Biogas production influenced by C/N ratio, biodegradability and batch modelling aspects

MASTER THESIS

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Abstract

Several decades may have passed where a lot of research activities respecting anaerobic digestion have been running and despite the complexity of the process, still remains a big point of interest. The relatively high degree of reduction of different organic matter along with the small increase in bacterial biomass as well as the production of biogas that is possible to be utilized for the generation of various forms of energy, make this process be worthwhile for further studies.

The main goal of this process is the gain of as high as possible yield of biogas from the organic matter that is being degraded. However, this procedure is highly influenced by the direct interaction among a variety of variables, such as pH, temperature, C/N ratio, concentration of nutrients and hydrolysis rate of the system.

In this work, the main emphasis was given to the interaction between the C/N ratio of the system and the biodegradability of the substrate. Three different types of substrate were tested according to how the biogas yield of each can be affected by a range of different C/N ratio.

Furthermore the biomodel that first introduced by Angelidaki et al, (1993) and used for continuous cases, was modified and after a parameter estimation was estimated for batch reactors. This mainly attempted in order is to give the availability to the model of estimating the methane yield and production of a BMP assay.

Περίληψη

Παρότι έχουν περάσει αρκετές δεκαετίες έρευνας σχετικά με την Αναερόβια Χώνευση και παρά την πολυπλοκότητα της διεργασίας, παραμένει ακόμα στο επίκεντρο του ενδιαφέροντος. Ο σχετικά μεγάλος βαθμός μείωσης των διαφόρων ειδών οργανικής μάζας μαζί με την μικρή αύξηση της βακτηριακής βιομάζας καθώς και την παραγωγή του βιοαερίου που είναι πιθανό να χρησιμοποιηθεί για την παραγωγή διαφόρων ειδών ενέργειας, προσδίδουν στη διεργασία αρκετό ενδιαφέρον για περεταίρω μελέτες.

Ο κύριος στόχος της διεργασίας είναι η επίτευξη όσο το δυνατόν μεγαλύτερης απόδοσης βιοαερίου από την οργανική ύλη που αποσυντίθεται. Ωστόσο, η διεργασία επηρεάζεται άμεσα από την αλληλεπίδραση ποικίλων μεταβλητών, όπως το pH, η θερμοκρασία (T), ο λόγος άνθρακα/αζώτου (C/N), η συγκέντρωση των θρεπτικών στοιχείων και ο βαθμός υδρόλυσης του συστήματος.

Στην συγκεκριμένη εργασία δόθηκε έμφαση στην αλληλεπίδραση του λόγου άνθρακα/αζώτου (C/N) του συστήματος και της βιοαποικοδομησιμότητας του υποστρώματος. Εξετάστηκαν τρία διαφορετικού τύπου υποστρώματα σχετικά με το πως επηρεάζεται η απόδοση του βιοαερίου στο καθένα απο μια σειρά διαφορετικών λόγων άνθρακα/αζώτου (C/N).

Επιπλέον το βιομοντέλο (biomodel) το οποίο πρώτα εισήχθηκε από την καθηγήτρια Angelidaki et al, (1993) και χρησιμοποιήθηκε για αντιδραστήρες συνεχούς λειτουργίας, τροποποιήθηκε κατάλληλα και μέσω εκτίμησης παραμέτρων χρησιμοποιήθηκε για αντιδραστήρες ημιδιαλείποντος έργου. Αυτό κυρίως δοκιμάστηκε με σκοπό να δοθεί η δυνατότητα στο μοντέλο να προβλέπει την παραγωγή, μέσω πειραμάτων μέτρησης, του Βιοχημικού Δυναμικού Μεθανίου.

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Table of contents

List of figures

List of tables

List of abbreviations

AD Anaerobic Digestion

B_{th} Theoretical Methane Potential

CSTR Continuous Stirred Tank Reactor

TAN Total Ammonia Nitrogen

TKN Total Kjeldahl Nitrogen

TS Total Solids

VFA Volatile Fatty Acid

VS Volatile Solids

1 Introduction

Nowadays it is already well known that the organic fraction of solid waste is recognized as a valuable resource and that can be converted into advantageous products *via* microbially mediated transformations.

Various processes exist for the treatment of organic waste but anaerobic digestion (AD) appears to be the most promising. AD process involves a series of consecutive metabolic reactions such as hydrolysis, acidogensis and methanogensis. It is considered as a natural process since it also occurs in natural environments, such as landfills, rice fields, sediments, and intestinal tracts of animals, where light and inorganic electron acceptors (nitrate, oxygen, sulfate, iron, etc.) are not present or limiting. For instance, when AD process takes place in landfills methane and carbon dioxide gases are produced and released into the atmosphere, which represents a burden to the environment. Under controlled conditions, the AD process has the potential to provide useful products such as biofuel and organic amendment (soil conditioner). Furthermore, methane is considered comparatively cleaner than fossil fuels. Thus, anaerobic digestion represents an opportunity to decrease environmental burdens, biogas can be used instead of fossil fuels for energy consumption (Khalid *et al*., 2011). While at the same time provides environmental benefits, such as reduction of greenhouse emissions and odors, and controlled waste disposal. Moreover, offers the possibility for recycling of nutrients (nitrogen and phosphorous), as the digested material can be applied on agricultural land as biofertilizer, replacing by that way the artificial fertilizers (Irini Angelidaki *et al*., 2011).

In order to have as much as possible a more effective AD process, the methane yield of each substrate that is used should be maximized. Biogas production depends on various parameters that directly affect the methane yield. Prominent among the factors are the type of substrate, temperature, concentration of slurry, pH and more importantly, the C/N ratio that controls the pH value of the slurry. The total solids, volatile organic matter and mineral concentrations are also important (Dioha *et al*., 2013).

Short-term (i.e. 1–2 months), batch-mode anaerobic digestion tests, such as the biochemical methane potential (BMP) assay, are primarily intended to determine methane yields and biodegradability of substrates. Furthermore, a considerable number of theoretical approaches has also been developed. In the early stages of anaerobic digestion, stoichiometrical based methods that predict the major final products of fermentation have been developed, like Symons and Buswell's equation (Symons and Buswell 1933). While most recent approaches are more complex models that simulate the biochemical and physicochemical reactions taking place during the anaerobic digestion process to predict the transient behaviour of the process and final products concentration of the fermentation process, like the model proposed by Angelidaki *et al.*,(1999).

Regardless of the theoretical method used, its accuracy will largely depend on the knowledge of the substrate composition, and particularly, on its biodegradable fraction. Thus apparently, there is the need for a simple, quick, and accurate method to estimate biomethane yields and the biodegradability of organic substrates (Labatut et al., 2011).

In this work, the effect of C/N ratio and biodegradability of three different substrates on biogas production and yield was determined by means of batch assays. Furthermore the AD process model, first introduced by Angelidaki *et al.* (1993), was modified and used to forecast the methane yield and the cumulative methane production in batch assays.

In the following chapter, chapter 3, is briefly described the AD process and some of the major factors that affect it. Mainly focusing on the influence of the C/N ratio of the system and the biodegradability of the substrates. Afterwards, follows an introduction about how the methane yield can be determined, starting from the basic well known theoretical method of Buswell and ending up to the real experimental procedure that

nowadays is going on. In both of the cases pros and con are also referred. Subsequently follows an introduction in the world of mathematical modelling of AD batch systems, where the basic models of AD and mainly the BioModel, first introduced by Angelidaki et al, (1993), is referred. In chapter 4, there is a briefly description of the substrates that were used in this study, as well the analytical methods and the two experimental set up that were accomplished. Finally the results of the experiments and the simulations can be found in chapter 5, as well the conclusions of them in the following chapter 6. In the appendices are provided the basic equations and a short description of the main biochemical pathways used in the BioModel. As well as, tables containing the results of the analytical methods that were used in the experiments.

2 Overall scope and objectives

C/N ratio and the biodegradability of the substrate are considered as two main factors that affect the methane yield and production in the AD process. In this study what was tried to be studied is how variant C/N ratio of the system can affect the methane yield of dissimilar substrates by means of different biodegradability. As well as the main scope was to investigate the interaction among the C/N ratio of the system and the biodegradability of the substrates.

Furthermore, mathematically, the degradation rate of each group of compounds can be described by a differential kinetic equation. The knowledge of the biodegradation kinetics and methane production could be helpful for the methane prediction of a specific substrate. Batch assays are used to predict the methane yield of mono or co-digestion of different substrates. However, in lab scale this demands time, thus more attractive could be the existence of a complete complex model that could be used instead. Until now the BioModel, first introduced by Angelidaki *et al*. (1993), is being used mainly for continuous cases, describing the AD process in CSTR reactors. A new attempt is tried in order to implement that for batch cases. The overall purpose of this attempt is to modify the model in case of estimating the methane yield and production of a BMP reactor.

3 Background

3.1 The anaerobic digestion (AD) process

Anaerobic digestion (AD) is a synergistic process of a sequence of microbes which can be classified along with a series of metabolic pathways. In Fig. 1 the major reactions of the process are shown. The first pathway during the AD process is known as hydrolysis and consists of reducing complex organic molecules (carbohydrates, proteins and lipids) into simple soluble molecules (sugars, amino acids, and long-chain fatty acids, respectively) by extracellular enzymes.

Then the reduced compounds are converted by fermentative bacteria into a mixture of short-chain volatile fatty acids (VFAs) and other minor products such as carbon dioxide, hydrogen and acetic acid. Acetogenic bacteria further convert the organic acids to acetate, carbon dioxide, and/or hydrogen, which are the direct substrates for methane production.

The final step of AD process is the methanogenesis, where a variety of methanogenic bacteria consume acetate, carbon dioxide, and hydrogen to produce methane. Methanogenesis pathway has been the focus of many AD studies due to its sensitivity to feedback inhibition by acidic intermediates (Li *et al.*, 2011).

The three major physiological groups of microorganisms in the AD process are (Angelidaki *et al*., 2011):

1. primary fermenting (hydrolytic–acidogenic) bacteria
2. anaerobic oxidizing (syntrophic–acetogenic) bacteria
3. methanogenic archaea

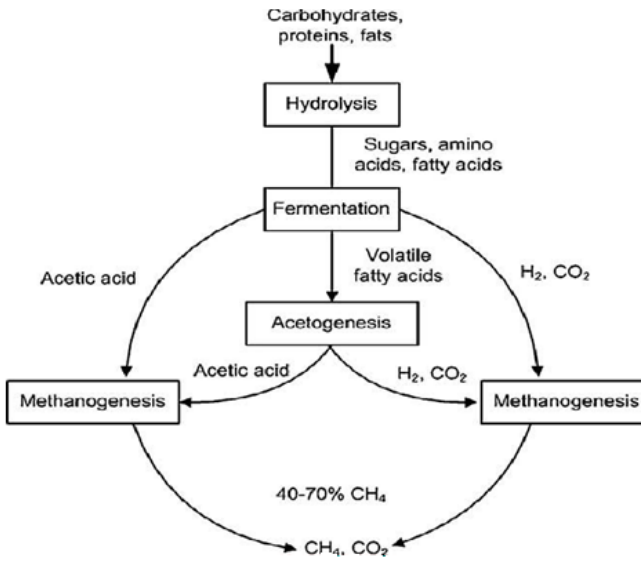

Figure 1 Process flow of the degradation of organic material through anaerobic digestion (Li et al., 2011).

An important step of the anaerobic biodegradation process is the hydrolysis of the complex organic matter. During the anaerobic digestion of complex organic matter the hydrolysis is the first and often the rate-limiting step. The hydrolysis can be defined as the breakdown of organic substrate into smaller products that can subsequently be taken up and degraded by bacteria. Substrate for hydrolysis can be directly present in the waste or can be formed by microbial activity such as internal storage products, or bacterial biomass.

Hydrolysis takes place extracellular by enzymes excreted by the biomass. The main mechanisms exist for the release of enzymes and the subsequent hydrolysis of the complex substrate are the following:

- The organism excretes enzymes to the bulk liquid, where they will either adsorb to a particle or react with a soluble substrate.
- The organism attaches to the particle, excretes enzymes into the surrounding area of the particle and next the organism will benefit from the released dissolved substrates.
- The organism has an attached enzyme that may also act as a transport receptor to the interior of the cell. This method requires the organism to absorb onto the surface of the particle (Angelidaki and Sanders 2004).

As aforementioned, during the hydrolysis pathway, hydrolytic bacteria reduce complex organic matter into soluble monomeric or dimeric substrates. In most cases, hydrolysis is the critical rate-limiting step that determines the conversion efficiency of the substrates. An example of an insoluble compound that undergoes enzymatic hydrolysis is cellulose, which is found in many agricultural and municipal wastes. Cellulolytic bacteria such as *Cellulomonas*, *Clostridium*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Baceriodes*, *Erwinia*, *Acetovibrio*, *Microbispora*, and *Streptomyces* can produce cellulases that hydrolyze complex molecules present in the biomass. In the meanwhile fermentative bacteria are responsible for the uptake of the soluble compounds resulting from hydrolysis pathway and convert them into various intermediates such as VFAs, hydrogen, carbon dioxide and alcohols. Some of the fermentation pathways that occur during AD process, along with their corresponding microorganisms, are shown in Table 1.

Except from the products of fermentation, acetate and carbon dioxide contribute the most to methane production. Acetogens or acetogenic bacteria are differentiated from acetate-forming fermentative bacteria mostly because of their capability to reduce carbon dioxide to acetate. There are exclusively acetogenic bacterial genera, such as *Acetobacterium* and *Sporomusa*, but also genera that contain both acetogenic and nonacetogenic bacteria, such as *Clostridium*, *Ruminococcus*, and *Eubacterium*. The vital role of acetate as a methanogen substrate, as well the ubiquity and diversity of the acetogenic bacteria makes AD a naturally robust process.

However, acetogens are obligate hydrogen producers that cannot survive in high hydrogen partial pressures, thus a symbiotic link exists between acetogens that produce hydrogen and methanogens that consume it.

Especially in solid state-AD systems, the heterogeneous nature of the substrate might create a multiplicity of ideal micro-environments for the growth of each of the microbial families required to perform the process. This means that fermentation processes might be well understood in conventional submerged cultures but can behave quite differently in solid substrates.

Table 1 Major genera of fermentative bacteria in anaerobic digestion (Li et al., 2011).

Fermentation pathway	Genera	Major products
Acetate fermentation	*Acetobacterium, Clostridium, Sporomusa*	Acetate, CO_2
Alcohol fermentation	*Saccharomyces*	Ethanol, CO_2
Butyrate fermentation	*Butyribacterium, Clostridium*	Butyrate, butanol, isopropanol, ethanol, CO_2
Lactate fermentation	*Lactobacillus, Streptococcus*	Lactic acid, CO_2
Propionate fermentation	*Clostridium*	Propionate, acetate, CO_2

As can also be seen in fig.1, the last step of AD process is, methanogenesis, follows two main pathways, these are:

- Hydrogenotrophic methanogenesis, which converts H_2 and CO_2 into CH_4. It takes place in close coexistence with acetogenesis and typically accounts for 30-40% of the CH_4, according to this overall reaction:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \text{ (3.1.1)}$$

- Aceticlastic methanogenesis, which converts acetate into CH_4 and CO_2. Typically generates 60-70% of the CH_4 and is one of the most sensitive processes in AD. It is also a highly specialized reaction and is mediated by two groups *Archaea*, *Methanosarcinaceae* and *Methanosaetaceae* within a single family *Methanosarcinales*. The overall reaction is the following:

$$CH_3COOH \rightarrow CH_4 + CO_2 \text{ (3.1.2)}$$

3.2 Conditions and parameters affecting the anaerobic digestion process

3.2.1 Substrate and biodegradability

The rate of anaerobic digestion is strongly affected by the type, availability and complexity of the substrate. Different types of carbon source support different groups of microbes. Thus, before starting a digestion process, the substrate must be characterized for carbohydrate, lipid, protein and fiber contents. Furthermore, the substrate should also be characterized for the quantity of methane that can potentially be produced under anaerobic conditions. Carbohydrates are considered as the most important organic component of municipal solid waste for biogas production. It is reported that the initial concentration and total solid content of the substrate in the bioreactor can significantly affect the performance of the process and the amount of methane produced during the process (Khalid *et al*. 2011).

Organic wastes consist also of nitrogenous compounds, which are usually proteins. Nitrogen is primarily required as a nutrient by the microorganisms in anaerobic digestion. Nitrogenous compounds are converted to ammonium by anaerobic digestion, by this form nitrogen contributes to the stabilization of the pH value in the bioreactor where the process is taking place. Microorganisms absorb ammonium for the production of new cell mass. For methanation a sufficient nutrient ratio of the elements C:N:P:S is considered to be 600:15:5:3.

However, ammonia in high concentration may lead to the inhibition of the biological process, it inhibits methanogenesis at concentrations exceeding approximately 100 mM. It has also found that the amount of ammonia in the digester may also affect the production of hydrogen and removal of volatile solids.

Total biogas production can be unaffected by small increases in ammonia nitrogen, but by higher increases the biogas production can be reduced up to 50% of the original rate. It is reported that "methanogenic activity is decreased by 10% at ammonium concentrations of 1670–3720 mg NH_4–N/L, while by 50% at 4090–5550 mg NH_4–N/L, and is completely inhibited at 5880–6000 mg NH_4–N/L" (Khalid *et al.*, 2011).

The terms of BMP, anaerobic biodegradability and digestibility are similar. They all deal with the degradation of organic matter (waste or forage) by microorganisms in anaerobic conditions. In anaerobic digestion degradation of organic matter leads to the production of biogas (mainly methane and carbon dioxide). At high gas production, less organic matter remains after fermentation, which indicates a higher biodegradability.

International standards for anaerobic biodegradability assays are not widely used, therefore, many different procedures are reported, which are making it more difficult to compare results with literature values of different authors (Costa *et al.*, 2014).

Biodegradability assays are based on the measurement of either formation of one or more products involved in the biological reaction under investigation or measurement of substrate depletion.

Methods based on product formation are monitoring either the end product (biogas) or intermediates production such as volatile fatty acids. Most of them though are based on monitoring biogas production.

Methods based on substrate depletion, require usually more complex analysis. Substrate depletion can be determined either as lumped parameter (volatile solids (VS), chemical oxygen demand (COD), dissolved organic carbon (DOC), etc.) or directly through quantification of the compound that is being used as substrate (Angelidaki and Sanders 2004).

3.2.2 Inoculum

Theoretically the BMP should be independent from the inoculum and blank's headspace, however according to (Angelidaki and Sanders, 2004) it is of great importance to find appropriate inoculum containing the necessary microorganisms for the degradation process to proceed. The most usual used inoculum is digested sludge, in our case though we used manure based inoculum since it is considered that it contains more nutrients than sludge. Nevertheless, in some cases, microorganisms adapted to specific conditions such as high ammonia concentrations are needed. Another important factor is the amount of inoculum added. Low amount of inoculum is often wished as inoculum also contributes to product formation (biogas) and thus can blur the results if biogas production from inoculum is relatively high compared to the substrate (or waste) under investigation. On the other hand a relatively small amount of inoculum can lead to overload of the process with acidification as a result.

Furthermore, according to Costa *et al.* (2014), it is shown that the BMP of a given substrate is highly influenced not only by the inoculum but the blank's headspace as well. Specifically it is reported that "the sludge effect can be endorsed to the cellulolytic and methanogenic activity, and to the pre-acclimation of the microorganisms to the tested substrate". It is also shown that the discount of the CH_4 produced in blank assay is crucial to assess the BMP and B_{th} of a given substrate. Moreover, regarding the influence of the volume of headspace, it's shown that it is possible due to the small volume of CH_4 produced and higher volume of

headspace to cause a propagation of errors, which ultimately will overestimate the methane produced from the residual substrate and underestimate the BMP of the tested substrate. So it seems that the best conditions to test is to perform the assays with raw inoculum in smaller vials' volumes for the blanks. However, in this study was assumed that having the same headspace for all cases, substrates and blank, would be more reliable for the comparison of the results

3.2.3 C/N ratio

The C/N ratio in the organic material plays a crucial role in anaerobic digestion. The unbalanced nutrients are regarded as an important limiting factor of anaerobic digestion of organic wastes (Khalid et al. 2011). As a result the production of biogas depends to a large extent, on the choice of feedstock and its carbon to nitrogen ratio.

The variation of the C/N values can affect the pH of the slurry. By increasing the carbon content in the system more carbon dioxide will be formed and the pH value will drop, while high value of nitrogen will enhance production of ammonia gas that could increase the pH to the detriment of the micro-organisms (Dioha *et al*., 2013).

There are different opinions and answers to the question on which is the optimal C/N ratio for anaerobic digestion. According to previous studies, the optimal C/N ratio ranges between 20 and 30 (Dioha *et al*. 2013). Another study (Nurliyana *et al*. 2015) provides the optimal C/N ratio to be in higher range of 45. On the contrary, another study shows that the optimal C/N ratio is between 9 and 30 (Sosnowski, 2003). Deviations of these standards can harm the digestion in different ways.

Most studies are currently based on chemically-measured carbon and nitrogen contents. However, some organic wastes can be composed of recalcitrant carbon fractions that are not bioavailable. Thus it would be preferable to know the anaerobic biodegradable organic carbon (BOC) since in general, all the BOC fractions are lower than the total organic carbon (TOC) and even lower than the total carbon (TC), which except the organic includes also the carbonate carbon. Therefore, the C/N ratios based on BOC are always lower than the total C/N ratio based on the TOC or TC measurement. The knowledge of the real bioavailable C/N ratio is crucial for the biological treatments of organic materials. (Puyuelo *et al*. 2011)

In this study more emphasize has been given to lignocellulosic materials where their chemical composition and structure hinders the rate of biodegradation of solid organic waste. It has been documented that "hydrolysis of the complex organic matter to soluble compounds is the rate-limiting step of anaerobic processes for wastes with a high solid content" (Khalid *et al*. 2011).

By changing the C/N ratio of the system but keeping constant the mass of the inoculum as well the C/N ratio of it, leads to have in each case different substrate/Inoculum ratio. Where the S/I ratio can be expressed as the amount of VS in the substrate per the amount of VS or amount of volatile suspended solid (VSS) originating from inoculum. (Chynoweth *et al*. 1993) reported that maximal methane yields were obtained with S/I ratios of 0.5 to 1.0 in anaerobic batch digestion of herbaceous and woody feedstock and municipal wastes. In addition, Hashimoto (1989) found that methane yields were lower at S/I ratios higher than 4.0 in a study on the influence of S/I ratio on BMP in wheat straw, using 20 different S/I ratios (0.03 to 10.91). Although S/I ratio highly influences methane yields from organic wastes, many BMP measurements for various feedstocks were made without considering it. (Yoon *et al*. 2014).

3.2.4 pH

Major part in anaerobic biodegradation plays the pH. It influences the activity of the hydrolytic enzymes and the microorganisms which are active within certain, usually narrow pH ranges. The anaerobic digestion process occurs in the pH interval of 6.0–8.3 (Angelidaki and Sanders, 2004). Each of the microbial groups involved in anaerobic degradation has a specific pH range at which they can grow optimally.

The methanogens and acetogens have an optimum pH of approximatelly 7, while acidogens have lower optimum pH around 6. Methanogens at pH lower than 6.6 grow very slowly (Christensen 2010).

According to Angelidaki and Sanders (2004) if the pH of the waste to be tested is outside the optimal range, and if enough buffer capacity is not present then the anaerobic process will be inhibited. This will lead to underestimation of the methane potential.

In an anaerobic reactor, instability can as a rule lead to accumulation of VFA, which subsequently can lead to a drop in pH (acidification). However, accumulation of VFA will not always be expressed as a drop of the pH due to the buffer capacity of some types of wastes. For instance in manure there is a surplus of alkalinity, which means that VFA accumulation shall exceed a certain point before this can be detected as a significant change in pH. Main factors affecting the pH are the organic acids and the carbon dioxide, which will decrease the pH while ammonia will increase it. Other compounds that are contributing to the buffering capacity are hydrogen sulfide and phosphate.

3.2.5 Temperature

Temperature affects survival and growth of microorganisms and also influences their metabolic activities. In general, higher temperatures that do not kill microorganisms result in higher metabolic activities. Temperature is assumed as the most important variable and controls the rate of microbial metabolism in anaerobic environments (Angelidaki and Sanders, 2004).

3.2.6 Inhibitors

Inhibition is a generalized term that means restriction of biological process. Speece (1996) divided the two inhibition process into: Toxicity and inhibition. The first is an adverse effect (not necessarily lethal) on microbial metabolism, and the second is an impairment of bacterial function. Batstone *et al*. (2002) further clarified these terms as following:

- Biocidal Inhibition. It is characterised by reactive toxicity and is normally irreversible. This type of inhibition matches Speece´s definition of toxicity, and the term reactive means that the toxic compound reacts with a functional component of the microbial cell, rendering it nonfunctional. Compounds which are generally biocidal to some or all anaerobes include detergents, cyanide and antibiotics. This main other potential toxic inhibitors found in solid waste are xenobiotics such a polyaromatic aromatic hydrocarbons. These also have an adsorption mechanism, but the technical difficulties involved with removal of the xenobiotics generally outweigh those caused by their toxicity to general anaerobic digestion.

- Biostatic inhibition. This is characterised by nonreactive toxicity and is normally reversible. Compounds that cause biostatic inhibition do not disable functional components, but rather disrupt cellular stasis, or change energy production. Microbes require intracellular conditions with redox potential, pH, and total salts within a small margin. Biostatic inhibiting compounds disrupt these conditions, and the microbe is required to spend energy on maintenance of stasis, rather than anabolism. Free acid and bases (e.g., VFAs, H_2S, NH_3), salts, and pH changes all cause biostatic inhibition. Biostatic inhibition is also caused by drops in thermodynamic yield caused by accumulation of product (e.g. hydrogen inhibition). Organisms with particularly marginal yields (e.g., acetogenic, hydrogen utilizing, and especially aceticlastic microbes) are particularly susceptible to biostatic inhibition.

- Product Inhibition. This kind of inhibition is caused by a drop in free energy available from catabolism, caused by an increase in product concentration. The most common product inhibition is hydrogen inhibition of acetogens, though acetate can also inhibit the same organisms at high concentrations (Christensen 2010).

3.3 BioMethane Potential (BMP)

Biomethane potential (BMP) assay is used to determine the ultimate methane yield from organic material by using an anaerobic batch reactor. It is expressed as the methane that is produced per unit of volatile solid (VS) content or per unit of chemical oxygen demand (COD) content at STP (standard temperature and pressure) conditions. Due to the importance of BMP of the organic material in the design, installation, and operation of an anaerobic digester, many researchers have proposed complete protocols for its determination (Owens and Chynoweth, 1993; Angelidaki and Sanders, 2004; Hansen *et al*., 2004; Angelidaki *et al*., 2009). In this study was used the protocol developed by Angelidaki *et al*., (2009). BMP determination for different organic wastes is essential because the anaerobic degradation process is highly influenced by the inherent substrate characteristics (Yoon *et al*., 2014).

Anaerobic biodegradability (BD) and BMP are too closely related meanings, which can also be expressed by Eq. (3.3.1).

$$BD = \frac{BMP}{350 * CODwaste} \quad (3.3.1)$$

Where

- COD_{waste} is the Chemical Oxygen Demand $\left(\frac{gCOD}{gVS}\right)$
- BMP the Biochemical Methane Potential $\left(\frac{mL\ CH4,STP}{gVS}\right)$ value expressed at Standard Temperature and Pressure (STP) conditions 273.15 K (0 ºC) and 100 kPa (1 atm) , respectively.

According to (Angelidaki and Sanders (2004) and Lesteur *et al*., (2010) is reported that 1 g L^{-1} of COD produces about 350 mL of CH_4. However, this biodegradability value is not accurate, because bacterial growth uses part of the organic matter that is consumed during methane production.

Even when various COD methods have been developed, interferences of other additional factors can occur when samples like manure are analyzed. Since we are talking about mostly solid state organic wastes, the samples have to be properly homogenized and diluted, because agricultural and household wastes contain much higher organic contents than samples like wastewaters.

3.3.1 Practical determination of BioMethane Potential

The experimental determination of biomethane potential in this study was done according to the protocol for batch assays proposed by Angelidaki *et al*., (2009). Where the main purpose of this paper is to determine some guidelines to researchers involved in such experiments, because nowadays anaerobic digestion technology is growing worldwide and a number of studies and research activities dealing with the determination of the biogas potential of solid organic substrates.

3.3.2 Problems associated with practical determination of BMP

Anaerobic digestion is considered as a highly complex and dynamic process, where microbiological, biochemical and physicochemical aspects are closely linked (Costa *et al.*, 2014).Therefore, it is easily possible to have problems during the estimation of BMP. Several problems could occur, for instance considering the nitrate, sulphate reducers and methanogens, sulphate reducers and denitrifiers are able to outgrow the methanogens. This is due to the higher energy gained by nitrate or sulphate reduction compared to methanogenesis. Thus, presence of high concentrations of sulphate or nitrate will result in determination of low methane potentials. Also sorption is an important mechanism that influences the fate and effect of organic compounds. When compounds stay in environments with high sorption capacity, they may become unavailable for anaerobic degradation and as a result can affect the determination of the methane potential. The experimental determination of the BMP can also be underestimated in cases where waste contains toxicants or the process is overloaded. Thus dilution of the waste is recommended, since it will result in a more accurate determination of the methane potential (Angelidaki and Sanders 2004).

3.4 Estimation of theoretical methane potential

The ability of theoretical methods to accurately estimate methane yields of complex substrates was estimated by comparing the observed methane yield ($B_{o,th}$) of selected substrates to the ultimate one (Bu). The observed or theoretical methane yield can be determined by the Buswell Formula.

Buswell Formula (Symons and Buswell 1933) is an equation that simply represents a balanced redox reaction where the only products of anaerobic digestion are methane, ammonia and carbon dioxide. It assumes that all

the electrons donated are exclusively used for metabolic energy, which means that cellular synthesis is neglected.

The method does not account for substrate biodegradability, i.e., it is assumed that all the electrons from the donor are available for the electron acceptors. The ability of the method to accurately estimate biomethane yields primarily depends on two fundamental substrate characteristics, namely chemical composition and biodegradability.

The calculations are based on the molecular formula of each substrates constituent. If the composition of the organic material is known and all the material is converted to biogas, the theoretical methane yield potential can be calculated from eq. (3.4.2) which is known as Buswell's equation (Buswell and Neave 1930):

$$C_nH_aO_bN_c + \left(n - \frac{a}{4} - \frac{b}{2}\right) H_2O \rightarrow \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4}\right) CH_4 + \left(\frac{n}{2} - \frac{a}{8} + \frac{b}{4}\right) CO_2 \quad (3.4.1)$$

This equation is derived from balancing the total conversion of the organic material to CH_4 and CO_2 with H2O as the only external source, i.e. under anaerobic conditions.

The specific methane yield, usually expressed as (STP L CH_4)/g VS might then be calculated as

$$B_{0,th} = \frac{\left(\frac{n}{2}+\frac{a}{8}-\frac{b}{4}\right)22.4}{(12n+a+16b)} \left[\frac{(STP\ L\ CH4)}{g\ VS}\right] \quad (3.4.2)$$

(Angelidaki and Sanders 2004).

If the chemical composition of the waste material contains also nitrogen, like straw and fibers, then the specific methane yield can be calculated with regard to it as well, by using the following equation:

$$C_nH_aO_bN_c + \left(n - \frac{a}{4} - \frac{b}{2}\right) H_2O \rightarrow \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4} + \frac{3c}{8}\right) CH_4 + \left(\frac{n}{2} - \frac{a}{8} + \frac{b}{4} + \frac{3c}{8}\right) CO_2 + cNH_3 \quad (3.4.3)$$

$$B_{0,th} = \frac{\left(\frac{n}{2}+\frac{a}{8}-\frac{b}{4}-\frac{3*c}{8}\right)22.4}{(12*n+a+16*d+14*c)} \left[\frac{(STP\ L\ CH4)}{gVS}\right] \quad (3.4.4)$$

Where in both of the equations 22.4 L/mol is the volume of 1 mol of gas at STP (standard) conditions (Lesteur et al., 2010).

According to literature ("Miljøstyrelsen" 2016) since the composition of Avicel is known ($C_6H_{10}O_5$) the Theoretical Biomethane Potential is 0.415 NL CH_4/gVS and after elemental analysis for the wheat straw that was used is 0.439 NL CH_4/gVS and for the fibers 0.448 NL CH_4/gVS.

3.5 Modelling of AD batch systems

In general modelling can be a very useful tool to design, evaluate and prototype anaerobic digesters. Apparently BMP is a powerful parameter widely used for waste characterization. However, measuring BMP is a time-consuming process, as more or less 60 to 90 days are required as a standard incubation time. Which at an industrial scale cannot be a practical management tool for anaerobic digestion optimization. Consequently, it is attractive to use faster methods to predict how much methane gas is possible to be produced from a given substrate. Understanding the kinetics of methane production from feedstocks is important for designing and evaluating anaerobic digestion process. Well known kinetic models that are being used to simulate anaerobic biodegradation in batch systems are the first order kinetic model and the Gompertz model.

First order kinetic model is the simplest model, nevertheless, this model does not predict the conditions for maximum biological activity and system failures. Hydrolysis is frequently assumed to be the rate-limiting step in anaerobic digestion, thus researchers have modeled batch BMP data using first-order hydrolysis models and obtained valuable explanations about hydrolysis kinetics.

The Gompertz model, on the other hand, was set on an exponential relationship between specific growth rate and population density, was originally developed to fit human mortality data and it has also been used to predict organ growth. The model is being also modified to a function that describes cell density during bacterial growth periods in terms of exponential growth rates and lag phase duration. The equation of the model has been defined as an assumption of methane production rate in a batch digester corresponding to the specific growth rate of methanogenic bacteria and identified as a good empirical non-linear regression model and commonly used in the simulation of methane accumulation (Kafle *et al*, 2016).

However, a more complex model with an integrated parameter database where can easily be defined the substrate composition as an input and forecast the methane yield and production could be more useful and attractive. Since it will be able to predict the methane yield of different substrate compositions, as well forecast any potential failure of the system.

3.5.1 Kinetics

The kinetics at each pathway of the anaerobic digestion process provide information on the extent of biodegradability and the rate of biodegradability of a particular substrate and/or combination of them. Thus, if the substrate is difficult to be degraded by hydrolytic enzymes (hydrolysis pathway), then the process will require more time to overcome or even worse can "stack" in the first step. On the other hand, if the substrate is easily degradable then the last step, methanogenesis, is supposed to be the rate limiting pathway. If both under the same conditions, the former pathway will take longer. Therefore, if the first AD pathway is accelerated then in principle, the overall biogas generation would be enhanced.

This means that when cells are introduced to a substrate in batch reactor the bacterial/microbial cell growth is based on time scale and consists of four steps, which are described below.

“Lag phase”. In this step hydrolytic bacteria are “waken up” and excrete enzymes owing to the injection of oxygen and their first interaction with substrate. If hydrolysis is fast and adequate, nutrients are produced and methanogens start the consumption faster, thus lag phase is short. At this phase the kinetics follow the zeroth order kinetics, which means that the substrate is consumed at a constant rate ($r_s = - k_1$, where k_1 is the zeroth order parameter in mass/volume*time units).

“Growth or Exponential phase”. During this phase methane yield rises rapidly. Cell consumes substrate in order to survive and not to divide. When microbes are robust and the nutrient substrate is abundant they follow a linear growth rate until the reduction of substrate. Thus the kinetics change to first order kinetics ($r_s = - k_2*S$, where k_2 is the first order kinetic parameter in $time^{-1}$ units and S is the substrate in mass/volume units). This indicates an accelerating hydrolytic and methanogenic activity which follows a linear increase as long as VFAs and more specifically acetate are abundant.

“Stationary phase”. It is assumed that after a pinpoint methanogens consumes the overall amount of acetate, and the most degradable part of substrate. Thereafter, methanogenic activity slows gradually and the microbial rise is equal to microbial death. Hence, microbes preserve themselves until their reduction because of starvation or adequate life growth.

“Death phase”. Afterwards follows the last step, where the live cell concentration starts dropping. This cell decay is due to the toxic by-products, harsh environments and/or depletion of nutrient supply, among others. It is obvious that the experiment continues until the stabilization of production instead of the drop of it, since due to the limited amount of nutrients it is logical that the drop of methane production will follow next.

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 approximately 5%.

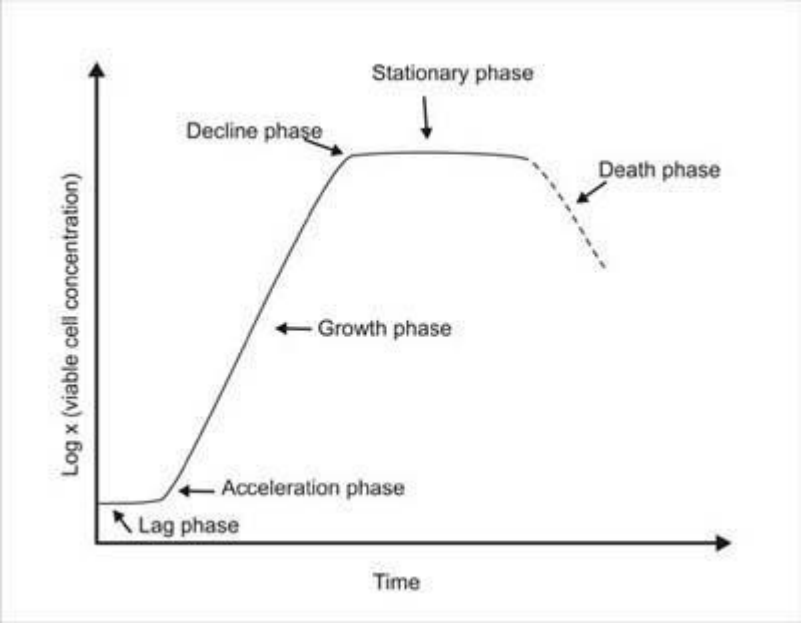

Figure 2 Phases of bacterial cell growth.

It has to be mentioned that this describes the behaviour of organisms in batch reactors. While, in continuous fed systems the lag phase is avoided since the substrate and the environmental conditions are already adjusted to the feed and the lag is damped with hydraulics. Furthermore, the two processes of death and respiration occur simultaneously and the uptake rate of substrate depends on the substrate concentration, with kinetics as for pure culture.

Therefore, there are two processes that have to be represented:

- Combined respiration and anabolism, where monod kinetics is generally used and it is first order at low substrate concentrations and zeroth order at high substrate concentrations. For activated sludge systems it is often expressed in the biomass growth rate:

  $$\mu_x = \frac{\mu_{max,X} S}{K_s + S} \quad (3.5.1.1)$$

  Where μ_x is the growth rate of biomass X on substrate S and $\mu_{max,X}$ is the maximum growth rate.

- Biomass decay, which is modelled as a first order equation

  $$rx, death = -K_{d.} * X \quad (3.5.1.2)$$

  Where $K_{d.}$ is the endogenous cell decay rate and X the biomass concentrations (g/L).

The Monod equation describes only situations where the substrate and biomass concentrations determine uptake rate. But respiration and anabolism can be influenced by a number of inhibitory factors that either decrease the amount of the available energy for respiration, so maintenance energy requirements increased, or just kill the microbes.

In general there are approximately 50 alternative relationships which describe substrate uptake or biomass growth, many of them incorporate inhibition kinetics. However, for inhibition, most recommended is the addition of each inhibition term to the basic Monod relationship.

The basic inhibition functions can be classified as non-competitive, uncompetitive and competitive inhibition. In AD modelling processes though almost only non-competitive functions are used, where Haldane inhibition is the most oftentimes used.

$$I = \frac{1}{1 + \frac{K_S}{S} + \frac{S}{K_I}} \quad (3.5.1.3)$$

$$\mu = \mu_{max} \left(\frac{1}{1 + \frac{K_S}{S} + \frac{S}{K_I}} \right) \quad (3.5.1.4)$$

Where:

- I : Haldane inhibition function
- K_S:: the half saturation coefficient (g/L)
- S: the substrate concentration (g/L)
- K_I: the half saturation inhibition constant (g/L)

This function is generally used to represent substrate inhibition since it allows Ks to be effectively reduced, which leads to the overall effect of causing low inhibition at low substrate concentrations and high inhibition at high substrate concentrations.

3.5.2 Physical-chemical equilibrium

An anaerobic digester is a dilute system, so most of the physicochemical reactions (*i.e.* those that do not involve biological catalysis) are into the general field of aquatic chemistry and normal dilute aquatic chemistry relationships are used to describe the physicochemical system.

The three general classes of physicochemical reactions that may occur in an anaerobic digester are

Liquid-liquid transformations: Here take part the very rapid reactions from acid to base, which are the association and dissociation of ions with water components (hydrogen ions, hydroxide ions and water molecules). These are normally modelled as instantaneous.

Gas-liquid transformations: In reality are transport reactions (transfer of liquid to gas phase) but they are in the class of physicochemical reactions and most of the generalised theory describes their behaviour well.

Liquid- solid reactions: Here the precipitation and solubilisation of ions from solution to a solid phase is being described. These reactions do not occur significantly in the majority of the reactors so they are not generally included in a model.

The concept of equilibrium establish whether the reaction is possible, and is the driving force for a reaction to occur. The rate at which the reaction will occur is determined by the resistance that may be to driving force. Almost all of the physicochemical reactions in aquatic systems are reversible, and the forward and backward reaction rates are defined by the rate constants (k_f and k_b for forward and backward reactions respectively):

$$aA + bB \underset{k_b}{\overset{k_f}{\rightleftharpoons}} cC + dD \quad (3.5.2.1)$$

For gas-liquid and liquid-solid transformations first order kinetics are often used.

3.5.3 BioModel

In 1993, after various simple or intermediate models describing certain aspects of the AD process by means of simplifications (only first-order kinetics and 2-3 microbial groups considered), the first complex model (the BioModel) able to give a good overview of most process variables, was introduced by Angelidaki ,*et al*.(2009). The model includes an enzymatic hydrolytic step, four bacterial steps (acidegonic, propionate acetogenic, butyrate acetogenic and aceticlastic step), involves 12 chemical compounds, two inhibitory effects (VFA, acetic acid), the pH and the temperature effects on microbial growth, limits the use of first-order equations only for calculating the hydrolytic step, and was now applies Monod-type kinetics for a more accurate bacterial growth calculation. The model focuses on ammonia inhibition and contains a detailed description of pH and temperature characteristics in order to accurately simulate free ammonia concentration. Acetate and free ammonia constitute the primary modulating factors in the model (Angelidaki, *et. al*1993).

The model characterization follows the chain of reactions starting from the composition of the substrate and the VFA (volatile fatty acid) that are being produced. Both of them determine the pH, which along with the temperature (T) regulate the ionization degree of NH_3. Then free NH_3 controls the methanogenic (aceticlastic) step, so whenever free ammonia (high for high pH) inhibits methanogenesis, acetic acid is accumulated. Right after this follows the inhibition of the acetogenic steps, resulting in propionate and butyrate accumulation (VFA accumulation). But VFA accumulation inhibits the hydrolytic step and depresses the pH, which it leads to a decrease of free NH_3 inhibition. The model is very good for describing the behaviour of manure fed digesters. The process is self-regulatory, unless the magnitude of the disturbance is larger than the system can withstand. When this occurs, the pH drops significantly, causing digester failure (Lyberatos and Skiadas, 1999).

A few years later the model was updated, including two more bacterial groups (lipolytic bacteria and long-chain fatty acid (LCFA) degrading acetogens). As a result inhibition caused by LCFA is also included on all steps of the process except hydrolysis. This model was used to simulate anaerobic codigestion of cattle manure along with olive oil mill effluent (OME). From the simulation data was indicated that lack of ammonia, which is needed as nitrogen source for synthesis of bacterial biomass and as an important pH buffer, could be responsible for the problems encountered when anaerobic degradation of OME alone is attempted, which could also be generalized for any full of lipids substrate.

In 1999 a more significant update was done by Angelidaki *et al*.(1999), including in total two enzymatic hydrolytic steps (one for hydrolysis of undissolved carbohydrates and another for proteins), eight bacterial steps (those being glucose-fermenting acidogens,lipolytic bacteria, amino acid-degrading acidogens, LCFA, propionic acid, butyric acid and valeric acid acetogens, and aceticlastic methanogens) and involving 19 chemical compounds. The decay of cell mass into consumable substrates, ammonia nitrogen as a growth-limiting co-substrate and two new types of inhibitors: namely ammonia affecting the growth of acetoclastic methanogens and the concentration of long-chain fatty acids influencing all microbial process steps were also included (Angelidaki *et al*., 1999). Similar to the first version, however, the conversion of hydrogen and glycerol were still not considered as separate elements of the model, due to their conversion dynamics being different from those defined in the model. Instead, they were accounted for being combined with other sections of calculations, in order to keep the mass balance.

Another important aspect of the BioModel is that its parameters were expressed in terms of gram component per litre substrate (g/L): the preferable way of characterising inhomogeneous substrates with high fibers and low water content (Lübken *et al*. 2007; Lindmark et al. 2012). The model was specifically developed for cases when manure was co-digested with other substrates (Angelidaki *et al*. 1997), thus it proved to be a suitable tool for simulating the anaerobic digestion of agricultural and many industrial wastes. In spite of its numerous advantages over its precursors, the BioModel was not developed markedly post its 1999 update, thus it could not reach a wider range of users and remained only for the use of researchers.

4 Material and methods

4.1 Substrates and inoculum

In the following subsections follows a briefly description of the structure of the substrates that were used in this study.

4.1.1 Avicel

Avicel is a microcrystalline cellulose powder ($C_6H_{10}O_5$). It is considered as a completely volatile component and high biodegradable. Avicel's native cellulose form is the same as that found in the fruits and vegetables. The chemical anaerobic reaction occurring for Avicel is:

$$C_6H_{10}O_5 + H_2O \rightarrow 3CH_4 + 3CO_2 \quad (4.1.1.1)$$

4.1.2 Wheat straw

Straw is the part of the cereal crop without the kernel and is an agricultural crop residue. It is built up of mostly cellulose, and to a smaller extent also hemicelluloses and lignin (Monlau *et al*. 2013). In literature, the TS and C/N varies between different cereals and also for a certain crop the values of these parameters differ. For instance, for wheat straw TS varies between 79,6-91,3 % (Chandra *et al*., 2012) and the C/N ratio is quite high C/N= 90 (Duong 2014). In this study, the wheat straw was provided by a farm in Sealand and the C/N ratio was 100.

4.1.3 Digested Fibers

The manure fibers that were used in this study were digested, which means that were sieved and taken from an inoculum of a thermophilic biogas plant. The composition of the inoculum was 60:40 pig and cow manure and industrial waste. By the term digested is meant that most of the carbohydrate part of the fibers consists of inert carbohydrates, which makes the hydrolysis process even more complicated. The C/N ratio of this substrate was 45.

4.1.4 Inoculum

The inoculum used in BMP was taken from the effluent of lab scale CSTR reactors where cattle manure and wheat straw were used as an influent. Prior to use the inoculum was sieved in order to remove all the remaining undigested fibers. The percentage of the inoculum that was used per each bottle was consciously really low (only 13%) in order to minimize any contribution of it in the effect of C/N ratio of the substrates. Moreover the inoculum should be "degassed", i.e. pre-incubated in order to deplete the residual biodegradable organic material present in it. Pre-incubation was done at 55 ^{0}C for around 10 days.

4.2 Analytical methods

In the following subsections are described all the analytical methods that were done before starting the BMP assays, where the respective results can be found in the appendix 5.2.

4.2.1 Total solids (TS), volatile solids (VS) and ash contents

Total solids (TS) and volatile solids (VS) were determined according to the Standard Methods (APHA, 1998). They were tested by weighing first the crucibles and then the samples (wheat straw, digested manure fibers and thermophilic inoculum) along with their crucibles. Then these were left in an 105°C oven for 24 hours and weighed again, at this part the TS measurement was taken. For the VS, that sample is put in an 550°C oven for 2h, in which all organic compounds are volatilized.

The calculations were determined by the following equations:

$$\%TS = \left(\frac{md-mc}{mw-mc}\right)100 \quad (4.2.1.1)$$

$$\%VS = \left(\frac{md-mv}{mw-mc}\right)100 \quad (4.2.1.2)$$

Where:

- mc: mass (g) of crucible
- mw: (Crucible + wet sample) initial mass of crucible and sample (g)
- md: (Crucible + dried sample) mass (g) of crucible and sample after 24h at 105°C
- mv: mass (g) of crucible and sample after 24h at 550°C

4.2.2 Total Kjeldahl and inorganic nitrogen

Determination of the amount of nitrogen in the samples was according to APHA, 1999. For assessing the total Kjeldahl nitrogen (TKN) content of substrate samples, a volume of 3 mL from each was digested in a Tecator™ 2020 digestor (produced by FOSS A/S), converting all nitrogen to mineralised form. In the next step, a FOSS Kjeltec™ 8100 distillation unit was used to extract ionised ammonia (NH_4^+) from both previously digested substrates and all untreated samples, as a means of defining their total amount of nitrogen (TKN) and inorganic nitrogen (TAN) content, respectively. Quantification of the nitrogen content of the different sample extracts – temporarily bound in the form of ammonium borate – was carried out by back titration, using a 0.1 M hydrochloric acid (HCl) solution. All samples were analyzed in triplicates. Final, the TKN was determined by the equation:

$$TKN = \frac{(V1-V2)*0.1*14.01}{Vs} \quad (4.2.2.1)$$

Where:

- TKN (total kjeldahl nitrogen) $\left(\frac{g}{L}\right)$
- V1: titration volume, (mL)
- V2: titration volume in blank, (mL)
- Vs: $sample\ volume(mL)$
- Factor 0.1 is 0.1 $mol\frac{molHCI}{L}$
- Factor 14.01 is the molar mass of nitrogen $\left(\frac{g}{mol}\right)$

4.2.3 Protein determination

In the BioModel the insoluble protein fraction needs to be determined, so the true protein content of samples was estimated using an average default factor of 5.6 taken from literature (Mariotti *et al*, 2008) and according to the following equation.

$$True\ protein = Org - N * 5.6 = (TKN - TAN) * 5.6 \quad (4.2.3.1)$$

Where *true protein* is the estimated protein content of the samples, also both TKN and TAN were derived by respective experimental analyses.

4.2.4 Volatile fatty acids

Short-chain or volatile fatty acid (VFA) concentrations of the thermophilic inoculum were analyzed similar to the *Gas Chromatographic Method* outlined in APHA, (2001). Each sample was prepared in duplicates and analyzed in a Shimadzu GC- 2010 gas chromatograph, fitted with a Shimadzu AOC-20i auto injector which was used for obtaining measurements. The individual VFAs measured were ethanol, 1-propanol, 1-butanol, iso-amylalcohol, 1-hexanol, acetate, propionate, isobutyrate, butyrate, isovalerate, valerate and 1-hexanoate.

Total VFA concentrations were calculated by adding up the concentrations of individual acids. In the case of modelling though the total VFA concentration is calculated by adding up acetate, propionate, butyrate and valerate, the other alcohol concentrations are not taken into consideration, due to their low significance.

4.2.5 pH

The pH of the thermophilic inoculum was measured and defined 7.9 by using a FEP20 – FiveEasy Plus™ digital pH meter, produced by Mettler-Toledo, LLC. Before the sample analysis, the equipment was calibrated with buffer solutions of pH 4 and 7, the choice of which being made due to expectations regarding the pH range of manure and industrial waste samples (Angelidaki *et al.*, 1993).

4.2.6 Total carbon (TC)

The total carbon of the substrates and the inoculum was measured by a LECO CS-200 analyzer, a microprocessor controlled instrument which is used for the determination of carbon and sulfur in metals, ores, soil, clay and other inorganic materials. A pre-weighed sample is combusted in a high- frequency induction furnace. The products of combustion are passed through a moisture trap to the sulphur IR cell, then the gases exiting the sulfur cell pass through a catalyst where any carbon monoxide (CO) is converted to carbon dioxide (CO_2). SO_2 is trapped out, and then carbon is measured as carbon dioxide in the carbon IR cell. The results are adjusted for sample weight and calibration factors and displayed.

The combustion method use a high frequency (HF) induction heated furnace (850 ^{0}C) for increased speed and accuracy. Approximately 0.01 g of samples were weighed and put in the crucibles, also one spoon of Alphacel II and one of Iron accelerator were added on the top of them. The purpose of the accelerator is to ignite the sample in order to oxidize the carbon and sulfur in the sample in a relatively short time frame. The use of an induction furnace is the preferred method of heating and combusting carbon and sulfur analysis.

4.2.7 Chemical oxygen demand (COD)

COD is determined in g O_2/litter, it describes the quantity of O_2 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 $Cr_2O_7^{2-}$ and a catalyst (silver sulphate) under heating conditions(148 ^oC, for 110 minutes). The main purpose is the reduction of Cr^{6+} to Cr^{3+} and the determination of the remaining Cr^{6+} by FAS (ferroammoniumsulfate) titration. For the titration a 25ml Brand digital II titration column was used, for measurement accuracy. It is noteworthy that, dilution was firstly followed in order to approach the appropriate COD range, we work in the high range detection (100-600 mg COD/L). Afterwards the samples were removed from the heating machine. The color of samples after the heating machine, was switched to bluish green from light orange, and after the FAS titration to red, due to the addition of the indicator.

The calculations were determined by the following equations:

$$M(FAS) = \frac{1{,}00\ (mL(K_2Cr_2O_7))*6*Conc.(K_2Cr_2O_7\ in\ M)}{mL\ FAS\ used\ for\ titration} \quad (4.2.7.1)$$

$$mg\frac{COD}{L} = [(mL\ FAS(average\ blank) - mL\ FAS(for\ sample)] * M(FAS) * \frac{8000}{mL\ sample} \quad (4.2.7.2)$$

4.2.8 Methane determination

Gas was tested for CH4 two times per week towards the end of the experiments. A Thermoscientific Trace 1310 GC with a HP-Plot/Q column (Agilent Technologies, USA, length 15 m, diameter 0.320 mm, film 20 µm) was used. The calibration was done each time using min. triple measurements of standard gases 100% CH_4, 60% CH_4 and 5% CH_4. Samples of 0.2 mL were injected into the GC. The calibrated GC then gives calculated values of %CH4 which has to be translated to ml CH_4/g VS at each measuring point, by using the equations eq. (4.2.8.1) and eq. (4.2.8.2).

$$BMP = VCH_4, sample - \frac{VCH_4,blank,avg}{VSadded,sample}\left[\frac{mlCH4}{gVS}\right] (4.2.8.1)$$

$$VCH4, sample = \frac{\%CH4}{100} * Vheadspace * \frac{273.15K}{(273.15K+15K)}\ [NmLCH_4] \quad (4.2.8.2)$$

Where:

- $VCH_{4,blank,avg}$ is the average gas volume produced by blank botles (mL CH_4),
- $VS_{added,sample}$ are the gram of VS of sample added to BMP bottles (g VS)
- $V_{headspace}$ is the headspace volume of BMP bottles (ml)

4.3 BMP assays

The anaerobic digestion of substrates was performed in batch mode based on the bio methane potential (BMP) assay proposed by Angelidaki *et al*. (2009). The ultimate methane potential of substrates was expressed as their specific methane yield (SMY) – defined as the total volume of methane produced during the digestion period per amount of substrate initially added at STP conditions (*i.e*. NmL CH_4/gVS_{added}). The duration of the BMP assay was specifically determined for each substrate, and the test was ended when the cumulative biogas curve reached the plateau phase, usually this happens after 30 days.

In this study, as far as the C/N ratio measurements, the total carbon (TC) of the inoculum and the substrates was measured by LECO analyzer and the percentage of nitrogen in each sample was determined by TKN analysis. Total carbon and total nitrogen content on weight basis of both samples were used to calculate the ratio. The calculation for the C/N ratio of the substrates mixture is as shown below (Eq. (4.3.1)),

$$C/N = \frac{(\%Cs*ms+\%Cin*\mathrm{min})}{(\%Ns*ms+Nin*\mathrm{min})} \quad (4.3.1)$$

Where:

- $\%C_s$, $\%C_{in}$ refer to the carbon content substrate and inoculum, respectively (g/L)
- $\%N_s$, $\%N_{in}$ refer to the nitrogen content substrate and inoculum, respectively (g/L)
- m_s, m_{in} the mass (g) of substrate and inoculum, respectively.

In both of the cases, in order to check how the biodegradability can affect the C/N ratio of the system and visa-versa, three different kinds of substrates were chosen and the main aim of choosing them was their difference in biodegradability, one high, the second one medium and the last one really low biodegradable. Respectively these substrates are Avicel, Wheat Straw and Digested Manure Fibers.

All the bottles were in triplicates, there were also triplicates of control and triplicates containing only inoculum, which included to account for background (i.e. endogenous) methane production.

4.3.1 Experimental set up 1

The first experimental set up was done due to the need of experimental results for the parameter estimation for the Biomodel. In this case triplicates of each substrate, the control and the blank were added to 320 mL bottles. The working volume was 60 mL and the percentage of thermophilic inoculum was used was 40%. At this setup, in each case the same amount of VS (of substrate) and inoculum were used. As a result the same Organic Load (4 gVS/L) was achieved, but in order to have as well the same C/N ratio in each case small and different amount of NH_4Cl needed to be added. At this point noticeable is that if the C/N ratio will be calculated only according to the substrates that were used is 30, but if it will be calculated according to the whole system (by using equation (4.3.1) is almost 5 for all the cases. Which also explain the low methane production that was observed in this case. The bottles were flushed with nitrogen, then sealed and incubated at 55 ºC.

Table 2 First experimental setup

Sample (g)		OL(sub) gVS/L	NH_4CL g	Inoculum (g)	water (g)
Glucose	0.24	4.00	0.0122	24	36
Straw	0.28	4.00	0.0105	24	36
Fibers	0.29	4.00	0.0064	24	36

4.3.2 Experimental set up 2

In the second experimental set up in order to check how the biodegradability can affect different C/N ratio of the system, the substrates were tested, not only in the optimum range of 20-30, but in C/N ratio of 5, 10, 20, 30, 40 and 50, while in the case of fibers until 30. Since the C/N ratio of this substrate was 45, value that did not let us to increase more the C/N ratio of the system. Known amounts of them and 13% of anaerobic thermophilic inoculum were added to 1000 mL bottles.

Important to mention is that no additional external nutrients/ trace elements were added to the BMP bottles as it was considered that all basic nutrient requirements for anaerobic microorganisms were provided by the manure-based inoculum.

Then the BMP bottles were prepared, where a mixture of each sample and the specific inoculum was placed in a sealed aluminum bottle with a working volume of 150 mL and incubated at 55 °C. Before closing them, the bottles were flushed with nitrogen gas to ensure anaerobic conditions.

Table 3 Second experimental setup

C/N	Inoculum g	Avicel g	OL(avicel) gVS/L	Straw g	OL(straw) gVS/L	Fibers g	OL(fibers) gVS/L
5	20	0,18	1,17	0,19	1,11	0,19	1,07
10	20	0,65	4,32	0,75	4,33	0,77	4,40
20	20	1,59	10,62	2,06	11,92	2,46	13,95
30	20	2,54	16,93	3,74	21,60	4,41	25,04
40	20	3,48	23,23	5,94	34,36		
50	20	4,43	29,53	8,98	51,93		

4.4 BioModel implementation for batch processes

As it has already referred the main scope of modifying the model was to implement that in batch conditions, which was achieved by changing the mass balance of the system but keeping the same pH simulation and kinetic equations. Furthermore, inserted the equations that are is being used both for the methane yield (mLCH4/ gVS) and the cumulative methane production. Thus first needed to run the simulation only for the case of the inoculum (blank) and then the mix of the inoculum and substrate. By subtracting then the mL CH_4 of the blank, the final methane yield (as well dividing by the gVS_{added} of the substrate) and the cumulative methane production were calculated.

AD modelling is the virtual representation of real-life processes, as is shown in table (2) there are two columns that should fulfilled in order to determine the input file of the simulation, the first column represents the component concentration while the second the biomass concentration. The inoculum is considered to originate from an ongoing digestion process, thus it is considered that it has bacterial activity inside already, in this case the biomass concentration should be determined. However, in a way to simplify model simulations, the assumption that no degrading activity is taking place in fresh substrates needed to be made.

Since the inoculum that was used in the experiments was the effluent from a lab scale CSTR reactor, in order to have more realistic results this case of the CSTR was simulated and the values of the final biomass concentration were taken and used for the inoculum.

The definition of the inoculum and the substrate plays a crucial role to the results. Thus in the experimental cases that were selected to be simulated tried to be as close as it could be to the experimental measurements of TKN,TAN and g VS_{added} in each case.

As far as the component concentration of the input sheet, could be filled via a series of experimental analyses. Values for the insoluble and inert glucose parameters could be taken from laboratory analyses according to the laboratory analytical procedure proposed by the National Renewable Energy Laboratory of the U.S. Department of Energy (Sluiter *et al*, 2008), while those for lipids could be determined by means of the Soxhlet method, similar to the process outlined by the US EPA *Method 3540C* (1996).

The dissolved T-NH_3 was derived by the total ammonium nitrogen (TAN). Acetic, propionic, butyric and valeric acid (denoted by *HAc*, *HPr*, *HBut* and *HVal*, respectively) can be taken from the VFA analysis and the amount of H_2PO_4 - (expressed on a *g-P/L* basis) could be derived from the methods described by Bader (2011) and Yang *et al.* (2013). It is also assumed that the concentration of CH_4 and H_2S in the substrate is zero, while CO_2 could be measured through titration (Lützhøft *et al*, 2014).

Finally, the value for cation concentration (Z^+) was provided from the cation-anion balance (CAB) given by the sodium (Na^+) and potassium (K^+) concentrations of the substrates, on a *g-K/L* basis, while the amount of anions (A^-) was assumed to be zero.

Nevertheless, the values of Lipids, LCFA, CO_2 H_2S, H_2PO_4, Z^+ and A^- were excluded from the present work and were estimated from literature. Since the units of the model are based on g VS/L, by using the equation (4.4.1) it's possible to determine values that probably are missing from experimental analyses, like in this case Lipids and LCFA.

$$OL\left(\frac{gVS}{L}\right) = CH.is + CH.in + CH.s + Lipids + LCFA + Prot.is + Prot.in + Amino\ acid + Total\ VFA \quad (4.4.1)$$

Crucial role to the results though plays the determination of the insoluble, inert and soluble part of glucose.

Table 4 Template for BioModel input

BioModel input parameter sheet (template)					
Component concentration	*Unit*	**IN/SU**	*Biomass concentration*	*Unit*	**IN/SU**
Insoluble glucose	*[g/L]*		Dead cell mass	*[g/L]*	
Inert glucose	*[g/L]*		Glucose hydrolising enzyme	*[g/L]*	
Soluble glucose	*[g/L]*		Protein hydrolising enzyme	*[g/L]*	
Lipids	*[g/L]*		Glucose acidogens	*[g/L]*	
LCFA	*[g/L]*		Amino acid acidogens	*[g/L]*	
Insoluble protein	*[g/L]*		Lipid acidogens	*[g/L]*	
Inert protein	*[g/L]*		LCFA acetogens	*[g/L]*	
Amino acids	*[g/L]*		Propionate acetogens	*[g/L]*	
HAc	*[g/L]*		Butyrate acetogens	*[g/L]*	
HPr	*[g/L]*		Valerate acetogens	*[g/L]*	
HBut	*[g/L]*		Methanogens	*[g/L]*	
HVal	*[g/L]*				
T-NH_3 (*dissolved*)	*[g-N/L]*				
CH_4 (*dissolved*)	*[g/L]*				
CO_2 (*dissolved*)	*[g/L]*				
H_2S (*dissolved*)	*[g/L]*				
Z+	*[g-K/L]*				
H_2PO_4-	*[g-P/L]*				
A-	*[g-K/L]*				

5 Results and discussion

5.1 BMP assay results

Below are following the results of the methane yield and methane production per each case.

5.1.1 Experimental set up 1

The results of the methane yield shown as it was expected to be higher for Avicel (almost equal to the theoretical value, which make sense since it's high biodegradable) and significant lower for the wheat straw and the digested fibers (also much more lower than their theoretical biomethane values respectively, as it was also expected, since the Buswell formula does not take into acount the biodegradability of the substrate). These results are going were used for the parameter estimation of the model.

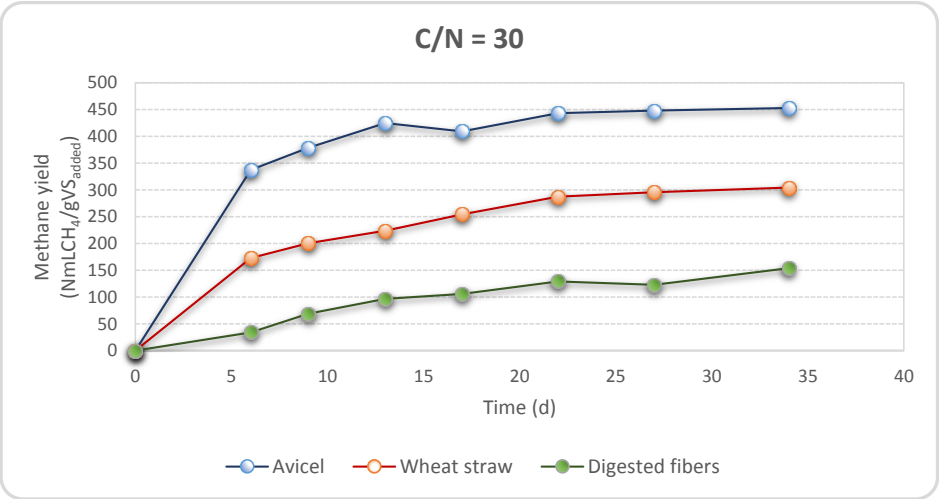

Figure 3 Methane yield (NmL CH4/g VS) of Avicel, Wheat straw and Fibers

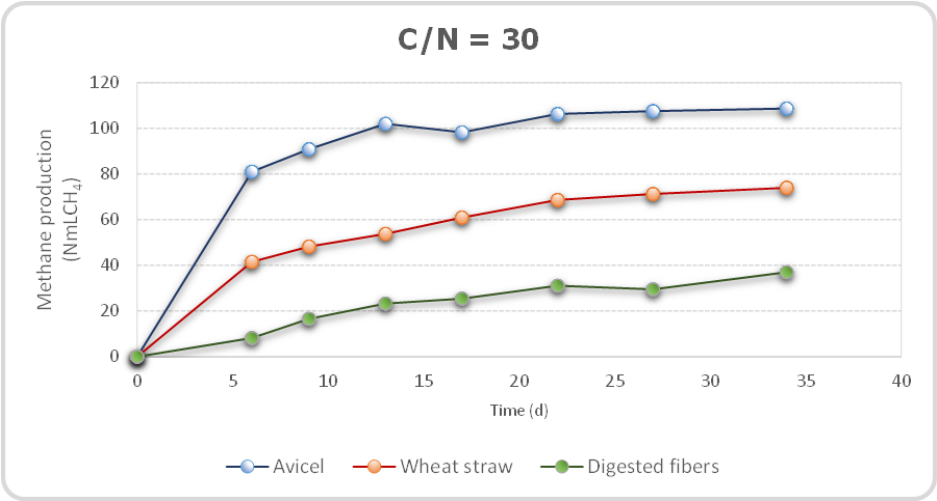

Figure 4 Methane production (NmL CH4) of Avicel, Wheat straw and Fibers

5.1.2 Experimental set up 2

Figures 5, 7, 9 shows the methane yield for a) avicel, b) wheat straw, and c) digested fibers. As observed in Figure 5 and 7 the highest methane yield for avicel (419 ± 4.7 $NmLCH_4$ $g^{-1}VS_{added}$) and wheat straw (262.1 ± 20.7 $NmLCH_4$ $g^{-1}VS_{added}$) was observed for the lowest C/N ratio; whilst the lowest was observed for C/N ratios of 30, 40, and 50 in both cases. In the case of the digested fibers though the results are close to each other, but as it is seen in the end the higher methane (99.4 ± 2.8 $NmLCH_4$ $g^{-1}VS_{added}$) is in the lowest C/N ratio

While figures 6, 8, 10 shows the methane production for a) avicel, b) wheat straw, and c) digested fibers. As observed in these figures the highest methane production for avicel and wheat straw was observed for the C/N ratio of 20; whilst the lowest was observed for C/N ratios of 5, 30, 40, and 50, where the results were close to each other in both cases. In the case of the digested fibers though the higher methane production is in the C/N ratio of 30.

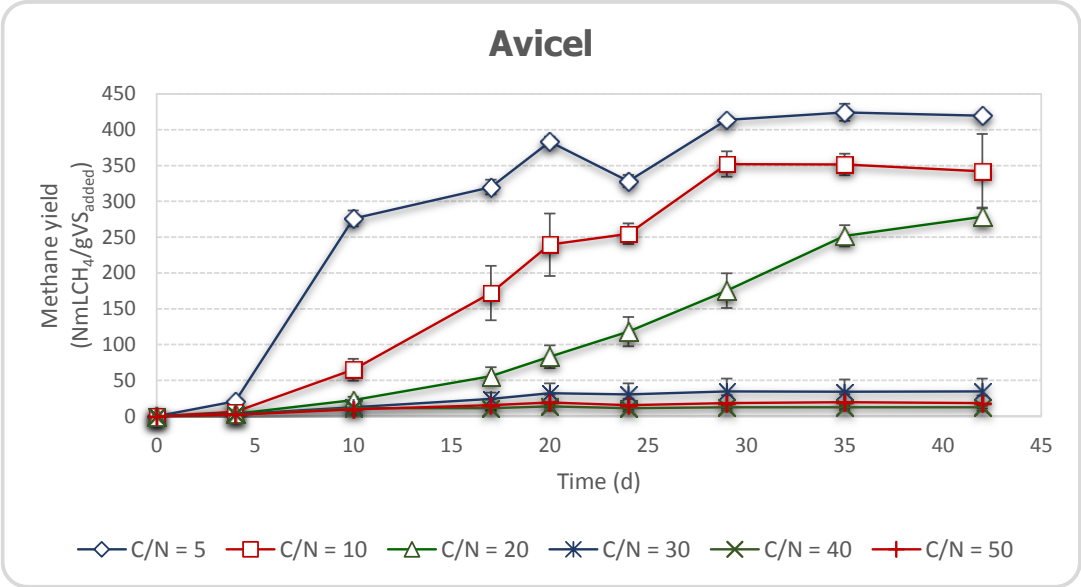

Figure 5 Methane yield (NmL CH4/g VS) of Avicel in C/N ratio 5, 10, 20 , 30, 40 and 50

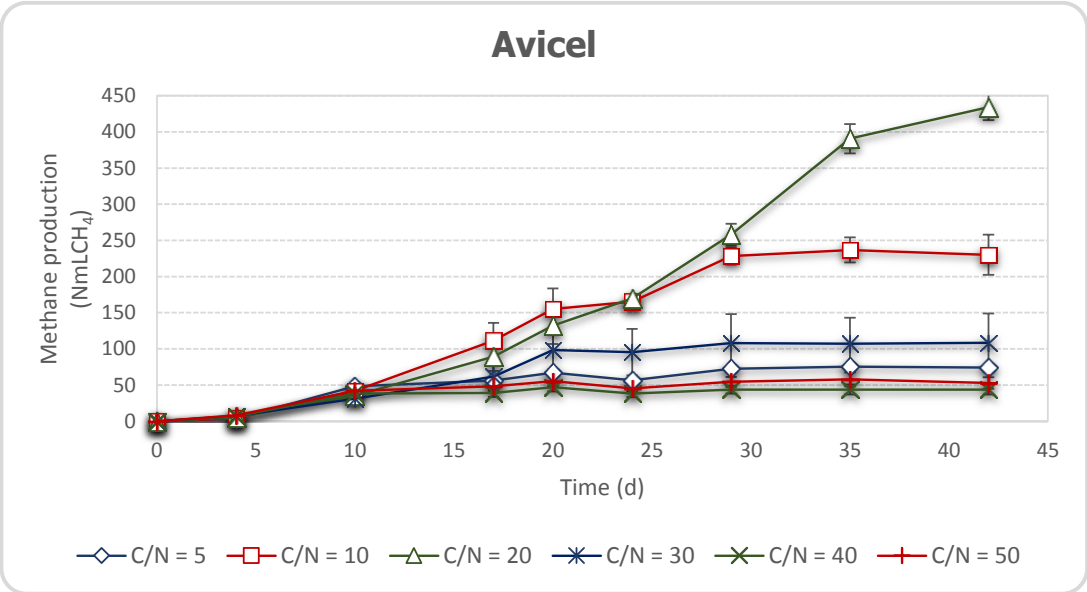

Figure 6 Methane production (NmL CH4) of Avicel in C/N ratio 5, 10, 20, 30, 40 and 50

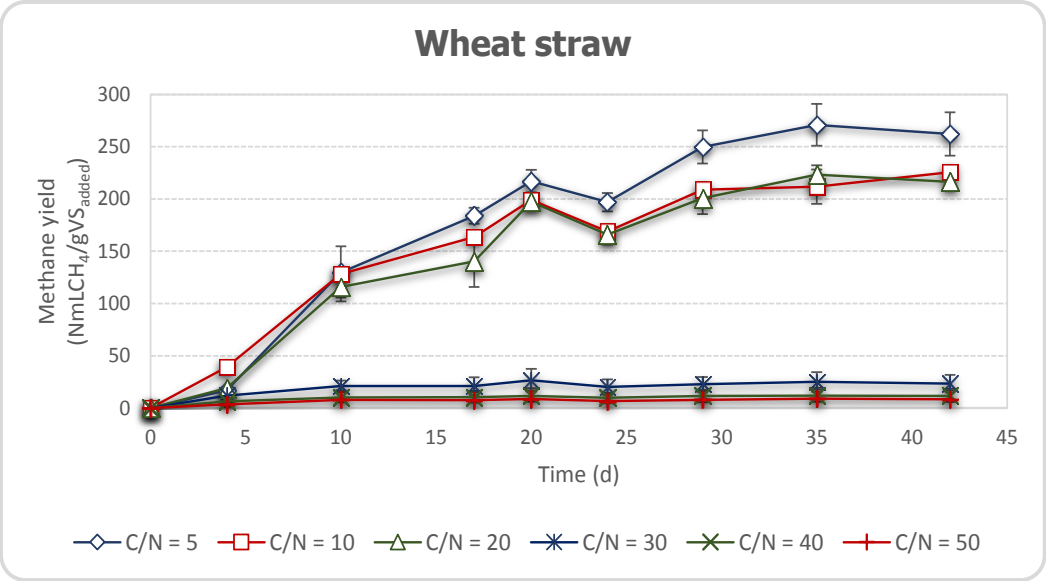

Figure 7 Methane yield (Nml CH4/g VS) of wheat straw in C/N ratio 5, 10, 20, 30, 40 and 50

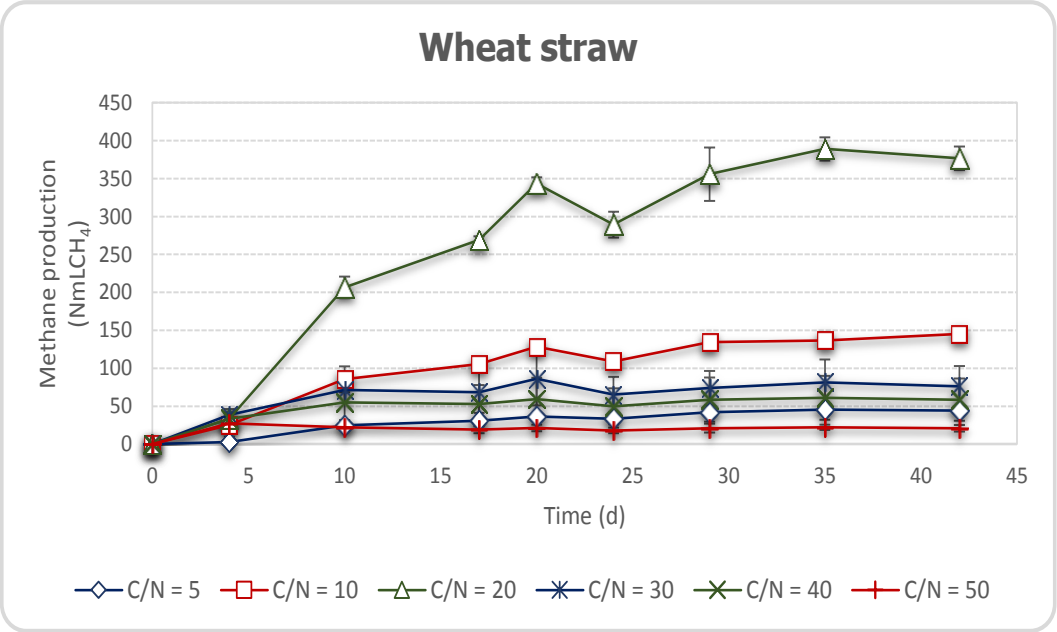

Figure 8 Methane production (NmL CH4) of wheat straw in C/N ratio 5, 10, 20 , 30, 40 and 50

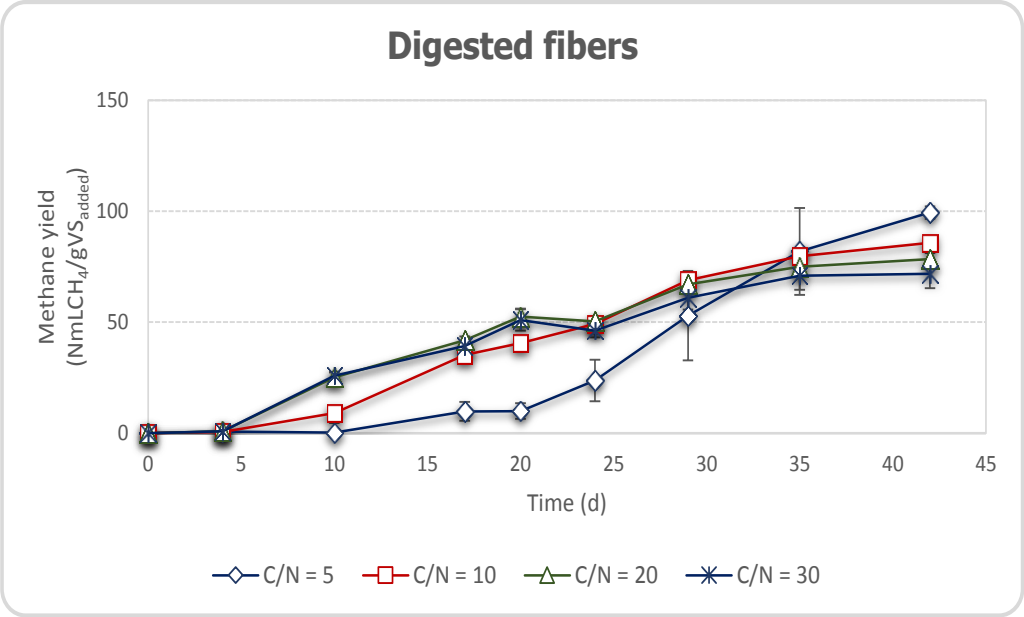

Figure 9 Methane yield (NmL CH4/g VS) of digested fibers in C/N ratio 5, 10, 20 and 30

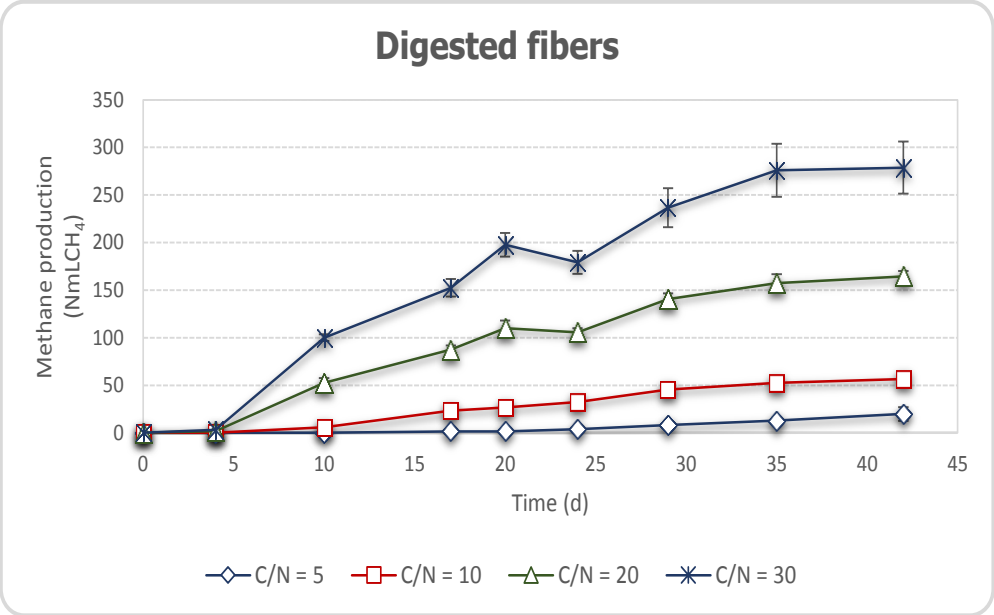

Figure 10 Methane production (NmL CH4) of digested fibers in C/N ratio 5, 10, 20 and 30

In this study as has already mentioned the C/N ratio of the inoculum was taken into consideration in the calculation of the total C/N ratio of the system. The C/N was determined by that way because the procedure is taken place in a closed system so the microorganisms are receiving the total amount of carbon and nitrogen that are having available in each case and are reacting proportionally.

In general a C/N ratio of 25 to 32 has been reported to have a positive effect on the methane yield (Biotech. .course, 2016)). However, as mentioned in the subsection 3.2.3, there are a lot of cases that it's not referred how the ratio has been determined. It has also been reported though that is being determined without taking into account the ratio of the inoculum, for instance in the study performed by Nurliyana *et.al*., (2015). In this report the determination of the optimum C/N ratio was also done according to the methane production and not the yield. While in the report of Yoon et al. (2014), where the S/I ratio was tested of different piggery slaughterhouse wastes, different S/I leads also to different C/N ratio of the system, the figures of methane production and methane yield did not follow the order. That means that if in this case the determination of the optimum C/N ratio of the system had to be done, the result would have been different if it was determined by the methane yield than if it was determined by the methane production. As can also be seen in this study, if the optimum C/N ratio is defined according to the yield is different than according to the production. This could lead to serious misunderstandings of the definition of the optimum C/N ratio of every substrate or co-substrate that is being used each time, thus important is to be defined how it has been calculated and defined in each case, in order to be possible to get equal comparisons.

However, in this case the values of methane yield of Avicel and wheat straw are extremely low, while the C/N ratio is increasing. This can show that there is inhibition in the process, while especially in the case of Avicel, since it is high biodegradable higher methane yield was expected.

In the case of the digested fibers though the results are seen more logical and quite expected, since for this substrate low methane yield was expected (already digested), and in the methane production apparently when the system is in the C/N ratio of 30 seems to be more productive.

As it has already mentioned major part in anaerobic biodegradation plays the pH, so for all the cases the pH of each system was measured right after the experiment. As seen in the table (3) there are crucial differences between the cases which are able to give explanations to the results. According to the literature anaerobic digestion process is limited to a relatively narrow pH interval, approx. is from 6.0 to 8.5, a pH value outside this range can lead to imbalance. pH inhibition at low pH levels, like in this case, is partly due to inhibition by free acids but mainly because a pH well outside the range, for energy-limited microbes such as acetogens and methanogens decreases the energy of anabolism and directs it towards maintenance.

Acid-overload is considered as one of the most common overloads in AD, is pH related and the self-reinforcing pattern that it follows is that overload causes accumulation of acetate. Where acetate accumulation causes drop in pH (normally below 7), but drop in pH inhibits aceticlasts, causing by this way further accumulation of acetate. While further accumulation of acetate leads to further drop in pH (pH drops below 6), since accumulated acetate inhibits hydrogenotrophs and acetogens. The system is fully inhibited and only acidogenesis can occur at a pH normally below 5, where all the COD is converted to acids instead of methane.

Systems that are fed mainly readily degradable organics are susceptible to overload, while systems fed with a significant fraction of the influent as protein are buffered against pH decreases by ammonia, produced from the proteins.

According to this experimental setup in order to increase the C/N ratio of each case more g VS of substrate were added, the system though does not seem able to control this mass of substrate and overcome the acid-overload. The pH in Avicel, the most degradable organic substrate that is used here so more sensitive to overload, starts dropping from the case of C/N ratio 10, while is completely inhibited in C/N ratios higher

than 20. Quite the same pH drop scale follows the wheat straw, which even if it's not completely degradable it's almost assumed that it does not contain proteins, so it's not buffered.

Man. Fibers though seem to have satisfactory pH results, all of the cases are in the limited interval of AD process, as well the graphs of methane yield and production were as expected. At this point is important to mention that in digestion of animal slurries like manure with high ammonia load, the pH can almost not decrease below 7, due to the high buffering capacity of ammonia. In these cases though the system is not fully breaking down but balances in an ''inhibited steady state'' condition, characterized by high VFA concentrations and low methane production yields. The pH of control and blank were also measured in order to ensure that the system without overload could work properly.

In order to strengthen the above explanation though, the VFA concentrations was also measured of all the samples (see appendix 4), where the results of Avicel and Wheat straw in C/N ratio of 30, 40 and 50 were apparently high (these samples need also to diluted and then multiplied by the factor of 2 in order to be in the range of the calibration curve). But in the case of the digested fibers, where also the pH is in the optimum range, the VFA concentrations were significant lower. In this case, this could lead to the conclusion that the low methane yields, which were observed, are mainly due to the low biodegradability of the substrate.

Table 5 pH measurements

C/N ratio	pH measurements		
	Avicel	Wh. Straw	Man. Fibers
5	7.56	7.75	8.20
10	6.97	7.20	7.60
20	6.35	6.70	7.25
30	4.55	4.70	7.00
40	4.50	4.62	
50	4.57	4.63	
CTL	7.28		
Blank	8.28		

Furthermore, by changing the organic load of the substrate leads also to the change of the substrate/ inoculum (S/I) ratio. According to (Yoon et al. 2014), where the methane production of piggery slaughterhouse wastes in different S/I ratio were tested, reported that the cumulative methane production at the lowest S/I ratio (0.1) for all piggery slaughterhouse wastes showed the lowest methane production, with production increasing as the S/I ratio rose from 0.1 to 1.5 with increased VS addition. While almost the opposite were shown in the plots of methane yield, higher yield was showed to the lowest S/I ratio and lower yield to highest one. It's also reported by Hashimoto (1989) that the methane yields were lower at S/I ratios higher than 4.0 in a study on the influence of S/I ratio on BMP in wheat straw, using 20 different S/I ratios (0.03 to 10.91).

Results that seem to agree to this study, while though in this experiment, since the percentage of the inoculum was decided to be only 13%, the differences between S/I ratio were significant bigger. As it can be seen in the following table, the S/I ratio starts from approx. 0.5 for the C/N ratio of 5 and ends up at 15 and 26.3 for avicel and straw respectively for the C/N ratio of 50 and 12.68 for the C/N ratio of 30 in the case of the fibers.

Table 6 S/I ratio

C/N	S/I (Avicel)	S/I (wh.straw)	S/I (man.fibers)
5	0.59	0.56	0.54
10	2.19	2.19	2.23
20	5.38	6.04	7.07
30	8.57	10.94	12.68
40	11.77	17.40	
50	14.96	26.31	

Apparently under this conditions it's not possible and reliable to compare the biodegradability of the substrates. The only cases that could be tested are when the C/N ratio is 5,10 and with prejudice the case of C/N 20, since at this case the S/I can be considered low and the pH starts dropping especially for avicel.

Table 7 Biodegradability of the substrates in the cases of C/N ratio 5,10 and 20, where Bth the theoretical methane potential of each substrate in mg VS/L and in the columns of C/N ratio the respective experimental methane potential in mg VS/L

Sample	Bth	C/N=5	C/N=10	C/N=20	%BD(C/N=5)	%BD(C/N=10)	%BD(C/N=20)
Avicel	415	419.6	343.1	278.4	**101.11**	**82.67**	**67.08**
Wh.Straw	439	262.1	225.1	216.3	**59.70**	**51.28**	**49.27**
Dig. Man. Fibers	448	99.4	85.8	78.4	**22.19**	**19.15**	**17.50**

The table shows the different percentages (%) of biodegradability between the three different substrates in each C/N ratio. It's shown that in each case by increasing the C/N ratio of the substrates the biodegradability decreases. These results though should be taken with caution since as shown above by increasing the C/N ratio the conditions of each system become more unfavorable. So, this might happened because of this inhibition (acidification) and not because in these C/N ratio more amount of substrate can be degraded.

5.2 BioModel bacth results

5.2.1 Parameter estimation

Initially, according to the experimental measurements the input file of each substrate and the inoculum was defined. Also the temperature and the working volume of the system.

In each kinetic step the biomodel contains 9 kinetic parameters, which are referred to the growth rates and the kinetic constants. In a try to investigate which of them influence more the methane yield, the maximum growth rate (μ_{max}) of each degrading step and the hydrolytic constant (k) were seen the most major.

The time-dependent increase in the microbial population in a closed system is stated as a growth curve, which fundamental to all predictive methods is a requirement to mathematically models. Either fully or partly, growth curves for micro-organisms are under a particular interest over a range of environmental conditions. ´´The maximum specific growth rate (μ_{max}) is an important parameter in modelling microbial growth rate under batch conditions´´ (Perni *et al*., 2005). Thus, since the inoculum has a weaker growth curve than when it is among with a substrate, in the model implemented an additional parameter matrix for the constants that are used in the kinetic equations for the case of blank.

Particular emphasis was given to the maximum specific growth rate of each kinetic equation, as well to the constants of the accumulation of enzymes referred to the enzymatic hydrolytic steps. For the case of the blank in order to achieve a satisfactory fitting the temperature dependent maximum specific growth rate ($\mu_{max(T)}$) and the hydrolytic constant (k) of each kinetic equation and hydrolytic step respectively needed to change, specifically needed to be 5% of the parameters that are implemented in the model. Subsequently, the biomodel was tested for the case of Avicel, where it shown a quite satisfying fitting for the methane yield and production, by using the already implemented growth rates and hydrolytic constants that were in the model. For the case of wheat straw though, these needed to change to 50% of the respective implemented. Except the growth rate of the carbohydrate and the propionate degraders, where better results shown when for the first degraders remain the initial value but for the second used only the 5% of it. For the case of the fibers the values went even lower, where the 25% of the initial was used, but keeping again the same values, that were used in the case of the straw, for the carbohydrate and the propionate degraders.

Except from the definition of the growth rate and the hydrolytic constants, as it's shown in the input file, the concentration of the carbohydrates is defined as insoluble, soluble and inert. Values which are affecting the yield and the production of the methane. So in each case, since the substrates that were tested have different biodegradability, the amount of the total carbohydrates that were available for the system had to be determined. Letting the 50% for avicel, the 40% for straw and the 20% for digested fibers to be the soluble part of the total value of the carbohydrates that were used.

In the case of the blank (inoculum system) the endogenous cell decay rate Kd was also increased (from 0.05 to 0.07 d^{-1}), since in this system there is not substrate and the decay rate is higher.

After the above parameter estimation, as it can be seen in the following plots, where the experimental and the simulated data are being compared, the BioModel can give quite satisfactory results. As it's shown it can predict the methane yield and production of three substrates with different component structure, which leads to different yields of biodegradable carbon.

First in figure 11, is shown the graph of the blank (the case with only inoculum), which further is being subtracted from the mix (inoculum + substrate) in order to get the pure methane yield and production of each case. Subsequently are following the figures of methane yield and methane production for avicel, wheat straw and digested fibers respectively.

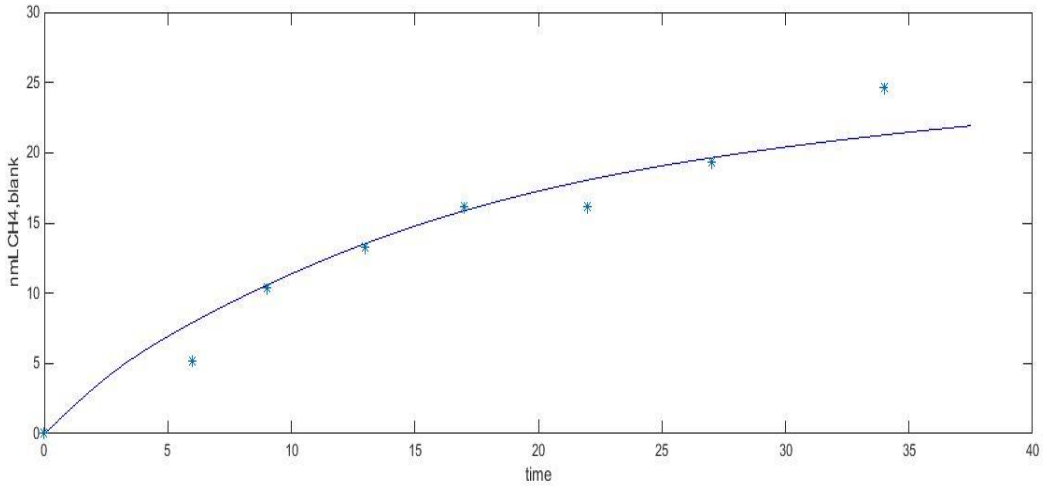

Figure 11 Methane production (NmL CH4) of the blank

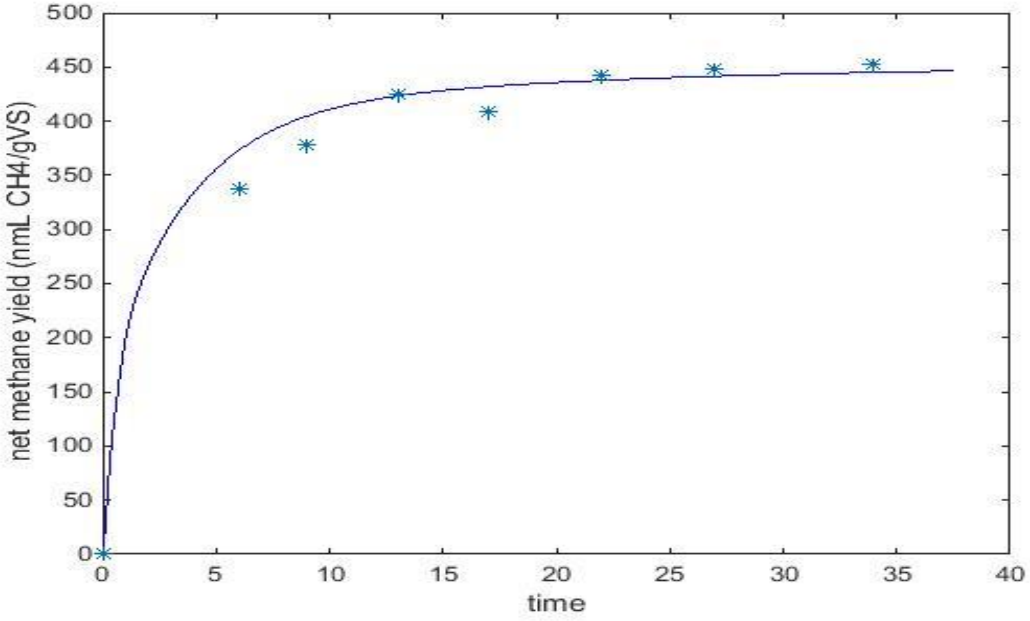

Figure 12 Methane yield of Avicel (NmL CH4 / g VSadded)

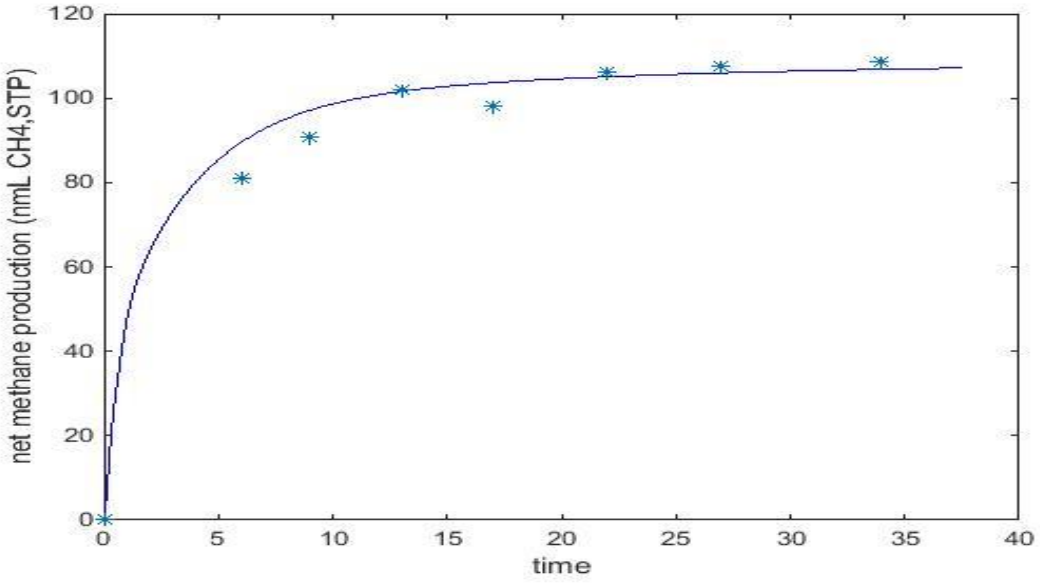

Figure 13 Methane Production of Avicel (NmL CH4)

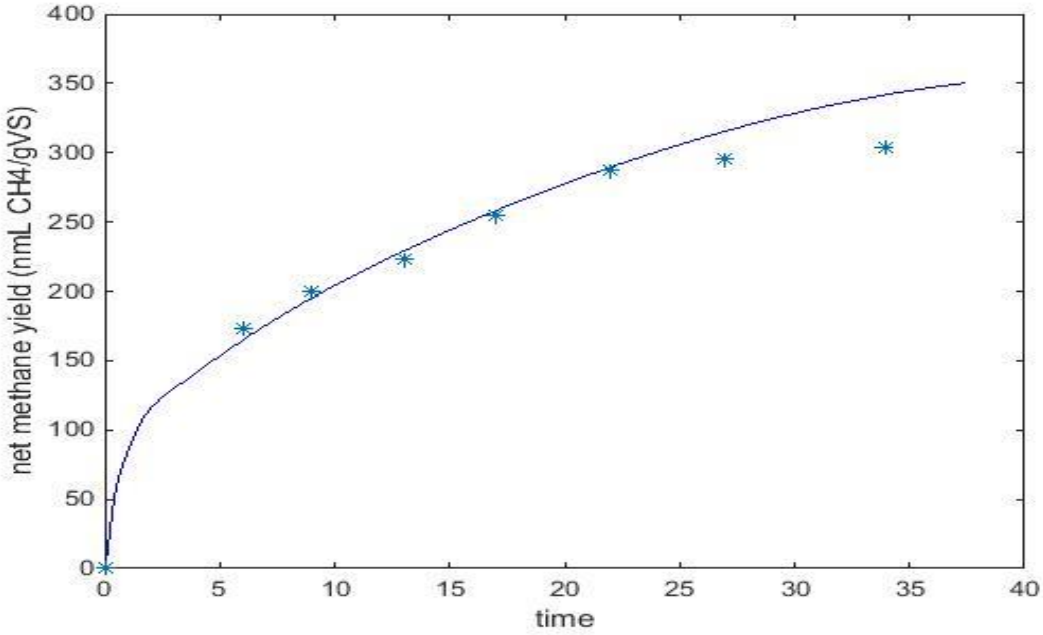

Figure 14 Methane yield of Wh.Straw (NmL CH_4/gVS_{added})

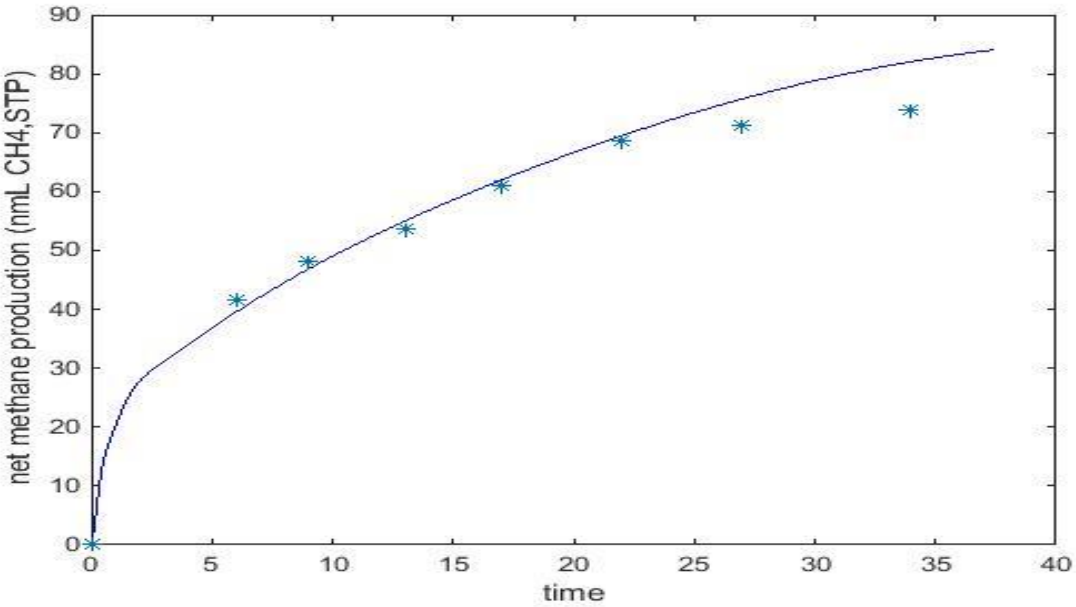

Figure 15 Methane Production of Wh. Straw (NmL CH_4)

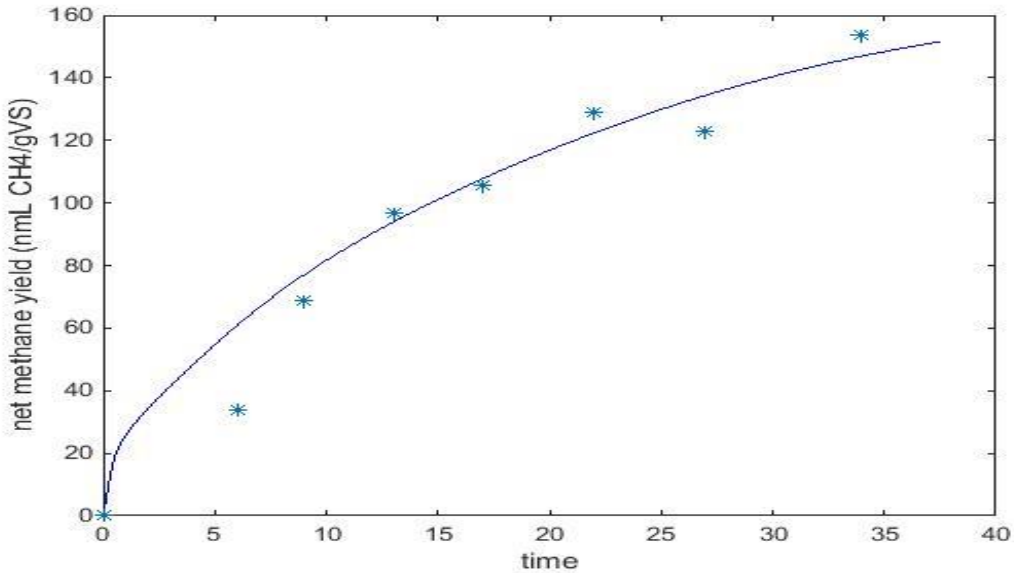

Figure 16 Methane yield of digested Man. Fibers (NmL CH_4 / g VS_{added})

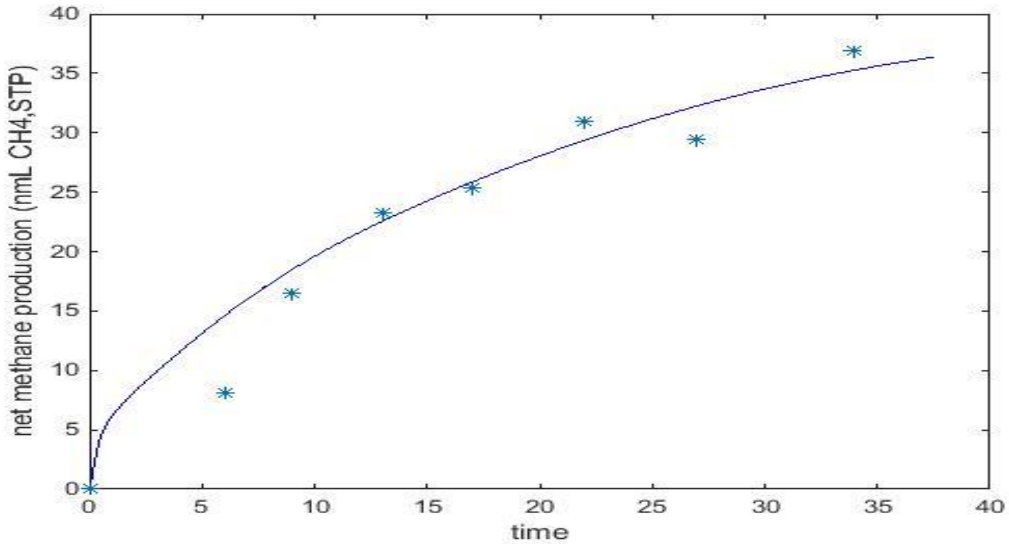

Figure 17 Methane Production of digested Man. Fibers (NmL CH4)

As is shown in the figures the biomodel predicts more satisfactory the case of the Avicel, both for methane yield and production. Moving to the next case where a substrate with medium biodegradability was checked, the wheat straw, as can be seen the fitting is good, but with an overestimation of the model in the stationary phase. While in the third case, where a low biodegradable substrate was simulated, the biomodel showed less satisfactory results, especially in the prediction of the lag phase. Which means that probably additional parameters have to be taken into account during the parameter estimation.

6 Conclusions and future prospects

6.1 Experimental

According to Angelidaki *et al*., (2011) it is known that different biomass feedstocks (organic wastes and residues) have different methanogenic potential, depending on the inherent degradability, as well as the carbon-oxidation state. The amount of biogas produced and the content of methane in the gas phase depend on the waste being degraded, both its degradability and its oxidation state. So the better degradability and the lower the oxidation state, the more methane will be produced, well this by comparing the different kinds of substrates in the lower C/N ratios was also shown in this study.

However due to the acid-overload in the higher C/N ratio this can't be shown. Apparently in order to get reliable and comparable results in each case, the system has to work under favorable conditions. Firstly this means that in order to avoid the acidification of the process in higher C/N ratio it's crucial the right amount of inoculum to be chosen. This could achieved by using again a well degassed inoculum but not so diluted (to be used higher percentage than 13%), but even if the percentage of it will be enough higher though like 80%, does not confirm us that the system especially in the readily degradable substrates will work properly in C/N ratio higher than 40. Because in the case of these substrates for example in order to achieve higher C/N ratio than 40, again high amount of organic load has to be added, which will lead to higher S/I ratio, and to acidification of the process.

So in order to ensure favorable conditions to the systems with high C/N ratio a possible scenario could be to run the experiment but this time by keeping a constant S/I ratio in all cases (apparently according to Hashimoto, 1989 this has to be below 4). Needless to say though that in this last case in order to keep constant the S/I ratio, the amount of the inoculum has to change in each C/N ratio. Which on the one hand changing two values in the experiment (mass of the substrate and inoculum) might not be preferable but on the other hand it could be safer since any inhibition will be avoided and the final effect of the biodegradability in higher C/N ratios will be shown.

6.2 BioModel

In general the biomodel is highly dependent on empirical parameters but it also provides a direct access to the affected parts through the MATLAB code. This gives the opportunity to change them, once more specific theoretical information becomes available about their calculation.

In this study during the parameter estimation more emphasize were given to the hydrolytic constants of carbohydrate and protein hydrolysis, to the growth of microorganic communities, specifically to the maximum growth rate and to the percentage of the biodegradable carbohydrates, all of them showed their sensitivity to the simulation results.

In the BioModel mainly are described details of pH and temperature characteristics in order to accurately simulate the free ammonia concentration which is one of the crucial factors for inhibiting the process. However, after the second experimental set up, which is shown how crucial can also be the acetate inhibition

in the methanogenesis step, it would have been really interesting to check how the acid overload can affect the simulations. In this current study, unlikely, due to the lack of time, this did not happen.

Furthermore, experimental data of different composition of substrates (with protein and lipid content) could also be used in order to estimate parameters and validate the BioModel.

So far, in this study it's shown that the BioModel except from working in continuous conditions is also possible to work in batch mode and predict quite successfully the methane yield and production of each case. Promising results which could lead to a future use of the model instead of BMP assays.

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Appendices

Appendix 1.

The flowsheet of the model is presented in Fig. 2 and is based on the description by Angelidaki et al (1993, 1999). As seen, the flow of the BioModel AD process can be distributed into five separate phases, which are:

"INPUTS". In this phase complex substrates are described in terms of their main organic and inorganic components. The organic fraction of each substrate is constituted by carbohydrates, proteins and lipids, which later degrade into fatty acids, CO_2 and other , while the inorganic fractions consists of NH_4^+, P, carbonate (CO_2), hydrogen sulphide (H_2S), anions (A^-) and cations (Z^+). By assuming that carbohydrates are present in the form of glucose, proteins in the form of gelatine and lipids in the form of glycerol trioleate (GTO), the model offers the possibility to use the results of common analytical measurements as input values, making quantification more straightforward. In addition, it accounts for the decay of dead bacterial cell mass, assuming that such biomass decomposes into carbohydrates and proteins and adding to the amount of these initial organic fractions. Although this step is not shown in the flowsheet.

"HYDROLYSIS". In the second phase of the process the enzymatic degradation (*yellow circles*) of carbohydrates and proteins to either inert material or soluble glucose and amino acids is introduced. The hydrolysis follows first order kinetics. In Fig. 2, *Carb.in* and *Prot.in* stand for inert glucose and gelatin, while *Carb.s* and *AA* represent soluble glucose and amino acids, respectively.

The stoichiometry of the carbohydrates' hydrolysis is

$$(C_6H_{10}O_5 * nNH_3)is \rightarrow Ye(C_6H_{10}O_5)s + (1 - Ye)(C_6H_{10}O_5\,.mNH_3)in + (n - (1 - Ye).m)NH_3$$

Where Ye is the enzymatic efficiency (yield factor), the subscript **is** represents the insoluble carbohydrates and the **s** the soluble part (in our case glucose). While subscript ***in*** represents the undegradable inert organic material (such as lignin, inaccesible structural cellulose, and other undegradable organic material).

The respective hydrolysis part for the proteins is

$$(Protein)is \rightarrow Yp(Amino\ acids) + (1 - Yp)(Protein)in$$

Where *Yp* is the degradability of protein.

"ACIDOGENESIS". In this phase soluble glucose, amino acids and GTO are consumed by acidogenic bacteria (*green circles*) and converted into VFAs and alcohols. As seen in the flowsheet, glucose is converted to acetic, propionic and butyric acid, while glycerol – the intermediate of GTO acidogenesis – is turned into propionic acid, under the assumption of instant degradation. Amino acids, on the other hand, can be converted to all three acids mentioned above, with the inclusion of valeric acid. As indicated in the flowsheet, butyric and valeric acid can be present both with their n-forms and isoforms. Alcohol formation is not shown in this visual representation.

In this phase the model has an important aspect of the extent of conversion on the hydrolysis step described previously. If the concentration of VFAs increases above to a certain level, the self-regulatory mechanisms of the system inhibit the further hydrolysis of the initial organic compound, in an attempt to reinstate the process equilibrium. However, if the VFA production exceeds the buffer capacity of the overall system, acidification might take place, leading to process failure and the death of pH sensitive microorganisms, such as methanogens. This regulatory effect is included in the model. Moreover, LCFA inhibition is also included (in the form of oleate inhibition), affecting every process phase in the model.

The exact stoichiometry of each component that takes place in this phase is shown below for glucose, GTO and Amino Acids respectively.

The soluble glucose degradation to VFA by acidogenic bacteria

$$(C_6H_{10}O_5)s + 0.1115NH_3 \rightarrow 0.1115C_5H_7NO_2 + 0.744CH_3COOH + 0.5CH_3CH_2COOH + 0.4409CH_3CH_2CH_2COOH + 0.6909H_2O$$

The overall reaction of GTO degradation, which it comes from combining the GTO lipolysis to oleate and glycerol (by glycerol-fermenting acidogenic bacteria) and the glycerol degradation to biomass and propionate.

$$C_{57}H_{104}O_6 + 1.90695\ H_2O + 0.04071\ NH_3 + 0.0291\ CO_2 \rightarrow 0.04071\ C_5H_7NO_2 + 0.0941843\ C_3H_6O_2$$

And the degradation of Amino Acids (CH2.03O0.6N0.3S0.001) to VFA by acidogenic bacteria.

$$CH_{2.03}O_{0.6}N_{0.3}S_{0.001} + 0.3006\ H_2O \rightarrow 0.017013\ C_5H_7NO_2 + 0.29742\ C_2H_4O_2 + 0.02904\ C_3H_6O_2 + 0.022826\ C_4H_8O_2 + 0.013202\ C_5H_{10}O_2 + 0.07527\ CO_2 + 0.28298\ NH_3 + 0.001\ H_2S$$

"ACETOGENESIS". Here, propionic acid, butyric acid, valeric acid and LCFA are converted by acetogens to acetic acid, while hydrogen (H2) and traces of CO2 are also generated. As opposed to the degradation of the VFAs, H2 is converted to methane (CH4) faster, hence this step was merged with the VFA degradation in the equations that describe the model. Nevertheless, Fig.4 shows H2 formation as a distinct step in the process flow. Acetogenesis is affected by the total concentration of acetic acid, and similar to the hydrolysis step, is subject to inhibition when this concentration exceeds the system buffer capacity. Acetic acid inhibition is represented by a non-competitive expression.

The respective stoichiometry of each component in this step is the following

The overall oleate degrading reaction, which is derived by combining the LCFA step (by LCFA acetogenic bacteria) and the hydrogen utilizing step.

$$C_{18}H_{34}O_2 + 7.7401\ H_2O + 4.0834\ CO_2 + 0.2537\ NH_3 \rightarrow 0.2537\ C_5H_7NO_2 + 8.6998C_2H_4O_2 + 3.4139\ CH_4$$

The overall propionate degrading acetogenic step which is derived by combining the propionic step with the hydrogen utilizing step.

$$CH_3CH_2COOH + 0.06198\,NH_3 + 0.134H_2O \rightarrow 0.06198C_5H_7NO_2 + 0.9345CH_3COOH + 0.6604CH_4 + 0.1607CO_2$$

The overall butyrate degrading acetogenic step which is derived by the combination of the butyrate step and hydrogen utilizing step.

$$CH_3CH_2CH_2COOh + 0.0653NH_3 + 0.5543CO_2 + 0.8038H_2O \rightarrow 0.0653C_5H_7NO_2 + 1.8909CH_3COOH + 0.4452CH_4$$

And finally the degradation of valerate degrading acetogenic step which was determined experimentally (Angelidaki et al.1999).

$$C_5H_{10}O_2 + 0.0653\,NH_3 + 0.5543\,CO_2 + 0.8045\,H_2O \rightarrow 0.0653\,C_5H_7NO_2 + 0.8912\,C_2H_4O_2 + 0.02904\,C_3H_6O_2 + 0.4454\,CH_4$$

"METHANOGENESIS". The final phase of the process where CH4 and CO2 are produced from the available acetic acid and H2. The final stoichiometry equation for this aceticlastic step is the following one.

$$CH_3COOH + 0.022NH_3 \rightarrow 0.022C_5H_7NO_2 + 0.945CH_4 + 0.945CO_2 + 0.066\,H_2O$$

To all of the above reactions the empirical formula C5H7N02 is used to express the corresponding cell mass in each bacterial step.

So far, it could be seen in the flowsheet that all bacterial groups – with the exception of amino acid degraders – utilise ionised ammonia for cell growth (*green arrows*), which applies to acetoclastic methanogens as well. For this reason, $NH4^+$ was introduced as a co-substrate in the model.

Methanogens are considered to be the most sensitive bacterial group among the ones involved in the AD system described by the BioModel, and as such, free NH3 inhibition is a major threat for their existence. Through the affection of methanogens, an increase in free NH3 concentrations can trigger the accumulation of VFAs, which eventually inhibits the enzymatic hydrolysis and reduces the system pH.

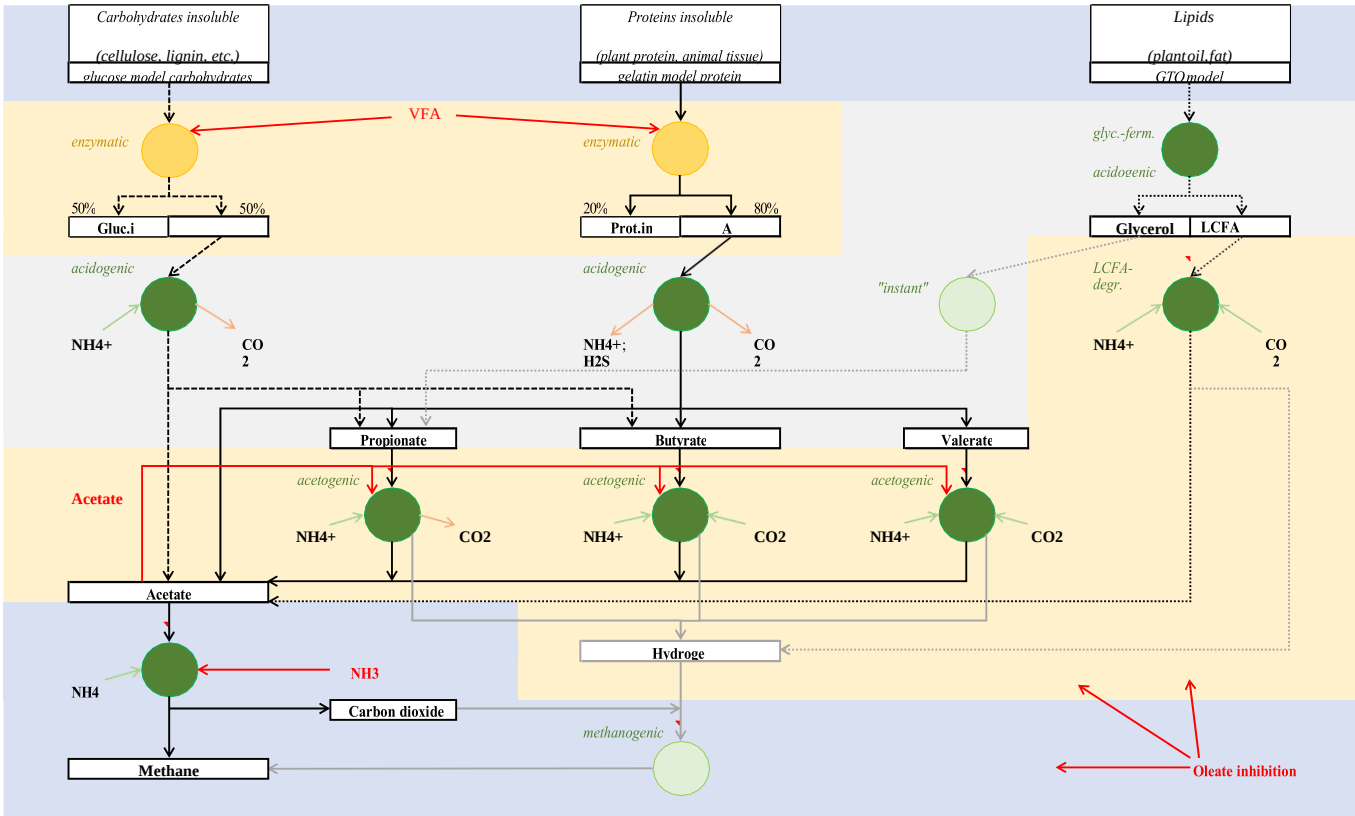

Figure 4: explaining flowsheet of the main biochemical pathways of the BioModel

Appendix 2.

pH simulation and kinetics used in the model.

pH simulation

The updated version of the BioModel in matlab follows almost strictly all the equations that were used in the first version of the complex model. According to Angelidaki et al, 1993 the pH of the procedure is calculated first by the ionic equilibrium of the components present in the liquid: $C0_2$, NH_3, HAc, HPr, HBut, H_2P0_4, and Z^+, An^-, which represent cations and anions, respectively. HS^- and LCFA have also been included. Each component is ionized to a degree determined by the pH, i.e.

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+ \qquad (K_{a1,CO_2})$$

$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+ \qquad (K_{a2,CO_2})$$

$$HAc \leftrightarrow Ac^- + H^+ \qquad (K_{a,HAc})$$

$HPr \leftrightarrow Pr^- + H^+$ $\quad (K_{a,HPr})$
$HBut \leftrightarrow But^- + H^+$ $\quad (K_{a,HBut})$
$NH_4^+ \leftrightarrow NH_3 + H^+$ $\quad (K_{a,NH_3})$
$H_2PO_4^- \leftrightarrow HPO_4^{2-} + H^+$ $\quad (K_{a_2,H_3PO_4})$
$H_2S \leftrightarrow HS^- + H^+$ $\quad (K_{a_{H_2S}})$
$LCFA \leftrightarrow LCFA^- + H^+$ $\quad (K_{a,LCFA})$
$H_2O \leftrightarrow OH^- + H^+$ $\quad (K_w)$

The resulting equilibrium pH was determined by iteratively solving the charge balance equation:

$$\begin{aligned}[H^+] - [OH^-] = Ch(pH) \\ &= [HCO_3] + 2[CO_3^{2-}] + [Ac^-] + [Pr^-] + [But^-] + [An^-] + [H_2PO_4^{2-}] + 2[HPO_4^{2-}] \\ &+ [HS^-] + [LCFA] - [NH_4^+]\end{aligned}$$

The derivative of Ch(pH) function according to H^+ is also calculated, then by following the Newton Raphson method the final value of H^+ is calculated and the value of pH is given by the equation:

$$pH = -\log_{10}(H)$$

Then according to the pH the different ion concentrations of the components are calculated.

The effect of pH on the growth rate was described by a Michaelis pH function, normalized to give a value of 1.0 as center value (Angelidaki et al. (1993), page 161):

$$F(pH) = \left(\frac{1 + 2 * 10\big(0.5 * (pKl - pKh)\big)}{\big(1 + 10(pH - pKh) + 10(pKl - Ph)\big)}\right)$$

Where pKl and pKh denote the lower and upper pH dropoff value, where the growth rates are approximately 50% of the uninhibited rate.

KINETICS

According to Angelidaki et al. (1999) the kinetic equations for all steps included in the model are summarized in the below table.

Conversion	Kinetic equation	
Enzymatic hydrolytic steps	$R_S = k\,S \quad \wedge \quad k = k_o \dfrac{k_i}{k_i + \Sigma VFA}$	(1)
Acidogenic glucose degrading step	$\mu = \mu_{max}(T)\left(\dfrac{1}{1+\dfrac{K_s}{[(C_6H_{10}O_5)_s]}}\right)\left(\dfrac{1}{1+\dfrac{K_{s,NH3}}{[T-NH3]}}\right)\left(\dfrac{1}{1+\dfrac{[LCFA]}{K_{i,LCFA}}}\right)$	(2)
Lipolytic step	$\mu = \mu_{max}(T)\left(\dfrac{1}{1+\dfrac{K_s}{[GTO]}}\right)\left(\dfrac{1}{1+\dfrac{K_{s,NH3}}{[T-NH_3]}}\right)\left(\dfrac{1}{1+\dfrac{[LCFA]}{K_{i,LCFA}}}\right)F(pH)$	(3)
LCFA acetogenic step	$\mu = \mu_{max}(T)\left(\dfrac{1}{1+\dfrac{K_{s,LCFA}}{[LCFA]}+\dfrac{[LCFA]}{K_{i,LCFA}}}\right)\left(\dfrac{1}{1+\dfrac{K_{s,NH3}}{[T-NH_3]}}\right)F(pH)$	(4)
VFA acetogenic step	$\mu = \mu_{max}(T)\left(\dfrac{1}{1+\dfrac{K_s}{[A]}}\right)\left(\dfrac{1}{1+\dfrac{K_{s,NH3}}{[T-NH_3]}}\right)\left(\dfrac{1}{1+\dfrac{[HAc]}{K_{i,HAc}}}\right)\left(\dfrac{1}{1+\dfrac{[LCFA]}{K_{i,LCFA}}}\right)F(pH)$	(5)
Aceticlastic methanogenic step	$\mu = \mu_{max}(T)\left(\dfrac{1}{1+\dfrac{K_s}{[HAc]}}\right)\left(\dfrac{1}{1+\dfrac{K_{s,NH3}}{[T-NH_3]}}\right)\left(\dfrac{1}{1+\dfrac{[NH_3]}{K_{i,NH3}}}\right)\left(\dfrac{1}{1+\dfrac{[LCFA]}{K_{i,LCFA}}}\right)F(pH)$	(6)

Where: S is the substrate for the insoluble part of carbohydrates or for the insoluble proteins; k is the reaction rate; Rs is the substrate utilization rate; $\mu_{max(T)}$ is the temperature-dependent maximum specific growth rate; K_i is the half-saturation constant; $Ks,NH3$ is the half-saturation constant for total ammonia; $[T-NH3]$ is the total ammonia concentration; Ki denotes inhibition constants and $F(pH)$ is the pH growth- modulating function.

Enzymatic Hydrolytic Steps

For the hydrolytic steps, first-order reaction rates were applied, which has been reported to be the best way to describe the kinetics of these steps (Pavlostathis et al., 1988). The hydrolytic reaction rates were assumed to be inhibited by the sum of VFA (acetate, propionate, butyrate, and valerate) taken on a molar basis.

Primary Substrate Growth Dependency

For all the bacterial steps Monod type kinetics were used with respect to their primary substrate. All bacterial steps require ammonia-N as nitrogen source for cell mass synthesis (i.e., all steps except amino acid degradation). Therefore, a Monod type ammonia-N cosubstrate dependency is included. Also LCFA inhibition has been introduced as a non-competitive inhibition expression in all steps except for the LCFA acetogenic step, where a Haldane-type substrate inhibition has been used.

Decay of Cell Mass

Bacterial death rate has generally been assumed to be 5% of the maximum growth rate.

$$celldeath = \mu_{\max\_T0.} * K_{d.} * X$$

Where Kd is the endogenous cell decay rate and X the biomass concentrations (g/L).

A first-order decay of dead cell mass with a rate of 0.01 h^{-1} is used (celldecay = 0.01*deadcells).

In order to keep all the results in the model a matrix is being created to hold the results of the derivation and all the components are changing according to the yield coefficient table.

Appendix 3.

Results from 1st BMP set up

According to Angelidaki et al. 2009, the BMP results should always be accompanied by a clear description of the components that were used, where in that case are inoculum, avicel, wheat straw and digested manure fibers.

TS, VS, TKN, TAN and TC were measured to all of them, apparently except from avicel which is a pure microcrystalline cellulose powder so it does not contain any Nitrogen ($C_6H_{10}O_5$). It is also assumed that is completely volatile and the volatile solid (VS) value is 1000 g VS/ kg substrate and the TS/VS is 100%.

In the below tables are following the values of the average of the triplicates that were measured in each case.

Sample	gTS/gwet sample	% (gTS/gwet sample)	% moisture	gVS/gwet sample	% (gVS/gwet sample)	% (gVS/gTS)	% (gash/gwet sample)	% (gash/gTS)
Inoculum	0.0240	**2.40%**	97.60%	0.0141	**1.41%**	**58.59%**	0.99%	41.41%
St.Dev	0.0003487	0.03%	0.03%	0.0003	0.03%	0.45%	0.01%	0.45%
Wh.Straw	0.9284	**92.84%**	7.16%	0.8672	**86.72%**	**93.41%**	6.12%	6.59%
St.Dev	0.0001	0.01%	0.01%	0.0039	0.39%	0.42%	0.39%	0.42%
Man. Fibers	0.8693179	**86.93%**	13.07%	0.8315555	**83.16%**	**95.66%**	3.78%	4.34%
St.Dev	0.0014949	0.15%	0.15%	0.0056914	0.57%	0.49%	0.42%	0.49%

Table: TS, VS measurements

Sample	TKN (g/kg)	TAN(g/kg)
Inoculum	**2.08**	**1.72**
St.Dev	0.18	0.15
Wh.Straw	**4.82**	**0.76**
St.Dev	0.08	0.01
Man. Fibers	**9.49**	**1.96**
St.Dev	0.05	0.27

Table: TKN, TAN measurements

Sample	TC (gC/ gwet sample)
Inoculum	0.65%
Wheat Straw	42.20%
Man.Fibers	45.80%

Table: TC measurements

Appendix 4.

Results from 2nd BMP measuremnts

As in the 1st experimental set up, the second one also was done according to the BMP protocol of Angelidaki et al. 2009. So TS, VS, TKN, TAN, COD and TC were measured to inoculum, avicel, wheat straw and digested manure fibers that were used this time.

Sample	gTS/gwet sample	% (gTS/gwet sample)	% moisture	gVS/gwet sample	% (gVS/gwet sample)	% (gVS/gTS)	% (gash/gwet sample)	% (gash/gTS)
Inoculum	0.0240	**2.40%**	97.60%	0.0141	**1.41%**	**58.59%**	0.99%	41.41%
St.Dev	0.0003487	0.03%	0.03%	0.0003	0.03%	0.45%	0.01%	0.45%
Wh.Straw	0.9284	**92.84%**	7.16%	0.8672	**86.72%**	**93.41%**	6.12%	6.59%
St.Dev	0.0001	0.01%	0.01%	0.0039	0.39%	0.42%	0.39%	0.42%
Man. Fibers	0.8693179	**86.93%**	13.07%	0.8315555	**83.16%**	**95.66%**	3.78%	4.34%
St.Dev	0.0014949	0.15%	0.15%	0.0056914	0.57%	0.49%	0.42%	0.49%

Table: TS, VS measurements

Sample	TKN (g/kg)	TAN(g/kg)
Inoculum	**2.08**	**1.72**
St.Dev	0.18	0.15
Wh.Straw	**4.82**	**0.76**
St.Dev	0.08	0.01
Man. Fibers	**9.49**	**1.96**
St.Dev	0.05	0.27

Table: TKN, TAN measurements

Sample	TC (gC/ gwet sample)
Inoculum	0.65%
Wheat Straw	42.20%
Man.Fibers	45.80%

Table: TC measurements

sample/C:N ratio	total VFA (mg/L)	stdev
Avicel/5	11,37	5,35
Avicel/10	6,42	1,31
Avicel/20	256,99	125,77
Avicel/30	1319,61	293,61
Avicel/40	1368,20	369,23
Avicel/50	3135,34	237,37
wheast straw/5	6,63	1,24
wheat straw/10	3,76	0,96
wheat straw/20	5,82	1,40
wheat straw/30	2803,70	97,86
wheat straw/40	3436,52	340,28
wheat straw/50	3742,41	1575,99
Fibers/5	28,46	28,70
Fibers/10	3,48	0,38
Fibers/20	5,26	1,52
Fibers/30	3,52	0,23
BLANK	60,08	23,59
CONTROL	2,05	0,22

Table: VFA measurements

According to Hill *et al* (1987) acetate concentrations higher than 13mM have been suggested to indicate imbalance.

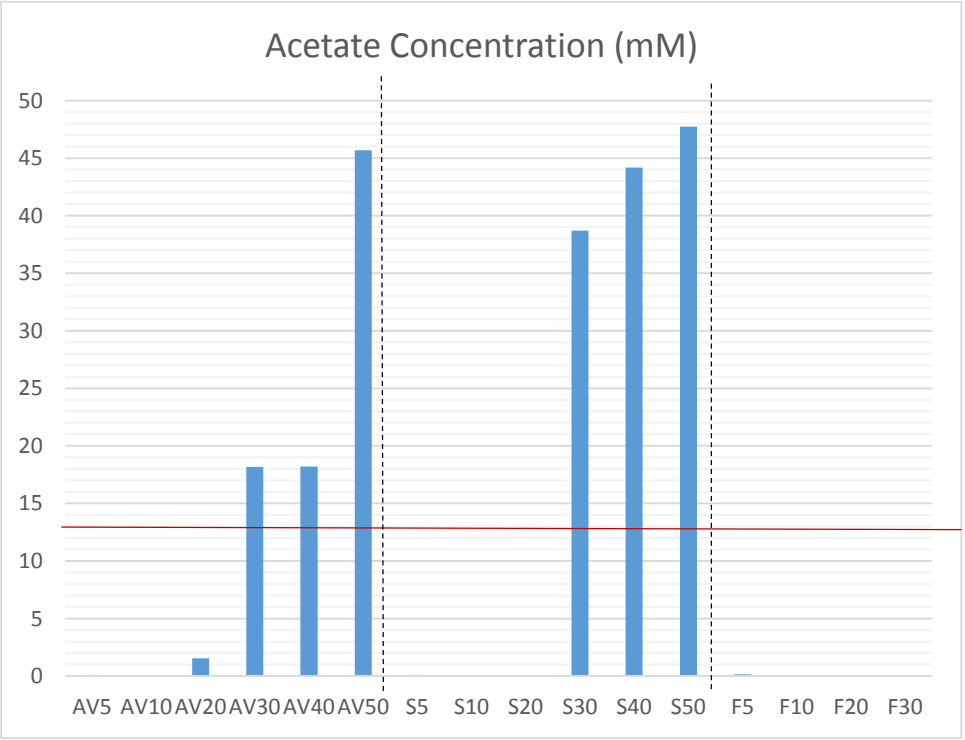

Figure: Acetate concentration

Appendix 5.

	BLANK	AVICEL (def.param.)	STRAW	FIBERS	% def.param.-Blank	% def.param.-Straw	% def.param.-Fibers
% Carbohydrate enzymes	6.25E-03	1.25E-02	6.25E-03	3.13E-03	50%	50%	25%
% Protein enzymes	2.08E-03	4.17E-02	2.08E-02	1.04E-02	5%	50%	25%
% Carbonhydrate degraders	1.06E-02	2.13E-01	2.13E-01	2.13E-01	5%	100%	100%
% Amino acid degraders	1.33E-02	2.66E-01	1.33E-01	6.65E-02	5%	50%	25%
% Lipid degraders	1.10E-03	2.21E-02	1.10E-02	5.52E-03	5%	50%	25%
% LCFA degraders	1.15E-03	2.29E-02	1.15E-02	5.73E-03	5%	50%	25%
% Propionate degraders	1.02E-03	2.04E-02	1.02E-03	1.02E-03	5%	5%	5%
%Butyrate degraders	1.40E-03	2.79E-02	1.40E-02	6.98E-03	5%	50%	25%
% Valerate degraders	1.44E-03	2.88E-02	1.44E-02	7.19E-03	5%	50%	25%
% Acetate degraders	1.25E-03	2.50E-02	1.25E-02	6.25E-03	5%	50%	25%

Table: Parameters that were changed in the biomodel.