluble protein for the 2.5% TS concentration was 177.1 ± 16.13 µg/ml. The mean of soluble protein for the 5% TS concentration was 239.08 ± 22.43 µg/ml. In figure 44 is presented the soluble protein release during Azotf36 consortium solublization of the feathers.

The hydrolysate was analysed for a total of 35 different amino acids but only 4 amino acids were above the limit of detection in the hydrolysate powder: Aspartic acid, Glutamic acid, phenylalanine and Taurine (Table 28).

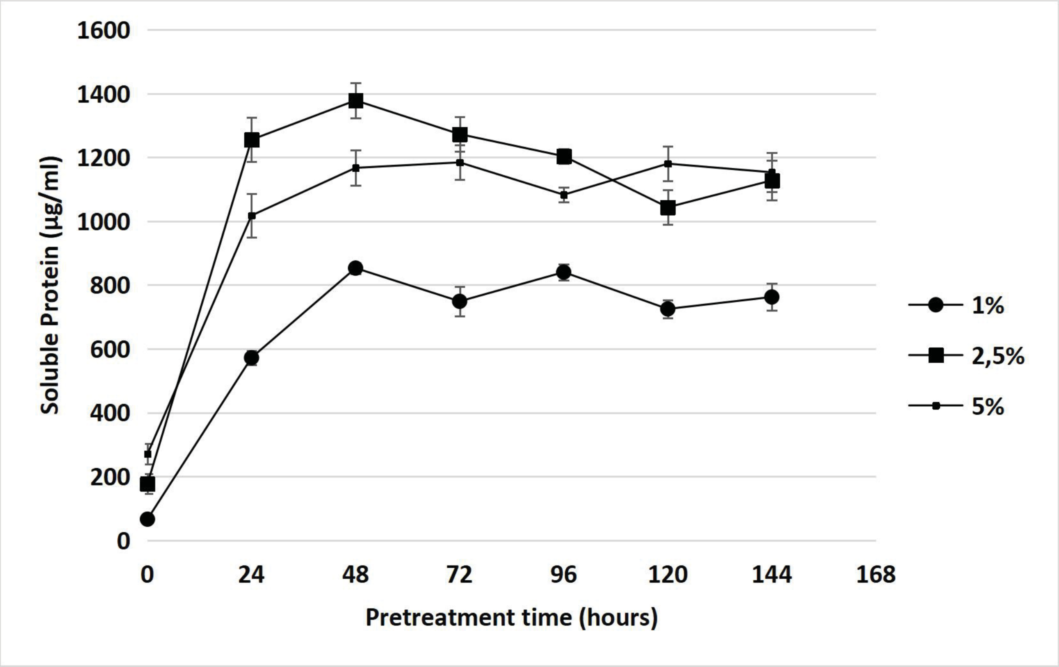


Figure 44. Soluble protein in the supernatant during Azotf36 consortium pretreatment. Initial soluble protein before pretreatment 1%TS (56.37 µg/ml) 2.5% TS (176.75 u µg/ml) and 5% TS (270.12 µg/ml)

Table 28. Identification and quantification of free amino acids in hydrolysate

Amino acids	Concentration (g/Kg hydrolysate)
Aspartic acid	0.3
Glutamic acid	0.3
Phenylalanine	0.7
Taurine	0.1
Total amino acids	6.3

3.4.2.6. | pH and Volatile organic products changes

The pH of Azotf36 hydrolysates for the three substrate concentrations of 1.0%, 2.5% and 5.0%, declined till day 2 (Figure. 45). The lowest pH values occurred at 48 hours for the three substrate concentrations 1.0%. 2.5% and

5.0%. The lowest pH values of the Azotf36 hydrolysates were for 8 7.6 and 7.2 for 1% 2.5% and 5%, respectively. The pH after 48h gradually increased in the three subtract concentrations reaching 8.6 in the 1% of TS and 8.6 and 7.5 in concentration 2.5% and 5% of TS respectively at the end of the pretreatment time. When substrate concentration was 2.5%. the pH hit the lowest value, 7.6. The decrease of pH values is directly correlated with the acidification of the medium related to the hydrolysis and production VFA.

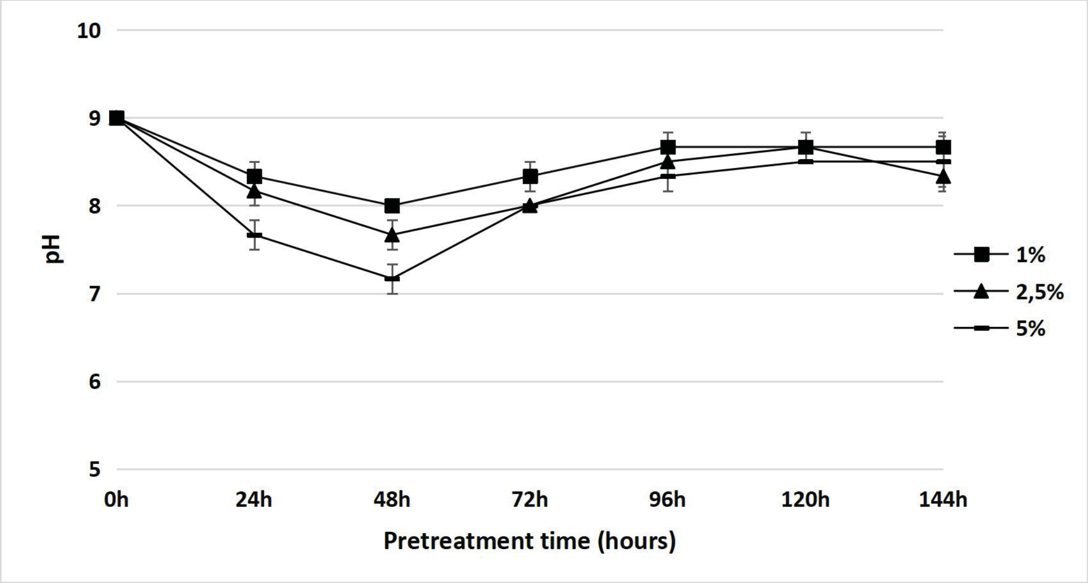


Figure 45. pH variation during pretreatment in the 3 concentrations

The monitoring of VFA during pretreatment was made with the previous selected 2.5% TS concentration, as it was the concentration tested in anaerobic assays (Figure 46). The concentration of VFAs was registered during 120 hours and a general raise was registered in the first days of pretreatment in the reactors with the consortium Azotf36. At the beginning of pretreatment, the concentrations of the three VFAs, in the bioreactors with the control and with our consortium Azotf36 were considered the same and as follows; Acetic acid (0.18 g/L), Propionic acid (0.26 g/L) and Butyric acid (0.20 g/L).

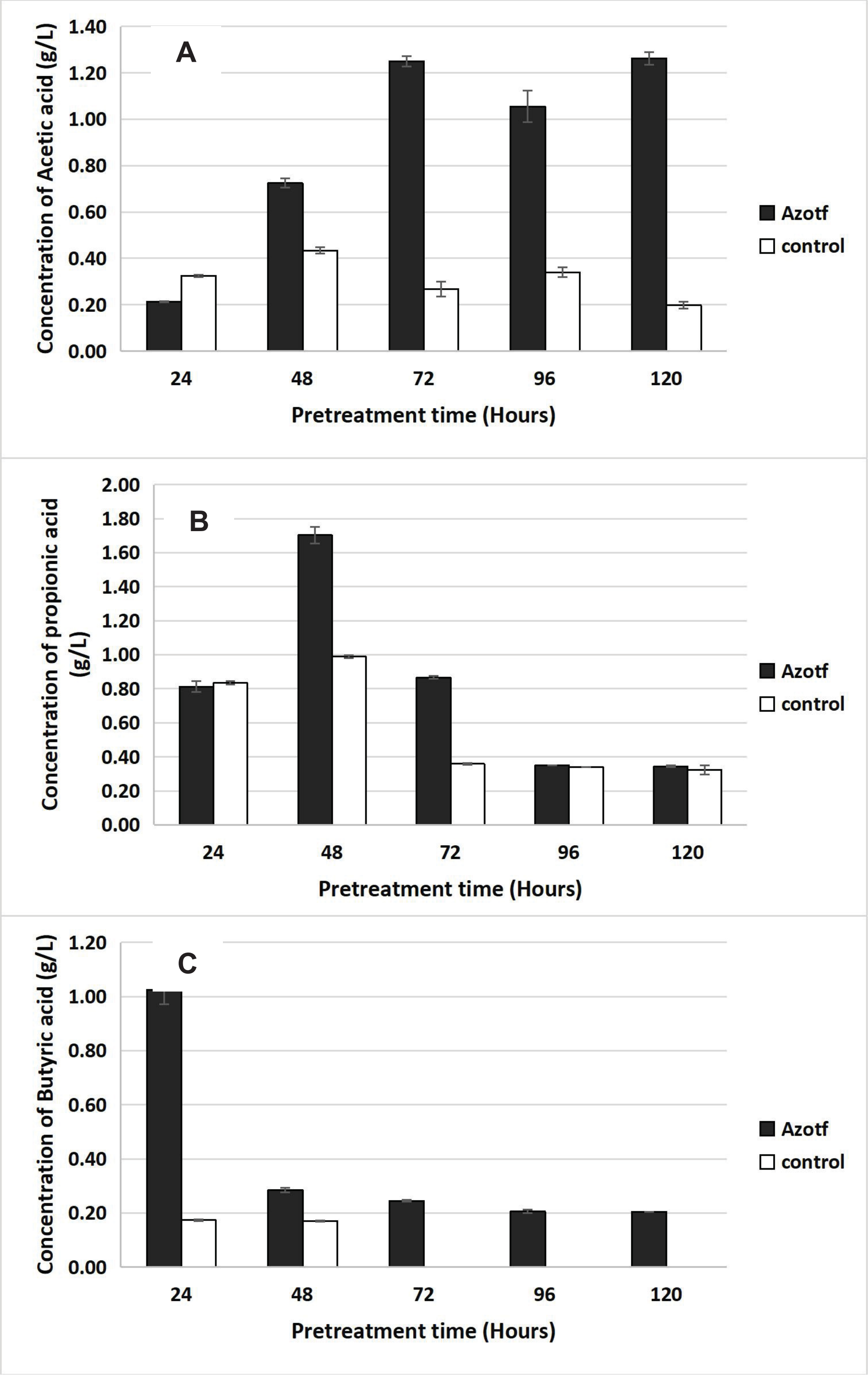


Figure 46. Quantitative analysis of major volatile organic products (VOPs) by GC during pretreatment. (A) acetic acid; (B) propionic acid; (D) butyric acid.

It was clear that the raise of VFAs and the initial high hydrolysis of the feathers contributed for the decrease of the pH. Although after the 48 hours the pH started to raise, this could be in consequence of VFAs general decrease after 48 hours of pretreatment. Analyzing the production of acetic acid in bioreactors with the consortium Azotf36, we saw an increase in the first 72 hours reaching a maximum of 1.25 g/L. Afterward acetic acid concentrations stabilized till the end of the pretreatment, the concentration of acetic acid in the controls varied between 0.18 and 0.43 g/L during the pretreatments.

The concentration of propionic acid had an increase during the first 48 hours, declining in the following days and stabilizing in the last two days. The concentrations fluctuate between 0.26 and a maximum of 1.70 g/L that was reached at 48 hours and afterward the concentration declined to the end of the pretreatment. The concentration of propionic acid in the controls were between 0.26 and 0.99 g/L having similar variation as the concentration of propionic acid in the Azotf36 reactors. The other VFA analysed was butyric acid, it started with 0.20 g/L reaching the maximum of 1.02 g/L at 24 hours. After 24 hours of pretreatment the concentrations of butyric acid decreased drastically and stayed between 0.20 and 0.28 g/L to the end of pretreatments. We only could detect butyric acid in the controls in the first 48 hours with values between 0.17 and 0.20 and after that butyric acid was no more detect till the end of pretreatments.

The VFA concentrations were not the first option for the selection of pretreatment stop time, the maximum values of total VFAs analysed were registered at 48 hours with 2.71 g/L, coinciding with the pretreatment stop time.

3.4.3. | Biogas production

3.4.3.1. | Biogas volumes

The total accumulated biogas yields for pretreated feathers at 2.5% TS concentration were higher than at untreated feathers. Differences in accumulated biogas volumes were more visible after day 14 till the end of the assay. The pretreated feathers produced 1.2 times more biogas than untreated feathers. At the end of the AD, bioreactors with pretreated feathers resulted in the production of 1125 ml of biogas. The volume of biogas production in bioreactors with unpretreated feathers were 945 ml total accumulated biogas (Figure 47). This result shows that Azotf36 pretreatment can enhance the biogas yields of feathers.

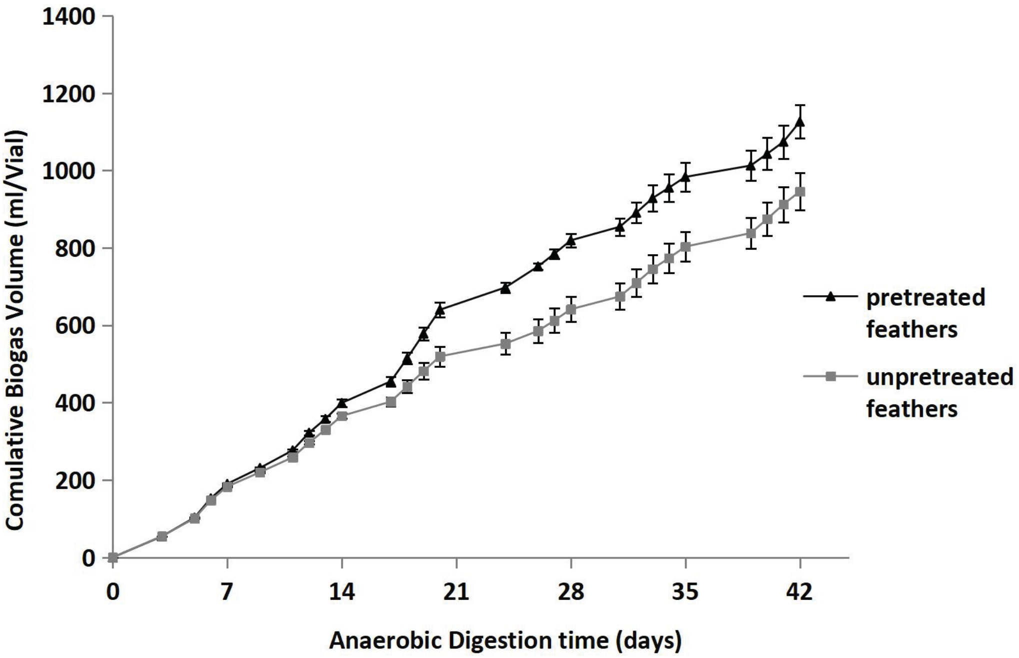


Figure 47. Total accumulated biogas volume during AD of pretreated and unpretreated feathers.

The daily accumulated biogas production shown in figure 48, revealed the days in which more biogas was produced. Analyzing the results, it was clear that in the first 7 days no differences in biogas volumes were noticed between pretreated and unpretreated feathers anaerobic reactors. Between the day 9 and the day 28, AD bioreactors with Azotf36 pretreated feathers produced higher volumes than the bioreactors with unpretreated feathers. After day 28 up until the end of pretreatment no differences were clear between the pretreated and unpretreated anaerobic bioreactors. The results indicate that our consortium pretreatment helps in the reduction of time for biogas production, therefore reducing hydraulic retention times of organic matter in the anaerobic bioreactors.

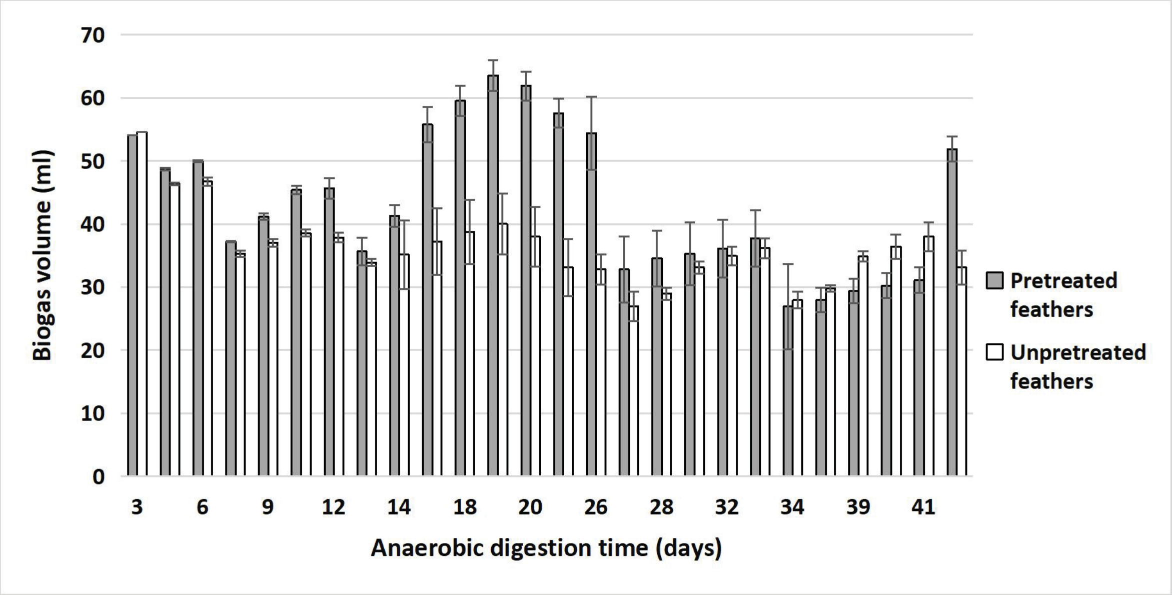


Figure 48. Mean of daily biogas volume production per vial during AD.

3.4.3.2. | Methane production

An increase in the methane production rate indicated that the pretreated feathers had become more readily biodegradable. The methane production rate of pretreated feathers was obviously faster than that of the unpretreated feathers specially between days 14 and 28 representing 60% of the total biogas produced (Figure 49). These results were in frame with the peaks of total biogas production that had its maximum values between days 9 and 28. A rapid methane production rate means short digestion times that could bring economic benefits through the increase of methane production efficiency.

At the end of the AD (day 42) the total methane content in bioreactors with pretreated feathers was 77% higher than in the controls without feather pretreatments. This result validates the efficiency of Azotf36 in the enhancement of methane production resulting in the efficient solubilization of feathers.

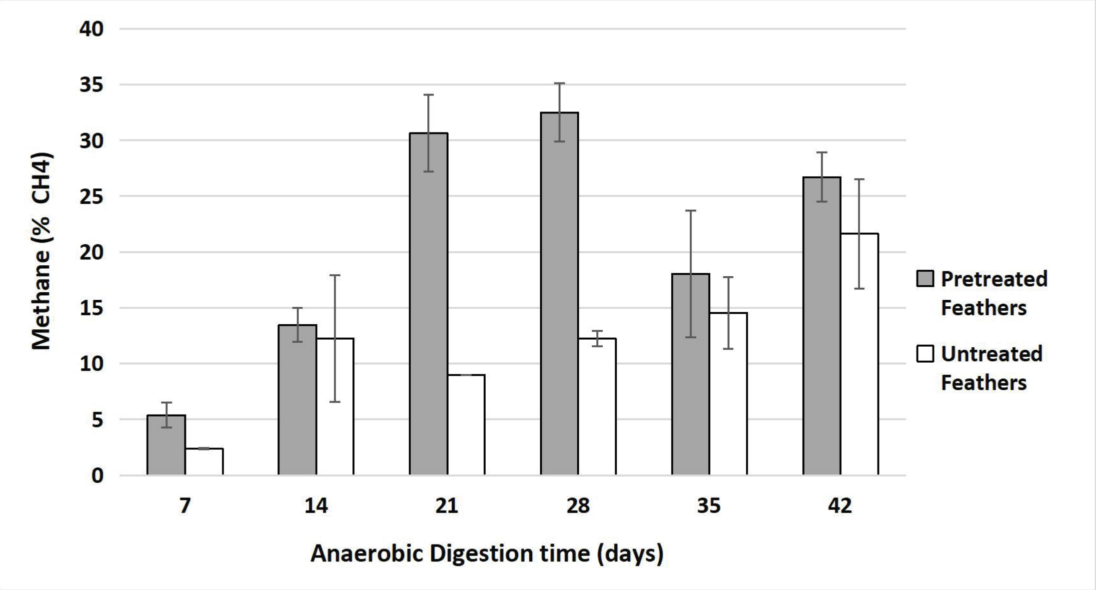


Figure 49. Methane content % in anaerobic reactors with and without feathers pretreatment with consortia Azotf36.

The maximum biochemical methane potential was 59.6 LCH_4/Kg VS added for the pretreated feathers with the consortium Azotf36. Where the untreated feathers biochemical methane production was 30.45 LCH_4/Kg VS added (Figure 50). This pretreatment resulted in an increase of methane potential. These values of methane were obtained of 95% more methane production per kilo of volatile solids of feathers added, when the sCOD reached the highest values at 48 hours of pretreatment with a concentration of 2.5% TS of feathers.

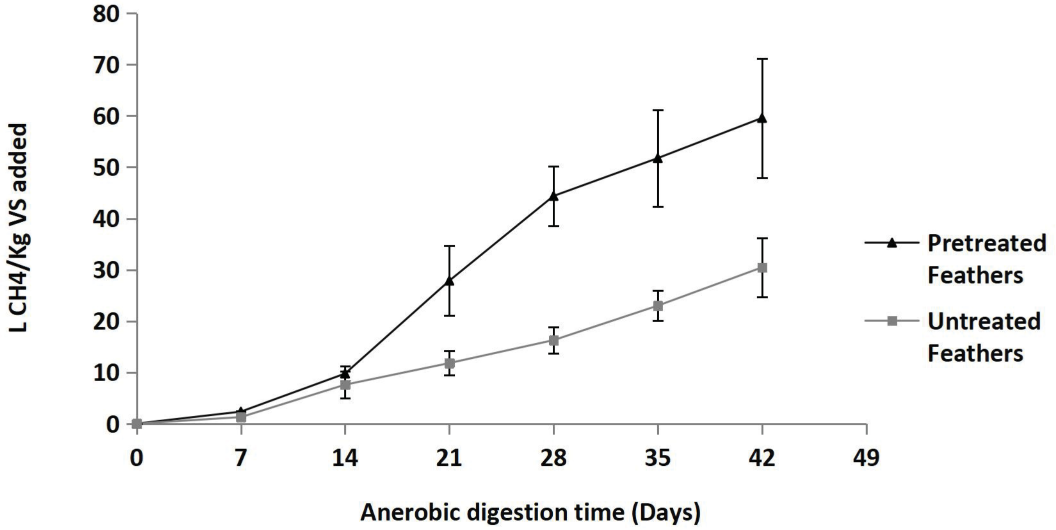


Figure 50. Biochemical Methane Potential curves for Azotf36 pretreated feathers and unpretreated raw feathers.

3.4.4. | Phylogenetic analysis of bacterial community

3.4.4.1. | Bacterial diversity and richness in Azotf36 consortium

The OTUs sequences from pyrosequencing were used to characterize and estimated the bacterial diversity, richness and relative abundance of the microbial consortium Azotf36. The adequacy of the sample size for the determination of diversity within the 16S rDNA library was evaluated by rarefaction analysis. As shown in figure 51, the calculated rarefaction curve was partially saturated at 3% divergence which indicates that the analysis had covered the bacterial diversity present in the consortium.

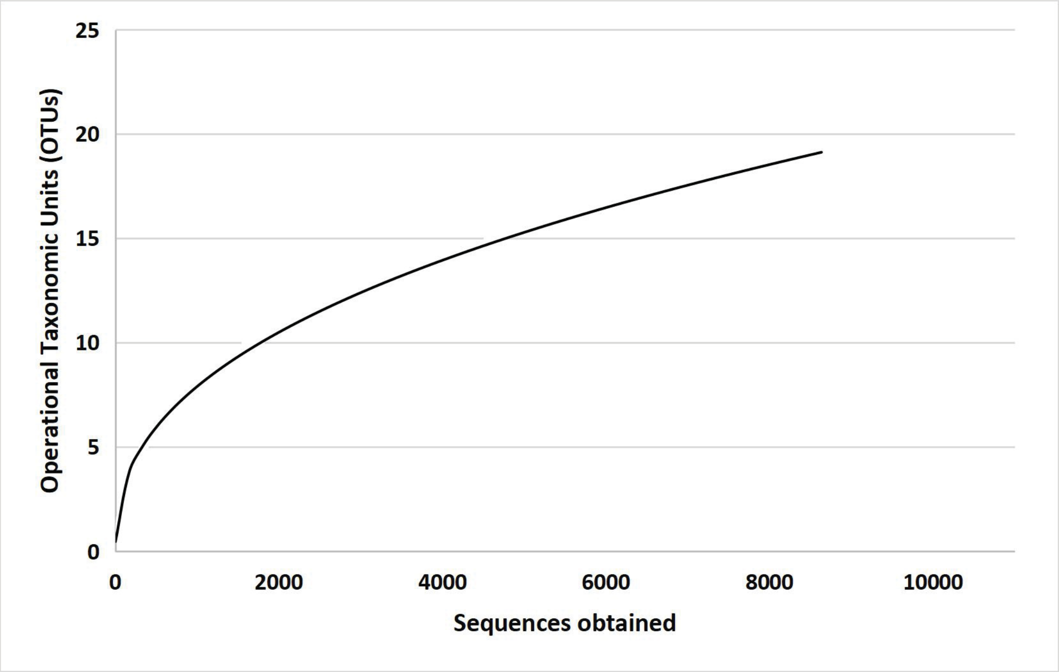


Figure 51. Rarefaction curves from 16S rRNA gene sequences of microbial consortia Azotf36. Sequences were clustered at a genetic distance of 3%. OTU values were calculated by the mean of replicates. Curve was based on a non-linear regression power model

Using Chao1 richness estimator, the maximum number of OTUs for the bacterial community expected at 3% genetic distance was 118, 139 and 139 for replicates G, H and I, respectively (Table 29). The obtained OTUs in replicate G was 14 covering 95.89% of the predicted Chaos richness estimator. In replicate H, we obtained 12 OTUs, representing 70.59% from the predicted Chaos results of 139 OTUs. In the replicate, I we obtained 21 OTUs covering 80.77% from expected chaos result of 26 OTUs.

Table 29. Diversity indices Chaos results of the 3 replicates from Azotf36

Consortia	Sample	Gene region	Chao expect	Chao obtained	Coverage %	Reads	OTUs
Azotf36	G	V3	14.60	14	95.89	8619	14
Azotf36	H	V3	17.00	12	70.59	6593	12
Azotf36	I	V3	26.00	21	80.77	12175	21

3.4.4.2. | Composition of the bacterial community in Azotf36 consortium

A total of 27 387 sequences were analysed in this study after reads sequences trimming the sequences of either short length or low quality and removing the chimeras, and selecting the reads without fail blast (Figure 52). The distribution of valid sequences in the replicates were as follows; replicate G with 8619 sequences, replicate H with 6 593 and replicate I with 12 175 sequences. The sequences were used to calculate percentage relative abundance at phylum and genus level of the microbial community from Azotf36.

The relative abundance of populations in Azotf36 were mainly from the phylum *Firmicutes* with 99.99%, and the phylum *Proteobacteria* with were represented with 0.01%.

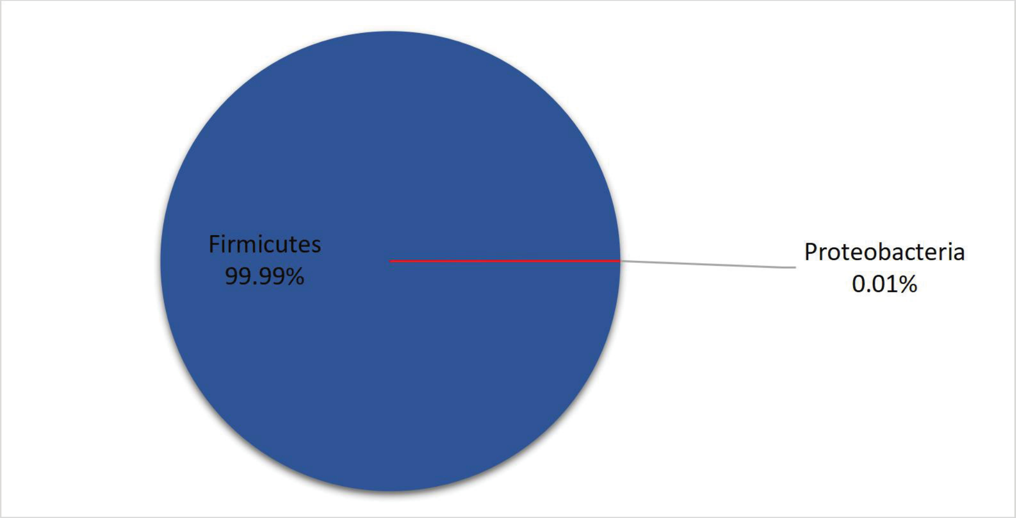
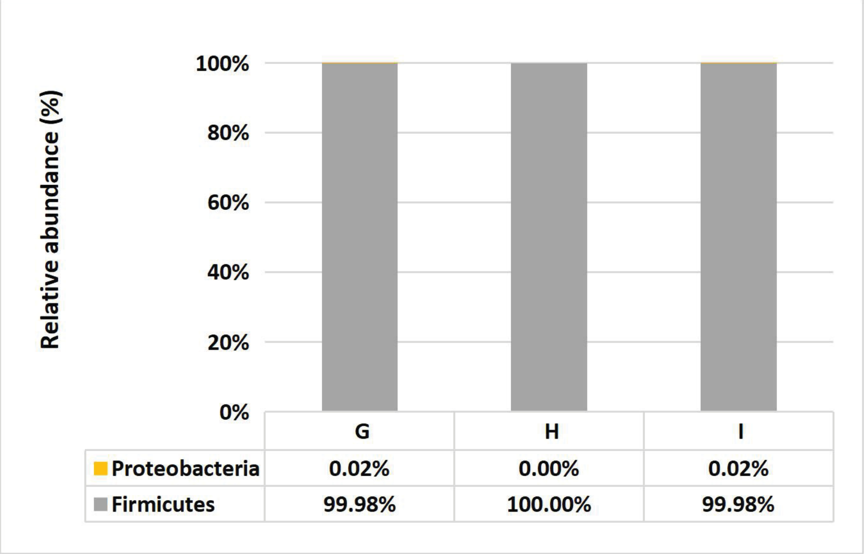


Figure 52. Percentage of mean relative abundance of sequences obtained in Azotf36 bacterial consortia at phylum level

Analyzing the 3 replicates the relative abundance was evenly distributed, for the phylum *Firmicutes* the values were above 99.7% of abundance in the three replicates and the phylum *Proteobacteria* with relative abundance below 0.21% (Figure 53).



	G	H	I
🟨 Proteobacteria	0.02%	0.00%	0.02%
▨ Firmicutes	99.98%	100.00%	99.98%

Figure 53. Relative abundance of sequences obtained in the three replicates of Azotf36 bacterial consortia at phylum level

The relative bacterial abundance in Azotf36 at genus level were mainly from *Brevibacillus* genus representing 99.20% of the consortium. The other six genus represented in the consortium had a representation below 1% and by decreasing order were; *Aneurinibacillus* (0.37%), *Geobacillus* (0.24%), *Aeribacillus* (0.04%), *Bacillus* (0.01%) and *Thermobacillus* with 0.005%. It was not possible to identify 0.13% of the sequences with any genus (Figure 54).

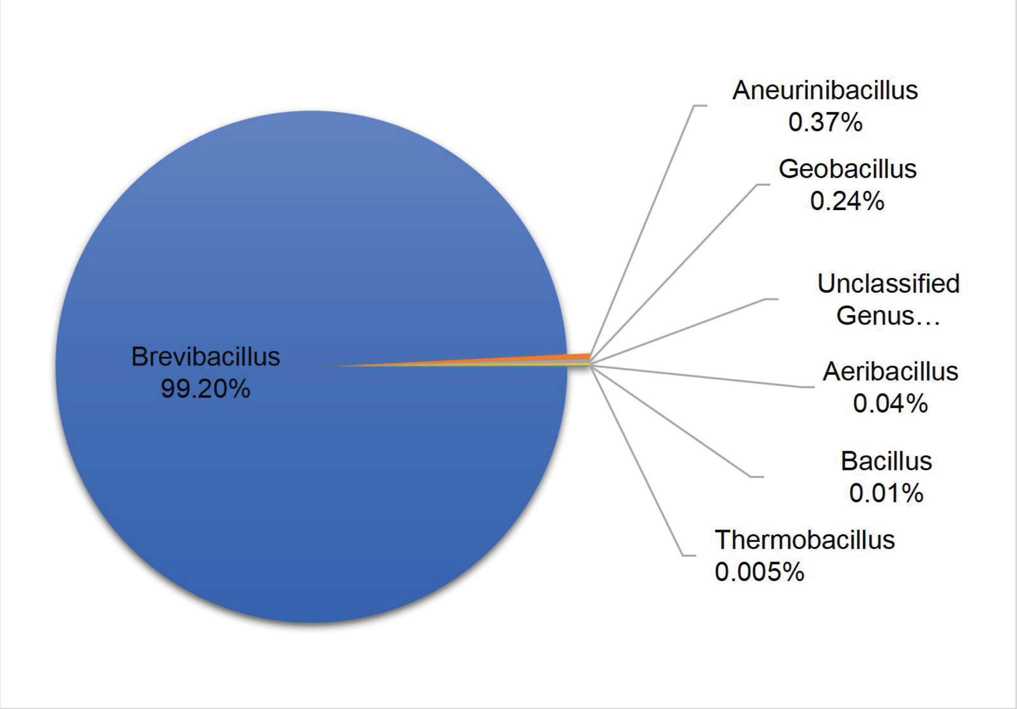


Figure 54. Mean of relative abundance of the total sequences obtained in Azotf36 bacterial consortia at genus level

Analyzing bacterial populations in Azotf36 at species level, we could identify 3 species (3 different OTUs) from a total 47 OTUs sequences (Table 30). All the 3-species identified belong in the genera groups with less than 0.67% of representation in our consortia.

Table 30. Bacterial genus and identified species with high relative representation (above 1%) in consortium Azotf36. Number of classified and unclassified OTUS at species level. The relative abundance percentage was based on the mean on sequences obtained.

Phylum>Class>Order>Family>Genus>Species	**Genus rel. abundance**	**Identified species**	**Total OTUs**
Firmicutes>Bacilli>Bacillales>Paenibacillaceae			
Genus Brevibacillus	99.20%	0	30
Genus below 1% "less represented"	0.67%	3	14
Unidentified Genus	0.13%	0	3
Total	100%	3	47

One of the OTUs was identified as a species that belong to the *Geobacillus* genus from the family *Bacillaceae*, phylum *Firmicutes* the OTU blast were identified as related to the specie of *Geobacillus thermodenitrificans* ID 33940. The other two OTUs were from *Aneurinibacillus* genus from family *Paenibacillaceae*, phylum *Firmicutes* and the OTUs blast revealed they were related to the specie of *Aneurinibacillus thermoaerophilus* ID143495 (Table 31).

Table 31. Bacterial Genus and identified species with relative abundance below 1% in consortium Azotf36 Genus. Number of classified and unclassified OTUS at species level. The relative abundance percentage was based on the mean on sequences obtained.

Phylum>Class>Order>Family>Genus>Species	**Genus rel. abundance**	**Identified species**	**Total OTUs**
Firmicutes>Bacilli>Bacillales>Bacillaceae			
Genus Aeribacillus	0.04%	0	3
Genus Bacillus	0.01%	0	3
Genus Geobacillus	0.24%	2	5
Geobacillus thermodenitrificans ID 33940		2	
Firmicutes>Bacilli>Bacillales>Paenibacillaceae			
Genus Aneurinibacillus	0.37%	1	2
Aneurinibacillus thermoaerophilus ID143495		1	
Thermobacillus	0.01%	0	1
Total	0.67%	3	14

3.4.4.3. | Isolation and molecular identification of hydrolytic active colonies

To identify the hydrolytic bacteria in our consortium we used skim milk agar and tributyrin agar plates to select the protease and lipases active isolates. We isolated 10 colonies with similar and distinct morphologic colonies from the Azotf36 consortium, from these 10 colonies we could identify 3 isolates presenting at least one of the two activities tested. Only 2 of the isolated bacteria revealed lipase activity on tributyrin substrate and all the 3 isolates presented protease activity. We tested all the 3 isolates that shown enzymatic for proteases and lipases activity in AEMAC medium with 1% of untreated feathers concentration. The results showed that only the isolate 14 could hydrolyse the feathers completely in 72 hours of incubation at 60 ˚C 250 r.p.m (Figure 55). The most closely related bacteria based on BLAST matches to 16S rRNA sequences and their enzymatic capability for production of proteases and lipases are presented in table 32.

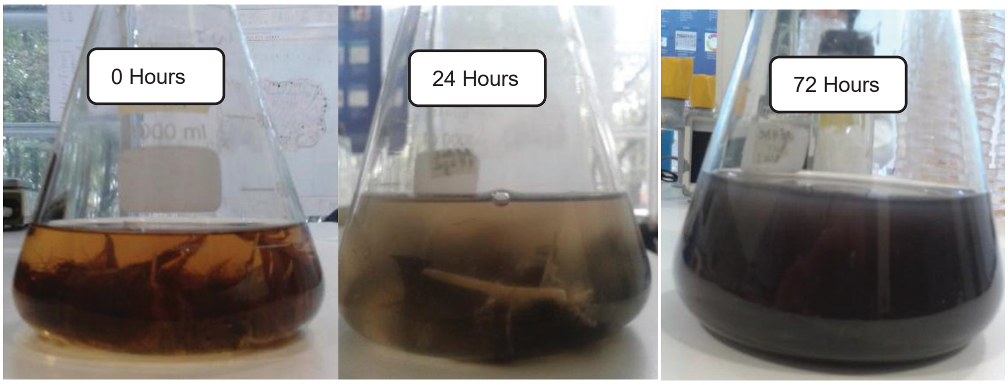


Figure 55. Assay to detect if the isolates that could hydrolysate untreated feathers (from the 3 isolates with proteolytic activity only Isolate 14 could hydrolyse feathers)

The isolate 4 showed protease and lipases activity, the blast results were revealed 97% of similarity with the strain *Aeribacillus pallidus* strain DSM 3670 and 94% of similarity with *Anoxybacillus vitaminiphilus* strain 3nP4. The isolate 10 only showed high proteolytic activity whereas the lipases activity was not detected. The blast for isolate 10 revealed a 99% of similarity with *Aeribacillus pallidus* strain DSM 3670 and 96% of similarity with *Anoxybacillus calidus* strain C161ab. In what concerns the isolate 14 is enzymatic activities were the highest for protease and lipase. This isolate 16SRNA sequence blast revealed 99% similarity with *Brevibacillus aydinogluensis* strain PDF25 and *Brevibacillus thermoruber* strain DSM 7064. These strains blast results revealed bacteria strains that were identified in our consortia at genus level using 454, pyrosequencing method.

Table 32. Molecular Identification and enzymatic characterization of the isolated bacteria.

Isolate	**Clearing zone diameter (mm)**		**Best match in GenBank database (accession number)**	**Similarity (%)**
	Skim milk	**Tributyrin**		
Isolate 4	15	5	*Aeribacillus pallidus* strain DSM 3670 (NR_026515)	97
			Anoxybacillus vitaminiphilus strain 3nP4 (NR_108379)	94
			Anoxybacillus calidus strain C161ab (NR_125532)	93
			Bacillus alveayuensis strain TM1 (NR_043013)	92
Isolate 10	20	NDt	*Aeribacillus pallidus* strain DSM 3670 (NR_026515)	99
			Anoxybacillus calidus strain C161ab (NR_125532)	96
			Geobacillus toebii strain R-35642 (NR_116984)	95
			Geobacillus thermoglucosidasius strain BGSC 95A1 (NR_043022)	94
Isolate 14	20	10	*Brevibacillus aydinogluensis* strain PDF25 (NR_117986)	99
			Brevibacillus thermoruber strain DSM 7064 (NR_112213)	99
			Brevibacillus fulvus strain K2814 (NR_125456)	97
			Brevibacillus borstelensis strain Logan B4029 (NBRC 15714)	97

3.4.5. | Remarks

The selection of a stable consortium that efficiently hydrolysate feathers was achieved in the present work. During the enrichment process only consortia Azotf36 enriched at pH 9 presented growth and protease activity throughout the twelve weeks, thus was selected for further assays.

The most efficient feather hydrolysis was achieved in concentration of 2.5% TS of feathers achieving 94% dry weight loss at the end the process.

The monitorization of the consumption of sCOD was performed to select the optimal pretreatment stop time. The highest value for sCOD (10 300 mg/L) was obtained at 48 hours of pretreatment in 2.5% TS. At this point the COD loss ratio percentage was 22.52%. Based on these data the pretreatment was stopped at 48 hours and pretreated feathers and hydrolysate were transferred to anaerobic bioreactors.

The consortium Azotf36 produced proteases and lipases capable of hydrolyse the feathers. The protease was essential to achieve the increase of soluble protein during pretreatment; the highest concentrations were 1378 mg/L registered at 48 hours in the concentration of 2.5% TS. At 48 hours of pretreatment our consortium had already 45% of feather COD solubilization.

The maximum value for VFAs (2.71 g/L) were registered at 48 hours, coinciding with the pretreatment stop time. The maximum concentrations of acetic acid and butyric acid in our pretreatment reactors were 1.25 and 1.02 g/L respectively. Propionic acid reached 1.70 g/L at 48 hours which is above limits for inhibition of methanogens.

The volumes of total biogas yield with pretreated feather substrates were higher than in the unpretreated, producing more 19.06% of total accumulated biogas at the end of the 42 days. This result shows that Azotf36 pretreatment can enhance the biogas yields of pretreated feathers substrate.

The methane content of pretreated feathers bioreactors was also higher than the unpretreated, this was noticed especially after day 14 up until the end of pretreatment. Content of methane in bioreactors with pretreated feathers were 77% higher than in the controls without feather pretreatment. The maximum biochemical methane potential of pretreated feather, at the end of the 42 days of AD was enhanced relatively to the unpretreated feathers. This pretreatment resulted in a raise of 95% of biochemical methane potential (59.6 LCH_4/Kg VS added) for pretreated feathers, at the end of the 42 days of AD.

The composition of Azotf36 bacterial community was represented essentially by the phylum *Firmicutes* (99.99%). At genus level the consortium was mainly composed by *Brevibacillus* (99.20%). In the two of the lowest represented *Geobacillus* and *Aneurinibacillus* genus, the pyrosequencing results identified three distinct OTUs. In the *Geobacillus* genus two strains were closely related to the specie *Geobacillus thermodenitrificans*. The OTU found in

Aneuribacillus genus was identified as being closely related to a *Aneurinibacillus thermoaerophilus*.

All together, these results show that the bacteria community present in the Azotf36 consortium was efficient in the solubilization and pretreatments of feathers, increasing total biogas, methane content and methane production rate. The biochemical methane production was also enhanced by 95% with pretreated feathers.

4. | DISCUSSION AND FINAL CONCLUSIONS

4.1. | DISCUSSION

4.1.1. | Pretreatment efficiency in wastes solubilization

The main objective of this work was the enhancement of biogas production focusing the hydrolysis as the limiting step in AD. We proposed a thermophilic bacterial pretreatment for three different wastes (Slaughterhouse sewage sludge, lignocellulosic grass mixtures and feathers) using a substrate enriched bacterial consortia. The consortia that resulted from the enrichment process grown and adapt in the chosen wastes, promoting the hydrolysis trough the production of enzymatic cocktails that increased soluble compounds. Thus, digestibility will be increased for bacterial anaerobic communities during AD further being converted in methane production.

First, we selected the best consortia for each waste based on the total dry weight loss. After this the pretreatments efficiency was accessed by measuring the released COD from the substrates to the soluble form and measuring compounds of interest for the anaerobic phase like proteins, free amino acids, reducing sugars and volatile fatty acids concentrations. Also, the enzymes present in the hydrolysate in the pretreatments were screened to better understand which enzymes were playing the main role in the complex solubilization of these wastes.

In this work, Azorean hot springs were used as a microbial source for the construction of consortia using a successive enrichment technique based on the use on the three wastes as the only source of carbon. Similar approaches have been attempt in using hot springs as a source of microbes to select consortia for waste decomposition (Hasyim et al., 2011; Zhao et al., 2014; Vishnivetskaya et al., 2015) however we don't found any reference with the objective of promoting pretreatments for biogas enhancement. Using the selected consortia Azotm36 for SSS, Azotdp36 for grass mixtures and AzotF36 for feathers, we achieved a substrates dry weight loss of 30.35%, 50% and 94%, respectively, which means an increase of nutrients for anaerobic bacteria. Also, similar results were found using a microbial consortium able of degradation straw that achieves 55% of degradation in 4 days (Haruta et al., 2002).

The results related to the dry weight loss shown that the substrate degradation rate was correlated to the substrate concentration. The degradation efficiency of Azotm36 on SSS achieved 30.2%, 34.7% and 9.9% of dry weight loss in 1%, 2.5% and 5% of TS, respectively. The lower value of degradation in this concentration (5%) cloud be associated with the lower available oxygen present in sewage sludge as concentration increases. The dry weight loss at different concentrations of grass mixtures caused by the consortium Azotdp36 also presented statistical differences, between concentration 1% and 5% of TS. Although the degradation rate difference between the two concentrations was only 7% suggesting that the consortia Azotdp36 could support higher grass mixtures concentration without losing reduction in degradation on grass substrate. The most efficient feather degradation with Azof36 consortium was achieved at 2.5% TS with 94% of feathers dry weight loss at the end of pretreatment assay.

One of the main questions in a pretreatment prior to AD is the duration time and the stop time to achieve an effective pretreatment. Soluble COD can be a good indicator for the pretreatment stop time (Yuan et al., 2012). Monitorization during the pretreatment allowed us to stop the pretreatment were the availability of soluble COD was higher and the consortia nutrient consumption was lower. It is established that the highest values of methane production are achieved when the sCOD values reached the maximum in pretreatments. The highest values for sCOD were achieved at different pretreatment times in our consortia, the Azotm36, Azotdp36 and Azotf 36 achieved the highest values of 70 680, 29 400 and 10 300 mg/L, respectively at 48 hours for Azotm36 and Azotf36 and at 120 hours for the Azotdp36 consortia. When the sCOD reached the maximums the COD loss ratio percentage was 1.6 and 36.2% for Azotm36 and Azotdp36 consortia, respectively. Only in the Azotf36 COD loss was higher than other concentration 22.52%, still we maintain these stop pretreatment time as the value of sCOD was the highest during all the pretreatment. Based on these results the pretreatments were stopped and pretreated wastes were transferred to anaerobic bioreactors.

In our work the percentage of solublization of the pretreatments based on COD values, were not at the maximum that the consortia could achieve. Up on the pretreatment stop time (48 hours) the consortia Azotm36 and Azotf36 presented a COD solubilization of 34% and 45%, respectively achieving 51% and 97% at the end of pretreatment. The Azotdp36 achieved a solubilization percentage of 53% at 120 hours and 61% at the end of pretreatment. The higher percentages of solubilization does not guaranty higher production of methane which relate to the accumulation of inhibitory products for methanogens such ammonium from protein decomposition during extended pretreatments times and at higher percentages of waste solubilization (Costa et al., 2014).

The success of bacterial consortia over bacterial isolates in biotransformation of wastes has been reported, demonstrating that a consortium can produce a mixture of enzymes for the hydrolysis, while such optimal enzyme mixture is difficult to be produce by a single microorganism. The symbiotic interaction between different bacteria in a consortium can accelerate and enhance the hydrolysis and solubilization of wastes (Zuroff & Curtis, 2012).

To better understand the results from the AD and additionally characterize the pretreatments, concentration of proteins, reducing sugars, enzymes of hydrolysates and VFA quantification were performed. The data about enzymes in the hydrolysates revealed that the consortia produced several enzymes capable of hydrolyzing different structural components of the wastes. It has been shown that bacterial consortia can produce more than 30 different enzymes working synergistically during the degradation of biomass.

The consortia Azotm36, produces proteases, lipases, amylases and alkaline phosphatase, acid phosphatase, phosphohydrolase, esterase C_4, esterase, lipase C_8, lipase C_{14}, trypsin, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Higuchi et al. (2005) stated that cell-bound alpha-amylase is mainly responsible for the hydrolysis of digested sludge. The proteases played an important role in the released proteins into the hydrolysates during the pretreatments, the concentration of soluble proteins was performed achieved a maximum value of 5770 mg/L at 48 hours. The high initial values of soluble protein in Azotm36 SSS concentration could be related to the sterilization procedure prior to the pretreatments that could enhanced the release of soluble protein.

The consortium Azotdp36 produced cellulases, xylanases and lipases, that hydrolyse cellulose and hemicellulose, the main constituents of plant cell walls. It was also detected the presence of Alkaline phosphatase, Phosphohydrolase, Esterase C_4, Esterase, Lipase C_8, Lipase C_{14}, Leucine amino-peptidase, Cystine amino-peptidase, Trypsin, β-glucosidase, N-acetyl-β-glucosaminidase and β-galactosidase in the hydrolysates. The chromogenic substrates differentiate between hydrolysis of C_4 esters (esterase activity) C_8 ester (ester lipase activity) and C_{14} ester (lipase activity). Hydrolysis of tributyrin correlates with the presence of lipases able to hydrolyse glycerides with C_4 fatty acids. It is known that esterase lipase is capable of hydrolyzing long chain fatty acid esters and some lipases have maximum activity towards medium or shorter acyl groups (C_8 to C_2). The hemicellulose and pectin structures of plant cell walls are constituted with a variety of side chains including acetyl groups that are attached to their backbone. Deacetylation during hydrolysis, shown that as the xylan becomes increasingly deacetylated, it becomes five to seven times more digestible. (Pereira et al., 2013). Synergism between xylanases and acetyl esterase has been reported to dramatically improve the release of sugars from xylan and glucan. This information coincided with the results obtained during pretreatment of grass mixtures for reducing sugars concentrations that achieved the highest concentrations of 1.23 g/L at 96 hours, an enhancement of 49.30% when compared with the unpretreated grass mixtures.

The consortium AzotF36 has shown to have high enzymatic activity in the keratin from feathers but not on keratin from hair. Other enzymes were also present in the consortium hydrolysate; lipases, alkaline phosphatase, acid phosphatase, phosphohydrolase; esterase C_4, esterase, lipase C_8, leucine amino-peptidase and β-glucosidase. The proteases are essential to achieve the increase of soluble protein and the amino acids during pretreatment; the highest concentrations of soluble proteins were 1378 mg/L at 48 hours. Keratin is the main component of feather, representing nearly 90% of feather weight, which shows an elevated content of the amino acids like glycine, alanine, serine, cysteine and valine but lower amounts of lysine, methionine and tryptophan (Onifade et al., 1998). Although the main constituent of amino acids from feather were below detection levels in the Azotf36 it as shown to be rich in Aspartic acid (0.3 g/Kg hydrolysate), Glutamic acid (0.3 g/Kg hydrolysate), phenylalanine (0.7 g/Kg hydrolysate) and Taurine (0.1 g/Kg hydrolysate).

Other important factor analysed during the pretreatment was the concentration of VFAs. The concentration of these acids can be a factor of enhancement or inhibition of AD, although there can't defined a standard concertation level for each VFA inhibitory effect on methanogens, as each reactor it's a different system, acetic acid and butyric acid concentrations of 2.4 and 1.8 g/L, respectively, did not inhibit the activity of methanogens, while a propionic acid concentration of 0.9 g/L resulted in substantial inhibition of the methanogens (Franke-Whittle et al., 2014). During the AD process, acetic acid, and butyric acid can be easier to use than propionic acid, because acetic acid can be directly used by methanogens, and the acetogenic rate of butyric acid is higher than that of propionic acid. Taking in consideration this information during pretreatments acetic acid, propionic acid and butyric acid were monitored in the bioreactors.

In Azotm36 the maximum values for VFAs were registered at 48 hours with 8.21 g/L. The maximum concentrations of acetic acid and butyric acid in our pretreatment reactors were 2.16 and 3.79 g/L respectively. At these time the propionic acid reached 2.26 g/L which has been reported to be above the limits (0.9 g/L) for inhibition of methanogens during AD (Ward et al., 2008). In general, the total accumulated concentrations of volatile acids were higher than in the controls reactors 1.8, 2.17 and 2 times higher for acetic acid, propionic acid and butyric acid, respectively. At 48 hours of pretreatment, the VFAs reach a maximum concentration of 8.21 g/L corresponding to the lowest values of pH in bioreactors with pretreated grass.

The Azotdp36 maximum concentrations of acetic acid and butyric acid in our pretreatment reactors were 0.60 and 1.10 g/L respectively, and propionic acid reached a maximum level of 1.35 g/L at 48 hours which is was also above inhibitory level for methanogens. Although we stop pretreatment at 120 hours and the values of propionic acid were below the inhibitory concentrations (0.61 g/L). Weak acids such as propionic acid, can loosen the structure of lignocellulose, leading to the increased accessibility of the substrate for the enzymes resulting in an improved overall hydrolysis rate. In general, the total accumulated concentrations of volatile acids were higher than in the controls reactors 1.7, 1.7 and 1.9 times higher for acetic acid, propionic acid and butyric acid, respectively. At 48 hours of pretreatment, the VFAs reach a maximum concentration of 2.72 g/L coinciding with the time of the lowest values of pH in bioreactors with pretreated grass. Although these maximum values of VFAs did not coincided with the pretreatment stop time.

The Azotf36 maximum concentrations of acetic acid and butyric acid in our pretreatment reactors were 1.25 and 1.02 g/L respectively, and propionic acid reached 1.70 g/L at 48 hours which is above of inhibition limits for methanogens. In general, the total accumulated concentrations of volatile acids were higher than in the controls the controls reactors. The maximum values for VFAs were registered at 48 hours with 2.71 g/L coinciding with the highest values obtained of VFAs and coinciding with the pretreatment stop time.

All together the data in pretreatments, show that the bacteria community present in the consortia Azotm36, Azotdp36 and Azotf36 can enhance the digestion and the solubilization and of SSS, grass mixtures and feathers.

4.1.2. | Bacterial consortium effect of the pretreatment in the enhancement of biogas

Many types of waste cannot be broken down sufficiently by AD. Despite the abundance of these types of waste material, AD remains inefficient because the anaerobic hydrolytic bacteria present in digesters are unable to break down long-chain molecules from wastes with more difficult digestion. The main purpose of pretreatment of these wastes materials is to alter the substrate composition, transforming them in small monomers that are more easily assimilated by methanogens enhancing biogas production. The data we present is of great value indicating us that the pretreatments with the consortia communities can enhance biogas production when comparing to unpretreated wastes during the first 42 days of anaerobic digestion.

During the anaerobic assays, we saw that the curves of BMP assays did not reach a stabilization curve at the end of pretreatment. These means that the

AD assays should be taken for a longer period till all the biomass present in the digester could be decomposed by the anaerobic bacteria.

Particularly the consortia Azotdp36 and Azotf36 enhanced the total biogas volumes when compared with the controls. The total biogas volumes yield for pretreated substrates in Azotdp36 and Azotf36 increased 18 and 19%, respectively, then the respective untreated wastes. Although the total volumes of biogas for consortia Azotm36 did not present such differences, presenting an enhancement of 5.23% of total accumulated biogas at the end of BMP essays.

This result shows that consortia pretreatments can enhance the biogas yields of pretreated substrates of grass mixtures and feathers but not for the SSS substrate.

Also, methane content was increased in the bioreactors with wastes after pretreatments with Azotdp36 and Azotf36. The values of methane content were enhanced after day 21 and day 14, respectively. The highest value of methane content was registered with the pretreated feathers substrate that registered an increase of 77% relative to the controls. These enhancement in methane content was achieved by adding the pretreated grass mixtures and feathers when the maximum sCOD was achieved, Although the highest values for the VFAs concentrations in the pretreated feathers bioreactors was coincident with the highest values of sCOD, the VFAs concentrations in pretreated grass mixtures did not. Regardless that other authors also reported that the highest values of methane content were achieved when sCOD were the maximum in the pretreatments not coinciding with the maximum concentrations of VFAs. In a recent study by Wall et al. (2014), the methane yields of pretreated grass silage was increased by 12%, while our pretreatments with Azotdp36 achieved 21%. Also in pretreated feathers with the consortium Azotf36 the methane content was higher when compared with past bacterial pretreatments.

The highest values for total biogas in bioreactors with substrates pretreated with these two consortia, were in concordance with the periods of highest methane content. These result reveals that the pretreatments with Azotd36 and Azotf36 consortium not only increased total biogas production but enhanced the methane production.

The lowest values of methane content were registered in pretreated SSS with the consortium Azotm36. The pretreated SSS did not enhanced biogas production, but achieved more 16% of methane content relative to the controls specially between day 21 and 28 of AD. The results of methane content for these pretreated SSS were achieved when the sCOD was at the maximum in pretreatments, also coinciding with the highest values of VFAs, the methane content was lower when compared with other pretreatments done in sewage sludge for the enhancement of methane production. These low biogas and methane content achieved with pretreatments of Azotm36 can be related to the high concentrations of propionic acid at the pretreatment stop times as propionic acids values were above the maximum inhibitory limits for the methanogenic Archaea. Also, the high content of protein can lead to the formation of high concentrations of ammonium that are also inhibitory for methanogenic Archaea.

Taking in consideration that the BMP curves did not reach a stabilization related with the production of methane it is not possible the conclude the final BMP values resulting from each pretreatment. Although we analyze the results of AD for the period of 42 days, comparing the BMP values of pretreated wastes with unpretreated. In the BMP assays the maximum methane production of pretreated wastes comparing with the respective controls were achieved in the pretreated feathers which resulted in 95% more biogas production per kilo of volatile solids of feathers (59.6 LCH_4/Kg VS added). The enhancement was achieved when the pretreatments solubilization achieved 45% of COD solubilization which was below of what the consortia Azotf36 could achieve during pretreatments demonstrating as elsewhere that lower solubilization percentages of feathers can achieve higher methane productions. When comparing the maximum BMP with other articles we can see that from feathers we can produce 123 L/ kg VS (Costa et al., 2012). These results were must higher than the ones we obtained in the present work suggesting that our anaerobic inoculum could not be in optimal conditions or the pretreatments resulted in the formation of inhibitory compounds.

The second most high results of BMP were achieved in the pretreatments of grass mixtures with the consortium Azotdp36, an enhancement 77.35% of more methane per kilo of volatile solids of grass added (168.36 LCH_4/Kg VS added) was achieved when comparing with the controls. The results of BMP in grass mixtures were achieved when the solubilization of the waste achieved 53% of COD solubilization as the maximum that the consortium could achieve 61% of total COD at the end of pretreatment.

The lowest values on BMP assays were produced with consortia Azotm36. In this case, here the AD was inhibited by the production of high concentrations of propionic acid accumulated during pretreatment or by the excess of protein that could lead to ammonium formation. The solubilization of SSS at the stop pretreatment time was 34% of total COD. This pretreatment only resulted in 20% more biogas production per kilo of volatile solids of grass (200.98 LCH_4/Kg VS added), at the end of the 42 days of AD.

The BMP data shown that an enhancement of biogas and methane production can be achieved without reaching the highest values of a waste solubilization for grass mixtures and feathers substrates using consortia pretreatments. It was determined that with the concentration of 5% TS of grass mixtures and with pretreatment time of 120 hours we could achieved an enhancement of methane production. In the case of Azotf pretreatments a concentration of 2.5% TS and a pretreatment time of 48 hours result in enhancement of methane production. The methane enhancing in the waste pretreated with aAzotm36 consortium resulted in the lowest values using a concentration of 2.5% TS of SSS at a pretreatment time of 48 hours.

Is suggested that other times of pretreatments should be tested in AD to ensure that the highest sCOD stop time correspond to the maximum methane production that we can achieve with our consortia pretreatments.

4.1.3. | Bacterial consortia diversity associated with wastes hydrolysis

The waste substrate type is the main driver of the structure of microbial consortia developing in enrichments side with the inoculums. Inoculum source strongly influences the composition of biomass-degrading microbial consortia, despite, stochastic factors can also affect the selection process and so driven the microbial diversity in the consortia. The growth and diversity at the end of enrichment process changes the structure of the original inoculated consortia, proving that substrate structure and composition has a major influence in the development of consortia communities (Cortes-Tolalpa et al., 2016).

During the enrichment process we observed that with the inoculum from the hot springs maintained growth or enzymatic activity during the enrichment in most of the wastes. The selection of communities capable of adapting to the selected wastes were achieved in DMSS, SSS, grass mixtures, feathers and olive oil, but not on the chitin substrate. These results suggest that the type of biomass where the consortia maintained growth is more closely related to the type of biomass present in the hot springs environment.

The composition of bacterial community in the selected consortia was very different from each other, although the starting inoculum was the same. The initial bacterial community changed during the enrichment according to the substrate given and at the end of enrichments we obtained three different consortia with distinct diversity, distinct hydrolysis efficiencies and different enzymatic activities. At genus level the consortia that presented the highest diversity was Azotdp36 followed by the Azotm36 and Azotf36 consortia. In the Azotdp36 were identified bacteria belonging to 18 different genera comparing with the 10 and 6 genera identified in the consortia of Azotm36 and Azotf36, respectively.

Based in the relative abundance of the sequences resulting from the pyrosequencing we can assume that the three consortia have at least one predominant genera in their composition. In the consortia Azotdp36 the predominant genus is *Thermobacillus* with a relative abundance higher than 1%, followed by the *Symbiobacterium*, *Brevibacillus*, *Hyphomicrobium* and *Geobacillus*, whereas 13 genera had a relative abundance below 1% (*Ureibacillus*, *Bacillus*, *Thermobispora*, *Paenibacillus*, *Caldalkalibacillus*, *Aeribacillus*, *Devosia*, *Thermoaerobacter*, *Mesorhizobium*, *Planifilum*, *Rhizobium*, *Thermobifida* and *Cohnella*). Many strains from the genera present in Azotdp36 were reported with hydrolytic capacities in wastes, particularly acting in the decomposition of lignocellulosic residues several strains were found to be able to enhance the hydrolysis of cellulose and hemicellulose by producing cellulases and xylanases enzymes. The single domain xylanase

XynA of *Thermobacillus xylanilyticus* can bind and hydrolyse insoluble xylan efficiently, which is an unusual feature for a single catalytical domain protein (Connerton et al., 1999).

The genera found in the Azotm36 consortium was mainly composed by *Ureibacillus*, followed by *Symbiobacterium*, *Geobacillus*, and *Thermobacillus*, having the other genera six genera; *Bacillus*, *Aeribacillus*, *Paenibacillus*, *Hyphomicrobium*, *Thermoaerobacter* and *Brevibacillus* representation below 1% in the Azotm36 consortium.

The two consortia Azotdp36 and Azotm36 present two distinct predominant genera, although the following most represented genera in the two consortia *Symbiobacterium* and *Geobacillus* were also present suggesting that the bacteria belonging to these two genera could have an important role in the hydrolysis of grass mixtures and SSS. As reported in recent research bacteria belonging to *Symbiobacterium* have a symbiotic relation with other bacteria favoring the decomposition of biomass by the production of enzymes. Past research revealed that the syntrophic growth of the specie *Symbiobacterium thermophilum* is supported by multiple functions of *Geobacillus. stearothermophilus*, by providing growth promoting factors, and removing growth inhibitory factors (Shiratori-Takano et al., 2014). The consortia Azotm36 and Azotdp36 presented both bacteria genus and strains of *Geobacillus* were identified in consortia Azotdp36. As the commensalism between the *Symbiobacterium* and *Geobacillus* species have been studied and reported, it is still unknown which symbiotic factors can *Symbiobacterium* delivery to the other species of *Geobacillus*, of if even the commensal strains can have symbiotic relations with the strains from genera *Thermobacillus* and *Ureibacillus* present in our consortia.

The diversity at genus level in the Azotf36 consortia was mainly composed by the *Brevibacillus* genus with 99.20% of relative abundance. The other genera present were, *Aneurinibacillus*, *Geobacillus*, *Aeribacillus*, *Bacillus* and *Thermobacillus* genera. Bacteria belonging to *Brevibacillus* with similar proteolytic activities in feathers, (Forgács, 2012) are also capable of hydrolyzing feathers but not achieving the highest decomposition rates (80% dry weight) as the consortia presented in the present work.

From the three bacterial consortia, the data of pyrosequencing resulted in the identification of 32 OUTs at species level. The 32 OTUs identified in the consortia revealed to be close related to the species of *Ureibacillus thermosphaericus*, *Thermoaerobacter subterraneus*, *Thermobispora bispora*, *Geobacillus thermodinitrificans* and *Aneurinibacillus thermoaerophilus*.

In Azotm36 from *Ureibacillus* genus with 20 OTUs only 12 species were identified all related to *Ureibacillus thermosphaericus* ID 51173. Past reports shown that bacteria from the genus *Ureibacillus* produced cellulases and xylanases at 60 ºC. Also, *Geobacillus* strains the third most represented genus in the Azotm36 consortia as shown have shown in past research to have lipolytic, esterase amylase and xylanase activities on the decomposition of manure compost (Charbonneau et al., 2012).

In Azotdp36 the identified species belong to the genera group with less representation in the consortia. A total of 52 OTUs blast in the genera and 17 from which 16 OTUs were identified at species level in genus *Thermobispora* from the family *Pseudonocardiaceae*, phylum *Actinobacteria*. The specie identified in genus *Thermoaerobacter* was assigned to *Thermaerobacter subterraneus* ID 175696 and the 16 species from genus *Thermobispora* were assigned to *Thermobispora bispora* ID 2006.

Finally, in the Azotf36 the OTU blast identified 3 species that were related to the specie of *Geobacillus thermodenitrificans* ID 33940, and the other two OTUs were related to *Aneurinibacillus* genus to the specie of *Aneurinibacillus thermoaerophilus* ID143495. Although the *Brevibacillus* genus was the most represented in the consortia and we could isolate a bacterium which blast results show similarity of 99% with *Brevibacillus aydinogluensis* strain PDF25 and *Brevibacillus thermoruber* strain DSM 7064. This last strain has been reported to exhibit keratinolytic activity in feathers with the production of volatile organic compounds (Bihari et al., 2010).

The use of the present selected natural consortia and the study of its symbiotic interactions for achieving the hydrolysis of waste materials, can be used as a model for further understanding bacterial interactions on biomass decomposition. The present consortia may prove particularly relevant in the study of bacterial relationships that can define the structure and stability of a stable hydrolytic microbial community. These consortia interactions are often extremely efficient in capturing small free energy differences and enhancing the extraction of wastes available energy.

4.2. | CONCLUSIONS

An initial challenge for discovering alternative waste for biogas production was one of the initial goals of this work. Enlarging the usable wastes feedstocks would allow a more continuous and stable biogas production during AD. This worldwide demand is needed to supreme the fluctuation in biogas production one of the main constrains to AD.

In the present work, we faced this challenge and selected three different wastes that are unusually used due to their high resistance to hydrolysis. The bacterial consortia obtained from the terrestrial hydrothermal hot springs were used to pre treat the wastes and achieve the maximum solubilization to enhance the methane production. One of the most active consortia achieved 97% of feathers solubilization. All the three consortia could solubilize the wastes enhancing methane production at different rates.

These results are the achievement of our main objective to find biological pretreatments for the enhancement of biogas production resulting in the accomplishment of our work.

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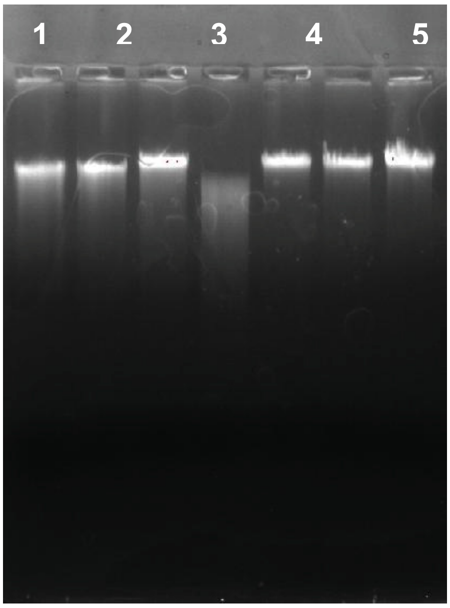
ANNEX



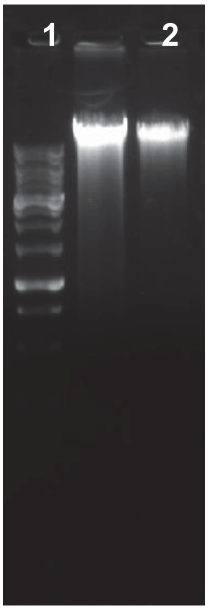


Figure A. Total gDNA agarose gel 0.8% (w/v). Lane; 1, azotdp36 (r1); 2, Azotdp36 (r2); 3, Azotdp36 (r3); 4, AzotF36 (r1); 5, azotm36 (r1); 6, Azotm36 (r2); 7, Azotm36 (r3).

Figure B. Total gDNA agarose gel 0.8% (w/v). Lane; 1, MW ladder 1Kb; 2, Azotf36 (r2); 3, Azot36 (r3).

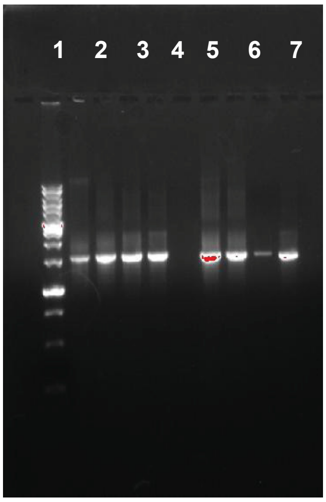


Figure C. 16S rRNA gene amplification of consortia gDNA agarose gel 1% (w/v). Lane; 1 MW ladder 1Kb; lane 2, Azotdp36 (r1); lane 3, Azotdp36 (r2); lane 4, Azotdp36 (r3); lane 5, Azotm36 (r1); lane 7, Azotm36 (r2); lane 8, Azotm36(r2); lane 9, Azotf36(r1); lane 10, Azotf36 (r2).

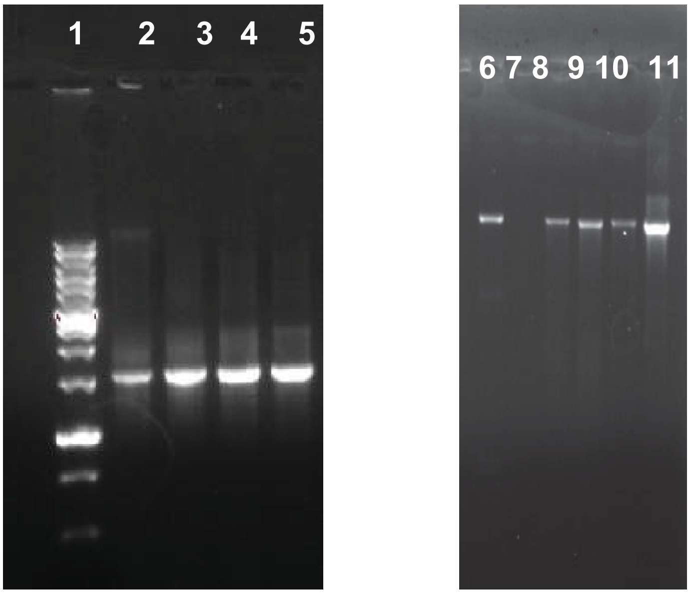


Figure D. 16S rRNA gene amplification genes of bacterial isolates from the consortia agarose gel 1% (w/v). Lane; 1 isolate1; lane 2, isolate4; lane 3, isolate6; lane 4, isolate2; lane5, isolate5; lane 7, isolate7, lane 8, isolate8; lane 9, isolate4; lane 10, isolate10, lane11, isolate14.

Table A. Registration made during the process of enrichment and construction of consortiums

Consortium	pH	Liquid medium	Substrate	Growth in Liquid medium	Growth in solid medium
Week 1					
Azotb1	3	AEMAC	DMSS	+	+
Azotb2	7	AEMAC	DMSS	+	+
Azotb3	9	AEMAC	DMSS	+	+
AzotbH1	3	H_2O	DMSS	+	+
AzotbH2	7	H_2O	DMSS	+	+
AzotbH3	9	H_2O	DMSS	+	+
Azotm1	3	AEMAC	SSS	+	+
Azotm2	7	AEMAC	SSS	+	+
Azotm3	9	AEMAC	SSS	+	+
AzotmH1	3	H_2O	SSS	+	+
AzotmH2	7	H_2O	SSS	+	+
AzotmH3	9	H_2O	SSS	+	+
Azoto1	3	AEMAC	Oilve oil	+	-
Azoto2	7	AEMAC	Oilve oil	+	+
Azoto3	9	AEMAC	Oilve oil	+	+
Azotdp1	3	AEMAC	Grass mixtures	+	-
Azotdp2	7	AEMAC	Grass mixtures	+	+
Azotdp3	9	AEMAC	Grass mixtures	+	+
Azotf1	3	AEMAC	Chicken Feathers	-	-
Azotf2	7	AEMAC	Chicken Feathers	+	+
Azotf3	9	AEMAC	Chicken Feathers	+	+
Azotc1	3	AEMAC	Chitin	+	-
Azotc2	7	AEMAC	Chitin	+	-
Azotc3	9	AEMAC	Chitin	+	+
Azots1	3	AEMAC	starch	+	+
Azots2	7	AEMAC	starch	+	+
Azots3	9	AEMAC	starch	+	+
Azotp1	3	AEMAC	Skim Milk	+	+
Azotp2	7	AEMAC	Skim Milk	+	+
Azotp3	9	AEMAC	Skim Milk	+	+
Azotn1	3	Nutrient broth	Nutrient broth	+	+
Azotn2	7	Nutrient broth	Nutrient broth	+	+
Azotn3	9	Nutrient broth	Nutrient broth	+	+
Week 2					
Azotb4	3	AEMAC	DMSS	+	+
Azotb5	7	AEMAC	DMSS	+	+
Azotb6	9	AEMAC	DMSS	+	+
AzotbH4	3	H_2O	DMSS	+	+
AzotbH5	7	H_2O	DMSS	+	+

AzotbH6	9	H_2O	DMSS	+	+
Azotm4	3	AEMAC	SSS	+	+
Azotm5	7	AEMAC	SSS	+	+
Azotm6	9	AEMAC	SSS	+	+
AzotmH4	3	H_2O	SSS	+	+
AzotmH5	7	H_2O	SSS	+	+
AzotmH6	9	H_2O	SSS	+	+
Azoto4	3	AEMAC	Oilve oil	+	-
Azoto5	7	AEMAC	Oilve oil	+	+
Azoto6	9	AEMAC	Oilve oil	+	+
Azotdp4	3	AEMAC	Grass mixtures	+	-
Azotdp5	7	AEMAC	Grass mixtures	+	+
Azotdp6	9	AEMAC	Grass mixtures	+	+
Azotf4	3	AEMAC	Chicken Feathers	-	-
Azotf5	7	AEMAC	Chicken Feathers	+	+
Azotf6	9	AEMAC	Chicken Feathers	+	+
Azotc4	3	AEMAC	Chitin	-	-
Azotc5	7	AEMAC	Chitin	+	+
Azotc6	9	AEMAC	Chitin	+	+
Azots4	3	AEMAC	starch	+	+
Azots5	7	AEMAC	starch	+	+
Azots6	9	AEMAC	starch	+	+
Azotp4	3	AEMAC	Skim Milk	+	+
Azotp5	7	AEMAC	Skim Milk	+	+
Azotp6	9	AEMAC	Skim Milk	+	+
Azotn4	3	Nutrient broth	Nutrient broth	+	+
Azotn5	7	Nutrient broth	Nutrient broth	+	+
Azotn6	9	Nutrient broth	Nutrient broth	+	+
Week 3					
Azotb7	3	AEMAC	DMSS	+	+
Azotb8	7	AEMAC	DMSS	+	+
Azotb9	9	AEMAC	DMSS	+	+
AzotbH7	3	H_2O	DMSS	+	+
AzotbH8	7	H_2O	DMSS	+	+
AzotbH9	9	H_2O	DMSS	+	+
Azotm7	3	AEMAC	SSS	+	+
Azotm8	7	AEMAC	SSS	+	+
Azotm9	9	AEMAC	SSS	+	+
AzotmH7	3	H_2O	SSS	+	+
AzotmH8	7	H_2O	SSS	+	+
AzotmH9	9	H_2O	SSS	+	+
Azoto7	3	AEMAC	Oilve oil	-	-
Azoto8	7	AEMAC	Oilve oil	+	+
Azoto9	9	AEMAC	Oilve oil	+	+

Azotdp7	3	AEMAC	Grass mixtures	+	-
Azotdp8	7	AEMAC	Grass mixtures	+	+
Azotdp9	9	AEMAC	Grass mixtures	+	+
Azotf7	3	AEMAC	Chicken Feathers	-	-
Azotf8	7	AEMAC	Chicken Feathers	+	+
Azotf9	9	AEMAC	Chicken Feathers	+	+
Azotc7	3	AEMAC	Chitin	-	-
Azotc8	7	AEMAC	Chitin	-	-
Azotc9	9	AEMAC	Chitin	-	-
Azots7	3	AEMAC	starch	+	+
Azots8	7	AEMAC	starch	+	+
Azots9	9	AEMAC	starch	+	+
Azotp7	3	AEMAC	Skim Milk	+	+
Azotp8	7	AEMAC	Skim Milk	+	+
Azotp9	9	AEMAC	Skim Milk	+	+
Azotn7	3	Nutrient broth	Nutrient broth	+	+
Azotn8	7	Nutrient broth	Nutrient broth	+	+
Azotn9	9	Nutrient broth	Nutrient broth	+	+
Week 4					
Azotb10	3	AEMAC	DMSS	+	+
Azotb11	7	AEMAC	DMSS	+	+
Azotb12	9	AEMAC	DMSS	+	+
AzotbH10	3	H_2O	DMSS	+	+
AzotbH11	7	H_2O	DMSS	+	+
AzotbH12	9	H_2O	DMSS	+	+
Azotm10	3	AEMAC	SSS	+	+
Azotm11	7	AEMAC	SSS	+	+
Azotm12	9	AEMAC	SSS	+	+
AzotmH10	3	H_2O	SSS	+	+
AzotmH11	7	H_2O	SSS	+	+
AzotmH12	9	H_2O	SSS	+	+
Azoto10	3	AEMAC	Oilve oil	-	-
Azoto11	7	AEMAC	Oilve oil	+	+
Azoto12	9	AEMAC	Oilve oil	+	+
Azotdp10	3	AEMAC	Grass mixtures	+	-
Azotdp11	7	AEMAC	Grass mixtures	+	+
Azotdp12	9	AEMAC	Grass mixtures	+	+
Azotf10	3	AEMAC	Chicken Feathers	-	-
Azotf11	7	AEMAC	Chicken Feathers	+	+
Azotf12	9	AEMAC	Chicken Feathers	+	+
Azotc10	3	AEMAC	Chitin	Stored	Stored
Azotc11	7	AEMAC	Chitin	Stored	Stored
Azotc12	9	AEMAC	Chitin	Stored	Stored
Azots10	3	AEMAC	starch	Stored	Stored

Azots11	7	AEMAC	starch	Stored	Stored
Azots12	9	AEMAC	starch	Stored	Stored
Azotp10	3	AEMAC	Skim Milk	Stored	Stored
Azotp11	7	AEMAC	Skim Milk	Stored	Stored
Azotp12	9	AEMAC	Skim Milk	Stored	Stored
Azotn10	3	Nutrient broth	Nutrient broth	Stored	Stored
Azotn11	7	Nutrient broth	Nutrient broth	Stored	Stored
Azotn12	9	Nutrient broth	Nutrient broth	Stored	Stored
Week 5					
Azotb13	3	AEMAC	DMSS	+	+
Azotb14	7	AEMAC	DMSS	+	+
Azotb15	9	AEMAC	DMSS	+	+
AzotbH13	3	H_2O	DMSS	+	+
AzotbH14	7	H_2O	DMSS	+	+
AzotbH15	9	H_2O	DMSS	+	+
Azotm13	3	AEMAC	SSS	+	+
Azotm14	7	AEMAC	SSS	+	+
Azotm15	9	AEMAC	SSS	+	+
AzotmH13	3	H_2O	SSS	+	+
AzotmH14	7	H_2O	SSS	+	+
AzotmH15	9	H_2O	SSS	+	+
Azoto13	3	AEMAC	Oilve oil	-	-
Azoto14	7	AEMAC	Oilve oil	+	+
Azoto15	9	AEMAC	Oilve oil	+	+
Azotdp13	3	AEMAC	Grass mixtures	+	-
Azotdp14	7	AEMAC	Grass mixtures	+	+
Azotdp15	9	AEMAC	Grass mixtures	+	+
Azotf13	3	AEMAC	Chicken Feathers	-	-
Azotf14	7	AEMAC	Chicken Feathers	+	+
Azotf15	9	AEMAC	Chicken Feathers	+	+
Azotc13	3	AEMAC	Chitin	-	-
Azotc14	7	AEMAC	Chitin	+	+
Azotc15	9	AEMAC	Chitin	+	+
Azots13	3	AEMAC	starch	ND	ND
Azots14	7	AEMAC	starch	ND	ND
Azots15	9	AEMAC	starch	ND	ND
Azotp13	3	AEMAC	Skim Milk	ND	ND
Azotp14	7	AEMAC	Skim Milk	ND	ND
Azotp15	9	AEMAC	Skim Milk	ND	ND
Azotn13	3	Nutrient broth	Nutrient broth	ND	ND
Azotn14	7	Nutrient broth	Nutrient broth	ND	ND
Azotn15	9	Nutrient broth	Nutrient broth	ND	ND
Week 6					
Azotb16	3	AEMAC	DMSS	+	+

Azotb17	7	AEMAC	DMSS	+	+
Azotb18	9	AEMAC	DMSS	+	+
AzotbH16	3	H_2O	DMSS	+	+
AzotbH17	7	H_2O	DMSS	+	+
AzotbH18	9	H_2O	DMSS	+	+
Azotm16	3	AEMAC	SSS	+	+
Azotm17	7	AEMAC	SSS	+	+
Azotm18	9	AEMAC	SSS	+	+
AzotmH16	3	H_2O	SSS	+	+
AzotmH17	7	H_2O	SSS	+	+
AzotmH18	9	H_2O	SSS	+	+
Azoto16	3	AEMAC	Oilve oil	-	-
Azoto17	7	AEMAC	Oilve oil	+	+
Azoto18	9	AEMAC	Oilve oil	+	+
Azotdp16	3	AEMAC	Grass mixtures	+	-
Azotdp17	7	AEMAC	Grass mixtures	+	+
Azotdp18	9	AEMAC	Grass mixtures	+	+
Azotf16	3	AEMAC	Chicken Feathers	-	-
Azotf17	7	AEMAC	Chicken Feathers	-	-
Azotf18	9	AEMAC	Chicken Feathers	+	+
Azotc16	3	AEMAC	Chitin	ND	ND
Azotc17	7	AEMAC	Chitin	ND	ND
Azotc18	9	AEMAC	Chitin	ND	ND
Azots16	3	AEMAC	starch	ND	ND
Azots17	7	AEMAC	starch	ND	ND
Azots18	9	AEMAC	starch	ND	ND
Azotp16	3	AEMAC	Skim Milk	ND	ND
Azotp17	7	AEMAC	Skim Milk	ND	ND
Azotp18	9	AEMAC	Skim Milk	ND	ND
Azotn16	3	Nutrient broth	Nutrient broth	ND	ND
Azotn17	7	Nutrient broth	Nutrient broth	ND	ND
Azotn18	9	Nutrient broth	Nutrient broth	ND	ND
Week 7					
Azotb19	3	AEMAC	DMSS	+	+
Azotb20	7	AEMAC	DMSS	+	+
Azotb21	9	AEMAC	DMSS	+	+
AzotbH19	3	H_2O	DMSS	+	+
AzotbH20	7	H_2O	DMSS	+	+
AzotbH21	9	H_2O	DMSS	+	+
Azotm19	3	AEMAC	SSS	+	+
Azotm20	7	AEMAC	SSS	+	+
Azotm21	9	AEMAC	SSS	+	+
AzotmH19	3	H_2O	SSS	+	+
AzotmH20	7	H_2O	SSS	+	+

AzotmH21	9	H_2O	SSS	+	+
Azoto19	3	AEMAC	Oilve oil	-	-
Azoto20	7	AEMAC	Oilve oil	+	+
Azoto21	9	AEMAC	Oilve oil	+	+
Azotdp19	3	AEMAC	Grass mixtures	+	-
Azotdp20	7	AEMAC	Grass mixtures	+	+
Azotdp21	9	AEMAC	Grass mixtures	+	+
Azotf19	3	AEMAC	Chicken Feathers	-	-
Azotf20	7	AEMAC	Chicken Feathers	-	-
Azotf21	9	AEMAC	Chicken Feathers	+	+
Azotc19	3	AEMAC	Chitin	ND	ND
Azotc20	7	AEMAC	Chitin	ND	ND
Azotc21	9	AEMAC	Chitin	ND	ND
Azots19	3	AEMAC	starch	ND	ND
Azots20	7	AEMAC	starch	ND	ND
Azots21	9	AEMAC	starch	ND	ND
Azotp19	3	AEMAC	Skim Milk	ND	ND
Azotp20	7	AEMAC	Skim Milk	ND	ND
Azotp21	9	AEMAC	Skim Milk	ND	ND
Azotn19	3	Nutrient broth	Nutrient broth	ND	ND
Azotn20	7	Nutrient broth	Nutrient broth	ND	ND
Azotn21	9	Nutrient broth	Nutrient broth	ND	ND
Week 8					
Azotb22	3	AEMAC	DMSS	+	+
Azotb23	7	AEMAC	DMSS	+	+
Azotb24	9	AEMAC	DMSS	+	+
AzotbH22	3	H_2O	DMSS	+	+
AzotbH23	7	H_2O	DMSS	+	+
AzotbH24	9	H_2O	DMSS	+	+
Azotm22	3	AEMAC	SSS	+	+
Azotm23	7	AEMAC	SSS	+	+
Azotm24	9	AEMAC	SSS	+	+
AzotmH22	3	H_2O	SSS	+	+
AzotmH23	7	H_2O	SSS	+	+
AzotmH24	9	H_2O	SSS	+	+
Azoto22	3	AEMAC	Oilve oil	-	-
Azoto23	7	AEMAC	Oilve oil	+	+
Azoto24	9	AEMAC	Oilve oil	+	+
Azotdp22	3	AEMAC	Grass mixtures	+	-
Azotdp23	7	AEMAC	Grass mixtures	+	+
Azotdp24	9	AEMAC	Grass mixtures	+	+
Azotf22	3	AEMAC	Chicken Feathers	-	-
Azotf23	7	AEMAC	Chicken Feathers	-	-
Azotf24	9	AEMAC	Chicken Feathers	+	+

Azotc22	3	AEMAC	Chitin	ND	ND
Azotc23	7	AEMAC	Chitin	ND	ND
Azotc24	9	AEMAC	Chitin	ND	ND
Azots22	3	AEMAC	starch	ND	ND
Azots23	7	AEMAC	starch	ND	ND
Azots24	9	AEMAC	starch	ND	ND
Azotp22	3	AEMAC	Skim Milk	ND	ND
Azotp23	7	AEMAC	Skim Milk	ND	ND
Azotp24	9	AEMAC	Skim Milk	ND	ND
Azotn22	3	Nutrient broth	Nutrient broth	ND	ND
Azotn23	7	Nutrient broth	Nutrient broth	ND	ND
Azotn24	9	Nutrient broth	Nutrient broth	ND	ND
Week 9					
Azotb25	3	AEMAC	DMSS	+	+
Azotb26	7	AEMAC	DMSS	+	+
Azotb27	9	AEMAC	DMSS	+	+
AzotbH25	3	H_2O	DMSS	+	+
AzotbH26	7	H_2O	DMSS	+	+
AzotbH27	9	H_2O	DMSS	+	+
Azotm25	3	AEMAC	SSS	+	+
Azotm26	7	AEMAC	SSS	+	+
Azotm27	9	AEMAC	SSS	+	+
AzotmH25	3	H_2O	SSS	+	+
AzotmH26	7	H_2O	SSS	+	+
AzotmH27	9	H_2O	SSS	+	+
Azoto25	3	AEMAC	Oilve oil	-	-
Azoto26	7	AEMAC	Oilve oil	+	+
Azoto27	9	AEMAC	Oilve oil	+	+
Azotdp25	3	AEMAC	Grass mixtures	+	-
Azotdp26	7	AEMAC	Grass mixtures	+	+
Azotdp27	9	AEMAC	Grass mixtures	+	+
Azotf25	3	AEMAC	Chicken Feathers	-	-
Azotf26	7	AEMAC	Chicken Feathers	-	-
Azotf27	9	AEMAC	Chicken Feathers	+	+
Azotc25	3	AEMAC	Chitin	ND	ND
Azotc26	7	AEMAC	Chitin	ND	ND
Azotc27	9	AEMAC	Chitin	ND	ND
Azots25	3	AEMAC	starch	ND	ND
Azots26	7	AEMAC	starch	ND	ND
Azots27	9	AEMAC	starch	ND	ND
Azotp25	3	AEMAC	Skim Milk	ND	ND
Azotp26	7	AEMAC	Skim Milk	ND	ND
Azotp27	9	AEMAC	Skim Milk	ND	ND
Azotn25	3	Nutrient broth	Nutrient broth	ND	ND

Azotn26	7	Nutrient broth	Nutrient broth	ND	ND
Azotn27	9	Nutrient broth	Nutrient broth	ND	ND
Week 10					
Azotb28	3	AEMAC	DMSS	+	+
Azotb29	7	AEMAC	DMSS	+	+
Azotb30	9	AEMAC	DMSS	+	+
AzotbH28	3	H_2O	DMSS	+	+
AzotbH29	7	H_2O	DMSS	+	+
AzotbH30	9	H_2O	DMSS	+	+
Azotm28	3	AEMAC	SSS	+	+
Azotm29	7	AEMAC	SSS	+	+
Azotm30	9	AEMAC	SSS	+	+
AzotmH28	3	H_2O	SSS	+	+
AzotmH29	7	H_2O	SSS	+	+
AzotmH30	9	H_2O	SSS	+	+
Azoto28	3	AEMAC	Oilve oil	-	-
Azoto29	7	AEMAC	Oilve oil	+	+
Azoto30	9	AEMAC	Oilve oil	+	+
Azotdp28	3	AEMAC	Grass mixtures	+	-
Azotdp29	7	AEMAC	Grass mixtures	+	+
Azotdp30	9	AEMAC	Grass mixtures	+	+
Azotf28	3	AEMAC	Chicken Feathers	-	-
Azotf29	7	AEMAC	Chicken Feathers	-	-
Azotf30	9	AEMAC	Chicken Feathers	+	+
Azotc28	3	AEMAC	Chitin	ND	ND
Azotc29	7	AEMAC	Chitin	ND	ND
Azotc30	9	AEMAC	Chitin	ND	ND
Azots28	3	AEMAC	starch	ND	ND
Azots29	7	AEMAC	starch	ND	ND
Azots30	9	AEMAC	starch	ND	ND
Azotp28	3	AEMAC	Skim Milk	ND	ND
Azotp29	7	AEMAC	Skim Milk	ND	ND
Azotp30	9	AEMAC	Skim Milk	ND	ND
Azotn28	3	Nutrient broth	Nutrient broth	ND	ND
Azotn29	7	Nutrient broth	Nutrient broth	ND	ND
Azotn30	9	Nutrient broth	Nutrient broth	ND	ND
Week 11					
Azotb31	3	AEMAC	DMSS	+	+
Azotb32	7	AEMAC	DMSS	+	+
Azotb33	9	AEMAC	DMSS	+	+
AzotbH31	3	H_2O	DMSS	+	+
AzotbH32	7	H_2O	DMSS	+	+
AzotbH33	9	H_2O	DMSS	+	+
Azotm31	3	AEMAC	SSS	+	+

Azotm32	7	AEMAC	SSS	+	+
Azotm33	9	AEMAC	SSS	+	+
AzotmH31	3	H_2O	SSS	+	+
AzotmH32	7	H_2O	SSS	+	+
AzotmH33	9	H_2O	SSS	+	+
Azoto31	3	AEMAC	Oilve oil	-	-
Azoto32	7	AEMAC	Oilve oil	+	+
Azoto33	9	AEMAC	Oilve oil	+	+
Azotdp31	3	AEMAC	Grass mixtures	+	-
Azotdp32	7	AEMAC	Grass mixtures	+	+
Azotdp33	9	AEMAC	Grass mixtures	+	+
Azotf31	3	AEMAC	Chicken Feathers	-	-
Azotf32	7	AEMAC	Chicken Feathers	-	-
Azotf33	9	AEMAC	Chicken Feathers	+	+
Azotc31	3	AEMAC	Chitin	ND	ND
Azotc32	7	AEMAC	Chitin	ND	ND
Azotc33	9	AEMAC	Chitin	ND	ND
Azots31	3	AEMAC	starch	ND	ND
Azots32	7	AEMAC	starch	ND	ND
Azots33	9	AEMAC	starch	ND	ND
Azotp31	3	AEMAC	Skim Milk	ND	ND
Azotp32	7	AEMAC	Skim Milk	ND	ND
Azotp33	9	AEMAC	Skim Milk	ND	ND
Azotn31	3	Nutrient broth	Nutrient broth	ND	ND
Azotn32	7	Nutrient broth	Nutrient broth	ND	ND
Azotn33	9	Nutrient broth	Nutrient broth	ND	ND
Week 12					
Azotb34	3	AEMAC	DMSS	+	+
Azotb35	7	AEMAC	DMSS	+	+
Azotb36	9	AEMAC	DMSS	+	+
AzotbH34	3	H_2O	DMSS	+	+
AzotbH35	7	H_2O	DMSS	+	+
AzotbH36	9	H_2O	DMSS	+	+
Azotm34	3	AEMAC	SSS	+	+
Azotm35	7	AEMAC	SSS	+	+
Azotm36	9	AEMAC	SSS	+	+
AzotmH34	3	H_2O	SSS	+	+
AzotmH35	7	H_2O	SSS	+	+
AzotmH36	9	H_2O	SSS	+	+
Azoto34	3	AEMAC	Oilve oil	-	-
Azoto35	7	AEMAC	Oilve oil	+	+
Azoto36	9	AEMAC	Oilve oil	+	+
Azotdp34	3	AEMAC	Grass mixtures	+	-
Azotdp35	7	AEMAC	Grass mixtures	+	+

Azotdp36	9	AEMAC	Grass mixtures	+	+
Azotf34	3	AEMAC	Chicken Feathers	-	-
Azotf35	7	AEMAC	Chicken Feathers	-	-
Azotf36	9	AEMAC	Chicken Feathers	+	+
Azotc34	3	AEMAC	Chitin	ND	ND
Azotc35	7	AEMAC	Chitin	ND	ND
Azotc36	9	AEMAC	Chitin	ND	ND
Azots34	3	AEMAC	starch	ND	ND
Azots35	7	AEMAC	starch	ND	ND
Azots36	9	AEMAC	starch	ND	ND
Azotp34	3	AEMAC	Skim Milk	ND	ND
Azotp35	7	AEMAC	Skim Milk	ND	ND
Azotp36	9	AEMAC	Skim Milk	ND	ND
Azotn34	3	Nutrient broth	Nutrient broth	ND	ND
Azotn35	7	Nutrient broth	Nutrient broth	ND	ND
Azotn36	9	Nutrient broth	Nutrient broth	ND	ND

Table B. Registration for consortium enzymatic activities during the process of enrichment and construction of consortiums in reactors at pH9

Consortium	Liquid medium	Substrate	Enzymes Bioreactors pH9			
			proteases	cellulase	xylanase	amylase
Week 4						
Azotb12	AEMAC	DMSS	+	ND	ND	-
AzotbH12	H_2O	DMSS	+	ND	ND	-
Azotm12	AEMAC	SSS	+	ND	ND	-
AzotmH12	H_2O	SSS	+	ND	ND	-
Azoto12	AEMAC	Olive oil	+	ND	ND	-
Azotdp12	AEMAC	Grass	+	+	+	-
Azotf12	AEMAC	Feathers	+	ND	ND	ND
Azotc12	AEMAC	Chitin	ND	ND	ND	ND
Azots12	AEMAC	starch	+	ND	ND	+
Azotp12	AEMAC	Skim milk	+	ND	ND	-
Azotn12	H_20	Nutrient broth	+	ND	ND	+
Week 8						
Azotb24	AEMAC	DMSS	+	ND	ND	-
AzotbH24	H_2O	DMSS	+	ND	ND	-
Azotm24	AEMAC	SSS	+	ND	ND	-
AzotmH24	H_2O	SSS	+	ND	ND	-
Azoto24	AEMAC	Olive oil	+	ND	ND	-
Azotdp24	AEMAC	Grass	+	+	+	-
Azotf24	AEMAC	Feathers	+	ND	ND	ND
Azotc24	AEMAC	Chitin	ND	ND	ND	ND
Azots24	AEMAC	starch	ND	ND	ND	ND
Azotp24	AEMAC	Skim milk	ND	ND	ND	ND
Azotn24	H_20	Nutrient broth	ND	ND	ND	ND
Week 12						
Azotb36	AEMAC	DMSS	+	ND	ND	-
AzotbH36	H_2O	DMSS	+	ND	ND	-
Azotm36	AEMAC	SSS	+	ND	ND	-
AzotmH36	H_2O	SSS	+	ND	ND	-
Azoto36	AEMAC	Olive oil	+	ND	ND	-
Azotdp36	AEMAC	Grass	+	+	+	-
Azotf36	AEMAC	Feathers	+	ND	ND	ND
Azotc36	AEMAC	Chitin	ND	ND	ND	ND
Azots36	AEMAC	starch	ND	ND	ND	ND
Azotp36	AEMAC	Skim milk	ND	ND	ND	ND
Azotn36	H_20	Nutrient broth	ND	ND	ND	ND

Table C. Registration for consortium enzymatic activities during the process of enrichment and construction of consortiums in reactors at pH7

Consortium	Liquid medium	Substrate	Enzymes Bioreactors pH7			
			proteases	cellulase	xylanase	amylase
Week 4						
Azotb11	AEMAC	DMSS	+	ND	ND	-
AzotbH11	H_2O	DMSS	+	ND	ND	-
Azotm11	AEMAC	SSS	+	ND	ND	-
AzotmH11	H_2O	SSS	+	ND	ND	-
Azoto11	AEMAC	Olive oil	+	ND	ND	-
Azotdp11	AEMAC	Grass	-	-	+	-
Azotf11	AEMAC	Feathers	+	ND	ND	ND
Azotc11	AEMAC	Chitin	ND	ND	ND	ND
Azots11	AEMAC	starch	+	ND	ND	+
Azotp11	AEMAC	Skim milk	+	ND	ND	-
Azotn11	H_20	Nutrient broth	+	ND	ND	+
Week 8						
Azotb23	AEMAC	DMSS	+	ND	ND	-
AzotbH23	H_2O	DMSS	+	ND	ND	-
Azotm23	AEMAC	SSS	+	ND	ND	-
AzotmH23	H_2O	SSS	+	ND	ND	-
Azoto23	AEMAC	Olive oil	+	ND	ND	-
Azotdp23	AEMAC	Grass	-	-	+	-
Azotf23	AEMAC	Feathers	ND	ND	ND	ND
Azotc23	AEMAC	Chitin	ND	ND	ND	ND
Azots23	AEMAC	starch	ND	ND	ND	ND
Azotp23	AEMAC	Skim milk	ND	ND	ND	ND
Azotn23	H_20	Nutrient broth	ND	ND	ND	ND
Week 12						
Azotb35	AEMAC	DMSS	+	ND	ND	-
AzotbH35	H_2O	DMSS	+	ND	ND	-
Azotm35	AEMAC	SSS	+	ND	ND	-
AzotmH35	H_2O	SSS	+	ND	ND	-
Azoto35	AEMAC	Olive oil	+	ND	ND	-
Azotdp35	AEMAC	Grass	-	-	+	-
Azotf35	AEMAC	Feathers	ND	ND	ND	ND
Azotc35	AEMAC	Chitin	ND	ND	ND	ND
Azots35	AEMAC	starch	ND	ND	ND	ND
Azotp35	AEMAC	Skim milk	ND	ND	ND	ND
Azotn35	H_20	Nutrient broth	ND	ND	ND	ND

Table D. Registration for consortium enzymatic activities during the process of enrichment and construction of consortiums in reactors at pH3

Consortium	Liquid medium	Substrate	Enzymes Bioreactors pH3			
			proteases	cellulase	xylanase	amylase
Week 4						
Azotb10	AEMAC	DMSS	+	ND	ND	-
AzotbH10	H_2O	DMSS	+	ND	ND	-
Azotm10	AEMAC	SSS	+	ND	ND	-
AzotmH10	H_2O	SSS	+	ND	ND	-
Azoto10	AEMAC	Olive oil	-	ND	ND	-
Azotdp10	AEMAC	Grass	ND	-	-	-
Azotf10	AEMAC	Feathers	ND	ND	ND	ND
Azotc10	AEMAC	Chitin	ND	ND	ND	ND
Azots10	AEMAC	starch	ND	ND	ND	ND
Azotp10	AEMAC	Skim milk	ND	ND	ND	ND
Azotn10	H_20	Nutrient broth	ND	ND	ND	ND
Week 8						
Azotb22	AEMAC	DMSS	+	ND	ND	-
AzotbH22	H_2O	DMSS	+	ND	ND	-
Azotm22	AEMAC	SSS	+	ND	ND	-
AzotmH22	H_2O	SSS	+	ND	ND	-
Azoto22	AEMAC	Olive oil	-	ND	ND	-
Azotdp22	AEMAC	Grass	ND	-	-	-
Azotf22	AEMAC	Feathers	ND	ND	ND	ND
Azotc22	AEMAC	Chitin	ND	ND	ND	ND
Azots22	AEMAC	starch	ND	ND	ND	ND
Azotp22	AEMAC	Skim milk	ND	ND	ND	ND
Azotn22	H_20	Nutrient broth	ND	ND	ND	ND
Week 12						
Azotb34	AEMAC	DMSS	+	ND	ND	-
AzotbH34	H_2O	DMSS	+	ND	ND	-
Azotm34	AEMAC	SSS	+	ND	ND	-
AzotmH34	H_2O	SSS	+	ND	ND	-
Azoto34	AEMAC	Olive oil	-	ND	ND	-
Azotdp34	AEMAC	Grass	-	-	-	-
Azotf34	AEMAC	Feathers	ND	ND	ND	ND
Azotc34	AEMAC	Chitin	ND	ND	ND	ND
Azots34	AEMAC	starch	ND	ND	ND	ND
Azotp34	AEMAC	Skim milk	ND	ND	ND	ND
Azotn34	H_20	Nutrient broth	ND	ND	ND	ND

Table E. List of formulas used in the present work

Identification	**Formula**
A	$PSprt = ((sCOD,Final) - (sCOD,Initial)/COD,total)*100)$ Where; sCOD, Final = (sCOD Available at pretreatment stop time or at final of pretreatment + Consumed (COD loss)). sCOD, Inicial (sCOD at beginning of pretreatment) COD total (total COD added at beginning of pretreatment).
B	$V0 = \frac{P * VH * T0}{P0 * T}$ Where; P= Pressure at time of reading (x mbar) VH= Volume in headspace (65 ml) T0= Standard temperature (273 Kelvin) P0 = Standard pressure (1013 mbar) T = Temperature of gas phase at time of reading (310,15 kelvin)
C	$COD - CH4 \ = mmol\, CH4\ (bioreactor) * 64\, mg\ COD * 1\ mmol\ CH4$ Where; COD-CH_4 is given in (mg COD-CH_4) Standard equivalents = 64 mg COD equals to 1 mmol CH4 (Neves, 2009)
D	$BMP=(COD-CH4)/mVS*035/1000$ Where, BMP is given in (L CH_4/kg VS added) Standard equivalents 1 g of COD equals to 0.35 L of CH_4 (Neves, 2009)