

EFFECT OF BIOAUGMENTATION WITH HYDROLYTIC BACTERIA AND MICROAERATION ON WHEAT STRAW BIODEGRADABILITY

Master Thesis

Aristotle Vasileiou

Exchange student from National Technical University of Athens

April 2016

Supervisors :

Hosting Institute (DTU)

- Professor Irini Angelidaki
- Researcher Panagiotis Kougias

Sending Institute (NTUA)

- Professor Gerasimos Lyberatos

Technical University of Denmark
Department of Environmental Engineering
Building 113



ACKNOWLEDGMENT

Firstly, I would like to thank my supervisors professor Rena Angelidaki(DTU) and Gerasimos Lyberatos(NTUA) for giving me the opportunity to come in Denmark and participate to the Bioenergy group of Environmental Engineering, DTU. I would also like to thank Researcher Panagiotis Kougias, my co-supervisor, for the guidance on critical parts of thesis and his palatable ideas. There are no words to thank Panagiotis Tsapekos for his absolute guidance and assistance during all the experimental period, without his precious collaboration this thesis would not be held with success.

I would also like to thank Hector Garcia and Hector Diaz, the two Biotech lab technicians, for their constant help on the lab facilities. Lot of thanks belong to Kim, the 113 building technician, for leading me the first days in Denmark and providing me significant assistance such as finding a place to stay and how to handle with the Danish sector.

Lastly I would like to thank all the colleagues and people from Bioenergy group who shared the same laboratory and office with high professionalism. Being a few months in the specific workplace provided by Bioenergy group I really felt part of an international community. The feeling of working with such innovative and passionate people from all around the world, from Mexico to China, gave me priceless memories which I will always remember. It was my honor to work in this group.

Last but not least, I would like to thank my family for the support which they all provided during my long stay in Denmark.



ABSTRACT

Looking back in history the biogas production was started as an alternative method to the post sanitation and stabilization of agricultural wastes. After the price soar, there was a need for implementation of alternatives in energy production. This led to the successful utilization of organic wastes (and not only wastes) in order to produce beneficial “green energy” in the form of biogas etc. Considering the benefits that organic waste degradation provide, such as Greenhouse gas emissions reduction and sanitation or fertilizing or storage, biofuels will never be replaced from alternatives such as solar or wind energy.

In order to ease the degradation of lignocellulosic material, a variation of pre-treatment and treatment methods have been investigated over the last years. Beyond the classic applications of physicochemical (such as steam explosion), or chemical (acid or alkali) pre-treatments, two promising methods are rising recently. The first one is microaeration which has resulted contradictory results, but is a very interesting and applicable method. Microaeration is the injection of trace amounts of oxygen which could boost hydrolysis in an AD system, always depending from the substrate. The target-microbes are the facultative anaerobic hydrolytic microbes which could be boosted to hydrolyze the recalcitrant lignocellulosic matter. In that way anaerobic process will not exceed the “anaerobic threshold” by just consuming rapidly the minor amounts of oxygen. In the present work this facultative consortia of microbes was enriched by the addition of “methano-compost” as [Scherer & Neumann \(2013\)](#) characterized it. Many other researchers had the opportunity over the last years to enhance the anaerobic procedure of degrading lignocellulosic biomass such as [Fu et al. \(2015\)](#) who gave the definition of “TMP” (thermophilic microaerobic pre-treatment) for the series of experiments of microaeration of corn straw under thermal environment, or [Botheju et al. \(2010\)](#) by providing a new model called “ADM-1 Ox”.

Moreover, an uprising treating method is the biological treatment such as bioaugmentation. By definition, bioaugmentation represents the injection of pure culture of microorganisms in a biochemical system in order to boost a specific process without inhibiting or disturbing others. In the following work the process-target was hydrolysis, and more precisely the demand from the injected microbes to adapt and excrete hydrolysing enzymes in order to enhance the overall biogas production by boosting hydrolysis.

Contents

ACKNOWLEDGMENT	1
ABSTRACT	2
LIST OF FIGURES AND TABLES.....	5
CHAPTER 1: PREFACE	7
1.1 Purpose of this work-Framework	7
1.2 Sustainability	8
CHAPTER 2: LITERATURE.....	10
2.1 AD systems	10
2.1.1 Steps of AD	10
2.1.2 Microbial community	13
2.1.3 Inhibitory factors	13
2.1.4 Process parameters.....	14
2.1.5 Monitoring parameters	15
2.2 Lignocellulosic structure.....	17
2.3 Pre-treatment methods.....	21
2.3.1Microaeration.....	23
2.3.2 Compost	25
2.3.3 Bioaugmentation	27
2.3.4 Microbial growth and metabolism.....	33
CHAPTER 3 : MATERIALS AND METHODS.....	34
3.1 Substrates.....	34
3.2 Analytical Methods.....	35
3.2.1 Solid phase measurements	40
3.2.2 Liquid phase measurements	42
3.2.3 Gas phase measurements	43
3.3 BMP	45
3.3.1 BMP Microaeration	46
3.3.2 BMP Bioaugmentation	51
3.4 Bioaugmentation CSTR	54
3.4.1 Assembly	54

3.4.2 Start-up period	55
3.4.3 Microorganisms injection	56
CHAPTER 4: RESULTS AND Discussion	57
4.1 Microaeration	57
4.1.1 VFA, sCOD, pH , ORP after Microaeration Period	58
4.1.2 Methane Production Results	62
4.1.3 VFA, sCOD, pH at the end of experiment	68
Complementary discussion on microaeration results in comparison with literature	71
4.2 Bioaugmentation	73
4.2.1 BMP Bioaugmentation	74
4.2.2 CSTR Bioaugmentation	77
Complementary discussion on bioaugmentation results in comparison with literature	82
CHAPTER 5: CONCLUSIONS –Interpretation	84
REFERENCES.....	86
APPENDIX.....	93

LIST OF FIGURES AND TABLES

FIGURE 1.1 LIGNIN BASED PUBLICATIONS OVER THE LAST DECADES, SOURCE FROM 2015 SOCIETY OF CHEMICAL INDUSTRY AND JOHN WILEY & SONS, LTD	9
FIGURE 2.1 CARBON FLOW FROM COMPLEX SUBSTRATE TO AD PRODUCTS DURING ANAEROBIC DIGESTION (ANGELIDAKI ET AL., 2002)	12
FIGURE 2.2 LIGNOCELLULOSE MATTER IN NATURE(RUBIN, 2008)	18
FIGURE 2.3 LIGNIN STRUCTURE	19
FIGURE 2.4 CELLOBIOSE (DIMER OF CELLULOSE, TWO GLUCOSEs CONNECTED)	19
FIGURE 2.5 CHAIN POLYMER CELLULOSE	20
FIGURE 2.6 EXAMPLE OF HEMI-CELLULOSE COMPLEXITY	20
TABLE 2.1 COMBINED PRE-TREATMENTS AND THEIR RESULTS ON LIGNOCELLULOSIC BIOMASS	23
FIGURE 2.7 COMPOST AFTER 1 MONTH IN AEROBIC CONDITIONS	26
TABLE 2.2 ALREADY KNOWN CELLULOSIC <i>CLOSTRIDIA</i> (KOTHARI 2015)	29
FIGURE 2.8 <i>C. THERMOCELLUM</i> ROD SHAPE	29
FIGURE 2.9 <i>C. THERMOCELLUM</i> SURFACE	29
FIGURE 2.10 CELLULOSOMES	30
TABLE 2.3 (PODOSKAYA ET AL 2013) PROPOSED THE FOLLOWING SUBSTANCES FOR UTILISATION BY <i>M. ROSEUS</i>	31
FIGURE 2.11 METABOLIC PATH OF <i>M. ROSEUS</i> (KARDICOV ET AL., 2013)	32
FIGURE 2.12 <i>M. ROSEUS</i> (KARDICOV ET AL., 2013)	32
TABLE 2.4 SUBSTRATES CHARACTERIZATION BEFORE PRE-TREATMENT, OR DIGESTION.	35
FIGURE 3.1 AND 3.2 HEAVY-DUTY CUTTING MILL SM 2000	36
TABLE 3.1 ELEMENTAL ANALYSIS	37
FIGURE 3.2 <i>VARIO MACRO CUBE</i>	37
FIGURE 3.3 AND 3.4 STEAM DISTILLATION UNIT (ON THE LEFT) AND SPECIAL HEATING SYSTEM(ON THE RIGHT)	38
FIGURE 3.5 AND 3.6 COLOURED SOLUTIONS BEFORE AND AFTER TITRATION	38
FIGURE 3.7 AND 3.8 AND 3.9 COD PREPARATION	39
TABLE 3.2 WHEAT STRAW CONTENTS	40
FIGURE 3.10 DIONEX ICS-5000 DUAL PUMP	41
FIGURE 3.11 PH METER	42
FIGURE 3.12 GC APPARATUS FOR VFA DETERMINATION AND SAMPLES	43
FIGURE 3.13 GC APPARATUS FOR BIOGAS DETERMINATION	44
FIGURE 3.14 WATER DISPLACEMENT	44
TABLE 3.3 PLAN FOR MICROAERATION	48
FIGURE 3.15 SETTING UP THE MICROAERATION EXPERIMENT	49
FIGURE 3.16 WHITE HOT ROT FUNGI ON COMPOST AFTER 1 MONTH ON AMBIENT CONDITIONS	51
TABLE 3.4 PLAN FOR BMP BIOAUGMENTATION	51
TABLE 3.5 <i>CLOSTRIDIUM THERMOCELLUM</i> STRAIN 1347 MEDIUM INGREDIENTS	52
TABLE 3.6 <i>M. ROSEUS</i> MEDIUM INGREDIENTS	53
FIGURE 3.17 CSTR SYSTEM	55
TABLE 3.7 VS, TS , OLR VALUES	55
FIGURE 3.18 BA MEDIUM , FIGURE 3.19 <i>M.ROSEUS</i> MEDIUM , FIGURE 3.20 <i>M. ROSEUS</i> (ON THE LEFT) AND C. THERMOCELLUM(ON THE RIGHT) MEDIUM	56
FIGURE 4. 1 TVFA BEFORE(BLUE BARS) AND RIGHT AFTER MICROAERATION(RED BARS)	58

TABLE 4. 1 VFA INCREASE IN A DAY FOR UNTREATED	59
TABLE 4. 2 VFA INCREASE IN A DAY FOR 5 ML O ₂ /GVS PER DAY	59
TABLE 4. 3 VFA INCREASE IN A DAY FOR 10 ML O ₂ /GVS PER DAY	59
TABLE 4. 4 VFA INCREASE IN A DAY FOR 15 ML O ₂ /GVS PER DAY	59
FIGURE 4.2 PH BEFORE (BLUE BARS) AND RIGHT AFTER MICROAERATION(RED BARS)	60
TABLE 4.3 SCOD AFTER MICROAERATION	61
FIGURE 4. 3 METHANE PRODUCTION FOR CONTROL BATCHES	63
FIGURE 4. 4 AGGREGATED METHANE POTENTIAL VALUE	63
TABLE 4. 5 AGGREGATED RESULTS FOR METHANE POTENTIAL	64
FIGURE 4. 5 METHANE YIELD FOR WHEAT STRAW 5-1-2	66
FIGURE 4. 6 METHANE YIELD FOR 10-1-1	67
FIGURE 4. 7 METHANE YIELD FOR 15-1-3	68
FIGURE 4. 8 TVFA AFTER DIGESTION	69
FIGURE 4. 9 PH CORRELATION BETWEEN UNTREATED (BLUE BAR) –TREATED(RED BAR) –DIGESTED(GREEN BAR).	69
TABLE 4. 6 SCOD TABLE	70
TABLE 4. 7 SCOD DIFFERENCE TABLE	70
FIGURE 4. 10 SCOD AFTER DIGESTION	70
FIGURE 4.11 OD FIGURES	73
FIGURE 4.12 METHANE YIELD FOR WHEAT STRAW MONO-DIGESTION	74
FIGURE 4.13 METHANE YIELD FOR CO-DIGESTION EXPERIMENT OF 10% SHARE OF WHEAT STRAW AND 90% OF CATTLE MANURE ON VS BASIS	75
FIGURE 4.14 AGGREGATED METHANE POTENTIAL FOR MONO AND CO- DIGESTION	76
FIGURE 4.15 PH AND VFA AT THE CONTROL REACTOR R1	77
FIGURE 4.16 METHANE YIELD AT THE CONTROL REACTOR-R1	78
FIGURE 4.17 PH AND VFA AT R2 BIOAUGMENTED WITH <i>C. THERMOCELLUM</i>	78
FIGURE 4.18 METHANE YIELD AT R2 BIOAUGMENTED WITH <i>C. THERMOCELLUM</i>	79
FIGURE 4.19 PH AND VFA AT R3 BIO-AUGMENTED WITH <i>M. ROSEUS</i>	80
FIGURE 4.20 METHANE YIELD AT R3 BIO-AUGMENTED WITH <i>M. ROSEUS</i>	81
FIGURE 4.21 METHANE YIELD FOR ALL THREE DIGESTERS	82

CHAPTER 1: PREFACE

1.1 Purpose of this work-Framework

The main purpose of this work was to examine the treatment of lignocellulosic substrate in order to enhance the hydrolysis step in anaerobic digestion (AD) process and subsequently increase the methane yield. In order to enhance hydrolytic step and biodegradability, two different methods were investigated under thermophilic anaerobic conditions, namely Microaeration and Bioaugmentation. Thus, this thesis is divided in two parts. The first part is focused in the microaeration of AD inoculum, compost inoculum and wheat straw through BMP assays. Whereas for the second part the effect of routine bioaugmentation to cattle manure and wheat straw is investigated through continuous reactors (CSTR) with two different strains of microbes, *Clostridium thermocellum* (DSMZ 1237) and *Melioribacter roseus* (DSMZ 23840). Additionally, in order to define the exact methane potential, series of BMP assessments were conducted.

In the first experiment 182 batches were prepared in order to probe the competence of facultative anaerobic fungi to hydrolyse wheat straw respiring small amounts of oxygen. The extended assessment of the aforementioned methods is following with main objective to indicate efficient alternative treatment processes for co-digestion of cattle manure and lignocellulosic energy crops such as wheat straw. Compost was used in terms of biological treatment in BMP assays, by means of “methano-compost” microflora.

Routine bioaugmentation has been reported by many authors as a promising method to interfere in a complex biological system. In the present thesis highly efficient hydrolytic bacteria were injected in order to excrete degrading enzymes and disrupt the recalcitrant part of wheat straw, lignin. More specifically, these strains were *Clostridium thermocellum* (DSMZ 1237) and *Melioribacter roseus* (DSMZ 23840). Both strains require for their growth anaerobic thermophilic conditions, except from *M. roseus* which is facultative anaerobic. The implementation and main objective of bioaugmentation was the long term infection and colonization in the new environment, avoiding the wash out of injected microorganisms. Nevertheless, the main aim was to obtain a new eco-system slightly adjusted by the new hydrolytic strains in aid of us.

1.2 Sustainability

From 1950, modern technologies and massive assessments were conducted for anaerobic digestion of organic wastes, which is until nowadays an emerging technology. Biogas production under anaerobic conditions figures many sustainable advantages which make it as one of the best alternative processes for green energy production. First exemplary merit of AD is the diminished odorous effluent due to the fact that the 99% of volatile odorous substances are decomposed (Smet et al., 1999). Additionally, the slurry effluent maintains agricultural nutrients, namely ammonia or other phosphoric or nitrate molecules, therefore, is a favourable alternative as a fertilizer. The most beneficial merit to the process is the degradation of organic matter to methane and carbon dioxide. Carbon dioxide and methane are a part of greenhouse gasses and contribute in the global warming and the overall greenhouse effect (Lansche, 2012). Moreover, carbon dioxide is a mineralization natural product, and normally it is recirculating in the carbon cycle and CH₄ is led to ignition for energy production. As far as it concerns, AD is preferred instead of combustion or other alternatives because of the lower energy demand and the production of bio-fuel, methane, instead of 100% CO₂ emissions. It must be noted that methane impacts stronger than CO₂ to the greenhouse effect if it is released in atmosphere before its ignition. Therefore, the slurry digested effluent should be strictly controlled. Simultaneously the bio-energy that is gained from biogas utilisation replaces natural gas which is a fossil fuel (Ward et al., 2008). Further on, AD is an important alternative to aerobic or half anaerobic processes such as composting. To prolong, the composting of organic wastes such as municipal or manure in extended landfills leads to the methane production in very slow rates and its release to atmosphere, contributing to the greenhouse effect (Bergland et al., 2014). However, the receiving effluent from AD process is not appropriate for direct exploitation, because of its humidity and the high volatile acids concentration. Therefore, it must be utilised with post treatments such as chemical additives or aerobic composting. Compared to fully aerobic composting, the overall effect of the combination AD-composting leads to the reduction of CO₂ emissions (i.e. 25-67%) and to the excess of electrical energy.

Over the years manure is mostly applied on the anaerobic digestion process. Rumen does not fully digest the cows' regime. Hereupon, the exploitation of surplus consisted by biofibers and other organic matter that manure contains is conducted. Nevertheless, in order to raise the methane potential of manure, the supplementary use (co-digestion) of rich-cellulose energy crops is turning as a promising method (Angelidaki & Ellegaard, 2003). Nowadays, there is an abundant amount of agricultural lignocellulosic wastes which are offered for utilization in order to produce biofuel. Moreover, cattle manure which is already exploited in order to produce biogas contains undigested fibbers which are constructed mostly by lignin. In a research by Eurostat, (2012), it was showed that more than 60% of biomass is appropriate for biofuel production in the European region. Although it is claimed that biomass is figured as only 10% of the source for bio-energy (Mata-álvarez, 2015). Of course this could be explained by the fact that lignin is not degraded leading to the rigid, recalcitrant nature of lignocellulosic biomass, makes it an avoided substrate for utilization. However, throughout the years after the exploitation of easily degradable biomass such as lipids, proteins and easily degradable carbohydrates, nowadays research community faces the challenge of recalcitrant matter utilization.

The second most abundant polymer in nature, after cellulose, is lignin which does not belong in the group of sugars, and it is considered to be non-degradable. Hence, lignocellulosic substrates are, recalcitrant to degrade. Lignin is an aromatic polymer which protects cellulose, and gives the characteristic rigid structure of lignin rich

plants. In nature lignin is very profitable for the plant because it protects it from water or insects, or microbial deconstruction. However for biotechnological purposes (i.e. Biogas production, paper recycling/produce) lignin is undesirable because it prevents process from microbial attack or mechanical deconstruction. As a result in order to reveal the hidden cellulose, strong acids and bases have been used for the pre-treatment.

The last decade due to the abundance of lignocellulosic material in nature, and the need of its exploitation, lignin rich energy crops are the most investigated organic wastes in order to be utilized for organic waste enrichment.

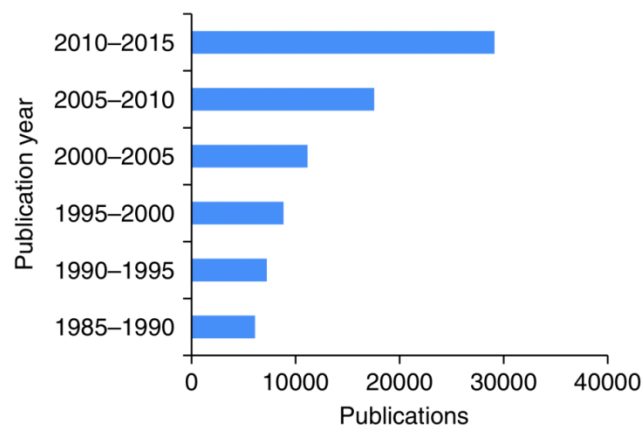


Figure 1.1 Lignin based publications over the last decades, source from 2015 Society of Chemical Industry and John Wiley & Sons, Ltd

CHAPTER 2: LITERATURE

2.1 AD systems

Amongst the AD products, the most valuable is the gas fraction, which is consisted mainly by methane (50-60%) and carbon dioxide (35-45%). Furthermore, inoculum which is the digested substrate from the effluent of a reactor, consists the liquid fraction and it can be used as a fertiliser because it maintains the N, P and nutrients after the digestion.

Biogas is the gas that is produced from anaerobic digestion through a very complex biochemical procedure. The digestion of high molecular organic wastes to, methane and carbon dioxide is a result of a simultaneous process which will be discussed in the present chapter. AD takes place normally in nature where a huge consortia of microorganisms under oxygen free conditions and a nutrient rich substrate produce biogas. Subsequently, the sector of science which deals with the utilisation of organic wastes for biogas production it belongs to environmental and chemical science, it uses biotechnology to produce bioenergy.

Kinetics in each step of this multistep process determine the overall time that is needed for the biogas production. Accordingly to the biomass that is used as substrate, there is effect on each step. Thus, if the substrate is difficult to degrade by hydrolytic microbes, then the process "stacks" in the first step. But if the implemented biomass is easily degradable then the last step of methanogenesis is supposed to be the rate limiting. In any case in the first situation it is rational the process to take more time, if both are operating in the same conditions. Therefore, as far as it concerns if the first AD step is accelerated then the overall biogas generation would be enhanced. In nature we can find hydrolytic bacteria in rumen (anaerobic conditions), hot springs, municipal wastes, sewage wastes, tree branches, piles, and in soil generally.

2.1.1 Steps of AD

The process of AD follows 4 concurrent steps

1st step: Hydrolysis

Hydrolysis is called the procedure in which hydrolytic extracellular enzymes are responsible for the degradation of high molecular polymeric compounds that consist the organic biomass. These polymeric compounds vary accordingly with the kind of waste. Consequently, food industry wastes could be rich in lipids (triglycerides fat or oil- $C_{57}H_{104}O_6$), proteins $(C_5H_7NO_2)_n$ and complex carbohydrates like polysaccharides ($C_6H_{10}O_5$). Macromolecules that consist the biomass are even bigger in size than microbes in theirself. Thus, due to the adequate biomass utilization, hydrolytic enzymes are produced by the relative fermentative bacteria. For instance, proteases and lipases disrupt proteinic and lipid polymers to glucose and Long chain fatty acids (LCFA) respectively. In the following work the most dominant and favoured enzymes are cellulases and xylanases which are secreted by cellulolytic and xylanolytic bacteria in order to disrupt recalcitrant biomass. Furthermore, polysaccharides such as

cellulose and pectin are hydrolysed to each monomeric form which is in general glucose (hexoses- $C_6H_{12}O_6$) or xylose (pentoses- $(C_5H_{10}O_5)$). Additionally, xylose is formed as a result of xylan disruption and is a very abundant hemicellulotic compound in straw which was confirmed also in this work.

In the agricultural sector which is the most common for biogas production, carbohydrates are the most common substrate. In the present work the selected biomass is lignocellulosic crop, hence hydrolysis is the most considerable and rate-limiting step. It is commonly known that lignin is a non-degradable aromatic, branched polymer. Whenever a recalcitrant biomass is used in an AD procedure, hydrolysis is the rate limiting step ([Eastman & Ferguson, 1981](#)). Nevertheless, all plants in nature are lignocellulosic and consisted by cellulose, hemi-cellulose and lignin in different compositions. For lignocellulosic substrates the main intermediates during the degradation are sugars. Thus, further degradation of cellulose and starch, leads to the formation of sugars like glucose. The enzymes that contribute to this formation are called cellulases which absorb on cellulose layers and destroy their crystal structure. Furthermore, acids which are simultaneously formed in this step decrystallize cellulose and enhance with their way the hydrolytic step. The following chapter describes the lignin structure more comprehensively.

2nd step: Acidogenesis

Generally, the best pathway to methane production is the direct utilization of hydrolysed products to acetate, CO_2 and H_2 . Nevertheless, only the 70% of the degraded polymers consist the aforementioned methanogenic nutrients and the rest 30% form intermediates such as volatile fatty acids (VFA), lactate and alcohols ([Angelidaki & Ellegaard, 2002](#)). Acidogenesis gains the advantage of a side-branched metabolism whereas can overtake the inhibitory H_2 accumulation which can occur due to the intense substrate oxidation.

Acidogens are responsible for the transformation of long chain fatty acids or sugars to short chain fatty acids and other intermediates. In particular, monosaccharides such as hexoses (glucose) and pentoses are further degraded to reduced electron carriers (NADH) which will significantly contribute in anaerobic fermentation for the production of fatty acids and alcohols, such as lactate, propionate, propionate, butyrate acetate and ethanol. From the aforementioned the most favourable is acetate as it consists the first order nutrient for aceticlastic methanogens.

3rd step: Acetogenesis

This is the step in AD process where fermented intermediary products are further degraded by acetogenic bacteria to acetate and H_2 , CO_2 ([McCarty, 1964](#)). Hydrogen producing bacteria use linear metabolism, and thus they are coerced to degrade VFA to acetate and H_2 . The measured and most abundant volatile fatty acids are acetate, propionate, butyrate, iso-butyrate valerate, iso-valerate. Whereas, due to the high negative free energy of acetate formation by glucose fermentation; the acetogenesis is more facilitated than propionate or butyrate formation.

4rd step: Methanogenesis

In this last step, obligate anaerobic methanogens use acetate, H_2 and CO_2 as substrate to grow and through their metabolic reaction they relieve methane and CO_2 as products.

As figure 2.1 depicts there are two different methanogenic archaea, the largest biogas volume comes from aceticlastic methanogens, approximately to 70% and the rest from hydrogenotrophic methanogens. In the first

group, microbes consume acetate (e.g. *Methanosarcina*) and in the second group they consume carbon dioxide and hydrogen (e.g. *Methanosaeta*). In comparison with the rest microbes methanogens are very vulnerable to environmental disturbances and to oxygen. Additionally, the second group of methanogens can utilize formate, which cannot be consumed by the aceticlastic methanogens. Regarding to the effect of temperature, it is stated that under mesophilic conditions the diversity of methanogens is higher (Karakashev et al., 2005).

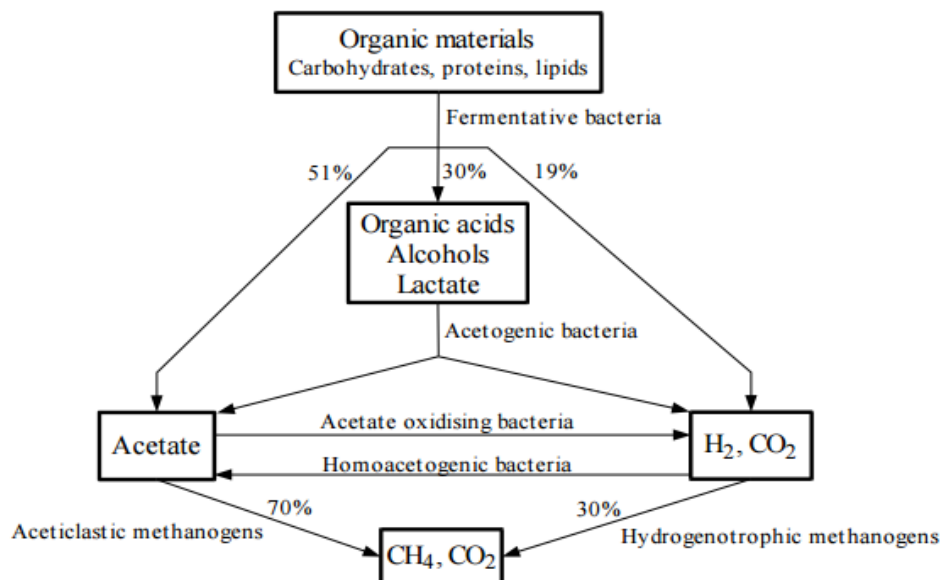


Figure 2.1 carbon flow from complex substrate to AD products during anaerobic digestion (Angelidaki et al., 2002)

4 stages of methane generation process in time

Bacterial/microbial life is divided in four steps. In the first step, the lag phase, cell consumes substrate in order to survive and not to divide, thus the kinetics follow the zeroth constant rate. When microbes are robust and the nutrient substrate is abundant they follow a linear growth rate until the reduction of substrate whereafter the stationary phase follows. At the stationary phase, microbial rise is equal to microbial death. Hence, microbes preserve themselves until their reduction because of starvation or adequate life growth.

Methane is produced from the last step of anaerobic digestion. Methanogens prioritize methanogenesis instead of doubling their mass, by means of carbon is led to methane production and not biomass generation and thus the archaea growth is slow. The carbon that is utilized to biomass is more or less 5%.

1st step. “Lag phase”. In this step hydrolytic bacteria are “waken up” and excrete enzymes due to the injection of oxygen and their first interaction with substrate. If hydrolysis is fast and adequate, nutrients are produced, and then methanogens start the consumption faster, thus lag phase is short.

2nd step. During the second step methane yield rises rapidly. This indicates an accelerating hydrolytic and methanogenic activity which follows a linear increase as long as VFAs and more specifically acetate are abundant.

3rd step. It is considered that after a pinpoint methanogens consumes the overall amount of acetate, and the most degradable part of substrate. Thereafter, methanogenic activity slows gradually until the last week.

4th step. Afterward, with the acetate/nutrients consumption it follows the last step, where methane yield stabilizes until it drops. It is obvious that the experiment continues until the stabilization of production instead of the drop of it. Of course due to the limited amount of nutrients it is rational that the drop of methane production will follow next.

2.1.2 Microbial community

In general the AD system is consisted by strictly anaerobic microbes such as clostridia , methanogenic archaea , and sulphuric consumers (Stamatelatou et al., 2011) .

Anaerobic hydrolytic bacteria could be divided in four phylum categories, *Firmicutes*, *Bacteroidetes*, *Fibrobacter*, *Thermotogae* and the minor subcategory of *Chlorobi*. The most abundant phylum in biogas plants are the first two categories (Campanaro et al., 2016). In the present work the implementation of *Clostridium thermocellum* and *Melioribacter roseus* turns our interest in phylum *Firmicutes* and *Chlorobi*, respectively (Sun et al., 2013a). Nevertheless, in previous cattle manure experiments, ubiquitous influence detected by genera *Clostridium* (Sun et al., 2013). However, slightly lower bacterial species richness was observed in digesters that obtained co-digestion with wheat straw and manure. In contrast, for archaea, no clear trend in species richness was observed when comparing the manure itself and the mesophilic digesters operating with manure, alone or combined with straw (Sun et al., 2013b). Although, in digesters which contained cattle manure and wheat straw *Bacteroidetes* were more abundant than in cattle manure digesters. Apparently, after the addition of wheat straw in the influent there was a rise of *Bacteroidetes*.

Karakashev et al. (2005) tried to clarify the different methanogenic communities in 7 full scale Biogas plant reactors which were fed with manure and various supplementary substrates. The most dominant methanogens were species similar to *Methanosarcinaceae* in rich ammonia, VFA and organic acid environment. However *Methanosarcinaceae* is an acetoclastic community which unlikely does not belong to bacterial gene, but in Archaea domain. Nevertheless, hydrogen-consuming bacteria or hydrogenotrophic, Methanobacteriales correspond favourably in manure digesters. On the other hand, sludge digesters contain different methanogenic species such as *Methanosaetaceae* and thereby contain lower VFA and organic acid concentrations than manure digesters. However it is known that in thermophilic anaerobic conditions, the hydrogenotrophic methanogens are dominant in rich ammonia environment (Sun et al., 2013b).

2.1.3 Inhibitory factors

As far as it concerns, there are less than a few common inhibitory factors for all the AD systems, although regarding to the feedstock, the inhibitory factors and toxic compounds are more diverse. For instance poor VS removal and low biogas yield are often associated with low C/N ratio, temperature instabilities, high $\text{NH}_3\text{-N}$, high VFA and low digester buffering capacity (Zeeman & Lettinga, 2006; Anderson et al., 2003). Moreover, a number of instabilities is counted due to unstable organic load or foam formation (Kougias et al., 2014).

Monitoring parameters indicate the stability of the process. Thus, temperature, pH, VFA, methane yield, biogas production and ammonia content are the main monitoring parameters to measure in an AD system. Additionally, associating parameters such as HRT (hydrolytic retention time), OLR (Organic load rate), substrate composition (TS,VS) should be clarified.

2.1.4 Process parameters

HRT

In broad terms, hydraulic retention time is defined as the average time that takes the reactor to change the working volume. In a biotechnological reactor the threshold of HRT is at least double the doubling time of microbes in order to avoid wash out (Yadvika et al., 2006).

$$HRT = \frac{Volume}{Flow}$$

OLR

It is measured in terms of gVS/L/day. It is reported that in co-digestion systems of recalcitrant substrates such as lignocellulosic, OLR reaches the amount of 1.5-3.5 gVS/L/day, which is low in comparison with the main co-digesting OLR of 5.5-7.3 g/L/day of cattle manure and "soft" organic wastes (Ruiz-Hernando et al., 2014; Lucas et al., 2015).

OLR disturbances could result many changes in the system. To monitor OLR in order to reach optimum conditions, it is needed to determine frequently the VFA, biogas, methane content or H_2 . Thus when the substrate is easily degradable, such as proteins or lipids, acetate and propionate are increased, biogas production is increased and methane content is decreased. As indicators the aforementioned reflect the steep increase of organic load (Boe et al., 2010). It is noteworthy that propionate captures the inhibition of anaerobic process in time even if inhibition is alleviated. By means of this, when propionate accumulates, it is not consumed adequately until the end of the experiment, even when conditions has turned optimum again (Boe, n.d.).

Temperature

Temperature is a crucial parameter, where even in slight changes system response immediately. For instance Chae et al. (2008) confirmed that even for 2 °C drop in temperature, biogas production responses by failing. There are

two main temperature ranges that anaerobic digestion usually implemented, thermophilic and mesophilic. Thermophilic conditions are ranging at 45-55 °C and mesophilic at 30-35 °C. In general thermophilic conditions are more preferable than mesophilic for AD purposes. Moreover, it is claimed that in higher temperatures pathogens are decomposed, Therefore the procedure is more stable and the receiving more sanitary product which is appropriate for using it as fertilizer (Sahlström, 2003). It is also observed that at 55 °C the rate of hydrolysis is greater and the amount of VFAs is bigger (Kim et al., 2010). Furthermore, there is an accelerating productivity of thermophilic hydrolytic bacteria in comparison with the mesophilic (Dumas et al., 2010).

Reactor configurations

Single stage CSTR reactors at thermophilic conditions perform higher acetate production than at mesophilic. Nevertheless thermophilic conditions are beneficial to the consortia of microorganisms especially to the methanogens, which accelerate their growth rate (Kim et al., 2002). Gannoun et al (2007) obtained greater methane yield and chemical oxygen demand (COD) removal in thermophilic than mesophilic conditions for co-digesting olive mill and abattoir. It is also observed in another research that in thermophilic conditions process reached similar methane yield in 11 days instead of 27 days which takes to mesophilic conditions (Hegde et al., 2007). This is in line with the HTR values even though HRT depends on microbial growth in order to suppress washing out microbes. Additionally, in high temperatures there are limitations in the system such as low process stability and rising of propionate concentration. Propionate belongs to volatile fatty acids and is added up to eliminating AD factors due to the positive ΔG .

Two stage reactors are beneficial when substrate is easily hydrolysed, thereby, acids accumulate and pH drops to approximately 5.5 developing an acidic environment in which methanogens are not able to grow up. In that case hydrolysis takes place in a separate reactor and the effluent is treated to the second reactor where methanogenesis is conducted.

2.1.5 Monitoring parameters

Hydrogen sulphide (H₂S)

Is the main reason for the odorous smell in every organic waste, and mostly in manure. In additional, it leads to inhibition with the consumption of trace metals which are used by the microbial consortium (Glass & Orphan, 2012).

LCFA

As it was mentioned above, the LCFA that are produced during the lipid hydrolysis could cause inhibition to the system by preventing more or less all microbes from their normal function (Angelidaki & Ahring, 1993). Thereby, higher concentration than 1 gr/L of Long-Chain Fatty Acids is inhibitory for the AD process (Vavilin et al., 2008). There is a group of bacteria that produce hydrogen and acetate, while consume propionate and LCFA; and when they are inhibited, LCFA and propionate accumulation is appeared. LCFA are absorbed to microbial surface, as result the transportation of nutrients through surface is blocked (Alves et al., 2001). It is reported that LCFA inhibition depends in many factors, such as the type of microorganism that affect, the type of surface, the surface area, the length of long chain fatty acids and of course the saturation of LCFA in the solution (Salminen & Rintala,

2002). It is reported from [Lalman & Bagley \(2002\)](#) that methanogens are more vulnerable in comparison with the acidogenic bacteria. However in thermophilic conditions there is a bigger susceptibility than in mesophilic, although the recovery of the microorganisms is faster. However, that is the most important fact of the LCFA inhibition: it is a reversible process. A recovery resolution is to dilute the system with active inoculum, and hence increase the proportion of biomass/LCFA. Another, but more expensive way to avoid the inhibition very fast is to add LCFA absorbents. This recovery action was tested from in semi-continuous fed system as result the reduction on recovery from 10 to 2 days.

VFA

Excess of volatile fatty acids could cause inhibition of methane production ([Nielsen et al., 2007](#)). Thus, the specific amount of them is of great importance for the stability of the biological AD system. In addition, the fraction of acetate /propionate serve as key parameter for the monitoring of a digester Therefore the proportion should not rise above the threshold of 1.4 ([Hill et al., 1987](#)). Accumulation of VFA leads to dramatic drop of buffering capacity and hereupon pH drops, inhibiting methanogenic step. The interval from VFA accumulation to pH drop could be devastating for the process. To this effect, researchers should rely firstly on buffering capacity and determine it initially. To alleviate an inhibition led by low buffering capacity, OLR should be reduced by diluting the working volume, or more drastically by the addition of bases or carbonate salts in order to increase pH and remove the excessive CO₂.

pH

Methanogens grow optimally in neutral pH ([Chandra et al., 2012](#)). Furthermore, alkalinity can be defined with pH measurement. At pH values less than 5.5, the process is severely damaged pinpointing the acidic accumulation. This is in line with the fact that acidogenesis is the most common step where anaerobic digestion is inhibited ([Karakashev et al., 2005](#)).

Ammonia

Inhibition of ammonia has been studied comprehensively the previous years ([Angelidaki & Ahring, 1993](#) ; [Liu & Chen, 2006](#)). Farm manures contain high concentrations of ammonia which in appropriate concentrations could give the inherent virtue of buffering capacity, and make dilute manure one of the most appropriate substrates for co-digestion with high organic energy crops.

However, regarding to the feedstock, if ammonia is greater than adequate proportion for microbial growth it can cause inhibition to AD. High concentrations of ammonia can be counteracted when used with other feedstocks which have low nitrogen concentrations such as wheat straw ([Hansen et al., 1998](#); [Sung & Liu, 2003](#)). It is known that, at high pH, dissolved ammonia is more dominant and inhibitory than the ionised ammonium (NH₄⁺), however, free ammonia should be calculated for investigation of inhibition ([Fotidis et al., 2013](#)).

2.2 Lignocellulosic structure

Lignocellulose structure is mainly consisted by cellulose repeating monomers $(C_6H_{10}O_5)_n$, hemicellulose $(C_5H_8O_4)_m$, lignin $C_9H_{10}O_3(OCH_3)$, pectins, extractives, and proteins (Sjostrom, 1993). Lignocellulosic biomass could be found in nature abundantly. Hence, it could be found in forest residues such as wood, or in agricultural sector's left overs such as sugarcane bagasse, silages (e.g. grass), and a big variety of straws (e.g. corn, wheat, rice), in general energy crops.

In order to approach the rigid lignocellulosic nature it must be clear that cellulosic and hemicellulosic component of the biomasses are tightly linked to the lignin. As a result the robust body is constructed by hydrogenic bonds. Hence for its better utilisation, under a treating method biomass could give a valuable result. As it seems, the interaction between lignin and holocellulose is the limiting factor for fiber degradation. The main parts of wheat straw are lignin, hemicellulose and cellulose in the approximate proportion of 15%-25%, 26%-31% and 33%-38% respectively (McKendry, 2002).

Cellulases are enzymes secreted by fermentative bacteria which access to amorphous cellulose more easily than in crystal cellulose and lead to its degradation. Thus, one parameter for the yield improvement is to implement a treating method which would degrade the crystal part of cellulose. Due to this fact the usage of hydrolytic bacteria for bioaugmentation would be compelling, as they secrete hydrolytic enzymes (cellulases) which degrade crystal cellulose.

Lignin firstly introduced by Anselme Payen in 1838 describing the rigid structure of lignocellulosic plants (Wisniak, 2005). Later in 1957 Adler introduces the first model of lignin in order to show that is a 16-phenylpropane structured polymer connected with C-O-C and C-C bonds. After many investigations on lignin, finally Sarikaya in 1983 proposed a figure for softwood lignin in 2-D depiction, as it follows. Sugars (xylose, glucose and arabinose), degradation products (furfural, hydroxymethylfurfural and soluble lignin) and acetic acid concentrations were determined for each set of hydrolysis conditions (Mussatto et al., 2008).

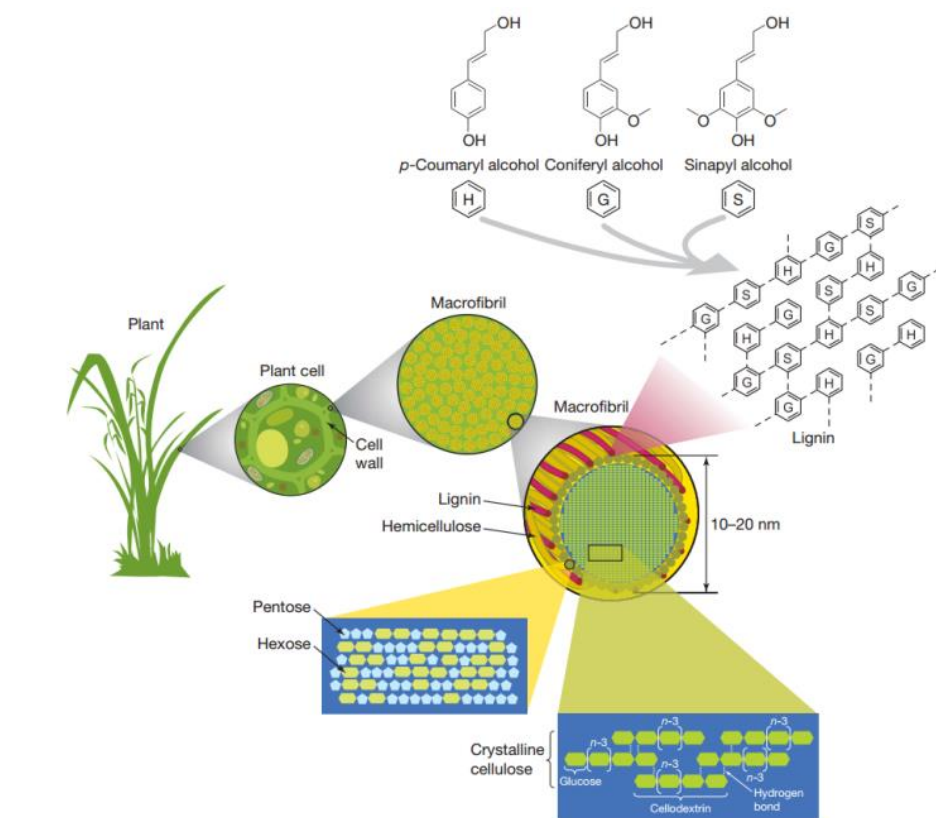


Figure 2.2 Lignocellulose matter in nature(Rubin, 2008)

Lignin structure

Lignin is a complex aromatic-phenol polymer bonded tightly with cellulose and hemicellulose with ester and hydrogen bonds. Lignin spreads in three dimensions with phenyl-propane networks which hydrolytic enzymes struggle to overcome and hydrolysis is delayed, or inefficient. It is considered as the dominant obstacle in the enzymatic hydrolysis of cellulose (Leonowicz et al., 1999).

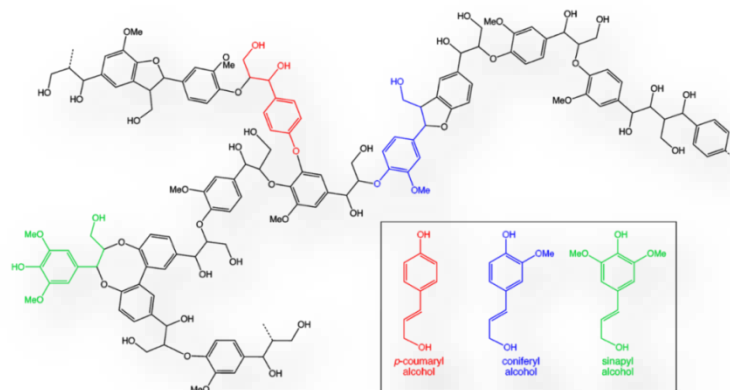


Figure 2.3 lignin structure

Cellulose structure (chain polysaccharides)

It is a linear chain D-glucose (C_6 -sugar polymer) polymer, and is more rigid than hemicellulose. Cellulose layers are imbedded in the lignin recalcitrant structure. Connection between the cellulose polymeric chains shape the overall 3 dimension structure which could be amorphous or crystal. The interaction with hemicellulose and lignin is direct in both physical and physicochemical way (Chen, 2014).

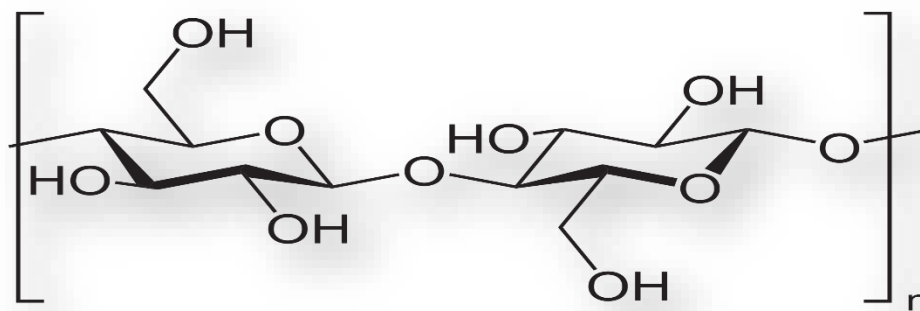


Figure 2.4 cellobiose (dimer of cellulose, two glucoses connected)

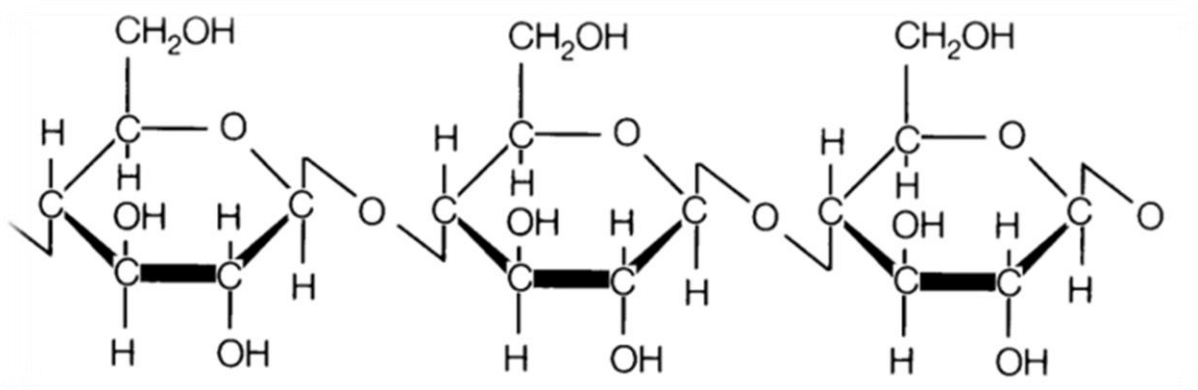


Figure 2.5 chain polymer cellulose

Hemicellulose structure(chain polysaccharides)

Hemicellulose is a branched heteropolymer which includes mostly pentoses such as D-xylose, D-arabinose. However, it also contains hexoses such as D-glucose, D-galactose and D-mannose and a variety of glucuronic acids. In soft wood hemicellulose rich in glucomannan is encountered, although xylan is the most dominant compound of hemicellulose. The interactive bonds between hemicellulosic layers are hydrogen bonds and it is considered to be degraded more easily in comparison with cellulose ([Hendriks et al., 2009](#)).

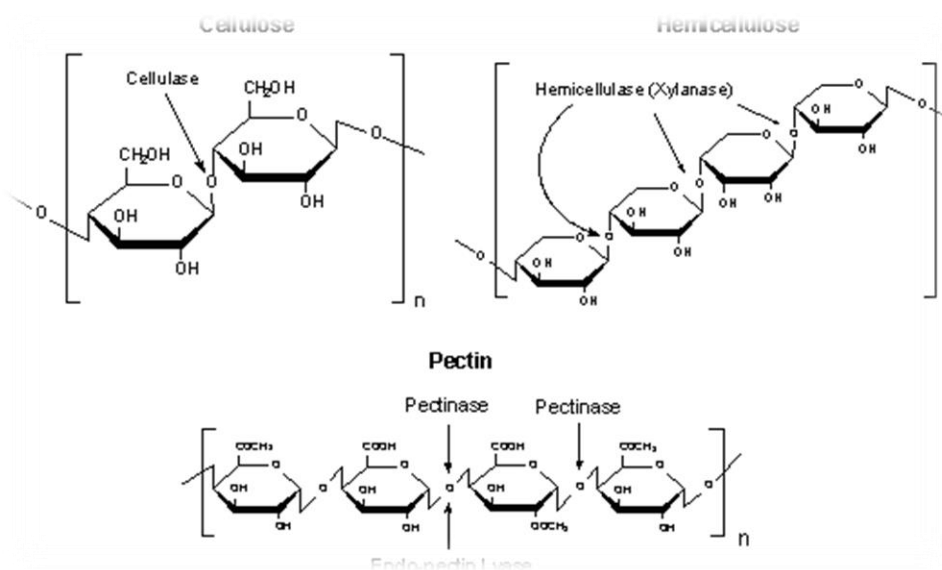


Figure 2.6 example of hemi-cellulose complexity

Co-digestion

Wheat straw is a lignocellulosic fibrous biomass, which is difficult to be degraded as it was aforementioned. Livestock manure also contains biofibers because ruminant animal regime contains fibrous grasses and wheat. Unfortunately these lignocellulosic fibers are not digested totally in the rumen. Moreover, it was found that when the fibrous content of manure is removed then the methane production is increased up to 20% (Angelidaki & Sanders, 2004). The co-digestion of wheat straw with manure has already been investigated from many researchers, such as (Wang et al., 2009). The researchers used as a feedstock 4.6 kg of wheat straw in 1L of swine manure in continuous operating system and the results were promising, indicating methane yield increase up to 10%. In contrary, Sun et al. (2013) did not resulted any improvement in methane yield by the co-digestion of cattle manure and wheat straw. It is clear that, as was mentioned before, regarding the quality of substrate the biogas production varies. In previous years, many assessments have been conducted for the implementation of enzymatic hydrolysis on lignocellulosic feedstocks such as dairy manure in order to enhance the deconstruction of the complex lignin matrix. Furthermore, a noteworthy application of wheat straw in anaerobic digestion is as biofilm carrier in packed bed reactors (Anderson et al., 2003).

2.3 Pre-treatment methods

Effective pre-treatment depends on many parameters. Firstly, the chemical use or the addition of nutrients makes the procedure more efficient but in overall, they are more costly and intricate since in the end of the procedure the surplus should be detracted. Furthermore, attention should be paid in the danger of inhibitory by-products.

Pore size is important wherein enzyme cannot reach cellulose or enzymes get trapped in pores. Moreover as greater as crystallinity and cellulose chain length are, degradation is much more difficult. Therefore one of the main purposes in pre-treatment methods is to decrease the crystallized cellulose in order to degrade a bigger portion of it, by means of degrading crystalline cellulose and amorphous cellulose.

On the other hand, Veecken et al. (2000) proposed that hydrolysis rate is totally related to the absorption of hydrolytic enzymes on the fiber waste. This is in line with Sanders et al. (2002) which were led to the same conclusions. Mata-Alvarez et al. (2000) tried to further enlighten the contribution of surface to the hydrolysis with the implementation of a surface based kinetic mathematic model. However, the total lignin removal or the quantity improvement of sugars is possible by maximising the cost. It is stated that the removal of lignin leads to desirable contribution for the further digestion of cellulose of lignocellulosic materials. Nevertheless, researchers have expressed the correlation of hydrolysis with the surface (Sanders et al., 2002). Additionally, the first order hydrolysis is expressed with the formula of $\frac{dX_{\text{degr}}}{dt} = -k_h \cdot X_{\text{degr}}$ (Eastman & Ferguson, 1981).

Examples

Alkali treatment, has been investigated for fiber degradation. The implementation of NaOH and NH₄OH showed an increase of methane potential. There was not a significant difference in results with further macerated straw. Although maceration by itself gives a great boost of methane yield due to the increase of surface. It has been reported 17% and 20% gradual increase due to the further fiber maceration to 0,35 mm, however there was no significant change in biogas potential for 5-20 mm chopped fibers ([Angelidaki & Ahring, 2000](#)). [Clarkson & Xiao \(2000\)](#) used alkali pre-treatment for the degradation of lignocellulosic biomass gaining important results, instead of the elevating temperature experiment which did not lead to any significant improvements. In general alkaline pre-treatment with NH₄OH, KOH, NaOH, Ca(OH)₂, NH₃, (NH₄)₂SO₃, NaOH contributes in wood fermentability to 55% with the lignin reduction to 20% for hardwood .

Solvent extraction: Organic solvent with water for instance EtOH- H₂O, But- H₂O, Benzene-H₂O.

AFEX (Ammonia fibre explosion method) diluted ammonia with high temperature leads to cellulose decrystallization and lignin removal ([Kim & Speece, 2002](#)).

Supercritical fluid pre-treatment : CO₂ (31°C, 7,4 MPa) leads to 100% fermentation from cellulose to glucose. It is also reported for the treated wheat straw an increase of sugars approximately to 15% ([Zabihi et al., 2010](#)).

Steam explosion(SE) or Steam pretreatment (ST) consist the most effective application for lignocellulosic biomass disruption During this method , heated steam at 240 °C and high pressure, leads to the solubilisation of lignocellulosic biomass ([Hendriks & Zeeman, 2009](#)).

HydroThermal: In the range of 130°C-230°C, warm water increases its pH values, and the diluted straw is degraded and greatens its surface area ([Thomsen et al., 2006](#)).

Biological: It is a mild pre-treatment because of the adjustment of microorganisms directly in the specific habitat. Hence, the temperature and pressure has to be maintained in low levels.

Compost and fungi

In the decade of 80' pure fungi was a promising alternative for energy production. However, in the next years the exploitation of biomass from food chain, such as white rot fungi, was forbidden leading to the need of other alternatives. Thereafter, compost was implemented on anaerobic processes. [Scherer & Neumann \(2013\)](#) used "methano – compost" for the pH recovery from acidic conditions and for the enrichment of biogas production. This team collected the microbial consortia by extracting with boiling water the hot-rot phase compost and injected the compost inoculum in an anaerobic system. The microbial analysis demonstrated the presence of methanogenic microbes and aerobic microflora, and the increase of hydrogenotrophic *Methanobacteriales*. Furthermore, the results indicated more promising tendency for thermophilic digestion instead of mesophilic.

Table 2.1 Combined pre-treatments and their results on lignocellulosic biomass

Pre-treatment	Results
Steam explosion in the following conditions : 198 °C, 10min, 1.5 MPa) and followed by alkaline peroxidase (4.0% H_2O_2) along with (1%NaOH)	Minor Hemicellulose Removal by 5% and lignin removal by 6% (Chen et al., 2008) .
"Steam explosion in the following conditions : 198 °C, 10min, 1.5 MPa followed by alkaline pre-treatment (1% w/v NaOH)	Hemicellulose removal by 6% and lignin removal by 15% (Chen et al., 2008)
"Steam explosion (4.5 min, 1.5MPa) followed by ethanol extraction (40% ethanol,fiber/liquor ratio 1:50 (w/v)at 180 °C for 20 min and 0.1%NaOH)	82% lignin removal (Liu & Chen, 2006)
Na_2CO_3	96% recovery of cellulose and 65% conversion of glycose (Klinke et al., 2002)

Oxidative delignification is also considered effective amongst various pre-treatments for lignin disruption. The main purpose is to oxidize the substrate by mixing with an oxidative gas. Typically some examples are ozon, air, oxygen, and hydrogen peroxide. The definition that is used in the literature is ozonolysis by means of the destruction of aromatic structure under "ozonic" conditions, something very convenient for the lignin disruption. Furthermore, used ozon (O_3) to disrupt and remove the lignin fraction from wheat straw and rye straw, achieving the significant yield of 89% instead of 57% compared with the theoretical enzymatic hydrolysis yield. Wet explosion of wheat straw at 180 °C and 10 bar combined with the use of H_2O_2 as oxidant (6 g H_2O_2 /100 g TS straw) led to decent results (Wang et al., 2009a).

2.3.1 Microaeration

Botheju (2010) first defined the injection of small amount of oxygen in anaerobic environment as "microaeration". However, another author defined the treating method as "TMP" (thermophilic microaerobic pre-treatment) presenting both thermal and microaerated conditions (Fu et al., 2015). For Wei & Wang (2013) microaeration is the controlled introduction of small amounts of oxygen into an anaerobic biochemical process to enable both anaerobic and aerobic biological activities to occur within a single bioreactor.

Regarding to the oxygen tolerance, there are four types of microbes. Firstly, the obligate anaerobes for which oxygen is suffocating, secondly facultative anaerobes who can tolerate and utilize small amounts of oxygen, thirdly the microaerophilic which require small amounts of O_2 and lastly fully aerobes, which can respire normally in fully aerobic conditions. Regarding the archaeal population, in the literature it is stated that *Methanosarcinaceae*, the hydrogenotrophic and carbon dioxide consuming methanogen is more tolerant in micro-oxygenic atmosphere

than the acetoclastic methanogens (Peters et al., 1995). Thus it is expected that H_2/CO_2 will not excess due to their instant consumption by *Methanosarcinaceae* and consequently this could lead to greater methane yield.

Generally, anaerobic systems desire free oxygen environment because oxygen prevents the normal function of acetogens and methanogens leading to decreased biogas production (Hungate, 1969). However due to the oxygen injection in an anaerobic habitat the sulphuric elements can be removed, avoiding the contingent inhibition by toxic sulphides, and reducing the odour and corrosion, two of the main problems in organic waste utilization plants (Zhou et al., 2007). Likewise, Diaz et al. (2010) described the removal of sulphide in sulphuric wastes digested in anaerobic fermenters under microaerophilic conditions. Microaeration obtains many benefits such as reduction of toxic AD products great values of ethanol or lactic acid. Charles & Walker (2009) observed enhanced activity of cellulose and protease hydrolytic enzymes with oxygen injection. On account of the microaerophilic conditions, pure oxygen is more proper to use instead of air for such minor oxygen loads (Diaz et al., 2010).

Thereupon, are illustrated some substrates that got through microaerobic pre-treatment such as sisal pulp showing that the pretreated substrate resulted in 26% higher methane yield (Mshandete et al., 2005). Botheju (2010) treated starch, conducting a rage up to 55% increase of methane yield by contrast to the untreated starch. Furthermore they reported a linear reduction of methane production under increasing oxygen conditions in the range of 0-10.1 % COD. Additionally, microaeration of brown water with food waste conducted 10-21% enhancement with extremely high oxygen load about 37.5% ml O_2 /L/days (Wei & Wang, 2013). Moreover, Fu et al. (2015) showed that under 5 ml O_2 /g $VS_{\text{corn straw}}$ the process is leaded to 16.24% higher methane yield and the result was perceived on the fifth day. After the pre-treatment they followed FT-IR analysis in order to observe the afterward degraded lignocellulosic structure. The results showed that crystalline cellulose was degraded, and lignin was partly disrupted. In itself, oxygen volume is the most dominant factor as the excess of it could lead to inhibition of methanogens. (Ramos, 2013) indicated that oxygen injection in an imbalanced habitat is an appropriate method to overtake the overloads. Due to hydraulic overloading, the micro-oxygenated environment gave rise to the action of hydrogenotrophic methanogens, preventing the inhibition of the system. Wei et al. (2013) performed series of experiments injecting oxygen in anaerobic acidogenic reactor (average pH 4). In that study of food waste utilization, the accumulation of TVFA indicated the destructive effect of enhanced hydrolysis on protein rich substrate. Moreover it was observed that under acidic conditions, *Firmicutes* (72%) and *Bacteroidetes* (24%) where the dominant phyla to obtain the hydrolytic step. Some conclusions that Wei & Wang (2013) stated in the results were, higher COD solubilisation, greater VFA accumulation and enhanced acetogenesis step with the increase of acetate.

As it was aforementioned, Botheju et al. (2010) have developed a new biochemical mathematical model to predict the dynamics for the oxygenated anaerobic environments in the procedure of AD. They converted the old familiar ADM1 into the ADM 1-Ox with in total 25 state variables and 22 biochemical paths. The new model also considered the contribution of solubilisation of organic matter and the inhibited action of obligatory anaerobic acetogens and methanogens. In the experimental part they injected a variance of oxygen loads to the mesophilic glucose solution. In the ADM1-Ox model are added 3 new procedures for the aerobic reaction of monosaccharides, long chain fatty acids and amino acids and hence they followed the Monod (1949) kinetic equations. Therefore, the produced curves from the model did not correspond precisely to the experimental curves, which indicated the lack of all the parameters that oxygen adds in the anaerobic system. The injected oxygen is consumed directly in a very rapid way due to the small amount, which is considered to be the reason of inhibition avoidance. In previous

investigations by the same author, [Botheju \(2010\)](#) the appropriate oxygen load was 1.5 % (by means of fed COD) in a CSTR semi-continuous feed mode. The CH₄/CO₂ was reduced with the oxygen injection and so the rise of CO₂ is indicated.

[Tang et al. \(2004\)](#) injected low oxygen load and observed an increase of microbial diversity, in thermal anaerobic bioreactors. The results showed the linear methane yield reduction in regard to the rising oxygen load as Botheju showed. In another research the oxygen was found to improve the hydrolytic step until a maximum point from where the hydrolysis was the dominant step in overall AD and the organic matter was converted into CO₂ instead of CH₄ ([Johansen & Bakke, 2006](#)). Even if the hydrolytic step could reach an increase of 50%, the ultimate methane potential could be dropped down to -50% due to the increase of CO₂ and decline of methane production.

[\(Bruni et al., 2010\)](#) used as a substrate for aerated biological treatment aerobic compost inoculum from straw and maize. However, the results were disappointing as the methane yield did not show any improvement under fully aerated conditions.

[Durrad \(1996\)](#) used two cellulose degrading fungi under microaerophilic environment and 30 °C for two different strains (*Trichocladium canadense*, *basidiomycete*). The results revealed that the examined strains could decompose cellulose, D-glucose, cellobiose and xylan. [Zitomer et al. \(1998\)](#) subsequently reported that oxygen addition did not inhibit the growth of methanogens, but increased their initial activity.

2.3.2 Compost

Lignin-degrading fungi grow on lignocellulosic materials in nature such as branches and leaf and white rot fungi encounter in nature on hardwood, such as branches of trees. The specific gene of fungi degrades the lignin surface by letting trees scraped. A number of fungi has been studied for implementation on lignocellulosic biomass disruption and many of them did not only secrete lignin enzymes but also hemicellulosic and cellulosic disrupting enzymes. Therefore, microbes that are used for the implementation should fulfil some criteria, for instance being preferably lignin-degraders and not strictly holocellulose degraders, otherwise no enhancement would be observed ([Miller & Trfisch, 1986](#)). The majority of fungi use oxygen in their metabolic path in order to grow ([Durrad, 1996](#)). It is reported that the hot-rotten phase of inoculated compost had considerably bigger effect in thermophilic conditions than in mesophilic ([Scherer & Neumann, 2013](#)). Hereupon, in the first case the gas yield was increased by 26.5% in comparison with mesophilic conditions where the effect was only in the range of 0-6%. In contrast to the non-inoculated waste, it was observed biogas enhancement on such a substrate sewage sludge with energy crops in both temperatures ([Scherer & Neumann, 2013](#)).

In recent research, [Kinet et al. \(2015\)](#) extracted cellulosic fungus from compost and used it as a substrate for BMP assessment. The compost was consisted by green lignocellulosic waste. Whilst, they did not implement microaeration, they encountered the pH drop due to the boost of hydrolytic step, and the overall digestion enhancement.

In another interesting investigation, white rot fungi were cultivated on solid wheat straw. [Miller & Trfisch \(1986\)](#) conducted some series of experiments in order to observe which kind of species favour the lignocellulosic surface

of wheat straw. The main purpose was to develop appropriate pretreated substrate for further biotechnological investigations. The “myco-straw” as they named it, was not implemented in the present work, although the relation between white rot fungi and wheat straw under anaerobic conditions reveals beneficial conclusions. The delignification experiments of myco-straw resulted reduction of maximum 60% of biomass. Moreover the faster lignin degrading white rot fungi were *Hapalopilus rutilans*, *Pleurotus ostreatus* and *Pleurotus sp florida*. Amongst others, the *florida* showed the biggest glucose generation. More specifically, the authors used wheat straw which was consisted of 28.0%, 42.9%, 16.0% and 0.87% of hemicellulose, cellulose, lignin and nitrogen respectively, and figured that after 30 days of fermentation in batch reactors the concentration of VFAs was higher than the untreated straw. They also stated as a fact that the most implemented fungus on wheat straw until present is *Phanerochaete chrysosporium*. Some examples of applied white-rot fungi are *Cyathus stercoletus*, *Ceriporiopsis subvermispora* and *Phanerochaete chrysosporium* (Hatakka, 1983). On the other hand Taniguchi et al. (2010) believed that the most effective white rot fungi for lignin removal is *Pleurotus osteratus*, showing 41% Klason lignin removal, and 48% hemicellulose reduction implemented on untreated rice straw Beyond the rice straw. Also, *Pleurotus osteratus* has been applied to wheat straw for five weeks by (Hatakka, 1983). This myco-straw resulted in reduction near to 35% of wheat straw to sugars, which is higher in comparison with the 12% reduction in untreated wheat straw. Therefore, the proportion of glucose in the produced sugars was 74% instead of 42% of the untreated wheat straw giving an overall higher amount of glucose in pretreated wheat straw. Among others, enzymatic saccharification was conducted with great success. Unfortunately, *Preutotus* is an important fungus in the food chain and cannot be considered as an alternative of extended implementation as a pre-treatment method for AD.

It is proved that with supplement of plant compost in acidified anaerobic sewage sludge environment there was a system recovery, turning conditions back to alkaline (Villavicencio et al., 2013). Additionally, Scherer & Neumann (2013) added hot rotten compost in fodder beet silage (FBS) under thermophilic conditions. Although the methane yield was not provided by the authors, they observed an increase of biogas yield up to 13.75 L, in contrast with the 10,87L of untreated batch reactor. The most industrial cellulase production is listed by fungi, and a considerable amount of bacterial cellulases are generated by metabolic engineering (Sukumaran et al., 2005; Olson et al., 2012).



Figure 2.7 compost after 1 month in aerobic conditions

2.3.3 Bioaugmentation

Bioaugmentation could be defined as the injection of pure culture of microorganisms in a biochemical system in order to enhance a number of processes without inhibiting others. In the biggest part of the literature bioaugmentation has been implemented on wastewaters treatment in order to alleviate the contaminant environment and to provide an efficient removal (Herrero et al., 2015). However, the selection and critical parameters researchers have applied in aerobic wastewater treatment are also applied for the anaerobic process. For instance the selection criteria are still the same, such as the injected culture to be competitive and subsequently compatible with the new environment (Martin-Ryals et al., 2015). Thus, in combination with the appropriate substrate it should colonize together with the integral microbial community, without exceeding or change the community due to inhibitions or competition by producing toxic by-products (Van Veen et al., 1997). Moreover a critical parameter for the selection of a microbe is to stay in the new system, enhancing it, with slow and steady degradation.

Many investigations have been conducted in recent years on bioaugmentation. For instance, Li et al. (2015) injected cultivations of *Clostridium cellulolyticum* in BMP batches with wheat straw AD inoculum and resulted higher methane potential. Additionally, Martin-Ryals et al. (2015) bioaugmented the first digester in a two-phase CSTR system with *Clostridium sp.* gene. The substrates were consisted by manure and maize. Furthermore, (Zhang et al., 2015) implemented fermentative bacteria instead of hydrolytic microbes. In particular *Acetobacteroides hydrogenigenes* in maize straw, resulted an increase of biogas potential up to 23%. *Caldicellusiruptor lactoaceticus* was injected in the first stage of two-stage reactors at 68°C, resulting in approximately 93 % in methane yield. However, the bioaugmentation did not achieve significant difference in the total methane yield at the end of one HRT (Nielsen et al., 2007). In two stage CSTR experiment, routine bioaugmentation achieved significantly greater soluble chemical oxygen demand (sCOD) generation (+25%) and methane production (+15%) compared to one-time bioaugmentation. In a continuous bench-scale system, routine bioaugmentation increased acid-phase sCOD by the range of 29–68% and acetic acid concentrations by 31–34%. Boosted hydrolysis and acetogenesis subsequently led to sustained increase in methane production (+56%) compared to non-bioaugmented habitat (Martin-Ryals et al., 2015). Moreover, the previous years wide investigations has been obtained on the external effect of hydrolytic microbes on the applied anaerobic habitats. Eventually, when a biological treatment is implemented in an AD system it is very important to gain the knowledge of which microorganisms could favour or survive in the selected environmental conditions. In broad terms, under intense conditions such as high pressure or high temperatures, AD habitat produce higher amount of inhibitors (Hansen et al., 1998).

Clostridium Thermocellum

Cellulose is degraded by the enzymes that hydrolytic bacteria secrete, thereafter, metabolites are absorbed into other bacteria or fungi and are further degraded into simpler monomers, such as sugars. Regarding the hydrolytic microbes, *Clostridium thermocellum* together with *Clostridium cellulovorans* consist the most studied cellulolytic bacteria, because of the extracellular enzymes which they produce. *C. thermocellum* favours anaerobic thermophilic conditions, and in nature is responsible for the plant tissue degradation (cotton bale). Significant structural characteristics are the rod shape, the gram positive and that it is endospore. This genus is obligatory anaerobic microbe, and belongs in cluster 3 and 4. Thus, it is very famous for its ability for degrading cellulose, with optimum pH range in 7.8-8.4 and as the name indicates it grows in thermophilic conditions (55-60°C). It is considered one of the most effective hydrolytic bacterium for these conditions, thus it is implemented for degradation on a big variety of cellulosic biomass. Gold & Martin (2007) investigated the activity of *C. thermocellum* utilizing Avicel and cellobiose on a proteomic study. The results indicated that 41 cellulosomal proteins were extracted, from which 29 were common for both Avicel and cellobiose samples. Moreover, more than 36 docking components were discovered. The protein structure is identified comprehensively until now. The activity in which it devastates the crystal cellulose is called Avicelase. Avicelase path, and is constructed by:

- Endo beta gluconases
- Four exogluconases
- One cellobiose phosphorylase
- One cellodextrin phosphorylase
- Two b-glucosidases

Cellulosomes is the multi complex enzyme consortia which is responsible for the cellulose degradation. Cellulosomes were first referred in the literature by Lamed et al. (1983) who observed them latched on *C. thermocellum* surface. Clostridia were put in niche regarding to their presence on the bacteria surface or to the temperature (Kothari et al., 2015). The first group is comprised by *Clostridium cellulovorans*, *Clostridium acetobutylicum* (all mesophilic) which produce enzymes that do not latch on the bacterial surface. In the second group there is only *C. thermocellum*, *Acetovibrio celluloticus* etc, which dominate in thermophilic conditions, are characterized with a diverse complexity due to the subsequent multiple scaffold-in interactions. Moreover they are present on bacterial surface. After many investigations it is not clear until nowadays which is the exact path of these enzyme-surface relation. However, it is known how bacteria adhere to crystal cellulose. Additionally, it is generally accepted that hydrolytic process of polymeric compounds under anaerobic conditions depends from the interaction of surface and hydrolytic bacteria (Sanders et al., 2002). Even a formation of a surface biofilm is needed in order to have an effective hydrolysis. Additionally, in a recent research (Wang et al., 2010) observed the latching of cellulosome on the grass surface forming a “hydrolytic” biofilm. This observation indicates that *C. thermocellum* is appropriate microbe for the degradation of lignocellulosic biomass under thermal, oxygen free conditions (Blumer-Schuette et al., 2013).

In general cellulosomes are consisted by a scaffold-in protein unit and an enzymatic unit. The role of the cohesin modules is to organize the cellulytic subunits into the enzyme consortia. As a result there is generated an interaction between cohesin-dockerin (Kothari et al., 2015). To prolong, for every type of dockerin there is a respective complementary type of cohesin. For instance, CipA is a scaffoldin protein which is present in *C. thermocellum*, and is consisted by nine modules of type 1 cohesins. The basic function that attains is the destruction of crystalline cellulose with an enzymatic complex named cellulosome which is consisted by "a central

noncatalytic scaffolin protein (CipA)" which bears nine catalytic subunits (Blumer-Schuetz et al., 2013). Each subunit carries a type one dockerin (Doc1) and attaches with one of the nine cohesin domains of CipA (Kruus et al., 1995). Subsequently, CipA is attached to the bacterium surface with a bond/interaction to type 2 dockerin with type 2 cohesin on one of the three S proteins such as SdbA, Orf2p, OlpB (Demain et al., 2005).

Table 2.2 Already Known cellulosic *Clostridia* (Kothari 2015)

<i>Clostridium thermocellum</i>	<i>Clostridium methylpentosum</i>
<i>Clostridium aldrichii</i>	<i>Clostridium papyrosolvens</i>
<i>Clostridium alkallicellulosi</i>	<i>Clostridium sporosphaeroides</i>
<i>Clostridium caenicola</i>	<i>Clostridium stercorarium</i>
<i>Clostridium cellobioparum</i>	<i>Clostridium straminisolvans</i>
<i>Clostridium cellulosi</i>	<i>Clostridium sufflavum</i>
<i>Clostridium clariflavum</i>	<i>Clostridium termitidis</i>
<i>Clostridium hungatei</i>	<i>Clostridium thermosuccinogenes</i>
<i>Clostridium leptum</i>	<i>Clostridium josui</i>

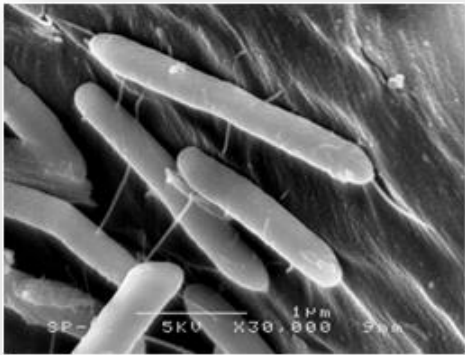


Figure 2.8 *C. thermocellum* rod shape

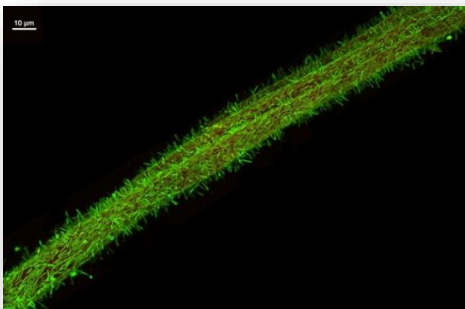


Figure 2.9 *C. thermocellum* surface

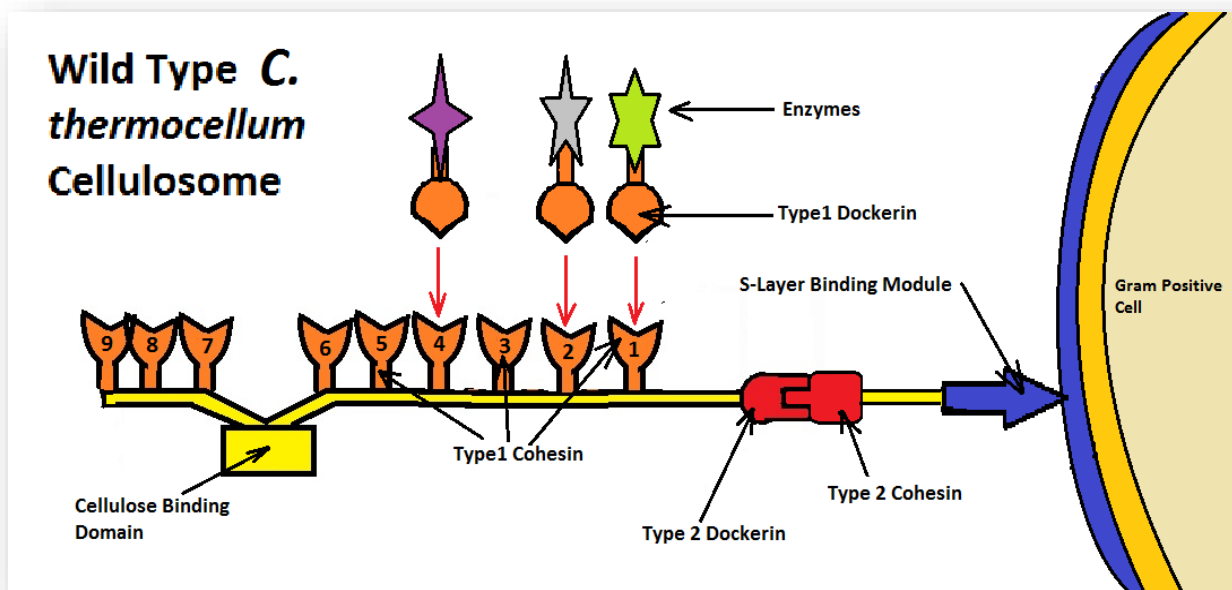


Figure 2.10 cellulosomes

Melioribacter Roseus

Melioribacter roseus is a facultative anaerobe and with the contribution of electron acceptors (i.e. Nutrients as acetate) can grow using two paths, fermentation, or anaerobic respiration. *M. roseus* favors aerobic conditions and it doubles only in 30 minutes, instead of 2.5 hours that takes under anaerobic conditions ([Podosokorskaya et al., 2013](#)).

This *Chlorobi* shows common characteristics with phylum *Bacteroidetes* and is reported extracting hydrolytic enzymes. *Chlorobi* are defined also as "green sulfur bacteria" ([Kadnikov et al., 2016](#)). *Bacteroidetes*, are similarly to *Chlorobi* (chemoorganotrophs) and one of their multi metabolic path is to hydrolyze long chain components. In general they are pathogenic for human health and in case of infection they symptom diarrhea or sinusitis, or periodontitis. Overall, it is stated that in comparison with *Firmicutes* and *Bacteroidetes* the other hydrolytic bacteria tend to be extinct in biogas plants. Although, when they are isolated and cultivated in similar conditions (temperature and pH) are developing considerably.

Species of *M. roseus* are curved rod-shaped, with an average length 85 µm and 0.30 µm width. They are reported to form a biofilm on the pick of their growth rate. This biofilm is black with colorless-yellowish media under free oxygen conditions and pink in aerated environment due to the generation of carotenoids, as it also formatted faster in aerated than in anaerobic environment. Additionally, they are acetate tolerant and degrade pentose, hexoses and polyoses likewise to *Acetobacteroides hydrogenigenes*.

[Podosokorskaya et al. \(2013\)](#) isolated the strain p3M-2T from a wooden surface of a chute from an oil research in a well in 2775 meters under the earth surface, in Tomsk region, Russia. It was investigated together with

Ignavibacterium album which belongs to the *Ignavibacteria* class. The condition in which they thrive is 46°C and 7.52 pH in the well and also, it flourishes in deep sea sediments. Nevertheless, they conducted in situ experiments in the lab in order to indicate the optimum conditions for the growth of this strain. In general the optimal range of temperature is 52-55 °C and optimal pH about 7.5. Additionally the optimal NaCl concentration was 0.6 g/L. More over the growth was inhibited in temperatures out of the range 30-65°C and/or for pH values 5.6 - 9.2, and/or above 65 g/L NaCl. It is also stated the vitamin solution was vital for the normal cell growth.

M. roseus can efficiently ferment cellulosic matter, protein and polymeric carbohydrates. The metabolites after the glucose fermentation under O₂ free environment are mainly hydrogen, CO₂ and acetate. In the metabolic system *M. roseus* also contains sugar transporters which indicate the ability for the formation of poly- and mono-saccharides. In case of not available electron acceptors, it ferments sugars in order to degrade pyruvate to acetate (Kadnikov et al., 2016). Appropriate feedstock are mostly carbohydrates, therefore *M. roseus* has been characterized as heterotroph "carbohydrate utilizer" (Podosokorskaya et al., 2013). It is known that cellulose is mostly degraded by some groups of enzymes such as endoglucanases, cellobiohydrolases and b-glucosidases in order to degrade crystalline cellulose to glucose (Lynd et al., 2002). *M. roseus* excrete a diverse amount of glycoside hydrolases, well known enzymes which degrade polysaccharides in order to grow. Beyond microcrystalline cellulose there are many substrates that *M. roseus* could degrade in order to grow, and these are carboxymethyl cellulose, starch, dextran, xylan, and their oligomers such as glucose, mannose cellobiose, ribose, xylose etc. Regarding with the substrate it is clear that *M. roseus* has a similar metabolic path with phyla *Bacteroidetes*.

In the following Table 4.8 are presented the favourable and undesirable organic compounds for *M.roseus*, as Podosokorskaya et al. (2013) proposed them.

Table 2.3 (Podoskaya Et al 2013) proposed the following substances for utilisation by *M. roseus*

Utilized substrate/nutrients	Unexploited substrate/nutrients
Yeast extract	Lignin
Lactose	Pectin
Cellobiose	Polyvinyl alcohol
Glucose	Lactate
Mannose	Propionate
Xylose	Ethanol
Arabinose	Butanol
Fructose	Sorbitol
Formate	Mannitol
Xylan	Propanol
Starch	
Maltose	
Microcrystalline cellulose(avicel)	
cotton wool(Crystallinity)	
Acetate	

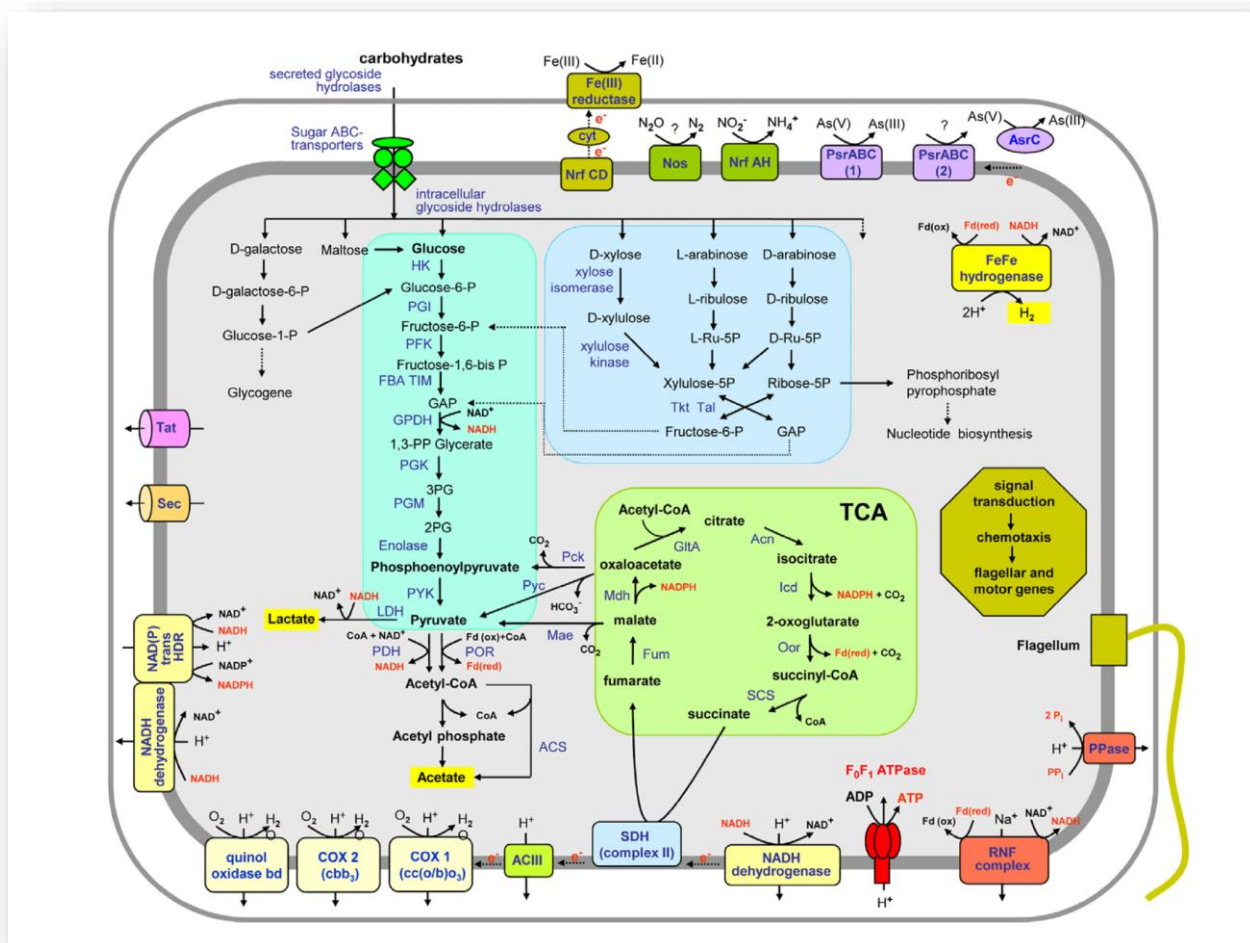


Figure 2.11 metabolic path of *M. roseus* (Kardicov et al., 2013)

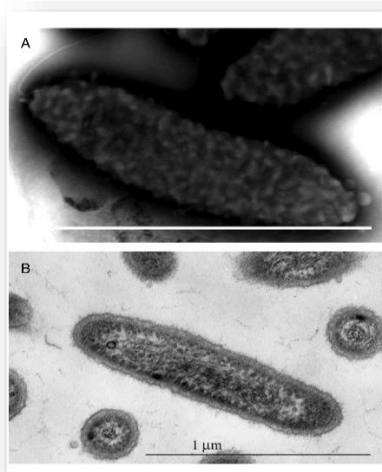


Figure 2.12 *M. roseus* (Kardicov et al., 2013)

2.3.4 Microbial growth and metabolism

Under optimal conditions, the calculation of number of bacterial cell generated in a specific amount of time could be conducted as follows

$$N = N_0 e^{\mu t}$$

e = euler number

N₀ = the initial number of cells in the growing cultivation

t = time

μ = growth rate

Practically the growth rate of cultivation could be observed with optical density (OD) measurement with a spectrometer. The measurement is based on the difference of the glowed light from the monochromator on the sample and the light that reaches the photoelectric wall. This difference is defined with the absorbed light on the cells. The OD plot cannot be predicted by a simple semilogarithmic equation due to the unstable initial growth after their storage in refrigerator. This is because microorganisms should restart their metabolism for the ATP, DNA, and RNA synthesis.

CHAPTER 3 : MATERIALS AND METHODS

3.1 Substrates

It is very important to determine the characteristics of the inoculum and substrate before starting an anaerobic process of organic matter such as livestock manure or energy crops. For instance C/N i.e. Carbon to nitrogen ratio, VS, COD, microbial kinetics and living conditions [Shanmugam et al. \(2009\)](#).

AD Inoculum

The initial AD inoculum with which both experiments were started was supplied from Snertinge, Denmark. When inoculum is very fresh, it is rich in undigested nutrients and has high organic load. Because of that, it was flushed and placed in incubator at 55 °C to digest the surplus of organic matter and degassed for 10 days. After its incubation, inoculum was completely homogeneous, the undigested leftovers (biofibers) were discarded and had the appropriate productivity.

Compost inoculum

It was provided by a compost plant from Zealand (Sjaelland), Denmark. It consisted by field organic waste such as grass, fallen leaves, flowers, branches, and its temperature ranged between 50-70°C. A succinct experiment was conducted in order to choose the most appropriate step from composting. Compost was collected from 3 steps, 1 week, 5-6 weeks and the last step. For the brief determination, 3 duplicates were prepared containing 0.8 ml acetate, 20 ml BA medium and 1 g compost. The best potential resulted by the second step from which the methano-compost was extracted.

Manure

The raw manure that was used in experiments was provided by a biogas farm plant in Snertinge, Denmark. In order to maintain cattle manure undigested until the feed in the reactor, it was stored in 5L bottles at -20 °C and when it was about to be used it was held in 4 °C for maximum one week.

Cattle manure, is common to be sieved in order to remove the remnant undigested biofibers in lab-scale experiments. After the sieving the TS, VS and subsequently, the OLR were reduced. Hereafter the lower TS, VS, OLR gave better estimation for the contribution of wheat straw in the final methane generation and a clearer picture of its biodegradability.

Wheat straw

Wheat straw was harvested by a field in Jutland. TS and VS were measured in the start of experiment. Wheat straw characteristics:

TS = 92.84% g/g_{wet sample} or equal to 928.4 gTS/kg

VS = 86.72% g/g_{wet sample} or equal to 867.2 gVS/kg

Substrate determination before treatment

Table 2.4 Substrates characterization before pre-treatment, or digestion.

Parameter	Wheat straw	Cattle manure	AD inoculum	Compost inoculum	Units
Moisture	7.16%	96.83%	97.25%	52.23%	%g/g _{wet sample}
TS	92.84%	3.17%	2.75%	47.77%	%g/g _{wet sample}
VS	86.72%	2.20%	1.71%	14.95%	%g/g _{wet sample}
VS/TS	93.41%	69.56%	62.79%	32.25%	%g/g _{wet sample}
Ash/TS	6.59%	30.44%	37.71%	67.75%	%g/g _{wet sample}
TKN	4.82	2.57	3.63	0.39	g/kg
ammonia	0.76	1.72	3.51	0.07	g/L
pH	-	7.69	8.91	8.2	-

It should be noted that overall methane potential varies amongst the variety of livestock. Hence, it is not trustworthy to compare directly the potential between two feedstocks, for instance chemical composition of cattle manure, which is provided from different forages, or wheat straw from different harvests could vary. Some agricultural factors for cows that must be considered are regime, stage of life, and type and bedding. Nevertheless for energy crops could be the season of harvesting and the different soil (different proportion of nutrients). For instance, winter wheat straw methane yield m^3 per kg volatile solids was 0,189, and in addition, dairy manure methane yield was stated from another work to 0.148 m^3 per kg VS and for beef cattle was 0.328 m^3 per kg VS. Although, when huge quantities of manure are stored in ambient conditions the amount of pre-digestion should be considered in final calculations.

3.2 Analytical Methods

Volatile Solids (VS) and Total Solids (TS)

VS and TS were determined following the Standard Methods for examination of Water and Waste Water ([APHA Methods, 1999](#)). The presented steps were followed successively:

- I. Dry the empty crucibles in the oven for 1h at 550 °C and afterwards cool down and weigh
- II. Put a specific amount of sample and weigh in the crucibles was putted initially 1g of wheat straw and 20 ml of inoculum or manure.)
- III. Dry the sample in the oven for 24 h at 105 °C in order to suppress humidity, after that cool down and weigh. Determine VS.
- IV. Put the sample in the preheated oven at 550 °C for 1 h, in order to scorch the organic matter. Following on, let cool down and weigh the ash. Determine TS. By definition ash is the inorganic fraction of sample which was left as a remnant after the organic ignition.

TS and VS were determined by the following formulas

$$VS = \frac{(\text{crucible} + \text{dried sample}) - \text{crucible}}{(\text{crucible} + \text{wet sample}) - \text{crucible}} \frac{g}{L}$$

$$TS = \frac{(\text{crucible} + \text{dried sample}) - (\text{crucible} + \text{ash})}{(\text{crucible} + \text{wet sample}) - \text{crucible}} \frac{g}{L}$$

Chopping / Maceration

The size of wheat straw was decreased in order to skip the frequent clogging in the effluent pipes and to increase the accessible surface area for enzymes adequately. Thus wheat straw was reduced to 0.5 cm. The equipment that was used was the "Heavy-Duty Cutting Mill SM 2000" by Retsch Company. Further mechanical pre-treatment can be avoided in order to skip higher operational costs. Substrate size is a significant parameter for the kinetics of the process. When the open surface is greater, consequently the amount of hydrolytic enzymes absorbed on it is greater, as a result there is overall faster solid degradation (Vasily et al., 2005).



Figure 3.1 and 3.2 Heavy-Duty Cutting Mill SM 2000

Elemental Analysis

The analysis was conducted with the model *vario MACRO cube* (Elemental Analyzer, Hanau-Germany). This analytic method exemplifies for accurate C%, N%, H%, O% and S%.

For the elemental determination the vario MACRO cube elemental analyser Hanau –Germany was used, which produced the following results regarding the specific wheat straw.

Table 3.1 elemental analysis

Wheat straw	values	Standard deviation
C/N	103	4.78%
C%	42.20%	0.02%
H%	6.23%	0.08%
O%	52.43%	Formula
VS	86.72%	0.38%
Ash	0.13	0.38%



Figure 3.2 vario MACRO cube

Total Kjeldahl Nitrogen (TKN)

The procedure which was followed for the TKN determination is based on Standard Methods (APHA, 1998). For statistical accuracy 5 samples of each concentration were prepared. Thus 5 samples were consisted by 1 g wheat straw (0.5 cm), 5 more with 2.5 ml of cattle manure, another five with 2.5 ml AD inoculum, and lastly five samples with 2.5 ml of compost inoculum. The preparation included, as always, the respective 5 control and blank solutions.

Due to the documentation the preparation steps were conducted as followed. For wheat straw, every tube contained 1 g wheat straw 2 Kjeldahl tablets, 4-5 glass beads, 16 ml hydrosulfuric acid (97%) and 50 ml of distilled water. Likewise, for manure and inoculum tubes, every tube contained 2.5 ml Inoculum, or manure, 4-5 glass beads, 1 Kjeldahl tablet, 10 ml hydrosulphuric acid (97%), and 50 ml distilled water. After the mixing, all tubes were placed in a special heating system for 1 h at 185 °C until humidity vaporization, afterwards, the digestion took place at 350 °C for 1.5 hours. Successively escalating temperature was chosen by means of overtaking the intense boiling. Distillation was followed, using the steam distillation unit in order to isolate for titration the new nitrated solution. The nitrate-receiving flasks contained 4 ml of boric acid (4% w/v) (enough to collect all the amount of ammonia), 50 ml distilled water and 3-4 drops of indicator (deep pink colour). Moreover, the group of blanks contained 8 ml NaOH, 50 ml H₂O and 3-4 drops of indicator (pink colour).

Formula for TKN calculation.

$$N \left(\frac{g}{L} \right) = \frac{[(V_1 - V_2) * 0.1 \text{ mol} \frac{HCl}{L} * 14.01 \left(\frac{g}{L} \right)]}{\text{sample volume}}$$

V_1 = HCl titr. Volume for samples,

V_2 = HCl titr. volume for blanks



Figure 3.3 and 3.4 Steam distillation unit (on the left) and special heating system (on the right)



Figure 3.5 and 3.6 coloured solutions before and after titration

Chemical Oxygen Demand (COD)

COD is determined in g O₂/litter and it describes the quantity of O₂ that is needed in order to oxidize both inorganic and organic fractions of an organic waste. This is supposed to be fulfilled with the addition of Cr₂O₇(2-) and a catalyst (silver sulphate) under heating conditions (148 °C, 110 minutes). The main purpose is the reduction of Cr(6+) to Cr(3+) and determination of Cr(6) surplus with FAS titration. For the titration we used a 25ml *Brand digital II titration column* for measurement accuracy. It is noteworthy that, intense dilution was firstly followed in order to approach the appropriate COD range. Afterwards the samples were removed from the heating machine. After the heating, their colour was switched to blueish green from light orange colour, and after the FAS titration they got red, due to the addition of indicator.

COD protocol : successive steps

- i. Centrifuge
- ii. Dilution 95% (1 ml diluted in 19 ml distilled water)
- iii. Collect 3.5 ml in 50 ml tubes.
- iv. Add 2 ml potassium dichromate , and 4.5 ml of dense sulphuric acid.
- v. Put the samples in special heating system to digest at 148 °C for 110 min.
- vi. Let cool down, add 5 ml of water, 3 drops FAS indicator, and titrate with FAS.

The calculations were deducted with the following formulas

$$\text{Conc. (FAS in M)} = \frac{1(\text{ml of K}_2\text{Cr}_2\text{O}_7) * 6 * \text{Conc. (K}_2\text{Cr}_2\text{O}_7 \text{ in M})}{\text{Volume (FAS in ml)}}$$

$$\text{COD} \left(\frac{\text{mg}}{\text{L}} \right) = \frac{\text{ml FAS (average blank)} - \text{ml FAS (for each sample)} * \text{M(FAS)} * 8000}{\text{Volume (sample in ml)}}$$



Figure 3.7 and 3.8 and 3.9 COD preparation

3.2.1 Solid phase measurements

Structural carbohydrates and Klason Lignin: the steps that were followed for the determination of sugars and lignin content of wheat straw are described below successively.

- i. 500 g material was dried for 24 h at 105 °C
- ii. 150 g of wheat straw in every tube, made in triplicates.
- iii. Added 1.5 ml of hydrosulfuric acid (72%) in each tube
- iv. Put the samples in the incubator at 35 °C for one hour and stir with Vortex every 10 minutes(in total 6 times)
- v. After the complete homogeneity, to ease the acidification we added 42 ml of distilled water, as a result the H₂SO₄ concentration from 72% dropped to 4%.
- vi. After that all the samples were sealed and putted in autoclave at 121 °C for 60 minutes.
- vii. Afterwards samples were cooled down in ambient conditions
- viii. For the lignin determination we injected 10 ml of each sample on vacuum filter to absorb the solid content, which was the lignin remnant. In order to take accurate measurements, every filter paper was firstly dried in oven at 105 °C for 60 minutes and weighed. After the filtration the filter paper held / absorbed the solid content which was mostly lignin and inorganic matter. To calculate the lignin and inorganic content each wet paper was dried at 105 °C for 24 h and weighed. Moreover, each sample was scorched at 550 °C for 1 hour, and weighed. The remnant that was left, was only inorganic, thus the lignin fraction could easily be calculated for each sample.
- ix. Separately, small quantity samples were centrifuged, diluted (1:50), filtered and stored in glass vials (1.5 ml content) in order to determine the sugar quality and content with IC machine.
- x. Sugars where determined with DIONEX ICS-5000 Dual Pump, Thermo SCIENTIFIC.

Each measurement was conducted in triplicates for statistical significance.

- Measured 5-carbon sugars : Arabinose, Xylose
- Measured 6-carbon sugars : Mannitol, Galactose, Glucose

Formulas of total cellulose and hemicellulose calculation

- Cellulose %TS = [C₆-sugars_{Released}] * (162/180) * 100% = 42,02%/gTS
- Hemicellulose %TS = [C₅-sugars_{Released}] * (132/150) * 100% = 30.75%/gTS

Table 3.2 wheat straw contents

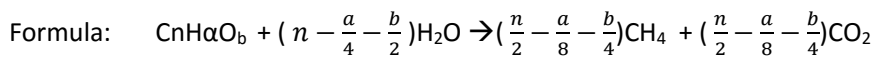
Wheat straw	Values%	Standard deviation
Klason Lignin	26.73%/gTS	2.35%
Cellulose	42.02%/gTS	0.72%
Hemicellulose	30.75%/gTS	0.52%



Figure 3.10 DIONEX ICS-5000 Dual Pump

Theoretical Methane Yield

Buswell's formula is used for the calculation of theoretical methane potential when organic mass is digested under anaerobic conditions without the basic oxidant factor, O_2 (Symons & Bushwell, 1933).



$$\text{Methane Potential: } B = \frac{\left(\frac{n}{2} - \frac{a}{8} - \frac{b}{4}\right) * V}{12n + a + 16b} STP \frac{l CH_4}{g VS}$$

$V = 22.4$ equals to the volume of 1 mole of gas at STP

For many reasons the produced methane is not consistent, and differs from the calculated methane potential. Firstly in Buswell's formula the organic matter that is utilized from microorganisms in order to make its body mass is not considered. Furthermore, lignocellulosic materials contain lignin, which cannot be consumed by microorganisms, but only deconstructed.

3.2.2 Liquid phase measurements

pH and ORP

Measurements were conducted with a pH-meter PHM 92 LAB which was calibrated before use and made sure the electrode was correctly filled with chemicals due to the accurate measurements. Sampling was obtained from the liquid phase of batch assays and from the bottom of CSTRs; and after each sampling in order to avoid the rapid oxygenation of alkaline by atmosphere the pH measurement was obtained quickly. ORP was measured with the METTLER TOLEDO (FiveEasy Plus).



Figure 3.11 pH meter

VFA

Similarly to pH, VFA sampling was obtained by the bottom of digesters, where an accumulation of undigested fibers was observed instead from the top. For the preparation of VFA samples, a considerable amount of substrate was collected from the bottom of the reactor and diluted by the half with water, adding 3 ml of distilled water. Additionally, 0.4 ml of Orthophosphoric acid (34%) was injected in the sample which was sealed and shaken up. Thereafter, the acidic form of VFAs was transferred divided in duplicates in plastic conical vials and was centrifuged at 13.4 rpm for 12 minutes. After the centrifuge the separated liquid fraction was collected and been stored in glass vials with 0.1 ml of Internal VFA standard. The vials were hermetically sealed and were stored in the fridge at -4 until their determination. Due to the intensity of stirring is assumed that samples were homogeneous and representative for the liquid fraction composition.

The apparatus that was used for the VFA concentration determination was the *Shimadzu gas chromatograph GC-2010* and the detection of VFAs was conducted by *Flame Ionization Detector*.



Figure 3.12 GC apparatus for VFA determination and samples

TEMPERATURE

Temperature was checked continuously, to make sure that was lying in the range of 54.5 - 55.5 °C. BMP batches were kept in incubators at 55 °C, while CSTR maintained their temperature at 55°C because of surrounding water jackets.

3.2.3 Gas phase measurements

Biogas-CSTR

Gas phase measurements are an easy way to observe directly the overall process. Biogas production was determined continuously with the water displacement method. Every 100 ml of biogas that passes from this system are summed and displayed on the gas meter. Every reactor has its own gas system. Daily just before the feeding, biogas production was recorded and afterwards both water displacement and gas meter were set to zero until the next day. Unfortunately the aforementioned system could not measure the biogas contents. Therefore, the most implemented machinery for biogas content determination was used, which is Gas chromatograph.

For CSTR biogas monitoring we used the Gas chromatograph Shimadzu. Before measurement, the chromatograph was initially calibrated with standards in order to take accurate measurements. The volume of each injection was 0.5 ml . After the injection carrier gas (H_2) carries down the biogas to the packed column and determine its concentration by a Thermo Conductivity Detector (TID) at 50 °C. The elements that were determined quantitatively were, CH_4 , CO_2 , and N_2

For BMPs, biogas composition was measured by GC Shimadzu GC-2010. Likewise the measuring method was quite similar by changing the calibrating method in the injection amount to 0.2 ml/injection



Figure 3.13 GC apparatus for biogas determination



Figure 3.14 water displacement

3.3 BMP

BMP theory and protocol-set up

BMP experiments take place in Batch reactors, which are sealed hermetically with rubber stoppers, thus the whole system is closed until the end of the experiment, when all the substrate is been utilized. Although, if there is need of either injecting or pulling liquid or gas, a 10 ml or 1 ml-syringe is used respectively. Furthermore, it is very convenient to control the internal pressure with degassing the produced biogas until it is alleviated to ambient pressure. In this way it is ensured that no explosions will take place during the experiment because of over-pressured conditions, and neither toxic compound would be produced. In order to avoid the often degassing great headspace volume has to be considered before the assembly of experiment.

Methane Potential calculations

The methane potential is usually measured in terms of methane per substrate such as $\text{ml CH}_4/\text{gVS}_{\text{substrate}}$ or $\text{ml CH}_4/\text{gCOD}_{\text{substrate}}$. Although for the examined substrates it is preferred to measure methane potential in $\text{L CH}_4/\text{gVS}_{\text{substrate}}$, which gives a better estimation for lignocellulosic fibers. On the other hand litter methane per g COD is more appropriate for waste waters than in slurry estimation. The determination of the methane yield could be conducted regarding the pressure in the bottle, or more accurately with Gas chromatography Chromatograph analysis with flame ionization. Considering that, the most appropriate way for methane determination is pulling a specific quantity of biogas from the headspace volume and injects it in Gas chromatograph.

With a thin needle adapted on 1 ml-syringe, a small quantity of biogas is collected from the headspace. The amount of biogas is in the ranges of 0.1 ml - 1 ml, depending on the Gas chromatograph and its calibration. Calibration of the chromatography is obtained with the injection of known composition (standard) gases containing methane in a specific concentration. Each sample peak from the batches is compared with the range of standard peaks, thus the precise methane concentration is determined.

In the present work BMP procedure was prepared according to the proposed protocol for solid organic wastes and crops ([Angelidaki et al., 2009](#)). As was detailed above, because of the diversity of feedstocks and the variety of experimental methods the methane productivity of a substrate should be measured in common metric units. Firstly, [Owen et al. \(1979\)](#) proposed the BMP assay, which indicates the biochemical methane potential, which is equal to the maximum of methane yield that a feedstock could reach under strict anaerobic conditions. Methane potential is measured in Litres and expressed with STP metric system in L/gVS .

Methane potential charts

For each sample the headspace methane was calculated with the presenting formula:

$$\text{Headspace (mlCH}_4\text{)} = \text{headspace volume(ml)} \times \text{methane concentration} \times \left(\frac{T_o}{T_{55}}\right)$$

$$\text{Headspace volume} = \text{Total volume} - \text{Working Volume}$$

Degassing was inevitable, because of the rapid biogas production for the 2 first weeks. Therefore, every time a specific amount of volume was degassed, it was added to the new measurement in order to observe the aggregated result.

For instance, 10th day of measurement includes degas from the 7th day, and the 14th day includes 7th and 10th day degased volume etc. Apparently in 20th day the production stabilizes in low levels and hereafter there is no need for degassing.

$$\text{Degas} = \text{predegas Headspace} - \text{after degas headspace volume}$$

3.3.1 BMP Microaeration

BMPs Microaeration- set up

In the present experiment the biochemical methane potential method was used for the preparation of 182 batch assays including by common a combination of AD inoculum, compost inoculum, water, and wheat straw. The total volume of batches was 547 ml and they were hermetically sealed with rubber stoppers and aluminium crimp. The effective (working) volume was 200 ml, hence, there was enough gas space for biogas in order to overtake overpressure difficulties which could lead to an explosion, or contribute to the production of toxic substances.

Each concentration was prepared in groups of triplicates for the purpose of ensuring the reproducibility of the statistical analysis ([Angelidaki et al., 2009](#)). Moreover, each of these triplicates was accompanied by blank triplicates containing 80 ml of AD inoculum, 80ml of compost inoculum and 40 ml of water and the equivalent oxygen load. According to the BMP protocol, the relative inoculum volume depends on the density of itself, otherwise is very common to do further dilution with water. In the following work both inoculums were very washy and diluted, however a further dilution was conducted.

Blanks

In the BMP procedure the final methane potential is measured in terms of CH₄ volume per weight of substrate and defines potential that a substrate possesses for biogas production. Further on, each blank contains the same compounds and oxygen load with its counterpart pretreated triplicate. In each pair the sample additionally includes the desired substrate.

Blank batch reagents:

- 80 ml inoculated white hot-rot fungi compost inoculum
- 80 ml inoculum from digested cattle manure inoculum
- 40 ml distilled water

All the blanks were microaerated with equivalent Oxygen load with their paired triplicates except the control counterpart, blank triplicate, which were not aerated.

Controls

Control batches were filled with 0.8660 g of microcrystalline cellulose i.e. 4.33 gVS/L. The most appropriate biomass substrate is the one that degrades rapidly and reaches the maximum activity of inoculum. Hence, cellulose is an appropriate biodegradable substrate to compare it with lignocellulosic substrates such as wheat straw.

Control Batch reagents:

- 80 ml inoculated white hot-rot fungi compost
- 80 ml AD inoculum
- 40 ml distilled water
- 0.08660 g microcrystallinity cellulose

Untreated wheat straw

To further enlighten the effect that inoculum has on the substrate, one triplicate of batches containing untreated wheat straw was prepared. Due to this, the results would show the combined effect on untreated wheat straw. Consequently, differences from results, microaeration and untreated batches would be evaluated.

Untreated Wheat straw batches:

- 80 ml inoculated white hot-rot fungi compost
- 80 ml AD inoculum
- 40 ml distilled water
- 1 g wheat straw

Wheat straw

The amount of added wheat straw in each batch was 1 g. Each triplicate set was aerated in very small amounts of oxygen in the purpose of investigating the methane potential under microaerated conditions.

All wheat straw batches initially had the same characteristics:

- 80 ml inoculated white hot-rot fungi compost
- 80 ml AD inoculum
- Oxygen load O₂ml/ gVS
- 40 ml distilled water
- 1 g wheat straw

Microaeration Plan

Main purposes were to observe how the microaeration affects a lignin-rich substrate such as wheat straw. Firstly in order to observe pulse effect, oxygen was provided in one, or two, or 3 injections per day, with the biggest amount of O₂ in the night.

The second parameter was Oxygen parameter, by which there was an increase of oxygen load to 5ml O₂/gVS per day, 10 ml O₂/gVS per day and 15 ml O₂/gVS per day. The last parameter was the increasing period of treatment (1, 2 and 3 days)

1) Pulse effect

2) Increasing O₂ load in 5ml O₂/gVS per day per day, 10 ml O₂/gVS per day per day and 15 ml O₂/gVS per day per day

3) Increasing days in 1, 2 or 3 days of microaeration

Table 3.3 Plan for microaeration

Sample	ml O ₂ /gVS per day per day	Injections per day	Days
Blank	0	0	3
Control	0	0	3
Untreated straw	0	0	3
Wheat straw 5-1-1	5	1	1
Wheat straw 5-1-2	5	1	2
Wheat straw 5-1-3	5	1	3
Wheat straw 5-2-1	5	2	1
Wheat straw 5-2-2	5	2	2
Wheat straw 5-2-3	5	2	3
Wheat straw 5-3-1	5	3	1
Wheat straw 5-3-2	5	3	2
Wheat straw 5-3-3	5	3	3
Wheat straw 10-1-1	10	1	1
Wheat straw 10-1-2	10	1	2
Wheat straw 10-1-3	10	1	3
Wheat straw 10-2-1	10	2	1
Wheat straw 10-2-2	10	2	2
Wheat straw 10-2-3	10	2	3

Wheat straw 10-3-1	10	3	1
Wheat straw 10-3-2	10	3	2
Wheat straw 10-3-3	10	3	3
Wheat straw 15-1-1	15	1	1
Wheat straw 15-1-2	15	1	2
Wheat straw 15-1-3	15	1	3
Wheat straw 15-2-1	15	2	1
Wheat straw 15-2-2	15	2	2
Wheat straw 15-2-3	15	2	3
Wheat straw 15-3-1	15	3	1
Wheat straw 15-3-2	15	3	2
Wheat straw 15-3-3	15	3	3



Figure 3.15 setting up the microaeration experiment

-Investigation for Pulse effect

1 injection in the day in the morning

Oxygen injection, ml	Oxygen injection , ml/gVSstraw
4	5
8	10
12	15

For 2 injections in a day

Oxygen injection, ml	Oxygen injection , ml/gVSstraw	Morning, ml/gVSstraw	Afternoon, ml/gVSstraw
4	5	2	2
8	10	4	4
12	15	6	6

For 3 injections in each day

Oxygen injection, ml	Oxygen injection , ml/gVS	Morning, ml/gVSstraw	Afternoon, ml/gVSstraw	Night ,ml/gVSstraw
4	5	1	1	2
8	10	2.5	2.5	3
12	15	3.5	3.5	5

Compost inoculum

Inoculum containing also white hot rot fungi was extracted from thermal compost from leafs and branches and the extraction was conducted with boiled water, instead of chemicals or solvents, making even “greener” the process.

In general the development of enzymes is a very expensive procedure therefore, in our case the appropriate fungal microbiome was extracted, without solvent, or any isolation. The type of compost which was implemented was organic waste such as leafs and branches which were collected from the public sector of Denmark near Copenhagen (Zealand). The collected compost was divided in 3 steps, 1 week old, 3 weeks old and 6 weeks old. The most appropriate for use was the 2nd-phase compost.

In the matter of extracting the desired facultative aerobic microflora from the compost, it was mixed in a shaker with boiling water, and afterwards it was filtered. Moreover, the mixture was homogeneous (VS content was similar to AD inoculum). Thus, 80 ml compost inoculum added with 80 ml cattle inoculum and 40 ml water leading to a convenient $VS_{sample} = V_{total} * OL = 200 \text{ ml} * 4.33 = 0.87 \text{ g}$. The compost was left in ambient laboratory temperature in fully aerobic conditions to grow up in order to clarify macroscopically the type of compost microflora. Apparently, from the figure 3.18 it is easily observed that white - hot rot fungi were thrive on the surface of hot compost.



Figure 3.16 white hot rot fungi on compost after 1 month on ambient conditions

3.3.2 BMP Bioaugmentation

The following Table 3.5 depicts the preparation of each triplicate. In total 10 different triplicates were prepared. Third and fourth group of triplicates were conducted in order to see the colonization of microorganisms in the same temperature consisted only by AD inoculum.

In fifth, sixth and seventh group the mono-digestion of wheat straw by each microorganism was observed. Apparently, the fifth group represented a "personal blank" for these series.

Eventually, the main purpose for the last three groups was to observe the effect of microorganisms to co-digesting substrate of cattle manure and wheat straw with the VS contribution 90:10, respectively. The total volume of all BMPs was 337 ml with 80ml working volume and 257 ml for headspace volume. The initial total organic load was 1.5 gVS/L.

Table 3.4 Plan for BMP bioaugmentation

Sample	substrate	Wheat straw, g	Water, mL	Inoculum, mL	Bioaugmentation, ml(20% of inoc. Volume)	Working volume, ml	Headspace volume, ml	Total volume, ml
Blank	0.00	0.00	16.00	64.00	-	80.00	260.00	337.00
Control	0.12(avicel)	0.00	15.84	64.00	-	79.96	257.04	337.00
Blank <i>M. roseus</i>	0.00	0.00	16.00	51.20	12.8	80.00	260.00	337.00
Blank <i>C. thermocellum</i>	0.00	0.00	16.00	51.20	12.8	80.00	260.00	337.00
Untreated Wheat straw	0.00	0.1384	15.86	64.00	-	80.00	257.00	337.00

Wheat straw- C. therm	0.00	0.1384	15.86	51.2	12.80	80.00	257.00	337.00
Wheat straw- M. roseus	0.00	0.1384	15.86	51.2	12.80	80.00	257.00	337.00
10:90 VS ratio	4.89(cattle manure)	0.01383	11.09	64.00	-	80.00	257.00	337.00
10:90 (C. therm)	4.89(cattle manure)	0.01383	11.09	51.20	12.80	80.00	257.00	337.00
10:90 (M. roseus)	4.89(cattle manure)	0.01383	11.09	51.20	12.80	80.00	257.00	337.00

3.3.2.2 Cultivation of microorganisms

Both microorganisms were provided by DSMZ GmbH, Germany. Hence the instructions for preparing the mediums were also available by the same company.

Table 3.5 Clostridium Thermocellum strain 1347 medium ingredients

Ingredients	quantity
(NH ₄) ₂ SO ₄	1.30 g
MgCl ₂ x 6H ₂ O	2.60 g
KH ₂ PO ₄	1.43 g
K ₂ HPO ₄	5.50 g
CaCl ₂ x 2H ₂ O	0.13 g
Na ₂ – glucerol - Phosphate x 4 H ₂ O	6 g
FeSO ₄ x 7 H ₂ O	1.10 mg
L-Glutathione redused	0.25 g
Resazurin	1 mg
Yeast extract	4.50 g
Cellobiose	5 g
Distilled water	1 L

Medium cultivation of *C. thermocellum* (strain 1237)

Two solutions were prepared for *C. thermocellum* cultivation. For the first, all ingredients except cellobiose were dissolved under anaerobic conditions (80% N₂ and 20 % CO₂) for 45 minutes, until pH reaches 7.0 - 7.2. The second solution was prepared under anaerobic conditions (100% N₂) and consisted by 10% (w/v) cellobiose (microcrystalline). The second solution was injected in the each tube by sterilized filter (20 µm). Afterwards, tubes were filled with the respective media and were autoclaved. Afterwards, in each new media, pure culture was injected and then, incubated.

Regarding the BMP experiments, after 74 hours of incubation, 12.8 ml of active culture was injected in each batch, counting almost 20% of inoculum volume. Hereafter, due to the growth rate, *C. thermocellum* white flocks appeared in 1 day. The growth was checked constantly by Optical Density with model. The OD was 0.5 when the bioaugmentation was occurred.

For the preparation of *C. thermocellum* cultures, after the incubation the culture could be centrifuged in order to blast away the cellulose. Nevertheless in the present work, residual cellulose was injected together with microbes, and the possible organic load of the digester was taken into account.

Medium cultivation of *M. roseus* (strain 23840)

As *M. roseus* is less implemented, few are known for the most appropriate preparation, however, for both *C. thermocellum* (DSMZ 1237) and *M. roseus* (DSMZ 23840) instructions from DSMZ were followed.

40 ml of the prepared medium was autoclaved in 120 ml bottles. Afterwards in ambient conditions we injected in the sterilized medium 3 ml of Na₂S (3%), 0.5 ml of Vitamin solution, with the help of 20 µm porous filters. Afterwards we injected 2 ml of pure culture on the rich nutrient substrate.

Table 3.6 *M. roseus* medium ingredients

Solution A	Quantity	Solution B	Quantity	Trace element solution	Quantity	"Vitamin solution"	Quantity
NaCl	1 g	Na ₂ S x 9 H ₂ O	0.3 g	FeSO ₄ (NH ₄) ₂ x 6H ₂ O	784 mg	Biotin	2 mg
MgCl ₂ x 6 H ₂ O	0.4 g	Distilled water	10 ml	ZnSO ₄ x 7 H ₂ O	143.6 mg	Folic Acid	2 mg
KCl	0.5 g			MnCl ₂ x 4 H ₂ O	99 mg	Pyridoxine-HCl	10 mg
CaCl ₂ x H ₂ O	0.1 g			Na ₂ SeO ₄	94.6 mg	Thiamine - HCl x 2 H ₂ O	5 mg
NH ₄ Cl	0.25 g			H ₃ BO ₃	6 mg	Riboflavin	5 mg
KH ₂ PO ₄	0.2 g			Na ₂ WO ₄ x 2 H ₂ O	33 mg	Nicotinic Acid	5 mg
Na ₂ SO ₄	4 g			CuCl ₂ x 2 H ₂ O	1.8 mg	D-Ca-pantothenate	5 mg

Yeast extracct	2 g			CoCl ₂ x 6 H ₂ O	238 mg	Vitamin B12	0.1 mg
NaHCO ₃	0.1 g			NiSO ₄ (NH ₄) ₂ SO ₄ 6 H ₂ O	395 mg	P-Aminobenzoic Acid	5 mg
Distilled water	1 L			HCl(conc.)	10 ml	Lipoic acid	5 mg
PH 7.2 – 7.8				Distilled water	990 ml	Distilled water	1000 ml

The first solution was autoclaved and afterwards mixed with the second and third solutions. The third solution was injected with sterilized filter. The cultivation conducted in autoclaved bottles containing medium and other nutrients. After four hours the flocks were seen visually, without the need of OD meter. Similarly, with *C. thermocellum*, for BMP experiments of *M. roseus* the injected volume was 12.8 ml.

It must be noted that during the injection of microbe media, a surplus of nutrients was subsequently provided in CSTR and BMP systems, on account of the fact that microbes were on the exponential phase and had not consumed all the amount of sugars, minerals and vitamins. For instance, potassium which is utilized by several enzymes, or sodium and other growth factors from media were injected in the reactors. Therefore, as it was expected, there was an overestimation in values of biogas production. Apparently in the present work the effect of the excess of nutrients was removed in order to have trustworthy conclusions, clearly reflecting the essential adaptation of microbes in the lignocellulosic AD system. Additionally the selected amount of ml culture injection was chosen empirically according to the literature, which may need a further optimization (Fotidis et al., 2014).

3.4 Bioaugmentation CSTR

3.4.1 Assembly

The experimental set up was conducted strictly under anaerobic conditions. The three 5-Litter CSTRs (Continuous Stirred Tank Reactor) had 3 L working volume and 2 L headspace volume. Each reactor was equipped with stirring motor to stir every 60 seconds and let the substrate being homogeneous. Warm water, at 55-56 °C was delivered in the external water jacket of each reactor, one after the other, continuously through a recirculating heating system. Moreover, the water flux was adequate and every rubber pipe and reactor was rapped with plastic insulator in order to minimize the heat convection to the cooler environment, and keep the temperature steady.

Feeding peristaltic pumps were calibrated first in order to have the most accurate and similar feeding in digesters. The feeding frequency was 100 ml every 12 hours in each 3 L working volume reactor, hence, the HRT was 15 days

for each. One hour before each feeding the system was adjusted to stir with a magnetic system the Influent bottles by means of the high homogeneity of manure-straw influent.



Figure 3.17 CSTR system

3.4.2 Start-up period

Initially, the influent was consisted by sieved cattle manure. After 5 HRTs the biochemical system within the reactor, and the product flow were steady. When the system was steady (day 63) in the influent we added the chopped wheat straw 8.46 g wheat straw / HRT (i.e. 0.56 g straw/day). Before the addition of wheat straw the OLR was 1.47 gVS/L/day. Thereafter, the impact was 0.17 gVS/L/day, in total the new OLR was 1.64 gVS/L/day. Every time we filled the influent conical bottles with 1 L cattle manure and 2.82 g wheat straw. Thus, in the daily feeding was 200 ml manure and 0.56 g wheat straw. It takes 5 days for 1 L to be injected in the reactor

$$OLR_i = VSi(g/ml) * \frac{0.2\ l}{3l}$$

Table 3.7 VS, TS , OLR values

VS	TS	OLR(gVS/L/day)
----	----	----------------

Cattle manure(first period)	22.08	32 (g/L)	1.47
Wheat straw	2.45(0.49 per day)	2.62 (g/day)	0.16
Co-Digestion(second period)	24.53(g/L/day)(2.45%)	34.62(3.46%)	1.64

3.4.3 Microorganisms injection

Routine Bioaugmentation was conducted for 6 days was conducted as treatment, signifying the start of third period. Reactor 2 and Reactor 3 were manually fed with 100 ml of cultivated culture by the bottom. In order to avoid the rising level and thus the wash out of the microorganisms, before every injection 100 ml of substrate were pulled out of the reactor, and the stirring was discontinued for 2 hours (half an hour before and 1.5 after injection). Of course, due to the adjustments the new HRT was being changed due to the dilution. Additionally, Reactor 1 was used as a control and was fed for 6 days with BA medium (abiotic augmentation) in order to equal the dilution with other reactors.

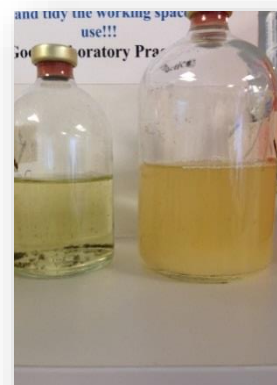


Figure 3.18 BA medium , Figure 3.19 *M.roseus* Medium , Figure 3.20 *M. roseus* (on the left) and *C. thermocellum*(on the right) Medium

CHAPTER 4: RESULTS AND Discussion

4.1 Microaeration

In the present work the amount of injected oxygen was named as Oxygen Load (ml O₂/gVS per day). From hereafter, OxL or ml O₂/gVS per day in terms of Oxygen Load is issued. Measurements of pH, VFAs and sCOD were conducted before microaeration, after microaeration (the last oxygen injection) and at the end of the experiment in order to evaluate the effect of oxygen on specific quantity of wheat straw.

Hydrogen

The results indicated low propionate; therefore, H₂ pressure was low. In order to testify this, hydrogen concentration was measured for the most and for the least promising BMP treatment. Finally the results did not show any steep increase of H₂. Otherwise, hydrogen is not an indicating parameter for BMP assays due to the stable OL, in comparison with continuous feeding where due to the increasing of OLR there is a reflected disturbance in H₂ ([Boe et al., 2010](#)).

Sulphate

In literature it is stated that microaeration could boost the activity of sulphide reducing bacteria. The aforementioned bacteria pre-exist in the anaerobic digester in low concentrations and are responsible for the depletion of sulphide by oxidizing it to elemental sulphur. However the increase of elemental sulphur in a digester could cause clogging in the pipes ([Krayzelova et al., 2015](#)).

Ethanol

Ethanol measurements were not essential because ethanol inhibits the utilization of propionate to acetate and as it was shown propionate maintained in very low amounts ([Lyberatos et al., 1997](#)). However ethanol concentration was measured by GC, resulting negligible amounts which were not represented in this chapter.

Temperature

Regarding to the self-heating properties that have been observed in previous studies with implementation of oxygen for delignification, in this case due to the low oxygen load was not expected an adequate self-heating and therefore, each sample was incubated properly at 55 °C. Of great importance was the low amount of propionate which was lower than acetate as shown in figures in the following chapter. This indicates that there was not inhibition due to the temperature or other factors. As it was expected, microaeration of the mixed aerobic and anaerobic culture does not unfold any self-heating ability.

4.1.1 VFA, sCOD, pH , ORP after Microaeration Period

At the end of microaeration period ORP, pH, VFA, sCOD and ORP were determined. Furthermore during the incubation period methane and carbon dioxide concentration were determined.

VFA

After microaeration a huge increase was observed due to the rapid hydrolysis. As illustrated in all figures, VFA showed stable accumulation after microaeration except from acetate, which was remarkably increased in the system in respect to the increase of the number of days and OxL. It is noteworthy that acetate increases and propionate stayed stable in all results. As regards to the aggregated amount of VFAs right after the treatment, each triplicate concentration is depicted in Figure 4.1. The 0-OxL. bars in the figure illustrate the amount of VFA that untreated straw resulted in the same time when the last injection of oxygen was implemented. It is easily observed that for each oxygen load there is an increasing trend with subsequent rising of injections and days. Towards all the samples, 5-3-3 had the highest production of VFA, resulting 955 mg/L, about 80.5 % higher than untreated 0-3-3.

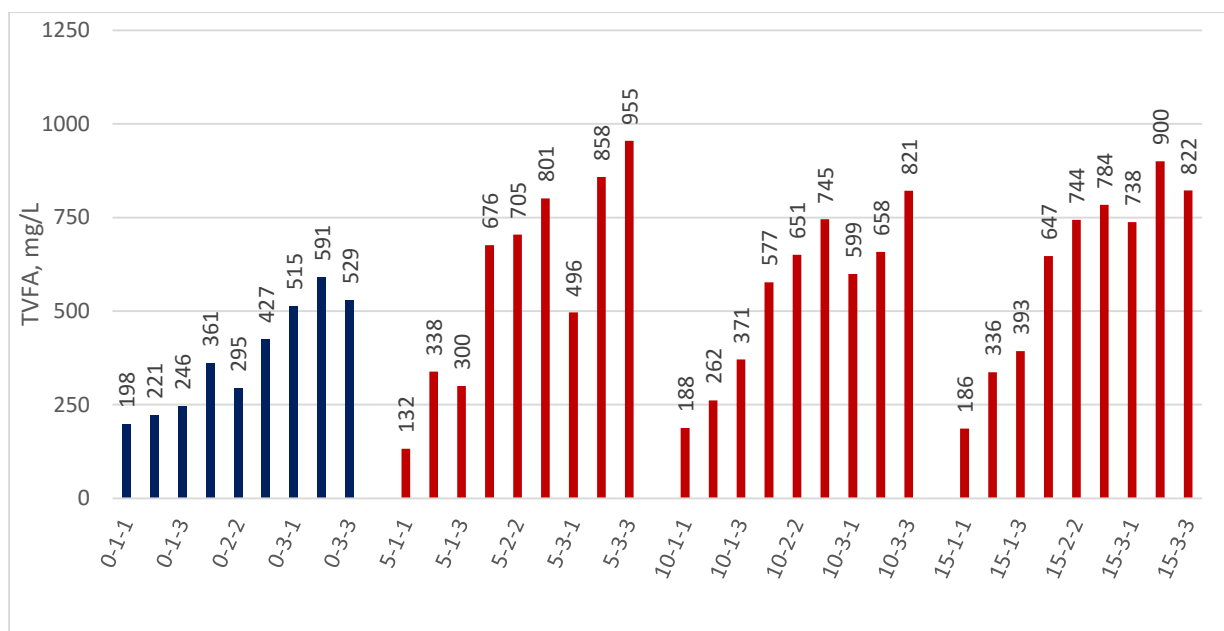


Figure 4. 1 TVFA before(blue bars) and right after microaeration(red bars)

Nevertheless, after microaeration, the greatest acetate production was presented for the samples that microaeration lasted the most days (3-days or 72 hours). The highest acetate concentration was resulted for the samples which were conducted with 3 injections and 3 day period, the average % acetate/VFA ratio was 81 % while the average proportion of propionate/VFA ratio indicated approximately 8 %. The lowest amount of acetate generation, was obtained in 1 inj- 1day samples and resulted an average acetate/VFA ratio equal to 59 %

It is easily observed that for each oxygen load there is an increasing trend of TVFA through days. Thereby, the Acetate/Propionate proportion was also increasing through the days. Overall, it is rational VFAs to be increased

due to the rising of days as subsequently the oxygenation of the closed system is rising. Regarding to the aforementioned explanation, it is generally observed in figures that every third day the TVFA were greater than other days. For untreated straw, VFA concentration was rising successively in time as it was expected. Hence, it is assumed that the hydrolysis for strictly anaerobic mixture showed good results in general.

Aggregated bar figures illustrated the enhanced production of acetate specifically for 15 ml O₂/gVS. It is also obvious that the 15 ml O₂/gVS samples consumed the same or even more amount of acetate than the others. This is not in line with the final methane yield, where by contrast 15 ml O₂/gVS seemed to be inefficient to produce methane in greater levels than untreated straw. As it was mentioned before, methanogens are very vulnerable to the oxygen.

4.1.1.2 *p*- parameter (Pulse effect)

VFA (p-parameter)

It is easily observed that for each oxygen load there is an increasing trend of TVFA during the day. The intensity did not contribute to the methane potential though. This showed that the amount of pulses does not have any impact on the methane potential. However, the amount of acetate and acetate/VFA proportion unfolded an increase due to the increase of the number of injections and O₂ load. For instance, in the next series of Tables 4.1-4.4 the differences due to the progression of injections and O₂L are portrayed for the same (first) day.

Table 4. 1 VFA increase in a day for untreated

Sample(untreated)	%Acetate/TVFA*	Difference in time
0-1-1(morning)	59.03%	
0-2-1(afternoon)	72.43%	+ 13.4%
0-3-1(night)	80.36%	+ 7.93 %

Table 4. 2 VFA increase in a day for 5 ml O₂/gVS per day

sample	%Acetate / TVFA*	Difference by means of injections
5-1-1(morning)	64.32 %	
5-2-1(afternoon)	74.81%	+10.49%
5-3-1(night)	82.49%	+ 7.68%

Table 4. 3 VFA increase in a day for 10 ml O₂/gVS per day

sample	% Acetate /TVFA*	Difference by means of injections
10-1-1(morning)	70.89%	
10-2-1(afternoon)	73.41%	+2.52%
10-3-1(night)	82.36%	+8.95%

Table 4. 4 VFA increase in a day for 15 ml O₂/gVS per day

sample	%Acetate / TVFA*	Difference by means of injections
15-1-1(morning)	70.13%	
15-2-1(afternoon)	77.26%	+7.13%
15-3-1(night)	88.06%	+11.20%

pH

pH captures the first estimation for the stability of AD process. However, to further investigate the substrate degradation results, as it was described before, VFA determination is required. In the present experiment, on the grounds of low buffering capacity, the system could not hold stable pH values. It is commonly accepted that when VFA concentration increases the pH drops, and as it was expected a pH drop was conducted due to this effect individually, whilst respiration was avoided as the methane proportion in biogas demonstrated values above 65%.

In Figure 4.2 the untreated samples are represented by blue bars, whereas after microaeration samples represented by red bars

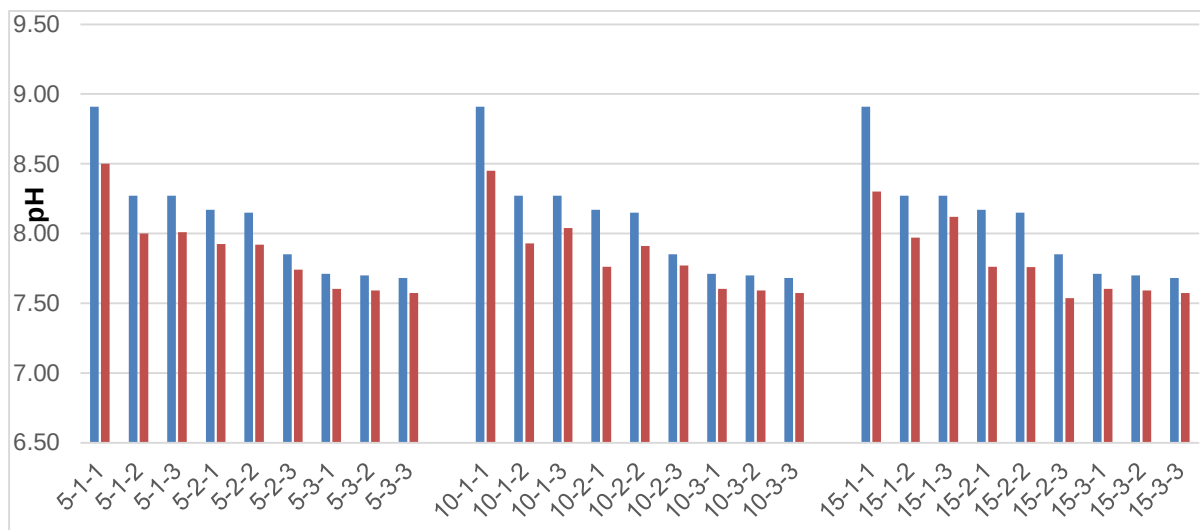


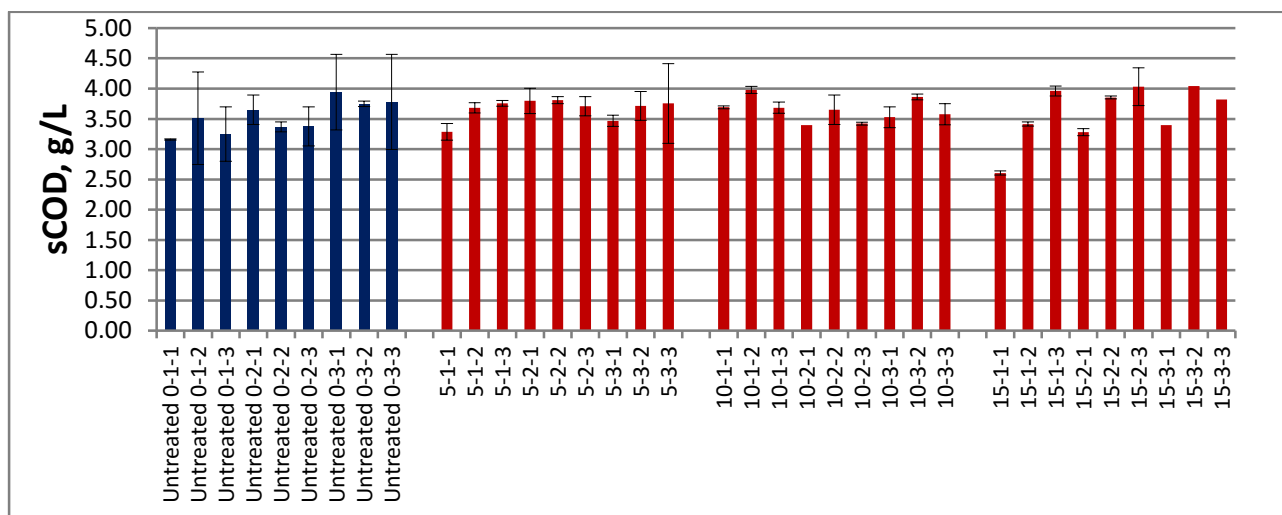
Figure 4.2 pH before (blue bars) and right after microaeration (red bars)

As illustrated in Figure 4.2, through the increase of days for each step of the process pH was dropping successively indicating an overall decrease of 20%. The untreated decline from the values of 8.9 to 7.5, whereas after microaeration shows similar decline from 8.5 to 7.6. Obviously for each oxygen load there is a similar drop of pH approximately 10% for the 1-injection and 2-injections bottles. It is considered that after the last day of treatment, hydrolysis step should be in its highest rate, therefore it was expected a sharp drop in pH, and subsequently after that period the stabilization of it. In particular, for 1 inj – 1 day there was a pH drop near to 0.40. Additionally for 1 inj – 2 days pH drop for every OxL was approximately 0.30. Likewise, in 2 inj – 2 days a common 0.20 pH drop was observed. As mentioned in the previous chapters, it is observed that for all VFA figures, acetate is increasing successively due to the intense of injections of O₂, but there is not difference with the trend of untreated.

sCOD

In general all the results showed that sCOD concentration slightly increased under microaeration. This is in line with previous similar researches (Wei & Wang, 2013). All 1st-day bars (appendix) showed that only a small amount of sCOD was generated, and this is rational due to the fact that the hydrolytic step is in its beginning and enzymes are not secreted yet adequately. It is very clear that with the rapid hydrolysis even for 15 ml OxL in the first day the solubilisation was limited. Furthermore, after microaeration in comparison with oxygen loads for the majority of figures, all the samples with 5 ml load/gVS obtained very limited but relatively higher sCOD values compared to untreated.

Table 4.3 sCOD after microaeration



ORP (oxidation-reduction potential)

In the present work ORP was measured during the microaeration in order to ensure that conditions did not switch from anaerobic to aerobic due to the intensity of respiration. In strict anaerobic conditions ORP fluctuates from -300 to -200mV, in opposition to fully aerobic conditions which indicate +50 mV (Jenicek, 2008). Although it was stated that the trace oxygen that already exist in the system from the start up, leads the system to ORP equal to -400mV (Stamatelatou et al., 2011).

In a recent research, Wei et al. (2013) obtained -278 mV to -220 mV under microaeration treatment. However in the present work during the highest 3-day microaeration of 15 ml O₂/gVS per day the ORP fluctuated between -65 and -72 mV from which is shown that anaerobic archaea were not severely inhibited by the presence of oxygen. As it was aforementioned the facultative wood rotting fungi that thrive on wood surface, excrete versatile enzymes which disrupt lignin. This enzymatic system, namely “feed-back”, is responsible for both disruption of lignin and cellulose (Eriksson et al., 1974). In this way, it could be assumed that under microaeration the boosting activity of these bacteria could lead to the lignocellulosic degradation. Eriksson & Pettersson (1974), also explained that during the fungal degradation of cellulose, beyond the effect of hydrolytic enzymes, additionally the produced oxidizing enzymes are contributing in the hydrolytic process.

It is noteworthy that during microaeration the anaerobic process should not change the basic function of redox reactions, and not be covered by the external electron acceptor O_2 , this will indicate the inhibition of normal functionality of anaerobic microbes.

4.1.2 Methane Production Results

Control

As it was mentioned above in this work the standard/control substrate was microcrystalline cellulose (Avicel).

Methane is produced from the last step of anaerobic digestion. Methanogens prioritize methanogenesis instead of doubling their mass, by means of carbon is led to methane production and not biomass generation and thus the archaea growth is slow. The carbon that is utilized to biomass is more or less 5%.

1st step. In this step hydrolytic compost and AD inoculum bacteria were “waken up” and excreted enzymes due to the injection of oxygen and their first interaction with substrate. If hydrolysis is fast and adequate, nutrients are produced, and then methanogens start the consumption faster, thus lag phase is short. In the present results it could be assumed that is a result from the slight inhibition of methanogens by trace amounts of oxygen (Li et al., 2013).

2nd step. The methane yield rose rapidly in the first 14 days. This indicates an accelerating hydrolytic and methanogenic activity which follows a linear increase as long as VFAs and more specifically acetate are abundant.

3rd step. Altogether, the charts indicated a successive drop of slope after the 14th day. Therefore, it is considered that between 10th and 14th day methanogens consumed the overall amount of acetate, and the most degradable part of substrate. Thereafter, methanogenic activity slows gradually until the last week. As it is depicted in the next bar figures, in the end acetate leftovers were minor.

4th step. Afterward, with the acetate consumption it followed the last step, where methane yield stabilized until it dropped. It is obvious that the experiment continues until the stabilization of production instead of the drop of it. Of course, due to the limited amount of nutrients it is rational that the drop of methane production will follow next.

The figure 4.3 below depicts the increasing methane yield in the first two weeks and the sharp stabilization after 14th day. Subsequently, there is a slower rise of the yield until the trend reaches the maximum potential of 373.82 ml CH_4 /gVS on the last week of experiment (35th day). Thus methane potential resulted the 84.5% of theoretical methane potential of wheat straw. Apparently, the overall progress of experiment was generated properly for specific microbial system under the thermal environment.

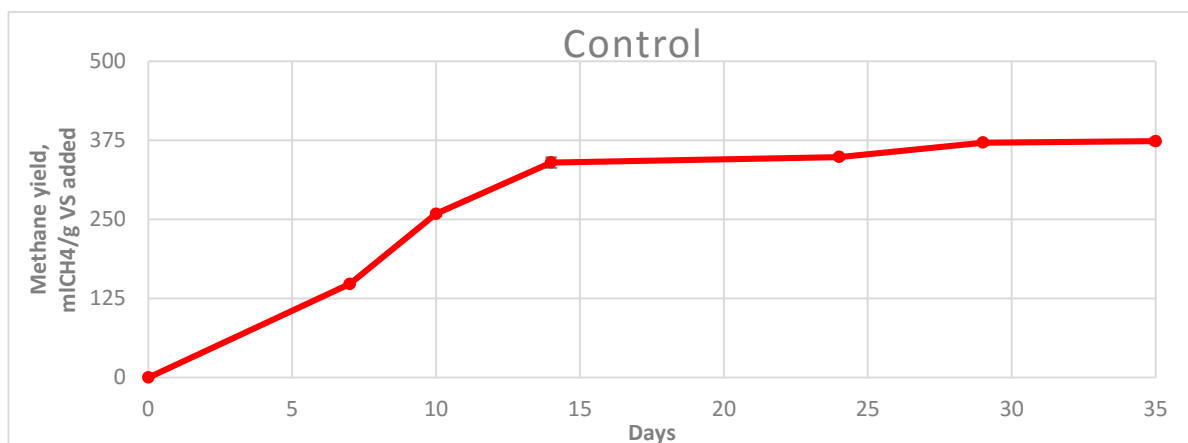


Figure 4. 3 Methane production for Control batches

Methane generation before the complete digestion

Apparently, the 5 and 15 ml O₂/gVS samples, during the first week, reflected a common lag phase, which was longer than untreated wheat straw. This could indicate the initial slight inhibition by O₂. Exceptionally, all 10 ml O₂/gVS samples resulted a converged tendency with untreated wheat straw for the first week. The results are illustrated more comprehensively in the next.

Methane generation after the complete digestion

Figure 4.4 illustrates the methane yield of each treated triplicate in comparison with the untreated at the end of microaeration period. It is obvious that the majority of 15 O_xL samples indicated lower values of methane potential, although the inhibition was not severe. Apparently, 5 ml and 10 ml resulted unstable overall results, fluctuating near the untreated potential.

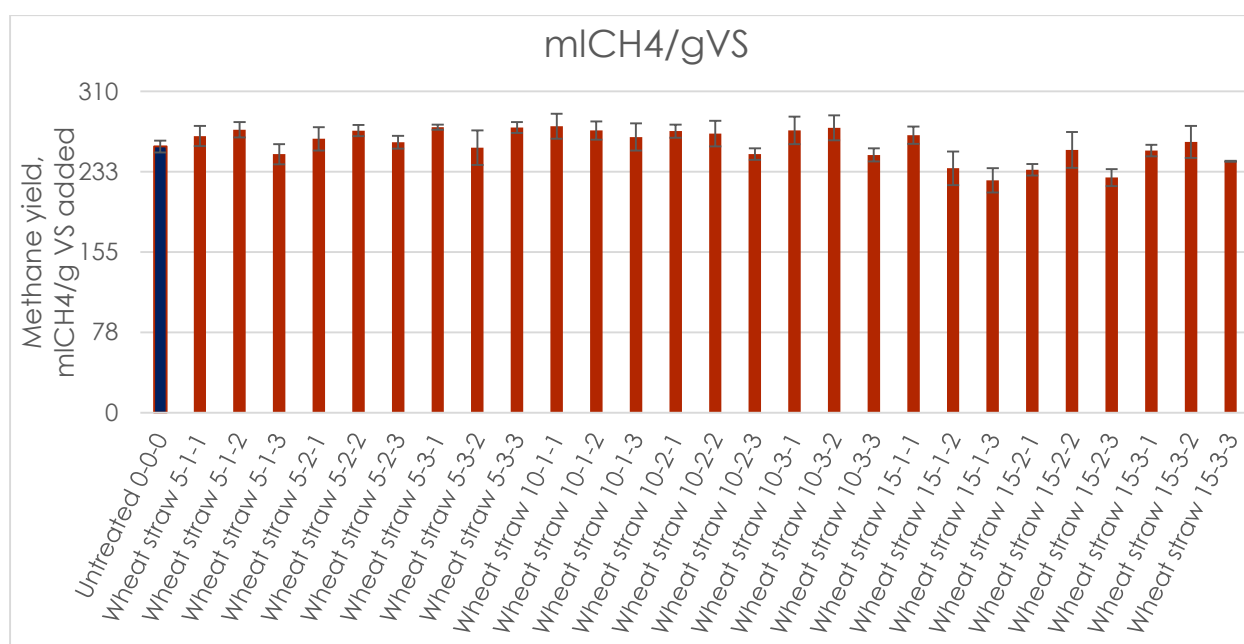


Figure 4. 4 Aggregated Methane potential value

In the following Table 4.5, the aggregated results for methane potential are presented.

Table 4. 1 Aggregated results for methane potential

Samples (ml O ₂ -day-injection)	Enhance	potential mlCH ₄ /gVS	STDEV
Untreated 0-0-0	-	257	6
Wheat straw 5-1-1	3.9%	267	10
Wheat straw 5-1-2	6.3%	273	7
Wheat straw 5-1-3	-2.9%	249	10
Wheat straw 5-2-1	2.9%	264	11
Wheat straw 5-2-2	6.0%	272	5
Wheat straw 5-2-3	1.6%	261	6
Wheat straw 5-3-1	7.2%	276	3
Wheat straw 5-3-2	-0.5%	256	17
Wheat straw 5-3-3	7.1%	275	5
Wheat straw 10-1-1	7.6%	276	12
Wheat straw 10-1-2	6.0%	272	9
Wheat straw 10-1-3	3.5%	266	13
Wheat straw 10-2-1	5.7%	272	6
Wheat straw 10-2-2	4.8%	269	12
Wheat straw 10-2-3	-2.9%	250	6
Wheat straw 10-3-1	6.1%	272	13
Wheat straw 10-3-2	7.0%	275	12
Wheat straw 10-3-3	-3.2%	249	6
Wheat straw 15-1-1	4.2%	268	8
Wheat straw 15-1-2	-8.2%	236	16
Wheat straw 15-1-3	-12.7%	224	12
Wheat straw 15-2-1	-8.8%	234	6
Wheat straw 15-2-2	-1.3%	254	17
Wheat straw 15-2-3	-11.7%	227	8

Wheat straw 15-3-1	-1.5%	253	5
Wheat straw 15-3-2	1.7%	261	15
Wheat straw 15-3-3	-5.6%	243	0

Lignin cannot be degraded, therefore, biodegradability for every lignocellulosic substrate, such as wheat straw, cannot reach 100% (Buffiere et al., 2006).

In the present work for the collected and implemented wheat straw, the theoretical methane potential was calculated by Buswell's formula and it was equal to 443 ml CH₄/gVS. Although, regarding to the lignin nature of wheat straw, methane reached lower values than theoretical maximum indicating lower biodegradability. The contribution of the previous could lead to the lower overall methane potential.

The biodegradability of untreated (unaerated) wheat straw was calculated to 58%. The highest methane potential occurred for the sample 5-3-1, indicating 276 ± 3 ml CH₄/gVS. Thus, the biodegradability of wheat straw in the present environment was 62%. On the other hand the lowest biodegradability was obtained for the sample 15-1-3 for which highest methane yield was equal to 224 ± 12 ml CH₄/gVS. In this case the ratio of measured to theoretical potential was only 51%. The overall range where samples degraded was from 51% to 62%. Towards all 15 ml O₂/gVS per day samples the highest biodegradability obtained the values of 60% for the 15-1-1 sample (268 ± 8 ml CH₄/gVS). For the rest 15 ml samples biodegradability of wheat straw was by far lower. Conversely, for 5 ml O₂/gVS per day and 10 ml O₂/gVS per day samples the biodegradability fluctuated in the range of 56% - 62%.

CH₄ results for 5 O₂L

All the methane figures depict an exponential increase for the first 2 weeks. The Figure 4.5 below illustrates the effect of 5 ml O₂/gVS load. The accelerating methane yield after day 15 was increased slower and a stationary phase was followed until the day 29 where the digestion period was stopped. Amongst others, sample 5-3-1 reached the highest methane production enhancement 7.2% (276 mlCH₄/gVS). Additionally, in compassion with all the figures only 5-1-2 illustrates the shorter time reaching the maximum methane yield in day 24 instead of 29. Additionally, 5-1-2 trend traces more aggressive line, which is clearly greater from untreated wheat straw during the stage, although the methane production increase was 6.3%.

The methane potential of untreated wheat straw was 257 mlCH₄/gVS, by means of 68.72% of the production achieved by control.

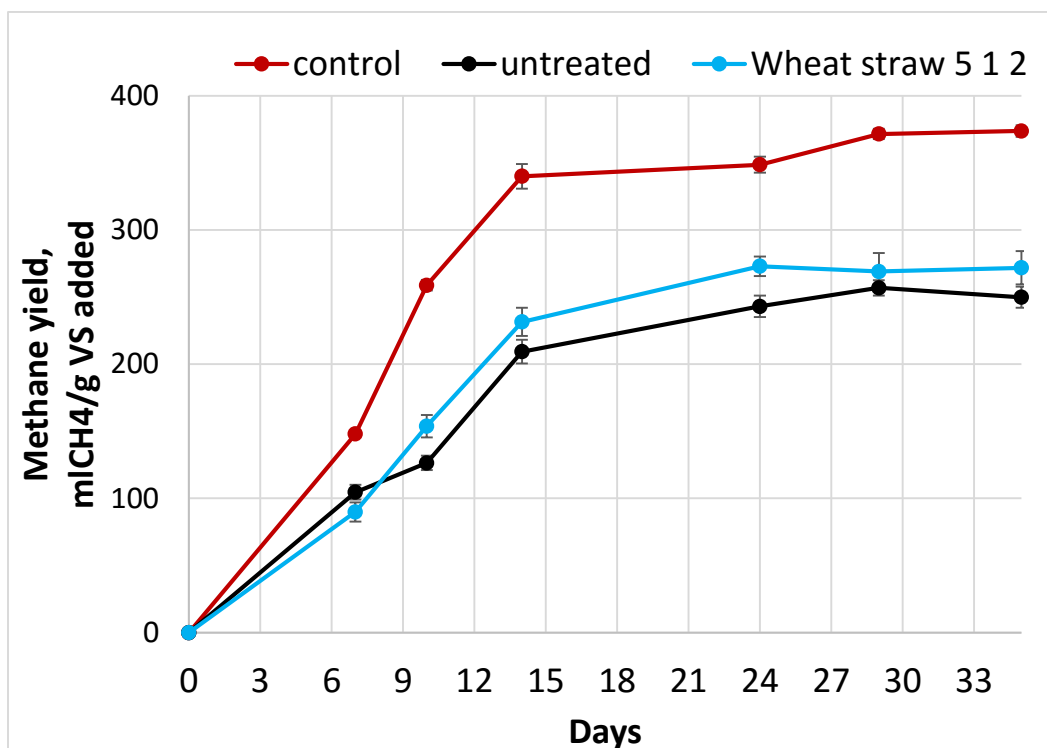


Figure 4. 5 methane yield for wheat straw 5-1-2

Apparently, in the first week, even for the most boosted samples, methane production was lower in comparison with untreated straw. This is explained by the fact that oxygen inhibits the methanogens, extending subsequently the time of lag phase (Wei et al., 2013). Nevertheless, during the lag phase hydrolytic facultative anaerobes consume oxygen while boosting the VFA production. However, when oxygen is consumed, methanogenesis is triggered and the slope of methane production was steep. It could be assumed something like this because obviously hydrolysis is boosted and nutrients have already formed, thus when the inhibitory for methanogens factor is consumed then it must be a rise of methane production

CH₄ results for 10 O_xL

In the next table the greatest enhancement of methane production is depicted. The sample 10-1-1 reached 7.6% with production of 276 mlCH₄/gVS. Moreover, the 10-1-1 trend outlines that in the first week, methane production was not lower than untreated straw. It should be mentioned that this is the only O_xL sample that its lag phase was covered untreated instead of being extended; nevertheless the difference was not remarkable. Thus it is evoked that methanogens did employ the hydrolysed substrate alleviating the oxygen inhibition.

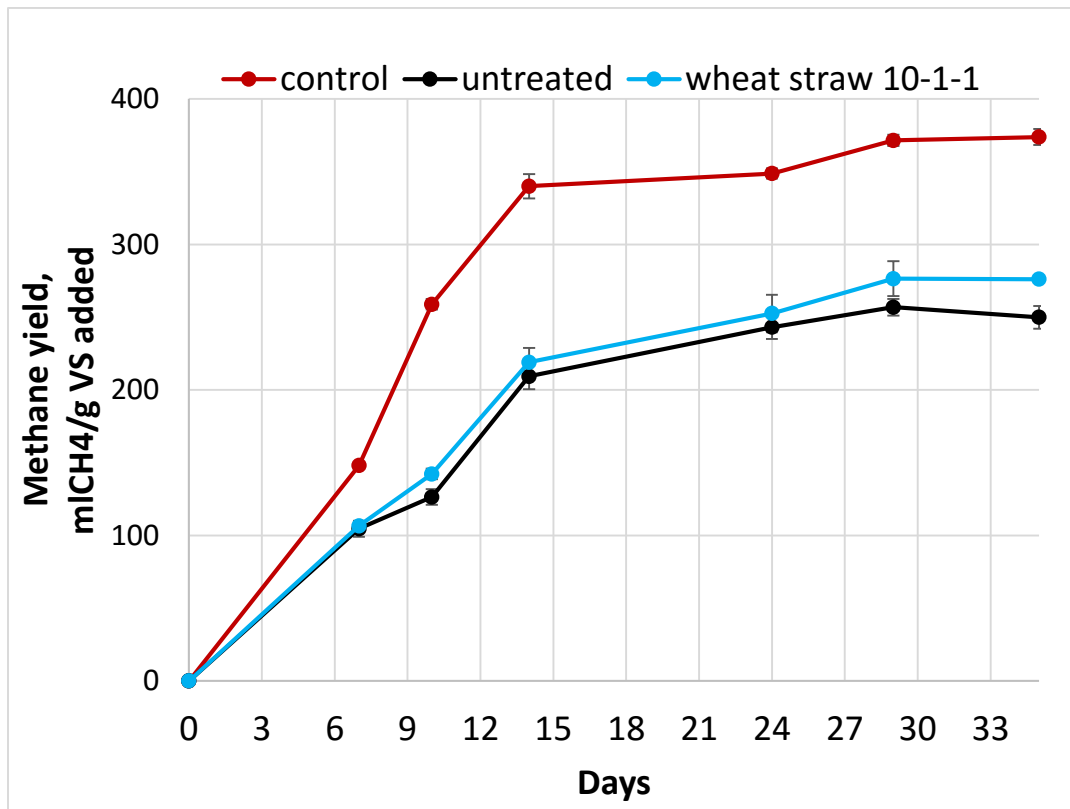


Figure 4. 6 methane yield for 10-1-1

CH₄ results for 15 O₂L

There is a decline in results concerning the effect of oxygen on the produced methane yield. Thus it could be assumed that due to the oxygen suffocation, methanogens could not generate greater methane than the untreated samples leading to a slight inhibition. In opposition with 5 ml O₂/gVS here there is not a greater increase from untreated, but the curve stays in lower levels. Therefore, it could be assumed that O₂ was not fully consumed from the facultative microbes, indicating a threshold.

The lowest methane potential is depicted in the following figure, dropping -12% in comparison with untreated.

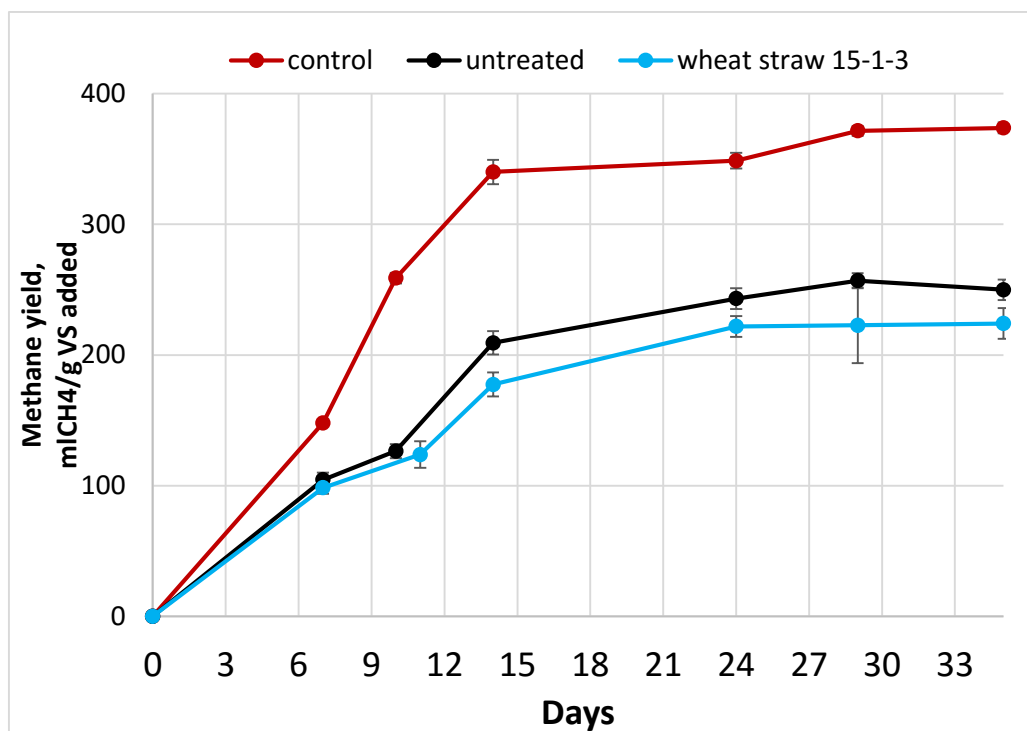


Figure 4. 7 methane yield for 15-1-3

As it was discussed previously, oxygen was consumed rapidly by facultative hydrolytic bacteria, increasing the solubilisation and the overall process. Nevertheless, when the amount of oxygen in the system is above a critical threshold the activity of methanogens and acetogens are inhibited, and hence methane yield is decreased (Xu et al., 2014). O_2 excess could also lead to the growth and over-reaction of aerobic methanotrophs, which exist in the extracted compost microflora, which already exist in the system in minor concentrations, as a result some of the produced methane is simultaneously consumed by these aerobes Zitomer et al. (1998), although, nothing similar was extracted in the present work.

4.1.3 VFA, sCOD, pH at the end of experiment

VFA

It is very clear that the AD process was very effective for all the samples. At the end of the experiment untreated and pre-treated samples reached the same minor VFA values. Thus, in the same period of days the amount of increased acetate was consumed by aceticlastic methanogens for biogas production. The main VFA was acetate with average amount of 30 mg/L. Hence, there was a general decrease over 500 mg/L for each sample. On the grounds that there was an acceleration of acetate consumption, methane generation rose respectively.

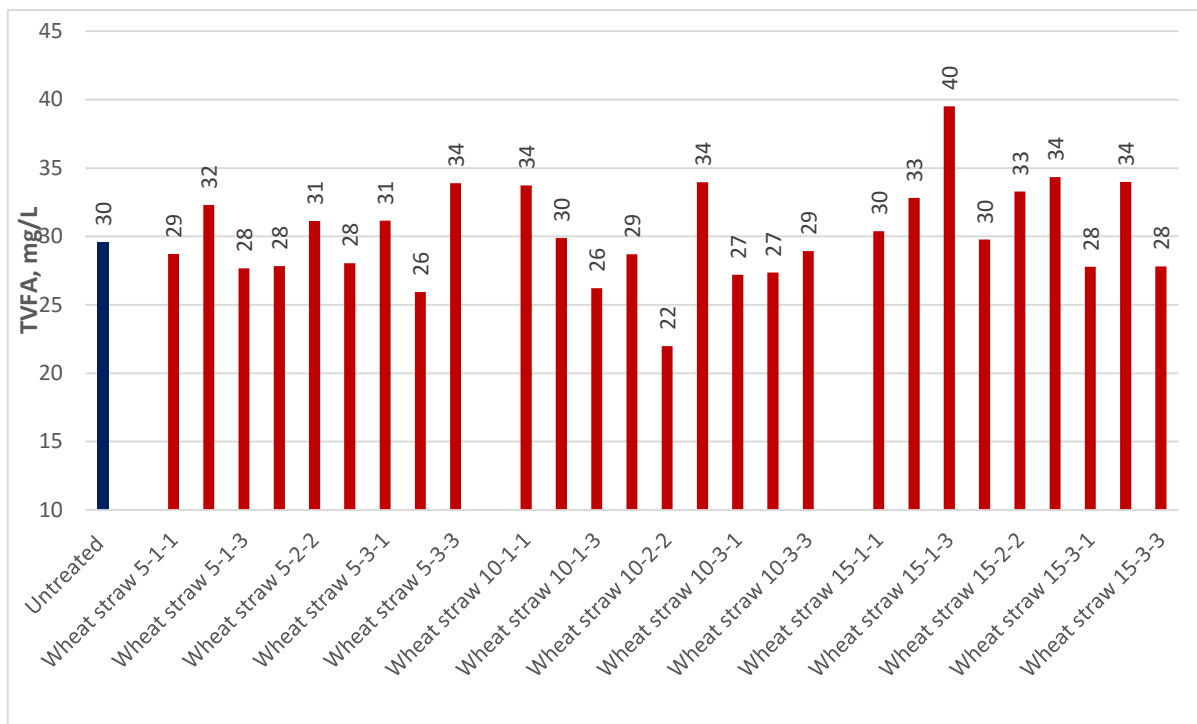


Figure 4. 8 TVFA after digestion

pH

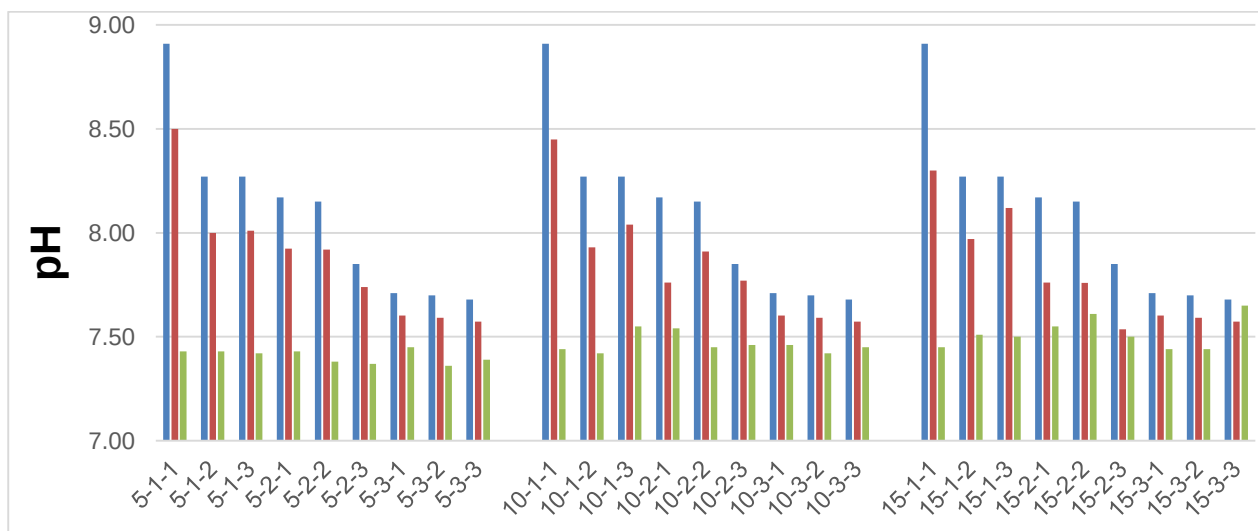


Figure 4. 9 pH correlation between untreated (blue bar) –treated(red bar) –digested(green bar).

After the 35th day pH measurements carried out in order to evaluate the general trend of pH in pre-treated wheat straw. The results illustrated common values of pH approximately 7.5 regardless the initial affection of oxygen in the system. This means that the system was stabilized in a desirable pH range of $7 < \text{pH} < 8$, which it is not a considerable variation of pH.

sCOD

As it was expected in the end of the BMP experiment, there was a rapid reduction for the sCOD with a common significant difference between untreated and treated groups. In comparison with 5 and 15 ml O₂/gVS the most reduced and effective sCOD values after digestion belong to 10 ml O₂/gVS.

Table 4. 6 sCOD table

10 -1-1	0.27 gCOD/L
10 -2-2	0.27 gCOD/L
10- 3 -2	0.24 gCOD/L
10- 3-3	0.13 gCOD/L

Considering the initial rise of sCOD higher than the untreated right after the microaeration and additionally the drop of them at the end of the anaerobic digestion it could be suggested that microaeration treatment was a favorable treatment for anaerobic digestion.

Table 4. 7 sCOD difference table

	After microaeration	After digestion(BMP protocol)	Reduction% gCOD/L
average untreated	3.53 gCOD/L	1.96 gCOD/L	-44.47%
average 5 OxL	3.66 gCOD/L	0.52 gCOD/L	-85.89%
average 10 OxL	3.64 gCOD/L	0.58 gCOD/L	-84.06%
average 15 OxL	3.60 gCOD/L	0.85 gCOD/L	-76.38%

The following figures depict the sCOD correlation between untreated and pretreated wheat straw after microaeration. The most desirable effect was achieved at the 2 inj – 2 days in which all the pretreated wheat straw bars had the highest sCOD comparatively with their relative untreated.

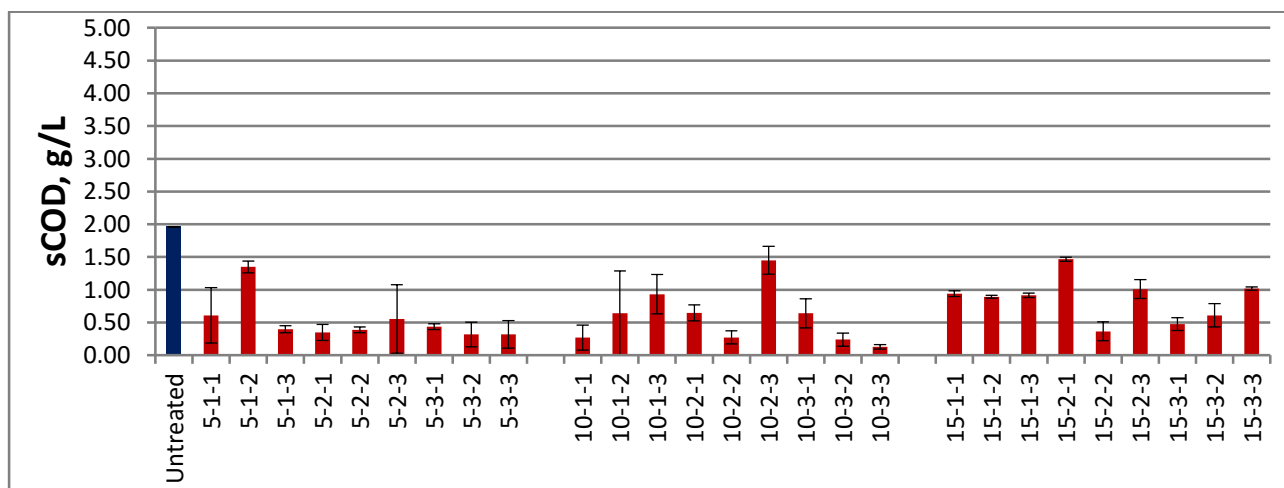


Figure 4. 10 sCOD after digestion

Statistical analysis

Each concentration was prepared in groups of triplicates for the purpose of ensuring the reproducibility of the statistical analysis (Angelidaki et al., 2009). After the experimental part, for the extraction of valuable results, Anova statistical analysis was conducted. More precisely Anova Analysis Tukey post hoc test: $p < 0.05$, occurred in order to render the different and significant variations among the treated samples. The results showed that only one sample (15-1-3) was significantly different illustrating $p < 0.05$, which consequently was the less desirable as it indicated the worst effect in the system. Methane potential was -12.7% lower than untreated for this individual sample. The results for the rest samples were not significant different ($p > 0.05$). The software that was used to perform the analysis was the Figurepad Prism 5 software package (Figurepad Software, Inc., San Diego, CA).

Complementary discussion on microaeration results in comparison with literature

Regarding the increase of VFA and solubilisation after microaeration in anaerobic system there are some authors who have ended up to similar conclusions with this work.

In combination with the inhibition of methanogens due to the presence of oxygen, methanogenic step was vitiated despite the soared hydrolysis.

In a recent study, (Fu et al., 2015) conducted lower lag phase after the injection of oxygen. Regarding to this work, microaeration could not occur depletion on lag phase, in opposition to Fu, there was a longer lag phase in the most samples. Although it must be noted that Fu et al. (2015) implemented microaeration on a completely different substrate. Nevertheless, the highest methane yield occurred when the oxygen load was 5 ml O_2 /gVS, 16.24% higher than the untreated group. Towards that, in 5 ml and 10ml oxygen loads the higher methane yield was observed. In the present work, although the similarity of conclusions in the 5 ml O_2 /gVS and 10 ml O_2 /gVS production, the lag phase was not shorter. Additionally, in the aforementioned study the VFAs profile were stable for each load, by contrast, in the current work after microaeration the amount of VFAs was increased.

Moreover, Wei & Wang (2013) proposed that microaeration prevents the accumulation of toxic products such as ethanol and lactic acid. In line with the aforementioned result, the reduction of propionate was stated in the present work. Additionally there was not any accumulation for the measured lactic acid or ethanol. Definitely Lim defined comprehensively through their work the same effects of microaeration when food waste was used as a substrate with brown water. In the same line with literature, in the present work the results after microaeration indicated higher COD solubilisation, greater VFA generation and conversion of VFA to acetate. Nevertheless on the grounds that acetate is the precursor for biogas generation, the increased production of acetate was adequate to make us assume that through this way the methane production was increased (Xu et al., 2014). Regarding to Miller & Trfisch (1986), 22 basidiomycetes degrade wheat straw with most favoured the Wight rot fungi. They used wheat straw which was consisted of 28.0%, 42.9%, 16.0% and 0.87% of hemi-cellulose, cellulose, lignin and

nitrogen respectively, and after 30 days of co-digestion of myco-straw with cattle manure in batch reactors they figured that concentration of Volatile fatty Acids was higher than untreated straw. And as it was reported the highest production of VFA reflected the increase of methane yield.

In contrast with this work, [Botheju & Bakke \(2010\)](#) showed that through oxygenation the VFAs were reduced, while they rendered oxygen injection as an appropriate method to avoid acidic inhibition. According to [Botheju et al. \(2010\)](#) who observed linear reduction of methane yield with the addition of the oxygen in the system, in the present work there was not a similar reduction. But it could be hypothesized that in another experimental series where oxygen load would be higher (for instance 20 ml O₂/gVS per day) then a linear reduction it could be illustrated, as the declined methane production was not rapid and it is possible to be extended more.

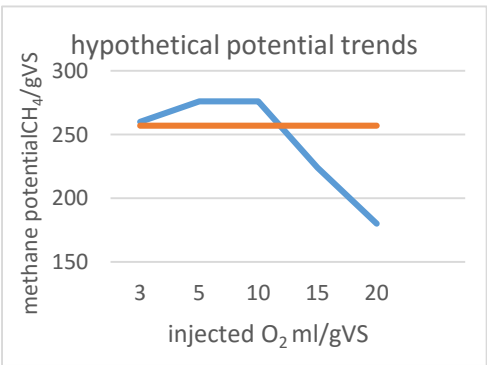
Future work

According to [Botheju et al. \(2010\)](#) who observed the linear reduction of methane yield with the addition of the oxygen in the system, in the present work there was not a similar linear reduction due to the low oxygen load. However, it could be hypothesized that with further addition of oxygen load (for instance 20 ml O₂/gVS per day etc.) a linear reduction could be illustrated.

It is noteworthy that the effect of injection of 5 and 10 ml O₂/gVS was very similar for both oxygen loads and did not show any remarkable reduction of methane potential with the oxygen increase, although methane potential reduction began with higher oxygen load of 15 ml O₂/gVS. Hence, it could be assumed that there is a pinpoint from where the excess of oxygen starts inhibiting in linear way the gas production. However, the methane yield tendency below 5 ml O₂/gVS is unknown, nevertheless, there must be a rising slope until the maximum methane yield. In the present work it could be assumed that the results indicated the maximum potential the system could provide under microaeration. Overall, it could be proposed a further optimization, of the ADM1-Ox by including the further effect of oxygen before the linear production which could be “drawn” as follows.

The overall trend in comparison with untreated wheat straw under microaerophilic conditions could be as follows. X-axis: oxygen load, y-axis: methane yield.

The blue trend traces the methane potential, of treated wheat straw and the orange the untreated wheat straw.



ml O ₂ /gVS	Methane potential(mlCH ₄ /gVS)
3	260*
5	276
10	276
15	224
20	180*

4.2 Bioaugmentation

Microorganism growth

Optical Density was measured frequently before the injection to the reactors or batches, in order to make sure that the injected culture in the system was grown adequately. Toward the two inoculated microorganisms only *C. thermocellum* was possible to observe through optical density. *M. roseus* OD was not determined due to the fact that creates biofilm on the bottom of the bottle which even if the bottle is stirred the mixture never goes completely homogeneous, but instead the cultivation is simply disrupted. Hence, *M. roseus* was observed by sight, although biofilm was escalated regarding to the literature. It was mentioned in the previews chapter that the doubling time for *M. roseus* under optimum anaerobic conditions is 2.5 hours, and for *C. thermocellum* 6.5 hours (Freier et al., 1988). With greater values than 0.5 the OD results overestimation. Therefore, as it can be seen the microbial growth occurs on the exponential phase when OD is in the range of 0.4-0.5.

The following figures depict the OD measurements for two *C. thermothellum* media samples. On the left is the first measurement of receiving DSMZ culture and on the right is the OD progress of next generation cultivation microbes in order to inject in the AD system.

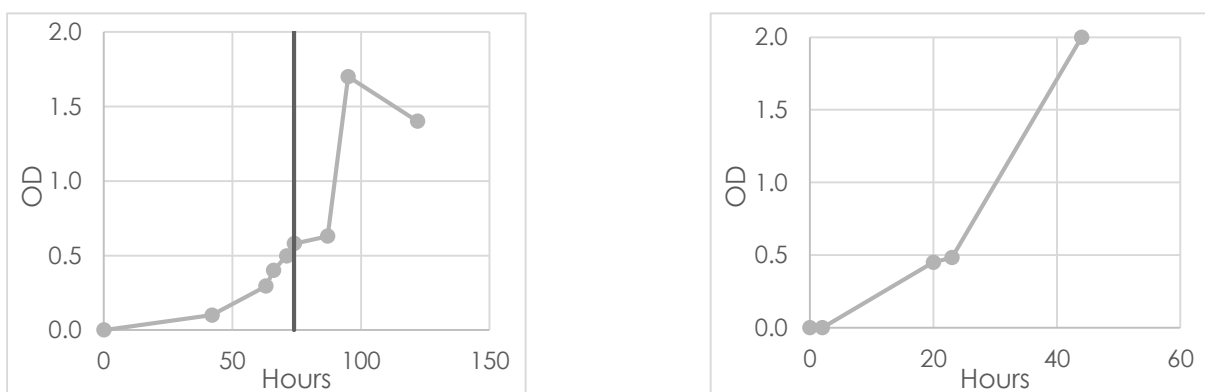


Figure 4.11 OD figures

4.2.1 BMP Bioaugmentation

In the following series of experiments, the effect of bioaugmentation with hydrolytic bacteria on the mono-digestion of wheat straw under batch assays was investigated. The first group of triplicates contained AD inoculum, and wheat straw as substrate with OL 1.5 gVS/L. In the following, (Figure 4.12) the comparison between untreated and bioaugmented samples, is illustrated. More precisely, due to the usage of *C. thermocellum* it is clear that the methane yield soared up from the first days. This steep rise of methane yield reached 305 ml CH₄/gVS, and comparatively with the untreated wheat straw which indicated 225 ml CH₄/gVS, the positive difference was up to 68.9%. The blue curve which represents *M. roseus* traces that it did not result good early adaptation to the system under specific conditions and environment.

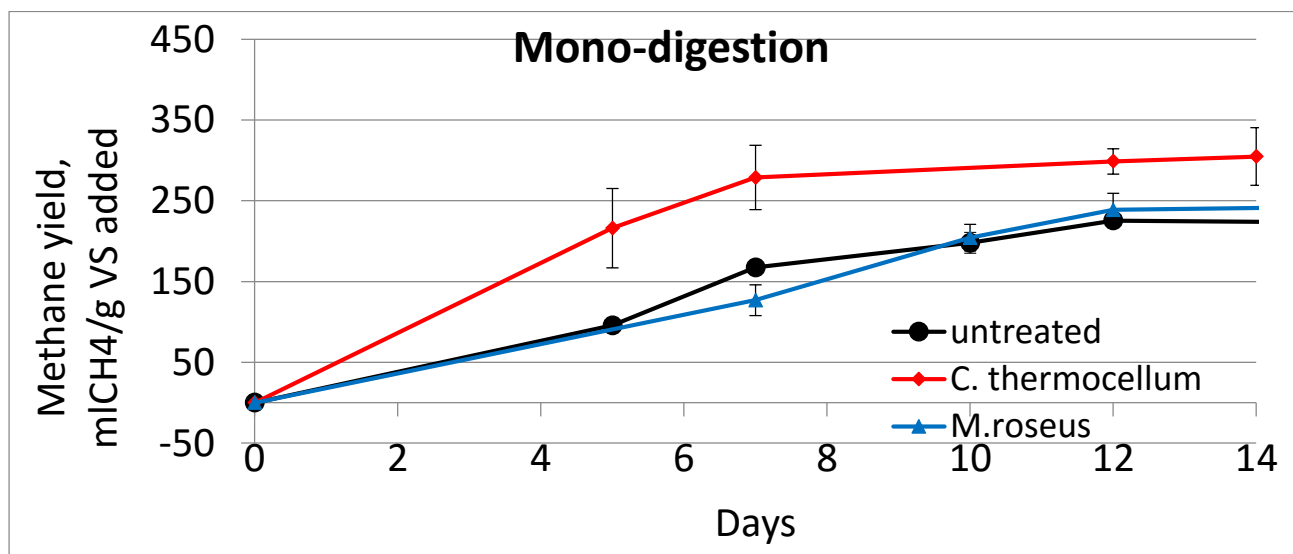


Figure 4.12 Methane yield for wheat straw mono-digestion

Co-digestion

In the present work as a premise to the CSTR experiment a set of BMP tests was conducted in order to have a picture of co-digesting cattle manure with wheat straw under bioaugmentation.

The Figure 4.13 below outlines the different methane production between the “infected” microbial anaerobic systems. It is conceivable that in early days *M. roseus* indicated a long lag phase and consequently it overtook the

biological “malfunction” one week later its implementation. In the 6th day methane yield was higher than the untreated samples. Similarly to the mono-digestion experiment of wheat straw bioaugmented with *M. roseus*, the same lag phase was observed as the methane production of the inoculated sample was higher after 6th day.

Nevertheless, *C. thermocellum* indicated greater methane yield in every phase of methane production, but slower in comparison with the mono-digestion curve.

Overall, the methane potential was 353, 418, and 377 ml CH₄/gVS, for untreated, *C. thermocellum* and *M. roseus* respectively. Thus, the final methane production enhancement for *M. roseus* and *C. thermocellum* were 7% and 18%, respectively.

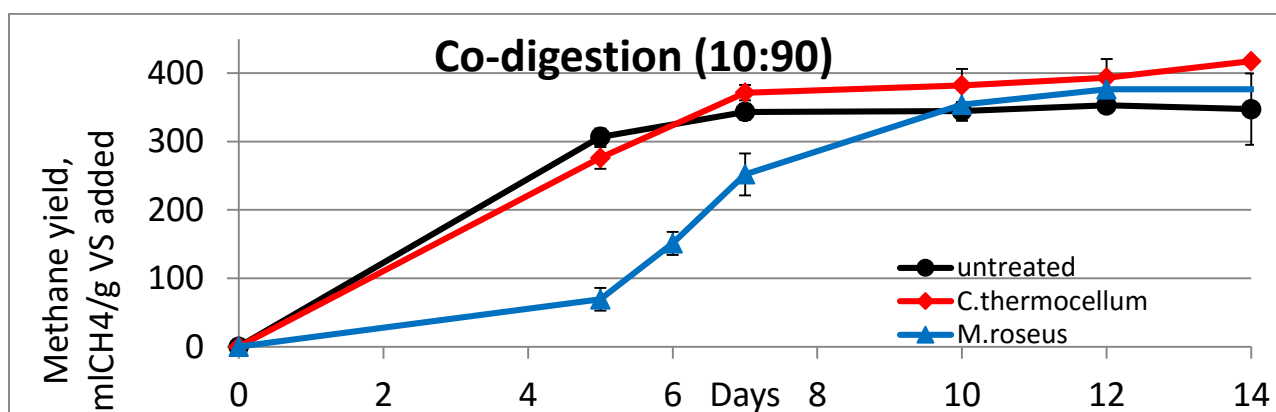


Figure 4.13 Methane yield for co-digestion experiment of 10% share of wheat straw and 90% of cattle manure on VS basis

Overall, it is noteworthy that in comparison with mono-digestion test, the enhancement of co-digestion with cattle manure was lower even with the same initial OL. This could be explained due to the cellulose rich wheat straw which was hydrolysed intensively by injected *C. thermocellum*, hence *C. thermocellum* was led to the lignin disruption and cellulose degradation. Hereafter, it could be assumed that *C. thermocellum* is a very promising microorganism for lignocellulosic degradation.

Aggregated results from BMP Bioaugmentation experiments

In figure 4.14 the first 3 bars illustrate the practical methane production of untreated wheat straw, *C. thermocellum* bioaugmentation and *M. roseus* bioaugmentation using wheat straw as substrate. Additionally the next three bars represent the practical methane production of wheat straw co-digested with cattle manure (VS_{share}: 10:90) compared to the bioaugmentation with the aforementioned hydrolytic bacteria.

However, for the second triplicate of bars, co-digestion bioaugmented with *C. thermocellum* indicated lower enhancement in comparison with wheat straw mono-digestion, enhancing the process by 18% instead of 35%. On the other hand, the same triplicate showed increased overall methane production in contrast to the first triplicate of bars. More specifically for untreated, the range between mono and co-digestion was from 225 to 305 ml CH₄/gVS, for *C. thermocellum* from 305 to 418 ml CH₄/gVS, and for *M. roseus* from 252 to 377 ml CH₄/gVS.

Eventually, it is clearly observed that the overall methane yield for the second triplicate of bars was higher than the first, indicating firstly the higher potential that co-digestion offers. The increased methane production at the co-digestion experiment is explained mainly from two reasons: a) cattle manure did not contain hardly degradable lignocellulosic fibers and is also considered as a nutrient rich substrate for AD and b) wheat straw is more difficult and recalcitrant to AD due to the lignin-polymer matrix. However, as it was aforementioned the enhancement of *C. thermocellum* in mono-digesting lignocellulosic environment was richer than in co-digestion.

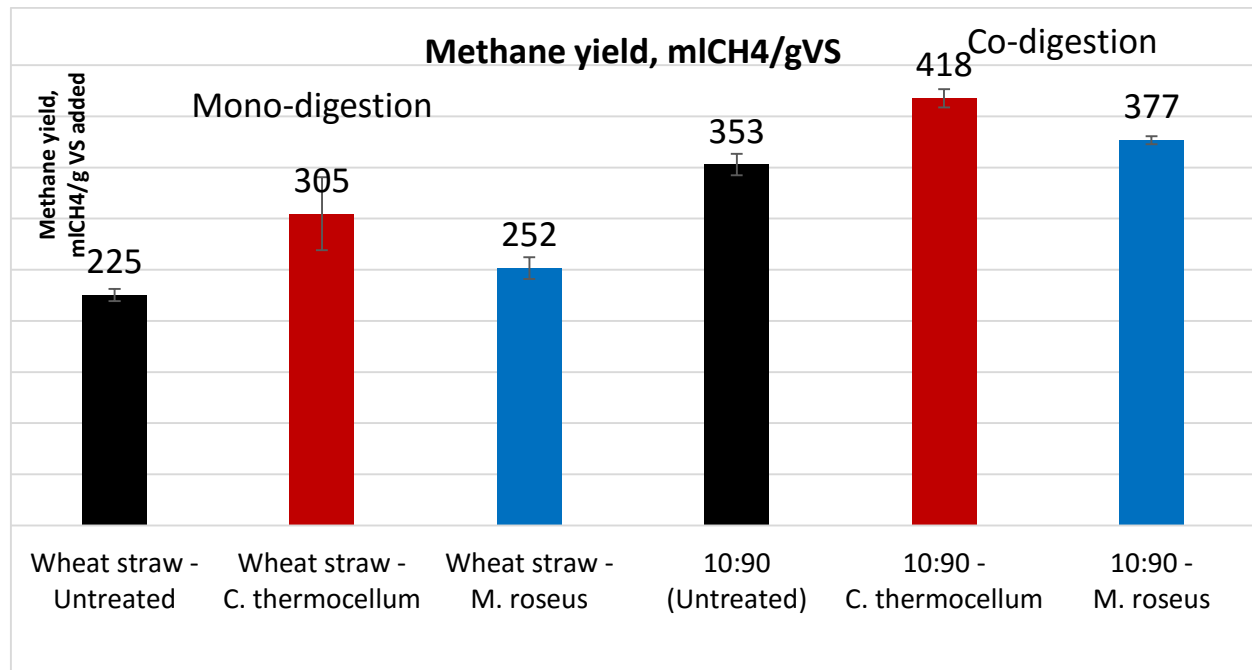


Figure 4.14 Aggregated methane potential for mono and co- digestion

Comparing to the theoretical methane potential which was calculated by Buswell's formula (443 ml CH₄/gVS), *C. thermocellum* showed a very similar value of 305 ml CH₄/gVS which indicates the overall exploitation of organic matter by the system. Lignin cannot be digested, thus biodegradability is lowered for every lignocellulosic substrate (Buffiere et al., 2006). Thus, the biodegradability of wheat straw in the bioaugmented habitat with *C. thermocellum* was 91.6%. On the other hand, *M. roseus* indicated much lower biodegradability reaching only 58%. However, both bacteria had a positive difference from untreated which indicated 50% biodegradability of wheat straw.

4.2.2 CSTR Bioaugmentation

After the set up for all three reactors, there was a big intermediate until they reached stationary conditions. Due to the normal progress of experiment, the indicating parameters such as VFA, pH was stable in normal levels during all periods. Biogas and methane showed different tendency due to the addition of recalcitrant lignocellulosic matter, wheat straw.

More precisely for the first period, when conditions were steady for more than 2 HRT, all three reactors maintained the same biogas yield and methane yield about to 351 ml biogas/gVS and 256 ml CH₄/gVS, respectively. At day 62, the same amount of wheat straw was added in each influent for all reactors, and the second period of experiment occurred until the injection of culture in day 107. For the second period there was a slight decline in biogas and methane results. More precisely, the overall methane yield was 236 mlCH₄/gVS, about 8.5% lower than the first period. The results for the bioaugmentation period are presented in the next for each reactor separately, and aggregated at the end of the chapter.

4.2.2.1 Reactor 1 (R1)- Abiotic bioaugmentation (control)

VFA and pH Progress

Total VFA maintained in very low levels during all the experimental period. More precisely propionate did not exceed 0.1 g/L and acetate did not exceed the upper unknown threshold. The upper concentration of VFAs that a digester could afford depends from the buffering capacity of the reactor. In the present work cattle manure has great capacity preventing any acidic irritation. As it seems the total amount of produced VFAs cascade with the amount of acetate, and the average production of TVFA was 0.4 g/L. Hence, during all three periods there was not any inhibition caused by acidic effect. In the same line, pH values were fluctuating in a very narrow range from 7.8 to 8.2 during all the period. As the figure 4.15 depicts below, the process for Reactor 1, abiotic augmented reactor, maintained in normal levels.

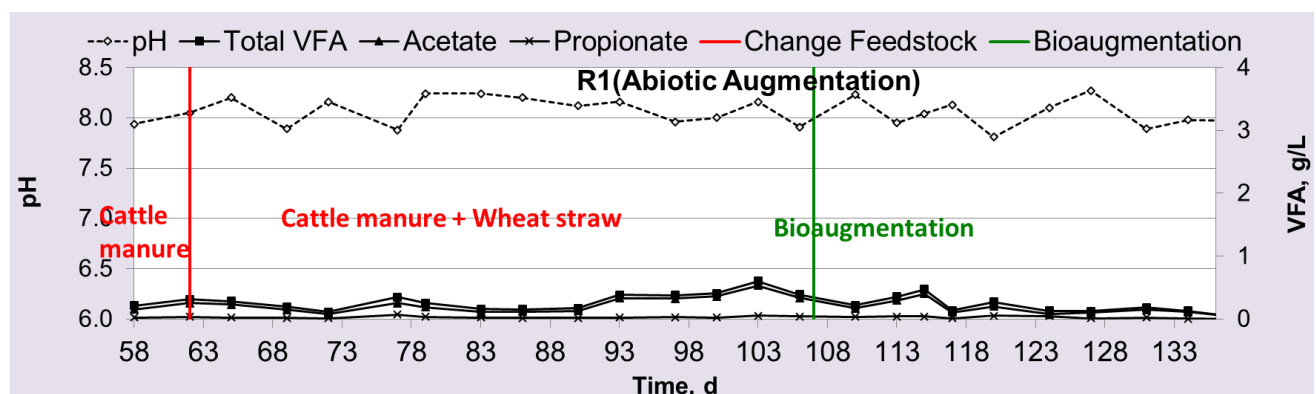


Figure 4.15 pH and VFA at the control reactor R1

Methane Generation

Initially, during the six days that abiotic augmentation took place by injecting BA media, the biogas yield slightly dropped, nevertheless, in the next days it returned properly to the previous generation values. As it was shown, in the first HRT after the injection of BA media, methane yield had a tendency of dropping from 228 ml CH₄/gVS to 202 ml CH₄/gVS (i.e. 12% decrease), and afterwards increasing successively to normal values of approximately 244 ml CH₄/gVS.

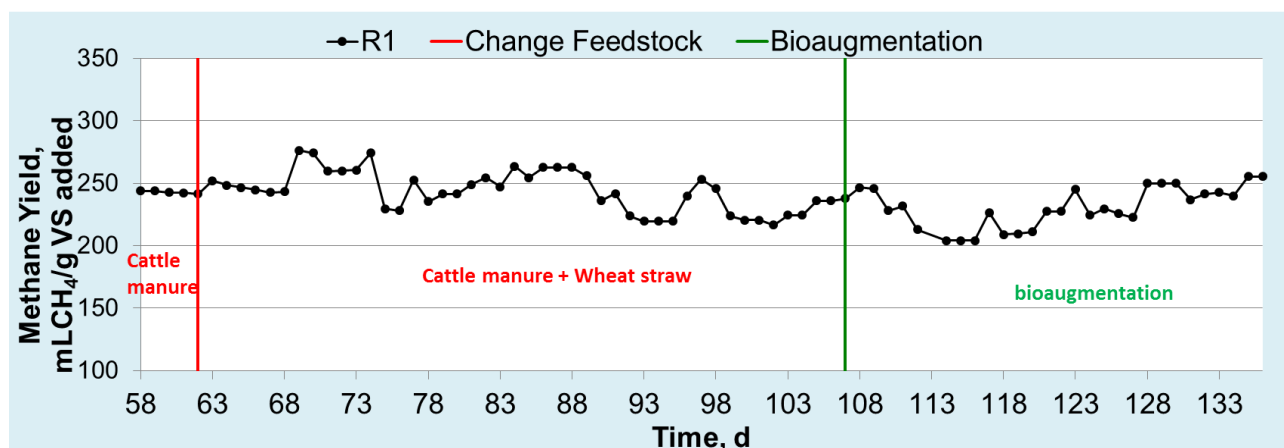


Figure 4.16 Methane Yield at the control reactor-R1

4.2.2.2 Reactor (R2)- Bioaugmentation with *C. thermocellum*

VFA and pH Progress

For the second reactor the total VFA maintained in low amounts despite the rapid hydrolysis from the injected *C. thermocellum*. It could be assumed that beyond the effective disruption of lignocellulosic matrix, the carbohydrates digestion that was followed was remarkable. Thus, the accelerated consumption of acetate from methanogens designated the enhancement of methane and biogas yield.

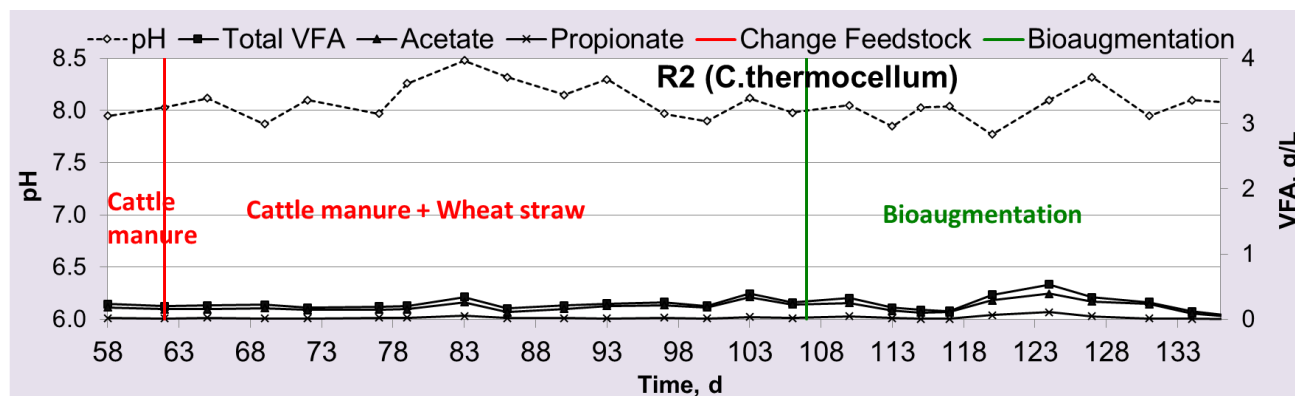


Figure 4.17 pH and VFA at R2 bioaugmented with *C. thermocellum*

Methane Generation

In the following figure 4.18, methane yield is depicted during all the entire AD process, and results for reactor 2 are illustrated besides to reactors 1 (control reactor) in order to correlate the tendency and efficiency of bioaugmentation.

The methane production for the first days of routine bioaugmentation rose remarkably, obtaining the highest methane yield from 234 ml CH₄/gVS reaching 298 ml CH₄/gVS, enhancing the process by +27.5%. The average methane yield during the six days of bioaugmentation was 269 mlCH₄/gVS (i.e. +15.1%). As far as it concerns, the accumulated, undigested, wheat straw into the reactor was degraded by injected *C. thermocellum* leading to an increased methane production during the first days after the bioaugmentation period. Moreover, as it was described in the previous chapter, the presence of nutrients with the injection of media, was considered and the resulted excess of methane yield was removed from the results.

After approximately one HRT the process was stabilized at 253 ml CH₄/gVS indicating a constant enhancement about +8.4 % comparing with the previous co-digestion period before bioaugmentation.

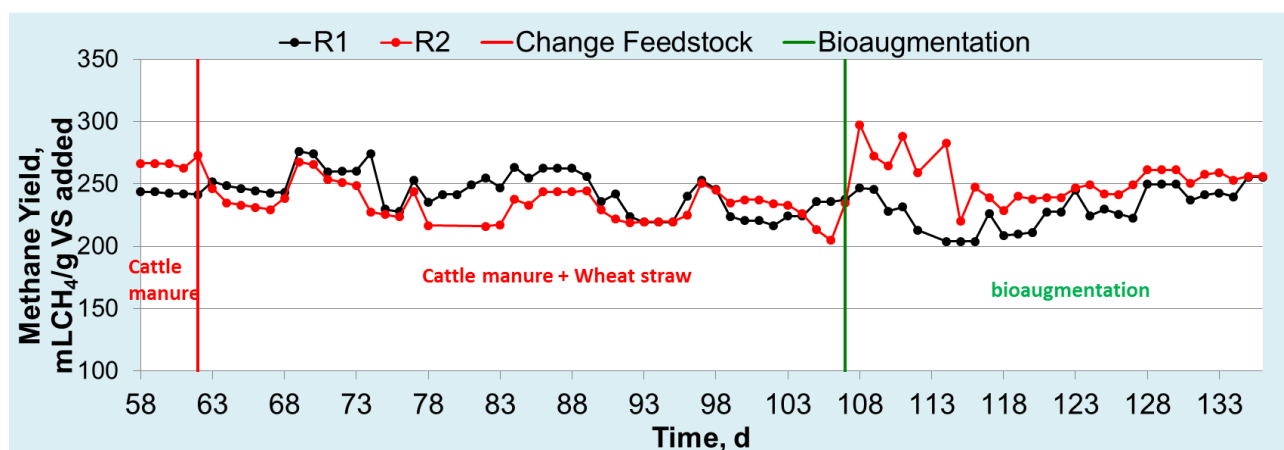


Figure 4.18 Methane Yield at R2 bioaugmented with *C. thermocellum*

By comparison with the control reactor (R1), the second reactor obtained a significant increase of methane and biogas production, until the end of the experiment. The constant, significant difference of yield compared with the previous period and with the control reactor, reflected the adequate adaptation of *C. thermocellum* in the system, avoiding the wash out. As results designated wheat straw was efficiently digested despite the high lignin proportion (about 26%/ gTS_{wheat straw}).

4.2.2.3. Reactor 3 (R3) Bioaugmentation with *M. roseus*

VFA and pH Progress

Regarding the third reactor, the determined amount of total VFAs was stable in low levels, approximately, 0.4 g/L, and pH was fluctuating between 7.8 and 8.2 during all the periods. Thus, the extracted conclusions provided a trustworthy process.

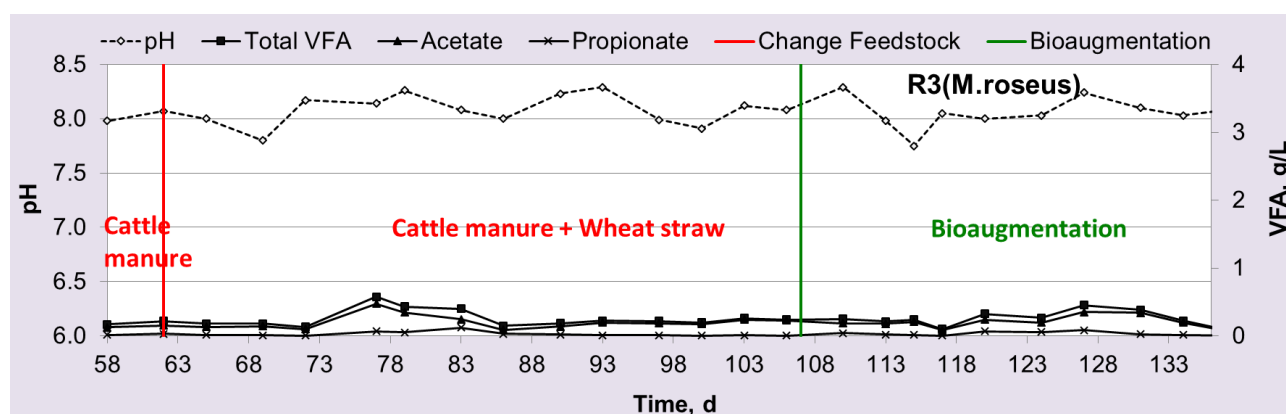


Figure 4.19 pH and VFA at R3 bio-augmented with *M. roseus*

Methane Generation

In the following figure, methane yield is depicted during all the process. Hence, results for reactor 3 are illustrated beside to reactor 1 (control reactor) in order to correlate the tendency and efficiency of bioaugmentation.

As illustrated in the figure 4.20, during day 113, methane yield rose from 232 ml CH₄/gVS to 248 ml CH₄/gVS indicating a maximum increase approximately to 6,8%. Thereafter, as was shown the *M. roseus* trend (blue trend) fell rapidly, even lower than control reactor for the bioaugmentation period of day 114 to day 118. However, after the 5-day lag phase, the hollow curve of the methane yield rose gradually until day 125 when the process was stabilized, 1.2% higher than previous period. Additionally, biogas yield decreased on day 114 until the day 118, even lower from the control reactor. Overall, this tendency could reflect competences leading to a slight inhibition of the process which was expressed by a long lag phase. It could be assumed that the decline was developed due to the competence of different enzymes which latch on the surface of lignocellulosic substrate (Converse & Optekar, 1993). Moreover, due to the fact that *M. roseus* thrives in very strict environment, approximately 3000 meter below the earth surface, it could be hypothesised that, initially, it was not colonized adequately due to competences with the overall consortia of microbes.

As the preliminary BMP experiment indicated, *M. roseus* experienced a 5-day lag phase ; in the same line, for CSTR experiment, after one week the injected bacterium adapted in the habitat providing a marginally enhanced tendency. Thus, it is clearly observed that *M. roseus* could not acclimatize adequately in the AD system of cattle manure and wheat straw. Nevertheless this outcome was expected due to the preliminary BMP results, for which *M. roseus* reflected a minor 17% enhancement.

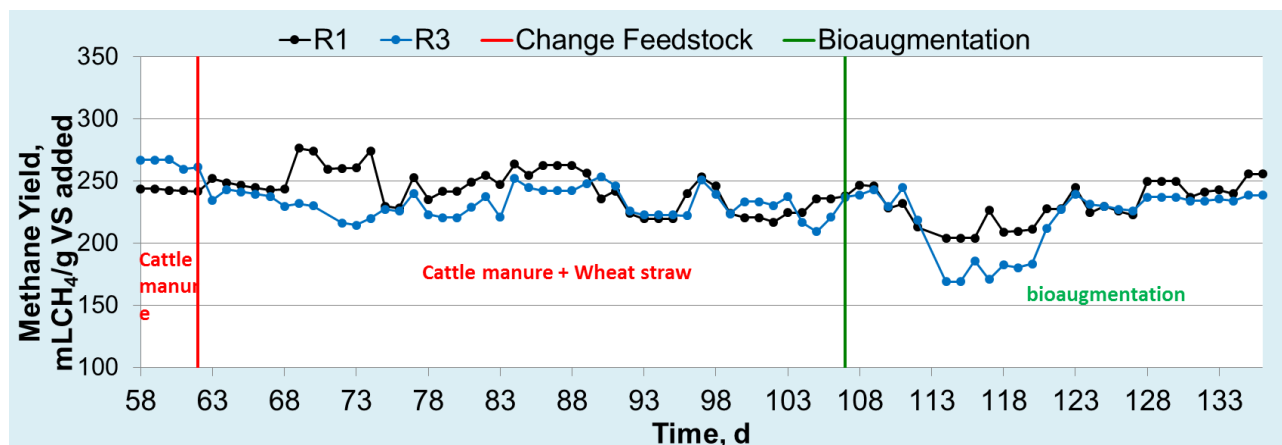


Figure 4.20 Methane yield at R3 bio-augmented with *M. roseus*

Aggregated results for CSTR bioaugmentation

The extracted conclusions about the VFA and pH results commonly indicated the expected buffering capacity that cattle manure provides in the habitat. In this way, all pH and VFA figures demonstrated the slight fluctuation in a very narrow range. For pH all reactors were fluctuating between 7.8 and 8.2 and total VFA between 0.2 and 0.8 g/L and the average was 0.4 g/L.

In the following Figure 4.21 all three curves of methane yield are presented in order to provide valuable conclusions. Firstly, the differences between first and second period reflected obviously the overall slight decrease of biogas and methane yield. Apparently, this depletion was expected due to the injection of stiff untreated lignocellulosic biomass in the reactor which could not be disrupted and digested from the pre-existing microbial consortia. Thereafter, when the system was steady for 1-2 HRTs the third period started with the bioaugmentation. For all three digesters the inoculation with BA media (R1), *C. thermocellum* media (R2) and *M. roseus* media (R3) provided valuable results.

As it is clearly shown in the Figure 4.21, *C. thermocellum* did not follow lag phase, instead it was adjusted perfectly in the lignocellulosic thermophilic environment, soaring the yield from the first days in extremely high values. In comparison, *M. roseus* reactor dropped beyond all, reflecting a significant lag phase, as preliminary BMP experiment showed, because of the initial abnormality. Nevertheless, after 15 days (1 HRT), all three digesters were stabilized in normal levels providing for the next month the clear long term effect. Beyond others, *C. thermocellum* obtained a significant increase of 10.4% above control reactor and 8.4%

above the pre-augmented period. In opposition *M. roseus* was stabilized slightly higher, about 1.2% increased than the previous period.

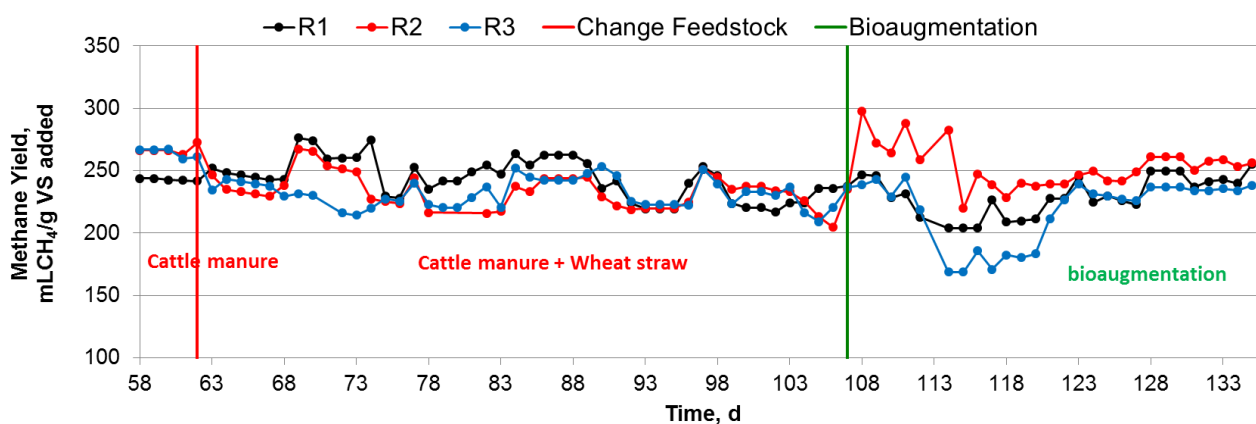


Figure 4.21 Methane yield for all three digesters

Complementary discussion on bioaugmentation results in comparison with literature

Nevertheless, it would be noteworthy to correlate the present results with the results that other researchers have obtained in order to interpret further the results.

Firstly, a critical parameter that characterizes the bioaugmented system is the critical amount ("critical biomass") of injected microbes in order to avoid the wash out from the digester (Fotidis et al., 2013). This parameter in combination with the optimum environmental conditions for the injected microbe would set the real success of treatment in time. In the present work, in order to prevent the hasty wash out, stirring was stopped for one hour before and after the injection, and additionally 100 ml were collected from the working volume of reactor, just the same amount of injected pure culture (100 ml). Fotidis et al. (2013) bioaugmented routinely for 6 days hydrogenotrophic methanogens in a lab scale CSTR system in order to observe the alleviation of ammonia inhibition. In the same line with the present work the methane yield decreased in the control reactor for the first days due to the abiotic injection.

Furthermore, the rapid production of *C. thermocellum* could be explained due to the synergies with the ambient consortia of microbes in the digester. More precisely, *C. thermocellum*, as it was aforementioned in the first chapter, favours hexose sugars, nevertheless, when it is in the same environment with another thermophilic anaerobic hydrolytic bacterium such as *Thermoanaerobacterium saccharolyticum*, it develops synergistic and syntrophic action (Wiegel, 1980). Hence, lignocellulosic matter was degraded by cellulosomes into 5-carbon and 6-carbon sugars, which were consequently further degraded with high efficiency to fermentation products such as acetate and lactate. Indeed, this synergy which was investigated by Herrero et al. (1985) and it was unflavoured for their results, because it covers the ethanol production due to the

remarkable acetate and lactate generation. Nevertheless, in the present work any microbial analysis for reactors, has been conducted in order to justify.

The initial steep increase of biogas yield during the first days of *C. thermocellum* injection could be explained also by the surplus of difficulty digested biomass in the reactor. Additionally, as [Ander et al. \(1990\)](#) proposed, the easily degrading cellulose molecules in a habitat would boost the delignification process. Moreover, [\(Li et al., 2015\)](#) injected cultivations of *Clostridium cellulolyticum* in BMP batches with wheat straw AD inoculum and resulted higher methane potential. More precisely the team conducted 2 series of experiments in order to see how the injected hydrolytic bacteria acclimatizes in the system with low OL and high OL, giving promising results. Furthermore, [Zhang et al. \(2015\)](#) implemented fermentative bacteria instead of hydrolytic microbes. In particular *Acetobacteroides hydrogenigenes* in maize straw, resulted an increase of biogas potential up to 23%. *Caldicellusiruptor lactoaceticus* was injected in the first stage of two-stage digesters at 68°C, resulting biodegradability approximately 91%. For [Nielsen et al. \(2007\)](#) the bioaugmentation did not achieve significant difference in the total methane yield at the end of one HRT. Eventually this is the most appreciable parameter, because it indicates the long term effect in the digester. The short effect reflects the wash out of the microbe from the reactor.

Additionally, [Martin-Ryals et al. \(2015\)](#) bioaugmented the first digester of two-phase continuous system with hydrolytic *Clostridium sp.* gene. The feedstock was consisted by manure and maize and in comparison with the present work, the acetic acid was increased due to the enhanced hydrolysis. However it is rational to get increased in the two stage reactor, in opposition to the one stage reactor in which acetate was consumed subsequently right after the production. In a two stage CSTR experiment, routine bioaugmentation achieved significantly greater soluble chemical oxygen demand (sCOD) generation (+25%) and methane production (+15%) compared to one-time bioaugmentation. In a continuous bench-scale system, routine bioaugmentation increased acid-phase sCOD by the range of 29–68% and acetic acid concentrations by 31–34%. Boosted hydrolysis and acetogenesis subsequently led to sustained increase in methane production (+56%) compared to non-bioaugmented system [\(Martin-Ryals et al., 2015\)](#).

Unequivocally, literature lacks of feedback for the present work. This is due to the variation of selected substrates or the combination of treatments, even due to the different target AD steps, which could be hydrolysis such as in our case, or methanogenesis. Eventually, the extracted results could give a promising feedback for the further application of the process in the future, even for industrial full-scale digesters.

CHAPTER 5: CONCLUSIONS –Interpretation

The main purpose in the present work was to obtain the adequate exploitation of a lignocellulosic matter such as wheat straw. The process that was used for the treatment and utilization of wheat straw was AD. It is well known that hydrolysis is the rate limiting step during the intricate procedure of AD when recalcitrant biomass is implemented. Therefore, the system was adjusted in order to accelerate hydrolysis and enhance the methane production. After the implementation of two methods for first time in the specific system, promising results were escalated.

For the microaeration method, series of batch experiments were performed in order to observe the methane potential of wheat straw combined with compost in an AD system. After microaeration (1,2 or 3 days), VFA and solubility increased, in contrary with pH which was decreased. Furthermore, all methane yield charts illustrated longer lag phase for the first week except for the 10 ml O₂/gVS samples which converged with untreated wheat straw.

At the end of experiment, the injection of trace amount of oxygen showed an increase of methane potential up to 7.6%, reflecting 62.3% biodegradability. Both 5 ml O₂/gVS per day and 10 ml O₂/gVS per day indicated a similar tendency to enhance slightly the methane potential in closed batch assays, by contrast with the 15 ml O₂/gVS per day which obtained a lower methane yield during the degradation of wheat straw, indicating the marginally inhibition by oxygen. Additionally, the adequate decrease of COD and VFA confirmed that microaeration could lead to disruption of lignocellulose matrix without severely inhibiting methanogens. Overall, microaeration could be considered as a reliable treating method for low concentrations of oxygen such as 5, 10 ml O₂/gVS per day.

For the BMP experiment on bioaugmentation, the remarkable increased methane potential was clear for the *C. thermocellum* resulting +85% of methane yield for lignocellulosic environment (mono-digestion). Conversely, *M. roseus* marginally acclimatized in the system indicating similar tendency for both mono digesting and co-digesting environments, by result the maximum methane yield was +17%. The aforementioned experiment was conducted as a premise work, giving promising results for further application in a continuous lab-scale CSTR system.

Thereafter, for the further investigation of tendency and massive adaptation in a continuous system with co – digestion of wheat straw and cattle manure, bioaugmentation was implemented in 3 CSTR digesters. Bioaugmentation resulted significant increase in biogas production for injected *C. thermocellum* under strict anaerobic conditions. Instantly few hours after the injection, the biogas production was soared from 1440 ml biogas/ day to approximately 2500 ml biogas/day, while methane concentration was fallen in 68% from 72%. This led to the maximum enhancement of methane yield about to 27.3% in the second day after bioaugmentation. Further on after 1 HRT, during steady state conditions methane enhancement was maintained at 8.4%. On the other hand *M. roseus* did not obtain with such good results as *C. thermocellum*, but instead is led to the minor steady enhancement of 1.2% after 1 HRT. Finally, it could be said that thermocellum is an appropriate hydrolytic bacterium to select in order to make biogas production rapid and seems a better alternative to boost the digestion of lignocellulosic substrate even in real biogas plant digester.

Future work

According to literature *M.roseus* is a facultative microbe and it favours aerobic environment despite the fact that it thrives in anaerobic sediments in nature (Podosokorskaya et al., 2013). The significant advantage is that it grows under both aerobic and anaerobic environment, which makes it very appropriate microorganism for microaeration. Hence, in order to exploit the aerobic abilities of *M. roseus*, the system could be boosted by the injection of minor amount of oxygen (i.e. microaeration).

Therefore, on the next, the combined bioaugmentation and microaeration is being tested. After the implementation of routine bioaugmentation and microaeration, the preliminary results were promising. More precisely, after the expected 1-week lag phase, which *M. roseus* repeated, biogas yield is rising gradually. However, the most promising result until now is that, as it seems, *M. roseus* could improve the AD process leading to biogas production with higher methane percentage. Although, the results and conclusions could not be extracted and interpreted yet, due to the fact that this experiment is still in progress.

Nevertheless, if the combined treatment in the 1-phase system fails then it could be implemented in a two-phase reactor system. More precisely microaeration together with the bioaugmentation could be implemented in the first, acidic reactor in order to prevent the inhibition of methanogens by oxygen or even to the possible acidification. Thus, the first acidic reactor would enhance hydrolysis and the second would utilize the produced nutrients. The feedstock would be the same with the CSTR system, cattle manure and wheat straw. Additionally, in order to let facultative microbes utilise all the amount of injected O₂ in the continuous system, a small adjustment would be implemented, adding gas phase recirculation.

Moreover, the remarkable ability of *C. thermocellum* disrupting adequately wheat straw, gave the evidence that even under higher OLR the same amount of injected pure culture would maintain the great utilisation of wheat straw. Therefore, the next step, a higher OLR would be examined by increasing the amount of injected wheat straw in the system. However, it should be more proper to use a two-stage CSTR system in order to separate the acidic phase, in order to achieve further enhancement.



REFERENCES

- Alves, M. M., Mota Vieira, J. A., Alvares Pereira, R. M., Pereira, M. A., & Mota, M. (2001). Effect of lipids and oleic acid on biomass development in anaerobic fixed-bed reactors. Part I: Biofilm growth and activity. *Water Research*, 35(1), 255–263. [http://doi.org/10.1016/S0043-1354\(00\)00241-4](http://doi.org/10.1016/S0043-1354(00)00241-4)
- American Public Health Association, American Water Works Association, & Water Environment Federation. (1999). Standard Methods for the Examination of Water and Wastewater. *Standard Methods*, 541.
- Ander, P., Mishra, C., Farrell, R. L., & Eriksson, K. L. (1990). Redox reactions in lignin degradation : interactions between laccase , different peroxidases and cellobiose : quinone oxidoreductase, 13, 189–198.
- Anderson, G. K., Sallis, P. J., & Uyanik, S. (2003). *Anaerobic treatment processes. The Handbook of Water and Wastewater Microbiology*.
- Angelidaki, I., & Ahring, B. K. (1993). Thermophilic anaerobic digestion of livestock waste: the effect of ammonia. *Applied Microbiology and Biotechnology*, 38(4), 560–564. <http://doi.org/10.1007/BF00242955>
- Angelidaki, I., & Ahring, B. K. (2000). Methods for increasing the biogas potential from the recalcitrant organic matter contained in manure. *Water Science and Technology*, 41(3), 189–194. Retrieved from <http://www.scopus.com/inward/record.url?eid=2-s2.0-0034057947&partnerID=tZOTx3y1>
- Angelidaki, I., Alves, M., Bolzonella, D., Borzacconi, L., Campos, J. L., Guwy, A. J., ... Lier, J. B. Van. (2009). Defining the biomethane potential (BMP) of solid organic wastes and energy crops : a proposed protocol for batch assays, 927–934. <http://doi.org/10.2166/wst.2009.040>
- Angelidaki, I., Alves, M., Bolzonella, D., Borzacconi, L., Campos, J. L., Guwy, A. J., ... Van Lier, J. B. (2009). Defining the biomethane potential (BMP) of solid organic wastes and energy crops: A proposed protocol for batch assays. *Water Science and Technology*, 59(5), 927–934. <http://doi.org/10.2166/wst.2009.040>
- Angelidaki, I., & Ellegaard, L. (2003). Codigestion of manure and organic wastes in centralized biogas plants: status and future trends. *Applied Biochemistry and Biotechnology*, 109(1-3), 95–105. <http://doi.org/10.1385/ABAB:109:1-3:95>
- Angelidaki, I., & Sanders, W. (2004). Assessment of the anaerobic biodegradability of macropollutants, 117–129.
- Bergland, W., Dinamarca, C., & Bakke, R. (2014). Effects of psychrophilic storage on manures as substrate for anaerobic digestion. *BioMed Research International*, 2014. <http://doi.org/10.1155/2014/712197>
- Blumer-schuetz, S. E., Brown, S. D., Sander, K. B., Bayer, E. A., Kataeva, I., Zurawski, J. V, ... Kelly, R. M. (2013). Thermophilic lignocellulose deconstruction. <http://doi.org/10.1111/1574-6976.12044>
- Boe, K. (n.d.). *Online monitoring and control of the biogas process*.
- Boe, K., Batstone, D. J., Steyer, J.-P., & Angelidaki, I. (2010). State indicators for monitoring the anaerobic digestion process. *Water Research*, 44(20), 5973–5980. <http://doi.org/10.1016/j.watres.2010.07.043>
- Botheju, D. (2010). Oxygen Effects in Anaerobic Digestion, 30(4), 191–201. <http://doi.org/10.4173/mic.2009.4.1>
- Botheju, D., & Bakke, R. (2011). Oxygen Effects in Anaerobic Digestion – A Review, (3901), 1–19.
- Botheju, D., Lie, B., & Bakke, R. (2010). Oxygen effects in anaerobic digestion - II. *Modeling, Identification and Control*, 31(2), 55–65. <http://doi.org/10.4173/mic.2010.2.2>
- Botheju, D., Samarakoon, G., Chen, C., & Bakke, R. (2010). An Experimental Study on the Effects of Oxygen in Bio-gasification – Part 2.
- Bruni, E., Peter, A., & Angelidaki, I. (2010). Bioresource Technology Comparative study of mechanical , hydrothermal , chemical and enzymatic treatments of digested biofibers to improve biogas production. *Bioresource Technology*, 101(22), 8713–8717. <http://doi.org/10.1016/j.biortech.2010.06.108>

- Buffiere, P., Loisel, D., Bernet, N., & Delgenes, J. P. (2006). Towards new indicators for the prediction of solid waste anaerobic digestion properties. *Water Science and Technology*, 53(8), 233–241. <http://doi.org/10.2166/wst.2006.254>
- Campanaro, S., Treu, L., Kougias, P. G., De Francisci, D., Valle, G., & Angelidaki, I. (2016). Metagenomic analysis and functional characterization of the biogas microbiome using high throughput shotgun sequencing and a novel binning strategy. *Biotechnology for Biofuels*, 9(1), 26. <http://doi.org/10.1186/s13068-016-0441-1>
- Chae, K. J., Jang, A., Yim, S. K., & Kim, I. S. (2008). The effects of digestion temperature and temperature shock on the biogas yields from the mesophilic anaerobic digestion of swine manure. *Bioresource Technology*, 99(1), 1–6. <http://doi.org/10.1016/j.biortech.2006.11.063>
- Chandra, R., Takeuchi, H., & Hasegawa, T. (2012). Methane production from lignocellulosic agricultural crop wastes: A review in context to second generation of biofuel production. *Renewable and Sustainable Energy Reviews*, 16(3), 1462–1476. <http://doi.org/10.1016/j.rser.2011.11.035>
- Charles, W., & Walker, L. (2009). Bioresource Technology Effect of pre-aeration and inoculum on the start-up of batch thermophilic anaerobic digestion of municipal solid waste. *Bioresource Technology*, 100(8), 2329–2335. <http://doi.org/10.1016/j.biortech.2008.11.051>
- Chen, H. (2014). *Biotechnology of Lignocellulose*. <http://doi.org/10.1007/978-94-007-6898-7>
- Chen, Y., Cheng, J. J., & Creamer, K. S. (2008). Inhibition of anaerobic digestion process : A review, 99, 4044–4064. <http://doi.org/10.1016/j.biortech.2007.01.057>
- Clarkson, W. W., & Xiao, W. (2000). Bench-scale anaerobic bioconversion of newsprint and office paper. *Water Science and Technology*, 41(3), 93–100.
- Converse, A. O., & Optekar, J. D. (1993). A synergistic kinetics model for enzymatic cellulose hydrolysis compared to degree-of-synergism experimental results. *Biotechnology and Bioengineering*, 42(1), 145–148. <http://doi.org/10.1002/bit.260420120>
- D??az, I., Lopes, A. C., P??rez, S. I., & Fdz-Polanco, M. (2010). Performance evaluation of oxygen, air and nitrate for the microaerobic removal of hydrogen sulphide in biogas from sludge digestion. *Bioresource Technology*, 101(20), 7724–7730. <http://doi.org/10.1016/j.biortech.2010.04.062>
- Demain, A. L., Newcomb, M., & Wu, J. H. D. (2005). Cellulase, Clostridia, and Ethanol†, 69(1), 124–154. <http://doi.org/10.1128/MMBR.69.1.124>
- Dumas, C., Perez, S., Paul, E., & Lefebvre, X. (2010). Combined thermophilic aerobic process and conventional anaerobic digestion: Effect on sludge biodegradation and methane production. *Bioresource Technology*, 101(8), 2629–2636. <http://doi.org/10.1016/j.biortech.2009.10.065>
- Durrad, L. R. (1996). Biodegradation of Lignocellulosic Materials by Soil Fungi Isolated under Anaerobic Conditions *, 189–195.
- Eastman, J. a, & Ferguson, J. F. (1981). Solubilization of particulate organic carbon during the acid phase of anaerobic digestion. *Journal of the Water Pollution Control Federation*, 53(3), 352–366. Retrieved from <http://www.scopus.com/inward/record.url?eid=2-s2.0-0019545815&partnerID=40&md5=f1a03ae0a5f5962e4a2870e7a28a3577>
- Eastman, J. a, & Ferguson, J. F. (1981). Solubilization organic phase of of carbon anaerobic particulate during the digestion acid. *Journal (Water Pollution Control Federation)*, 53(3), 352–366.
- Eriksson, K., & Pettersson, B. (1974). S-1 14 86, 49(2), 282–285.
- Eurostat. (2012). *Europe in Figures. State of the Union 2010*. <http://doi.org/10.2785/20539>
- Fotidis, I. A., Karakashev, D., Kotsopoulos, T. A., Martzopoulos, G. G., & Angelidaki, I. (2013). Effect of ammonium and acetate on methanogenic pathway and methanogenic community composition. *FEMS Microbiology Ecology*,

83(1), 38–48. <http://doi.org/10.1111/j.1574-6941.2012.01456.x>

- Freier, D., Mothershed, C. P., & Wiegel, J. (1988). Characterization of *Clostridium thermocellum* JW20. *Applied and Environmental Microbiology*, 54(1), 204–211.
- Fu, S. F., Wang, F., Yuan, X. Z., Yang, Z. M., Luo, S. J., Wang, C. S., & Guo, R. B. (2015). The thermophilic (55°C) microaerobic pretreatment of corn straw for anaerobic digestion. *Bioresource Technology*, 175, 203–208. <http://doi.org/10.1016/j.biortech.2014.10.072>
- Fu, S., Wang, F., Yuan, X., Yang, Z., & Luo, S. (2015). Bioresource Technology The thermophilic (55 ° C) microaerobic pretreatment of corn straw for anaerobic digestion. *BIORESOURCE TECHNOLOGY*, 175, 203–208. <http://doi.org/10.1016/j.biortech.2014.10.072>
- Gannoun, H., Othman, N. Ben, Bouallagui, H., & Moktar, H. (2007). Mesophilic and thermophilic anaerobic co-digestion of olive mill wastewaters and abattoir wastewaters in an upflow anaerobic filter. *Industrial and Engineering Chemistry Research*, 46(21), 6737–6743. <http://doi.org/10.1021/ie061676r>
- Glass, J. B., & Orphan, V. J. (2012). Trace metal requirements for microbial enzymes involved in the production and consumption of methane and nitrous oxide. *Frontiers in Microbiology*, 3(FEB), 1–20. <http://doi.org/10.3389/fmicb.2012.00061>
- Gold, N. D., & Martin, V. J. J. (2007). Global View of the *Clostridium thermocellum* Cellulosome Revealed by Quantitative Proteomic Analysis. *Journal of Biological Chemistry*, 282(19), 6787–6795. <http://doi.org/10.1074/jbc.M610082-07>
- Hansen, K. H., Angelidaki, I., & Ahring, B. K. (1998). Anaerobic digestion of swine manure: Inhibition by ammonia. *Water Research*, 32(1), 5–12. [http://doi.org/10.1016/S0043-1354\(97\)00201-7](http://doi.org/10.1016/S0043-1354(97)00201-7)
- Hatakka, A. (1983). o = ; Applied Microbiology and Biotechnology Pretreatment of Wheat Straw by White-Rot Fungi for Enzymic Saccharification of Cellulose, 350–357.
- Hegde, G., & Pullammanappallil, P. (2007). Comparison of thermophilic and mesophilic one-stage, batch, high-solids anaerobic digestion. *Environmental Technology*, 28(4), 361–369. <http://doi.org/10.1080/09593332808618797>
- Hendriks, A. T. W. M., & Zeeman, G. (2009). Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technology*, 100(1), 10–18. <http://doi.org/10.1016/j.biortech.2008.05.027>
- Herrero, M., & Stuckey, D. C. (2015). Chemosphere Bioaugmentation and its application in wastewater treatment : A review. *Chemosphere*, 140, 119–128. <http://doi.org/10.1016/j.chemosphere.2014.10.033>
- Hungate, R. E. (1969). Chapter IV A Roll Tube Method for Cultivation of Strict Anaerobes. *Methods in Microbiology*, 3(PART B), 117–132. [http://doi.org/10.1016/S0580-9517\(08\)70503-8](http://doi.org/10.1016/S0580-9517(08)70503-8)
- Jenicek, P., Koclik, F., Maca, J., & Bindzar, J. (2008). Use of microaerobic conditions for the improvement of anaerobic digestion of solid wastes. *Water Science and Technology*, 58(7), 1491–1496. <http://doi.org/10.2166/wst.2008.493>
- Johansen, J. E., & Bakke, R. (2006). Enhancing hydrolysis with microaeration. *Water Science and Technology*, 53(8), 43–50. <http://doi.org/10.2166/wst.2006.234>
- Kadnikov, V. V., Mardanov, A. V., Podosokorskaya, O. A., & Gavrilov, S. N. (2016). Kadnikov VV , Mardanov AV , Podosokorskaya OA , Gavrilov SN , Kublanov IV , Beletsky AV et al .. Genomic analysis of *Melioribacter roseus* , facultatively anaerobic organotrophic bacterium ... Genomic Analysis of *Melioribacter roseus* , Facultatively Anaero, (March). <http://doi.org/10.1371/journal.pone.0053047>
- Karakashev, D., Batstone, D. J., & Angelidaki, I. (2005). Influence of Environmental Conditions on Methanogenic Compositions in Anaerobic Biogas Reactors Influence of Environmental Conditions on Methanogenic Compositions in Anaerobic Biogas Reactors. *Applied and Environmental Microbiology*, 71(1), 331–338. <http://doi.org/10.1128/AEM.71.1.331>
- Kim, M., Ahn, Y. H., & Speece, R. E. (2002). Comparative process stability and efficiency of anaerobic digestion;

mesophilic vs. thermophilic. *Water Research*, 36(17), 4369–4385. [http://doi.org/10.1016/S0043-1354\(02\)00147-1](http://doi.org/10.1016/S0043-1354(02)00147-1)

- Kim, M., & Speece, R. E. (2002). Reactor configuration--Part II. Comparative process stability and efficiency of thermophilic anaerobic digestion. *Environmental Technology*, 23(6), 643–654. <http://doi.org/10.1080/09593332308618380>
- Kim, W., Hwang, K., Shin, S. G., Lee, S., & Hwang, S. (2010). Effect of high temperature on bacterial community dynamics in anaerobic acidogenesis using mesophilic sludge inoculum. *Bioresource Technology*, 101(SUPPL.1), S17–S22. <http://doi.org/10.1016/j.biortech.2009.03.029>
- Kinet, R., Destain, J., Hilgsmann, S., Thonart, P., Delhalle, L., Taminiau, B., ... Delvigne, F. (2015). Bioresource Technology Thermophilic and cellulolytic consortium isolated from composting plants improves anaerobic digestion of cellulosic biomass : Toward a microbial resource management approach. *BIORESOURCE TECHNOLOGY*, 189, 138–144. <http://doi.org/10.1016/j.biortech.2015.04.010>
- Klinke, H. B., Ahring, B. K., Schmidt, A. S., & Thomsen, A. B. (2002). Characterization of degradation products from alkaline wet oxidation of wheat straw. *Bioresource Technology*, 82(1), 15–26. [http://doi.org/10.1016/S0960-8524\(01\)00152-3](http://doi.org/10.1016/S0960-8524(01)00152-3)
- Kothari, V., Surti, P., & Kapadia, D. (2015). Cellulosomes- A robust machinery for cellulose degradation Cellulosomes - A Robust Machinery for Cellulose Degradation, (October).
- Kougias, P. G., De Francisci, D., Treu, L., Campanaro, S., & Angelidaki, I. (2014). Microbial analysis in biogas reactors suffering by foaming incidents. *Bioresource Technology*, 167, 24–32. <http://doi.org/10.1016/j.biortech.2014.05.080>
- Kruus, K., Wang, W. K., Ching, J., & Wu, J. H. D. (1995). Exoglucanase activities of the recombinant *Clostridium thermocellum* CelS, a major cellulosome component. *Journal of Bacteriology*, 177(6), 1641–1644.
- Lalman, J., & Bagley, D. M. (2002). Effects of C18 long chain fatty acids on glucose, butyrate and hydrogen degradation. *Water Research*, 36(13), 3307–3313. [http://doi.org/10.1016/S0043-1354\(02\)00014-3](http://doi.org/10.1016/S0043-1354(02)00014-3)
- Lamed, R., Setitser, E. V. A., & Bayer, E. A. (1983). Characterization of a Cellulose-Binding , Cellulase-Containing Complex in *Clostridium thermocellum*, 156(2), 828–836.
- Lansche, J. (2012). Life cycle assessment of energy generation of biogas fed combined heat and power plants : Environmental impact of different agricultural substrates, (3), 313–320. <http://doi.org/10.1002/elsc.201100061>
- Leit??o, R. C., Van Haandel, A. C., Zeeman, G., & Lettinga, G. (2006). The effects of operational and environmental variations on anaerobic wastewater treatment systems: A review. *Bioresource Technology*, 97(9), 1105–1118. <http://doi.org/10.1016/j.biortech.2004.12.007>
- Leonowicz, A., Matuszewska, A., Luterek, J., Ziegenhagen, D., Cho, N., Hofrichter, M., & Rogalski, J. (1999). Biodegradation of Lignin by White Rot Fungi, 185, 175–185.
- Li, A., Chu, Y., Wang, X., Ren, L., Yu, J., Liu, X., ... Li, S. (2013). A pyrosequencing-based metagenomic study of methane-producing microbial community in solid-state biogas reactor. *Biotechnology for Biofuels*, 6(1), 3. <http://doi.org/10.1186/1754-6834-6-3>
- Li, L., He, Q., Ma, Y., Wang, X., & Peng, X. (2015). Bioresource Technology Dynamics of microbial community in a mesophilic anaerobic digester treating food waste : Relationship between community structure and process stability. *Bioresource Technology*, 189, 113–120. <http://doi.org/10.1016/j.biortech.2015.04.015>
- Liu, L., & Chen, H. (2006). Enzymatic hydrolysis of cellulose materials treated with ionic liquid [BMIM]Cl. *Chinese Science Bulletin*, 51(20), 2432–2436. <http://doi.org/10.1007/s11434-006-2134-9>
- Lucas, R., Kuchenbuch, A., Fetzer, I., Harms, H., & Kleinstuber, S. (2015). Long-term monitoring reveals stable and remarkably similar microbial communities in parallel full-scale biogas reactors digesting energy crops. *FEMS Microbiology Ecology*, 91(3), 1–11. <http://doi.org/10.1093/femsec/fiv004>

- Lyberatos, G., Gavala, H. N., & Stamatelatou, A. (1997). An integrated approach for management of agricultural industries wastewaters. *Nonlinear Analysis: Theory, Methods & Applications*, 30(4), 2341–2351. [http://doi.org/10.1016/S0362-546X\(97\)00309-X](http://doi.org/10.1016/S0362-546X(97)00309-X)
- Lynd, L. R., Weimer, P. J., Zyl, W. H. Van, & Pretorius, I. S. (2002). Microbial Cellulose Utilization : Fundamentals and Biotechnology, 66(3), 506–577. <http://doi.org/10.1128/MMBR.66.3.506>
- Martin-ryals, A., Schideman, L., Li, P., Wilkinson, H., & Wagner, R. (2015). Bioresource Technology Improving anaerobic digestion of a cellulosic waste via routine bioaugmentation with cellulolytic microorganisms. *BIORESOURCE TECHNOLOGY*, 189, 62–70. <http://doi.org/10.1016/j.biortech.2015.03.069>
- Mata-álvarez, J. (2015). Anaerobic Digestion of Organic Solid Wastes . An Overview of Research Achievements and Perspectives, 74. [http://doi.org/10.1016/S0960-8524\(00\)00023-7](http://doi.org/10.1016/S0960-8524(00)00023-7)
- Mata-Alvarez, J., Macé, S., & Llabrés, P. (2000). Anaerobic digestion of organic solid wastes. An overview of research achievements and perspectives. *Bioresource Technology*, 74(1), 3–16. [http://doi.org/10.1016/S0960-8524\(00\)00023-7](http://doi.org/10.1016/S0960-8524(00)00023-7)
- Mccarty, P. L. (1964). Anaerobic Waste Treatment Fundamentals. *Public Works*, 95(9), 107–112.
- McKendry, P. (2002). Energy production from biomass (part 1): overview of biomass. *Bioresource Technology*, 83(1), 37–46. [http://doi.org/Pii S0960-8524\(01\)00118-3](http://doi.org/Pii S0960-8524(01)00118-3) [http://doi.org/10.1016/S0960-8524\(01\)00118-3](http://doi.org/10.1016/S0960-8524(01)00118-3)
- Miiller, H. W., & Trfisch, W. (1986). Applied Microbiology Biotechnology Screening of white-rot fungi for biological pretreatment of wheat straw for biogas production, 180–185.
- Mshandete, A., Bjo, L., Kivaisi, A. K., Rubindamayugi, S. T., & Mattiasson, B. (2005). Enhancement of anaerobic batch digestion of sisal pulp waste by mesophilic aerobic pre-treatment, 39, 1569–1575. <http://doi.org/10.1016/j.watres.2004.11.037>
- Mussatto, S. I., Fernandes, M., Milagres, A. M. F., & Roberto, C. (2008). Effect of hemicellulose and lignin on enzymatic hydrolysis of cellulose from brewer ' s spent grain, 43, 124–129. <http://doi.org/10.1016/j.enzmictec.2007.11.006>
- Nielsen, H. B., Mladenovska, Z., & Ahring, B. K. (2007). Bioaugmentation of a Two-Stage Thermophilic (68 - C / 55 - C) Anaerobic Digestion Concept for Improvement of the Methane Yield From Cattle Manure, 97(6), 1638–1643. <http://doi.org/10.1002/bit>
- Olson, D. G., McBride, J. E., Joe Shaw, A., & Lynd, L. R. (2012). Recent progress in consolidated bioprocessing. *Current Opinion in Biotechnology*, 23(3), 396–405. <http://doi.org/10.1016/j.copbio.2011.11.026>
- Owen, W. F., Stuckey, D. C., Healy, J. B., Young, L. Y., & McCarty, P. L. (1979). Bioassay for monitoring biochemical methane potential and anaerobic toxicity. *Water Research*, 13(6), 485–492. [http://doi.org/10.1016/0043-1354\(79\)90043-5](http://doi.org/10.1016/0043-1354(79)90043-5)
- Peters, V., Conrad, R., Peters, V., & Conrad, R. (1995). Methanogenic and other strictly anaerobic bacteria in desert soil and other oxic soils . These include : Methanogenic and Other Strictly Anaerobic Bacteria in Desert Soil and Other Oxic Soils, 61(4), 1673–1676.
- Podosokorskaya, O. A., Kadnikov, V. V, Gavrilov, S. N., Mardanov, A. V, Merkel, A. Y., Karnachuk, O. V, ... Kublanov, I. V. (2013). Characterization of Melioribacter roseus gen . nov ., sp . nov ., a novel facultatively anaerobic thermophilic cellulolytic bacterium from the class Ignavibacteria , Ignavibacteriae, 15, 1759–1771. <http://doi.org/10.1111/1462-2920.12067>
- Podosokorskaya, O. A., Kadnikov, V. V., Gavrilov, S. N., Mardanov, A. V., Merkel, A. Y., Karnachuk, O. V., ... Kublanov, I. V. (2013). Characterization of Melioribacter roseus gen. nov., sp. nov., a novel facultatively anaerobic thermophilic cellulolytic bacterium from the class Ignavibacteria, and a proposal of a novel bacterial phylum Ignavibacteriae. *Environmental Microbiology*, 15(6), 1759–1771. <http://doi.org/10.1111/1462-2920.12067>
- Ramos, I. (2013). Bioresource Technology The potential of oxygen to improve the stability of anaerobic reactors during unbalanced conditions : Results from a pilot-scale digester treating sewage sludge. *Bioresource Technology*, 140,

80–85. <http://doi.org/10.1016/j.biortech.2013.04.066>

- Rubin, E. M. (2008). Genomics of cellulosic biofuels, *454*(August), 841–845. <http://doi.org/10.1038/nature07190>
- Ruiz-Hernando, M., Mart??n-D??az, J., Labanda, J., Mata-Alvarez, J., Llorens, J., Lucena, F., & Astals, S. (2014). Effect of ultrasound, low-temperature thermal and alkali pre-treatments on waste activated sludge rheology, hygienization and methane potential. *Water Research*, *61*, 119–129. <http://doi.org/10.1016/j.watres.2014.05.012>
- Sahlström, L. (2003). A review of survival of pathogenic bacteria in organic waste used in biogas plants. *Bioresource Technology*, *87*(2), 161–166. [http://doi.org/10.1016/S0960-8524\(02\)00168-2](http://doi.org/10.1016/S0960-8524(02)00168-2)
- Salminen, E., & Rintala, J. (2002). Anaerobic digestion of organic solid poultry slaughterhouse waste--a review. *Bioresource Technology*, *83*(1), 13–26. [http://doi.org/10.1016/S0960-8524\(01\)00199-7](http://doi.org/10.1016/S0960-8524(01)00199-7)
- Sanders, W. T., Zeeman, G., & Lettinga, G. (2002). Hydrolysis kinetics of dissolved polymer substrates. *Water Science and Technology*, *45*(10), 99–104.
- Scherer, P., & Neumann, L. (2013). “Methano-compost”, a booster and restoring agent for thermophilic anaerobic digestion of energy crops. *Biomass and Bioenergy*, *56*, 471–478. <http://doi.org/10.1016/j.biombioe.2013.05.021>
- Shanmugam, P., & Horan, N. J. (2009). Bioresource Technology Simple and rapid methods to evaluate methane potential and biomass yield for a range of mixed solid wastes, *100*(2), 471–474. <http://doi.org/10.1016/j.biortech.2008.06.027>
- SJÖSTRÖM, E. (1993). Lignin. *Wood Chemistry*, 71–89. <http://doi.org/10.1016/B978-0-08-092589-9.50008-5>
- Smet, E., Van Langenhove, H., & De Bo, I. (1999). The emission of volatile compounds during the aerobic and the combined anaerobic/aerobic composting of biowaste. *Atmospheric Environment*, *33*(8), 1295–1303. [http://doi.org/10.1016/S1352-2310\(98\)00260-X](http://doi.org/10.1016/S1352-2310(98)00260-X)
- Stamatelatos, K., Antonopoulou, G., & Iyberatos, G. (2011). *12 - Production of biogas via anaerobic digestion. Woodhead Publishing Series in Energy* (Vol. 1895). Woodhead Publishing Limited. <http://doi.org/http://dx.doi.org/10.1533/9780857090492.2.266>
- Sukumaran, R. K., Singhanian, R. R., & Pandey, A. (2005). Microbial cellulases - Production, applications and challenges. *Journal of Scientific and Industrial Research*, *64*(11), 832–844.
- Sun, L., Müller, B., & Schnürer, A. (2013a). Biogas production from wheat straw : community structure of cellulose-degrading bacteria. *Energy, Sustainability and Society*, *3*(15), 1–11. <http://doi.org/10.1186/2192-0567-3-15>
- Sun, L., Müller, B., & Schnürer, A. (2013b). Biogas production from wheat straw : community structure of cellulose-degrading bacteria, 1–11.
- Sung, S., & Liu, T. (2003). Ammonia inhibition on thermophilic anaerobic digestion. *Chemosphere*, *53*(1), 43–52. [http://doi.org/10.1016/S0045-6535\(03\)00434-X](http://doi.org/10.1016/S0045-6535(03)00434-X)
- Symons, G. E., & Bushwell, A. M. (1933). The methane fermentation of carbohydrates. *Journal of the American Chemical Society*, *55*(5), 2028–2036. <http://doi.org/10.1021/ja01332a039>
- Tang, Y., Shigematsu, T., Ikbai, Morimura, S., & Kida, K. (2004). The effects of micro-aeration on the phylogenetic diversity of microorganisms in a thermophilic anaerobic municipal solid-waste digester. *Water Research*, *38*(10), 2537–2550. <http://doi.org/10.1016/j.watres.2004.03.012>
- Taniguchi, M., Takahashi, D., Watanabe, D., Sakai, K., Hoshino, K., Kouya, T., & Tanaka, T. (2010). Effect of steam explosion pretreatment on treatment with *Pleurotus ostreatus* for the enzymatic hydrolysis of rice straw. *Journal of Bioscience and Bioengineering*, *110*(4), 449–452. <http://doi.org/10.1016/j.jbiosc.2010.04.014>
- Thomsen, M. H., Thygesen, A., Jørgensen, H., Larsen, J., Christensen, B. H., & Thomsen, A. B. (2006). Preliminary results on optimization of pilot scale pretreatment of wheat straw used in coproduction of bioethanol and electricity. *Applied Biochemistry and Biotechnology*, *129-132*(1), 448–460.

<http://doi.org/10.1385/ABAB:130:1:447>

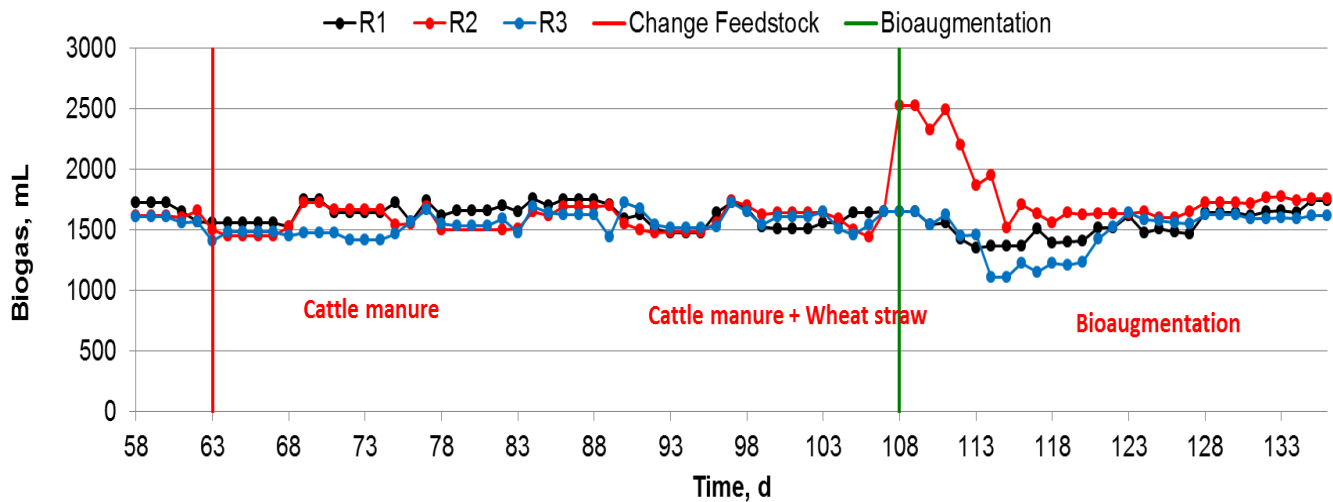
- Van Veen, J. A., Van Overbeek, L. S., & Van Elsas, J. D. (1997). Fate and activity of microorganisms introduced into soil. *Microbiology and Molecular Biology Reviews*. Retrieved from <http://www.scopus.com/inward/record.url?eid=2-s2.0-0030740090&partnerID=tZ0tx3y1>
- Vavilin, V. A., & Angelidaki, I. (2005). Anaerobic degradation of solid material: Importance of initiation centers for methanogenesis, mixing intensity, and 2D distributed model. *Biotechnology and Bioengineering*, 89(1), 113–122. <http://doi.org/10.1002/bit.20323>
- Vavilin, V. A., Fernandez, B., Palatsi, J., & Flotats, X. (2008). Hydrolysis kinetics in anaerobic degradation of particulate organic material: An overview. *Waste Management*, 28(6), 939–951. <http://doi.org/10.1016/j.wasman.2007.03.028>
- Veeken, A., Kalyuzhnyi, S., Scharff, H., & Hamelers, B. (2000). Effect of pH and VFA on Hydrolysis of Organic Solid Waste. *Journal of Environmental Engineering*, 126(12), 1076–1081. [http://doi.org/10.1061/\(ASCE\)0733-9372\(2000\)126:12\(1076\)](http://doi.org/10.1061/(ASCE)0733-9372(2000)126:12(1076))
- Villavicencio, H., Hernández, C., Gómez, A., Cózar, J. M., Rodríguez-Antolín, A., Prieto, L., ... Angulo, J. C. (2013). Tratamiento del cáncer de próstata y cáncer renal con fármacos orales (abiraterona y antiangiogénicos): posicionamiento de la Asociación Española de Urología. *Actas Urológicas Españolas*, 37(6), 321–323. <http://doi.org/10.1016/j.acuro.2013.04.001>
- Wang, G., Gavala, H. N., Skiadas, I. V., & Ahring, B. K. (2009a). Wet explosion of wheat straw and codigestion with swine manure : *Waste Management*, 29(11), 2830–2835. <http://doi.org/10.1016/j.wasman.2009.07.004>
- Wang, G., Gavala, H. N., Skiadas, I. V., & Ahring, B. K. (2009b). Wet explosion of wheat straw and codigestion with swine manure : Effect on the methane productivity. *Waste Management*, 29(11), 2830–2835. <http://doi.org/10.1016/j.wasman.2009.07.004>
- Wang, H., Vuorela, M., Ker?nen, A. L., Lehtinen, T. M., Lensu, A., Lehtom?ki, A., & Rintala, J. (2010). Development of microbial populations in the anaerobic hydrolysis of grass silage for methane production. *FEMS Microbiology Ecology*, 72(3), 496–506. <http://doi.org/10.1111/j.1574-6941.2010.00850.x>
- Ward, A. J., Hobbs, P. J., Holliman, P. J., & Jones, D. L. (2008). Bioresource Technology Optimisation of the anaerobic digestion of agricultural resources, 99, 7928–7940. <http://doi.org/10.1016/j.biortech.2008.02.044>
- Wei, J., & Wang, J. (2013). Enhanced hydrolysis and methane yield by applying microaeration pretreatment to the anaerobic co-digestion of brown water and food waste. *Waste Management*, 33(4), 813–819. <http://doi.org/10.1016/j.wasman.2012.11.013>
- Wiegel, J. (1980). Formation of ethanol by bacteria. A pledge for the use of extreme thermophilic anaerobic bacteria in industrial ethanol fermentation processes. *Experientia*, 36(12), 1434–1446. <http://doi.org/10.1007/BF01960144>
- Wisniak, J. (2005). Anselme Payen. *Educación Química*, 16(4), 568–580. Retrieved from <http://biblat.unam.mx/en/revista/educacion-quimica/articulo/anselme-payen#.U00NJHy4wX4.mendeley> http://www.researchgate.net/profile/Jaime_Wisniak/publication/236232917_Anselme_Payen/links/00463519dcf987c485000000.pdf
- Xu, S., Selvam, A., & Wong, J. W. C. (2014). Optimization of micro-aeration intensity in acidogenic reactor of a two-phase anaerobic digester treating food waste. *Waste Management*, 34(2), 363–369. <http://doi.org/10.1016/j.wasman.2013.10.038>
- Yadvika, Yadav, A. K., Sreekrishnan, T. R., Satya, S., & Kohli, S. (2006). A modified method for estimation of chemical oxygen demand for samples having high suspended solids. *Bioresource Technology*, 97(5), 721–726. <http://doi.org/10.1016/j.biortech.2005.04.013>
- Zabihi, S., Alinia, R., Esmaeilzadeh, F., & Kalajahi, J. F. (2010). Pretreatment of wheat straw using steam, steam/acetic acid and steam/ethanol and its enzymatic hydrolysis for sugar production. *Biosystems Engineering*, 105(3), 288–297. <http://doi.org/10.1016/j.biosystemseng.2009.11.007>

- Zhang, J., Guo, R., Qiu, Y., Qiao, J., Yuan, X., Shi, X., & Wang, C. (2015). Bioresource Technology Bioaugmentation with an acetate-type fermentation bacterium *Acetobacteroides hydrogenigenes* improves methane production from corn straw. *BIORESOURCETECHNOLOGY*, 179, 306–313. <http://doi.org/10.1016/j.biortech.2014.12.022>
- Zhou, W., Imai, T., Ukita, M., Li, F., & Yuasa, A. (2007). Effect of limited aeration on the anaerobic treatment of evaporator condensate from a sulfite pulp mill. *Chemosphere*, 66(5), 924–929. <http://doi.org/10.1016/j.chemosphere.2006.06.004>
- Zitomer, D. H., & Shrout, J. D. (1998). Feasibility and benefits of methanogenesis under oxygen-limited conditions. *Waste Management*, 18(2), 107–116. [http://doi.org/10.1016/S0956-053X\(98\)00008-7](http://doi.org/10.1016/S0956-053X(98)00008-7)

APPENDIX

Bioaugmentation

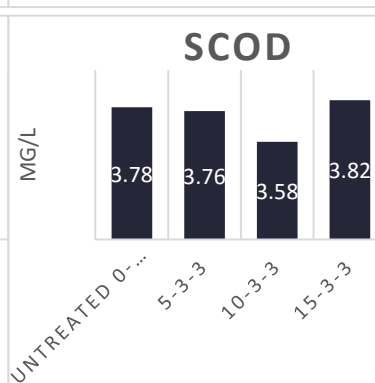
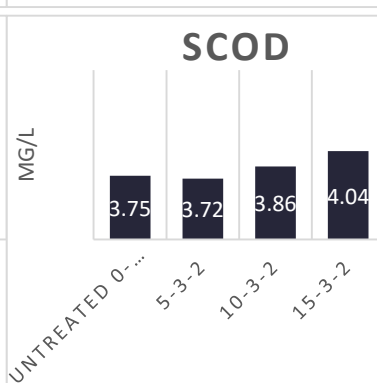
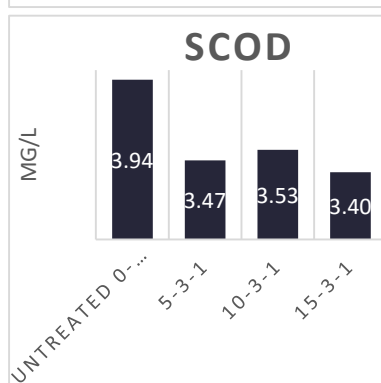
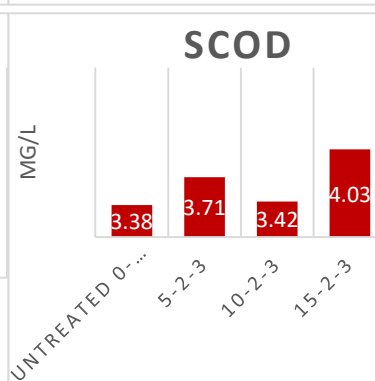
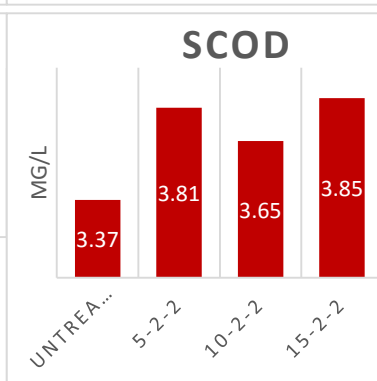
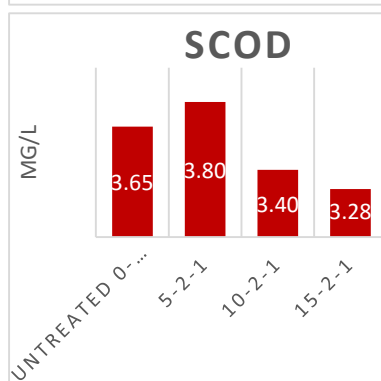
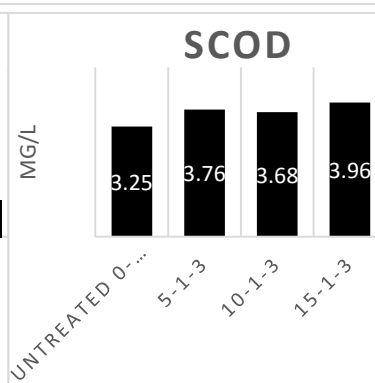
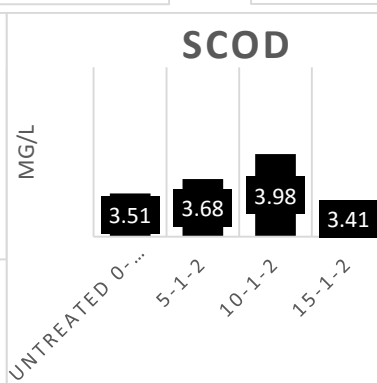
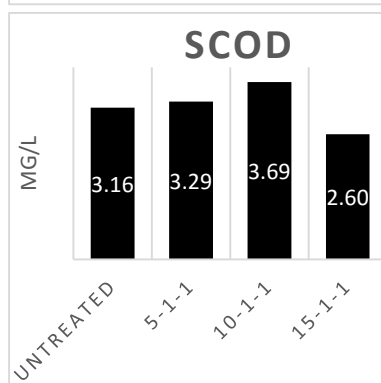
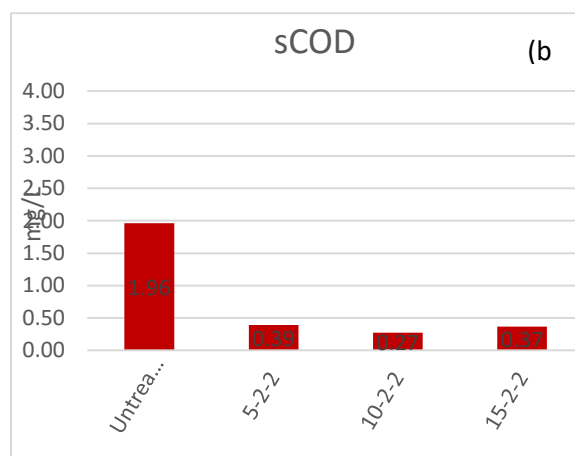
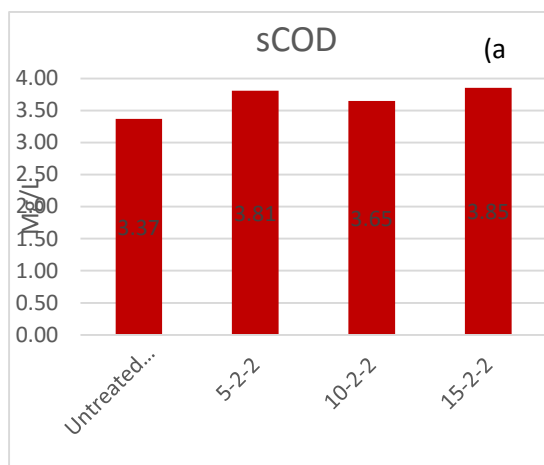
Biogas production during CSTR bioaugmentation



Microaeration

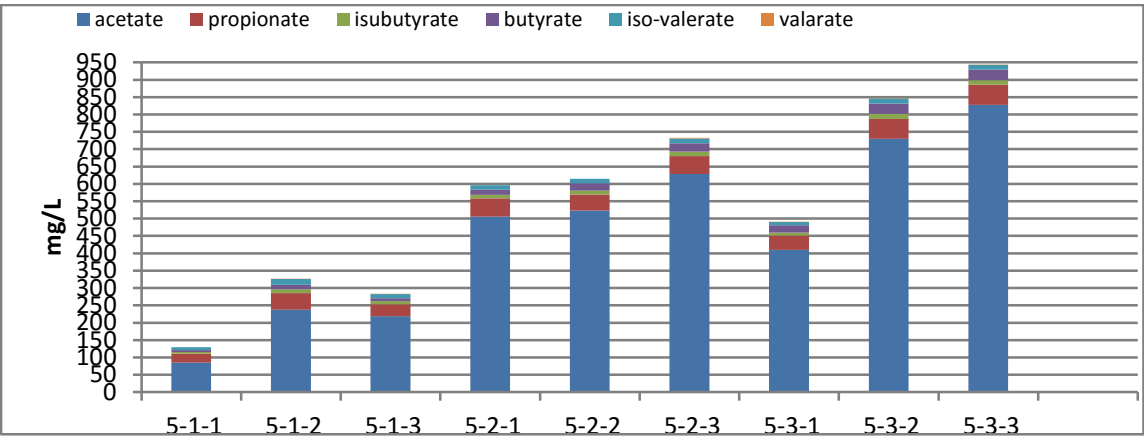
sCOD

sCOD after microaeration (on the left), sCOD at the end of the experiment(on the right) for the most remarkable samples 2 inj-2 days.

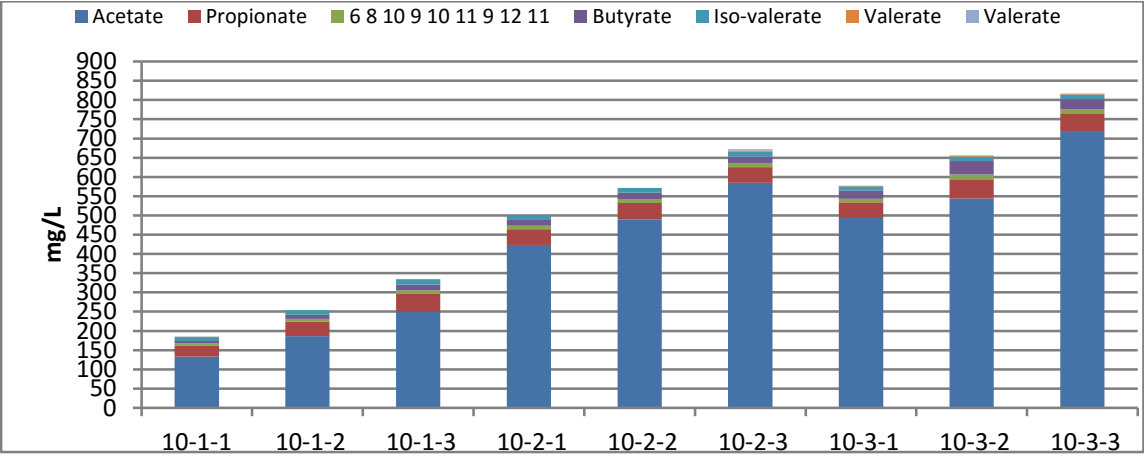


VFA

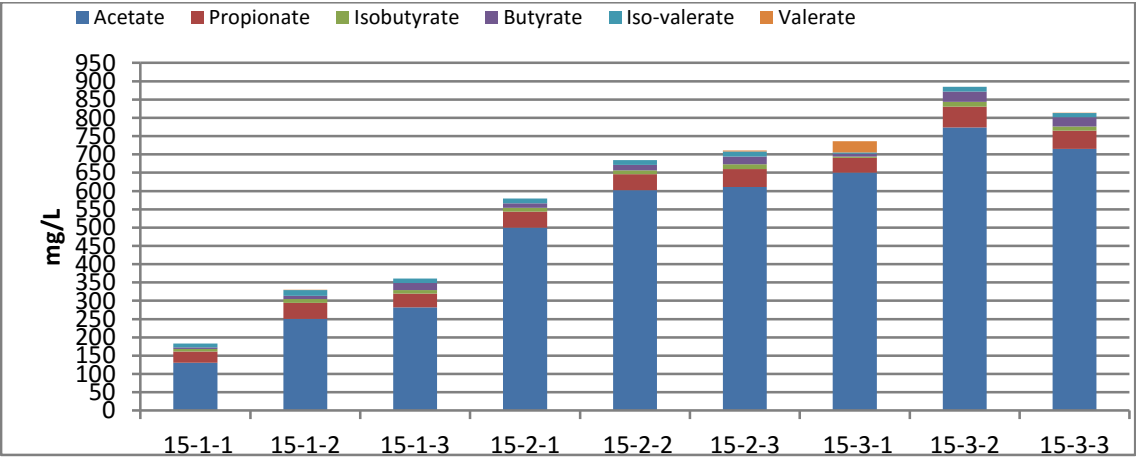
5 ml O₂/gVS



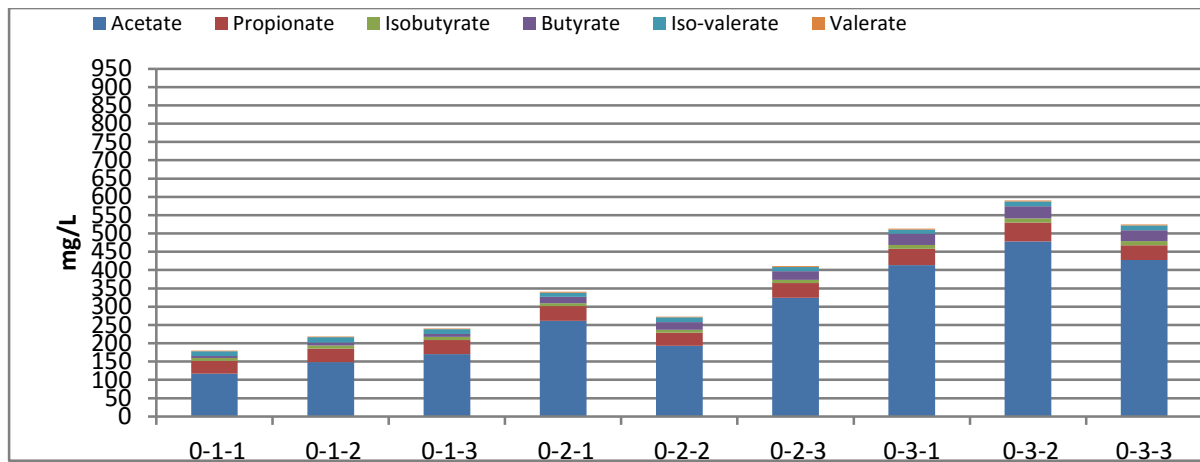
10 ml O₂/gVS



15 ml O₂/gVS



Untreated



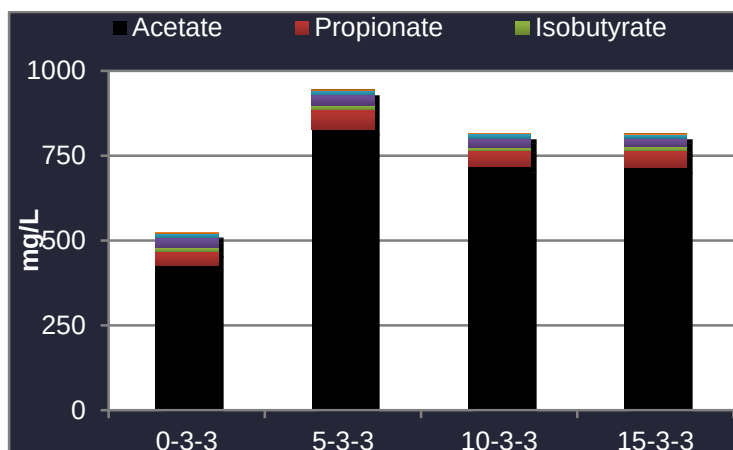
The greatest acetate production was resulted for 3 inj – 3 day , and the lowest acetate for 1inj-1day.

For 3-3 samples after microaeration (3 days-72 hours)

Average % Acetate/TVFA = 80.8 %

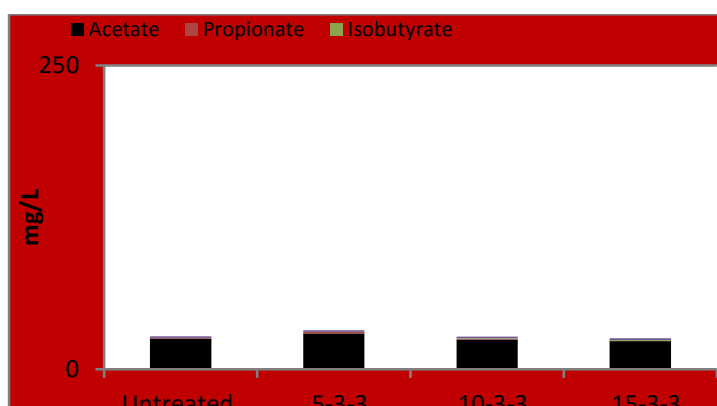
Average % Propionate/TVFA = 7.66 %

	0-3-3	5-3-3	10-3-3	15-3-3
	3			
Acetate	428	828	717	715
Propionate	41	57	46	50
Isobutyrate	12	14	11	12
Butyrate	29	30	27	26
Iso-valerate	14	14	13	11
Valerate	3	0	1	1



VFA microaerated samples After the digestion (840 hours)

	0-3-3	5-3-3	10-3-3	15-3-3
Acetate	25	30	24	23
Propionate	1	1	1	0
Isobutyrate	0	0	0	1
Butyrate	1	1	1	2
Iso-valerate	0	0	0	0
Valerate	0	0	0	0



For the lowest amount of acetate generated, which was obtained in 1 inj- 1 day samples

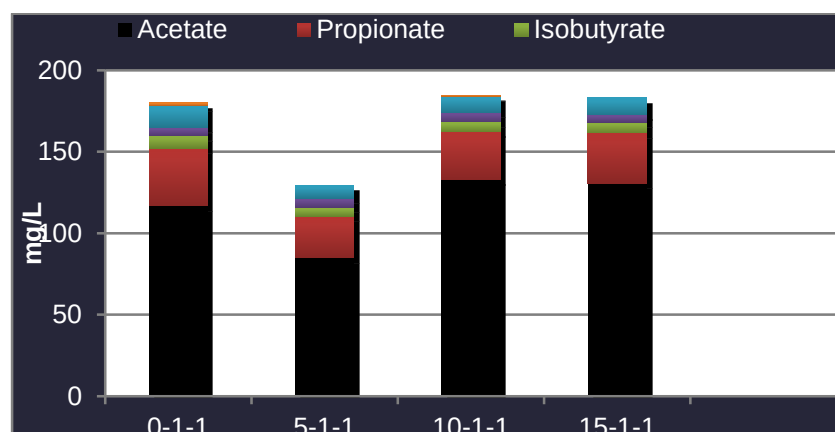
After microaeration (3 days or 72 hours)

	0-1-1	5-1-1	10-1-1	15-1-1
Acetate	117	85	133	131
Propionate	35	26	29	31
Isobutyrate	8	5	6	7
Butyrate	5	6	6	5
Iso-valerate	13	8	10	10

Valerate	2	0	1	0
-----------------	---	---	---	---

Average %Acetate/VFA = 59.037 %

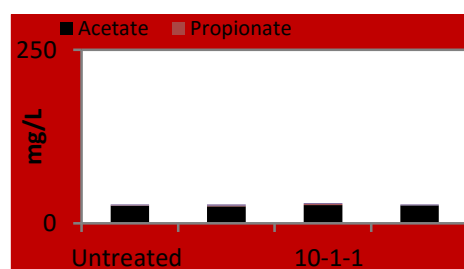
Average %Propionate/VFA = 17.838 %



Untreated				
		5-1-1	10-1-1	15-1-1
Acetate	25	25	27	26
Propionate	1	1	1	0
Isobutyrate	0	0	0	0
Butyrate	1	2	1	2
Iso-valerate	0	0	0	0
Valerate	0	0	0	0

Average % Acetate/VFA = 86.02 %

Average % Propionate/VFA = 2.2 %



Methane yield charts for microaeration period

