

Integrated Master in Chemical Engineering

Master´s Thesis

Kinetic studies of biogas generation using enzymes for hydrolyze

**Institute of Food Technology and Bioprocess Engineering
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March, 2009

Course: Integrated Master in Chemical Engineering

Document type: Master's Thesis

Developed within the discipline of: Final Project in Foreign Institution

Title:

Kinetic studies of biogas generation using enzymes for hydrolyze

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March, 2009

Acknowledgements

The Master's Thesis is an intensive work that creates the bridge between being student and engineer. All studying levels that I already overcame made me achieve a higher level of knowledge. Reaching the Master's level and at the same time having the privilege of working on a project on a foreign institution is something for which I need to thank to my family that always tried to provide me the best education possible.

I would like to thank my supervisor Dipl.-Ing. Anett Werner for the warm reception that made me feel at home, for the support in the difficult moments, for the help provided during the project execution, for all the time spent on me and above all for all the teaching that I received. I also would like to thank to Eva-Maria Kneschke for all the patience she had with me in the laboratory, particularly with the HPLC technique. My gratitude also to Tatyana Khaham for all the work developed in the glucose biosensor. Without her the analysis would not be possible. I would also like to extend my gratitude to Fraunhofer Institut Keramische Technologien und Systeme (IKTS), in particular to Björn Schwarz, for creating the possibility of making biogas yields experiments in their biogas laboratory plant.

Finally: I would like to thank to all the staff and students of Institute of Food Technology and Bioprocess Engineering of the Technical University of Dresden for receiving me so tenderly and for all the help offered. In particular I would like to thank to Dr.rer.nat.habil. Thomas Bley for accepting me on this project, thus offering me a new chapter in my life.

Abstract

Specific optimization of hydrolysis in maize silage for biogas production can easily lead to an improvement in biogas yield and consequently in an increase of process efficiency.

In the present study, the enzymatic hydrolysis of ensiled maize was studied. Batch reactors at room temperature with constant steering were used to perform the hydrolysis step, usually during 21 h. Different enzymes were used to test the effectiveness in ensiled maize: Novozyme 188, Methaplus L100, Celluclast 1.5L, Viscoflow MG and Sigma hemicellulase. Different analytical methods were used to determine sugars concentration: DNS method, glucose biosensor and HPLC. Novozyme 188 was found to be the best enzyme for maize silage, showing after 48 h a maximum release of 14.73 g/L of reducing sugars for maize silage treated by the extruder (particle size reduction). This value represents a release of 23.6 % of reducing sugars in VS and was the biggest sugar release obtained. Pre-treatment of maize silage was performed: wash and particle size reduction. Wash step did not improved sugar yields, unlike particle size reduction that improved the sugars release by 1.5 to 4 times. Biogas and methane yield were tested for optimal enzymatic hydrolysis conditions. Batch reactors were used at 38 °C without steering. Enzymes were added to the hydrolysis step or directly in the reactor Without enzyme, 567 l/kg VS of biogas with 61.7% of methane was produced. For the project conditions, no improvements on biogas yield were observed with the addition of enzymes.

Key Words: enzymatic hydrolysis; pretreatment; maize silage; cellulase; biogas.

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Abbreviations

MSW	Municipal Solid Wastes
LCA	Life Cycle Assessments
VFA	Volatile Fatty Acids
LCFA	Long Chain Fatty Acids
OLR	Organic Loading Rates
EC	Enzyme Commission (number)
E	Enzyme
S	Substrate
ES	Enzyme-Substrate complex
EP	Enzyme-Product complex
P	Product
I	Inhibitor
EI	Enzyme-Inhibitor complex
ESI	Enzyme-Substrate-Inhibitor complex
IKTS	Institut Keramische Technologien und Systeme (Fraunhofer)
TS	Total Solids
VS	Volatile Solids
NVS	Non Volatile Solids
CP	Crude Protein
MEVM	Methane Energy Value Model
AFEX	Ammonia Fiber Explosion
rpm	Rotations Per Minute
lpm	Laps Per Minute
DNS	Dinitrosalicylic Acid
GOD	Glucose Oxidase
POD	Peroxidase

Introduction

The production of renewable energies is nowadays one of the most important fields of research for scientists. The increasing demand for energy, the pollution originated by the use of fossil fuels as well as their influence on the global economy, forced researchers to redirect their efforts to renewable energy sources. The search for new renewable energy sources and the optimization of current energy processes are extremely vital for a sustainable grow and management of a society, as for its future survival.

Lignocellulosic materials are the most abundant carbon source on earth and they can be used as source of fuel, food and chemicals. In biogas production, cellulase and hemicellulase can be hydrolysed to single sugars, which can then be used by bacteria to produce biogas through anaerobic digestion. However, cellulose and hemicellulose hydrolysis rates are extremely low without the help of catalysts or pretreatments. To overcome this problem, scientific community directed their research efforts to specific enzymes and substrate pretreatment. Enzymes can catalyse the hydrolysis reaction and boost the reducing sugars release. Pretreatment can help to increase the substrate susceptibility to enzymatic attack by opening the lignocellulosic structures (lignin removal, reduction of cellulose crystalline structure, higher specific surface area).

Further investigation is needed in the renewable energies field to create viable alternatives to fossil fuels. Production of biofuels from biomass is a promising technology, especially if substrates such as organic wastes or lignocellulosic residues can be efficiently used. Applied enzymatic hydrolysis for biofuels is still an immature technology; however, promising efforts are already being made especially on the production of bioethanol from cellulose. A viable enzymatic hydrolysis where cellulose and hemicellulose could be converted to simple sugars (such as glucose) would open a new horizon on biofuels. Research about enzymes behaviour in real substrates, such as maize silage, will result in process optimization, through the use of new enzymes specific to substrate, enzymes synergy and pre-treatment of substrate to improve enzyme activity.

In the present study, the enzymatic hydrolysis of ensiled maize was studied. Different enzymes were used to test the effectiveness in ensiled maize. Pre-treatment of maize silage was performed: wash and size reduction. Biogas and methane yield were tested for optimal enzymatic hydrolysis conditions.

Thesis organization

This thesis is organized in seven chapters.

The first chapter introduces biomass theory and some energy production processes that are commonly used.

The second chapter introduces the anaerobic digestion as a process for biogas production. Anaerobic digestion stages are presented, feedstocks for biogas production and process designs. The relevancy of cellulosic feedstocks is also discussed.

The third chapter specifically introduces enzymatic hydrolysis of cellulose, which is the main theme studied on this project. Enzymes and pre-treatment parameters on hydrolysis of cellulose are discussed and a more incisive bibliographic review is made about these themes.

The fourth chapter presents the methodology used in all the experiments made on this project: substrate characterization and pre-treatment, enzymatic hydrolysis experiments and biogas production.

The fifth chapter presents the results of the experiments made and their discussion.

The sixth chapter contains the most important conclusions observed on this project.

The seventh and final chapter presents the evaluation of all the work developed.

1. Biomass as a feedstock for energy production

One of the most available resources in nature is biomass. Biomass can be described as wood, crops, animal manures, agricultural residues (sugarcane baggasse, corn stover, rice hulls, straw), sludges from municipal wastewater treatment or even municipal solid waste (food residues, paper). Biomass can be more precisely defined as “all nonfossil organic materials that have an intrinsic chemical energy content. This includes all water- and land-based vegetation and trees, or virgin biomass, and organic components of waste materials such as municipal solid waste (MSW), municipal bio solids (sewage) and animal wastes (manures), forestry and agricultural residues, and certain types of industrial wastes” (Paisley 2003). The fossil fuels are not considered biomass, although they have animal and vegetal origin. The big period of time necessary for their creation leads to a time delay between the trap and release of the carbon, thus the reserves are limited and they also produce a big impact on the carbon equilibrium on the atmosphere when used (long carbon cycle).

Biomass has the advantage of being a renewable energy source. During their life cycle, plants constantly capture the CO₂ in the atmosphere and capture solar energy with photosynthesis. This potential energy is used by animals as well as men, not only for food but also as energy source. As long as there is sunlight and enough living beings capable of using it, the biomass will be constantly renovated without depleting the reserves. Thus, the use of biomass as energy source can: reduce the dependence of fossil fuels, help with the management of organic wastes and in the reduction of greenhouse gas emissions. Life cycle assessments (LCA) studies demonstrated that the production of energy based on biomass have a net negative balance in total greenhouse gas emissions (carbon dioxide, methane, nitrous oxide) because the removal of biomass from the fields prevents the release of methane to the atmosphere. On the other hand, fossil fuels can produce 40-250 % more greenhouse gas than biomass (Paisley 2003).

The production of energy and bio-fuels using biomass can be achieved through various processes. The most simple and common is the direct combustion of wood, which is used for simple heating in our homes (low efficient process) or in simple industrial processes (average efficient process when optimized by the use of industrial equipments). Biomass wastes can also be used to produce energy by combustion in “mass burn” systems. These kinds of process usually have big maintenance costs due to the wide variety of compounds in the waste which affect the normal operation of the equipments. Due to the big variety of components in the wastes, emissions of hazardous contaminants are also a problem. Treatment steps can be

implemented to improve the process by removing unnecessary residues and to remove contaminants in the exhaust. However the extra equipment represents an extra capital investment that can invalidate the economic viability of the process.

Another very well known process is gasification, used widely in the last century to produce gas for heating. In this process the biomass is converted to a gas by oxidation. A flow of oxidant (air or oxygen) passes through the biomass, producing an outlet stream of low or medium calorific gas that can be used in a wide range of applications such as fuel for heating. The calorific capacity of the gas depends of the biomass, temperature, pressure, oxidant and type of reactor used. The pyrolysis is a process identical to gasification. The main difference is that occurs in anaerobic conditions, creating the conditions to convert the biomass into primary liquid hydrocarbons (bio oil) that can be used in furnaces, boilers and engines.

Biodiesel is another common bio-fuel that can be used standalone or blended with diesel as diesel fuel engine. Biodiesel is a renewable fuel derived from vegetable oils or animal fats. Biodiesel can be produced by numerous oils from plants and seeds like rapeseed, soybean and sunflower oil. Waste vegetable oils can also be used, with the convenient of environmental, social and economical advantages: solve the problems in residual water treatment stations originated by the flush of vegetable oils; reduce the dependence of fossil fuels; reduced particulate emissions, sulfur dioxide and aromatic hydrocarbons; renewable energy, thus having an improved balance on greenhouse gases. Biodiesel is produced from a transesterification reaction. The triglycerides in the oil react with a short chain alcohol, usually methanol, in the presence of a catalyst (acid, base (KOH and NaOH are the most used due to economic reasons), supercritical alcohols and enzymatic (lipases)) producing methyl esters and a secondary valuable product, glycerol. The methyl esters have a similar composition as the normal diesel, so the adaptation to diesel fuel engines is quite easy.

Ethanol can be produced through fermentation process (93% of world production by fermentation, 7% by synthetic method), using biomass as substrate, especially crops such as wheat, corn and sugar cane (Badger 2002). Ethanol is a very flexible fuel that can be blended in normal gasoline or used entirely pure and its one of the most promising biofuels, having already made a big impact in Brazil and United States. The fermentation process is anaerobic and can be divided in 3 steps: hydrolysis of biomass into sugars, (it shares this step with the anaerobic fermentation process like in biogas production), fermentation of sugars, purification of ethanol. It can be produced by acid hydrolysis, enzymatic hydrolysis or thermochemical processes.

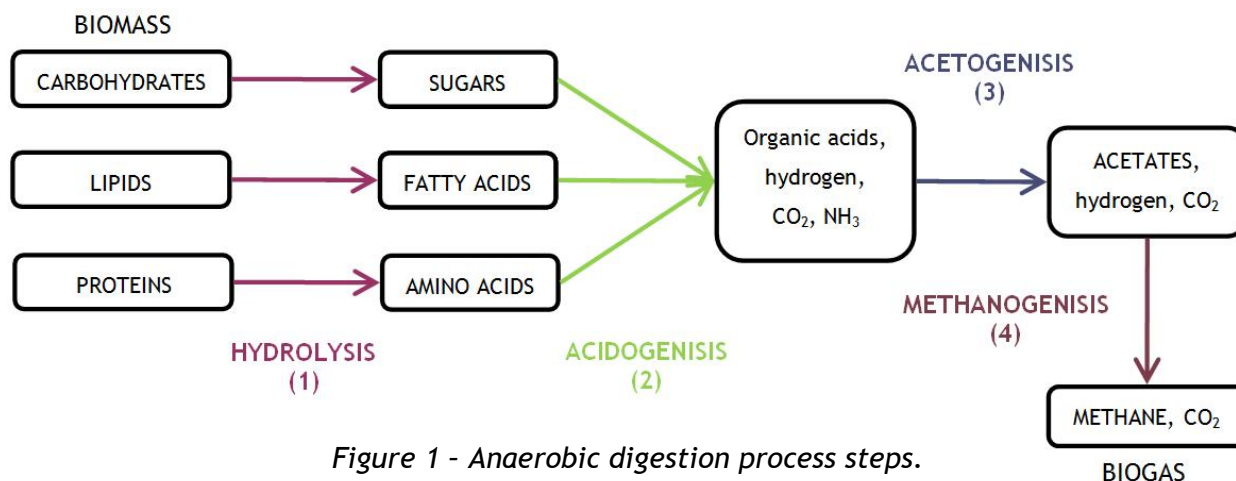
2. Biogas production by anaerobic digestion

Anaerobic digestion is one of the most used processes to treat/convert biomass. It consists on the biological digestion of biomass (commonly energy crops, wastewater sludge's, organic wastes and cattle manures) in the absence of oxygen. The main product of anaerobic digestion is biogas and it consists mainly in methane (40-80%) and carbon dioxide (20-50%). It can be used for heat and electricity production. It can also be used in cogeneration systems where the produced heat can be used for heating (ex: heating the digesters).

2.1. Anaerobic digestion stages

Has shown in Figure 1, the anaerobic digestion process has four bacterial reaction steps that complement each other and allow the transformation of biomass in biogas: (1) hydrolysis, (2) acidogenesis, (3) acetogenesis and (4) methanogenesis.

- (1) Hydrolysis performs the reduction in size of big organic molecules present in the biomass, such as carbohydrates, lipids and proteins, in smaller units like sugars (especially monosaccharide's like glucose), fatty acids and amino acids.
- (2) Acidogenesis step is the responsible for the transformation of these smaller molecules in organic acids (volatile fatty acids (VFA) and long chain fatty acids (LCFA)), hydrogen, carbon dioxide and ammonia.
- (3) In Acetogenesis the organic acids are converted to acetates (acetic acid), hydrogen and carbon dioxide.
- (4) Methanogenesis finally converts the acetates, hydrogen and carbon dioxide in methane and carbon dioxide.



2.2. Feedstock

In the biogas production one of the most important factors for increasing the methane production is the feedstock. All types of biomass can be used as a source of carbohydrates, lipids and proteins. Although cellulosic materials as wood are biomass, the microorganisms have difficulty in the degradation of lignocellulosic materials. Energy crops, organic wastes and animal manures are common substrates. In developing countries biogas production from wastes is a good way to reduce/treat organic wastes like cattle manures, waste water sludge or municipal organic wastes and to get a low-cost and decentralized energy source. In the most developed countries, the attentions are turned to energy crops due to their potential for biogas production. Maize is one of the most important feedstock in Central Europe for having one of the highest methane potential comparing to other energy crops (Amon, Amon et al. 2007a). In Table 9 (on Appendix A) are presented different energy crops for biogas production as well as their biogas and methane yields.

Each feedstock has its own advantages, depending not only of its specific characteristics but also social, geographical and economical situations of the countries. Each feedstock has different organic and inorganic content which is an important factor in the methane yield and for process and reactor design. The characterisation of dry matter, acids, cellulose, hemi-cellulose, lignin, protein, fat and ash defines the substrate content. This content is influenced by the variety, cultivation, stage of maturity at harvesting time and storage (fresh or ensiled) for crops or variety, feeding quality and intensity for animals (Amon, Amon et al. 2007a).

Silage process is a way for fresh cutted crops conservation. The method is usually used to conserve crops, such as maize, to ensure an annual feedstock for animals with minimum

nutrient loss. The search for new substrates for biofuel production led to the use of silages, which were found to be an adequate substrate (Thomsen, Holm-Nielsen et al. 2008). The entire plant is harvested and stored in a silo. The material is compressed to create an anaerobic environment which leads to anaerobic fermentation. Acetic and lactic acids are formed which ensure the material preservation and allow nutrient conservation for a longer period of time (Encyclopaedia Britannica, 2009). The fermentation process starts normally 48 h after ensiling and stops 2 weeks later due to the fall of pH values.

It is also common to co-digest sewage sludge or animal manures with an energy crop or crop residue. This process called “seeding” is made to reduce the time of the process; the microorganisms normally take some time to reach the optimal stability, so the introduction of materials with existing bacterial cultures reduces the start-up time. Examples are co-digestion of pig manure, maize and glycerine (Kryvoruchko, Amon et al. 2004) or maize with cattle manure (Amon, Amon et al. 2007a). The sewage and manure, alone, do not have a big methane potential, due to be residues with less potential than fresh materials.

2.3. Process Design

2.3.1. Reactor configuration

The characteristics of the anaerobic digesters can be suited to different situations and project demands.

The simplest digester is batch, where all the biomass is introduced at once. The batch digester is sealed to promote anaerobic digestion and is opened just in the end of the process, while in a continuous or semi-continuous process the reactor content is being renovated constantly or for certain periods of time (days), respectively. In continuous and semi-continuous process, the biogas production is constant, while in batch process the biogas yield is formed over time.

The process temperature has to be suited to the organisms for an optimal anaerobic digestion. There are 2 common temperature levels: mesophilic and thermophilic. Mesophilic organisms can develop at ambient temperatures (20-45 °C), with an optimal performance between 37 and 41 °C; thermophilic bacteria can develop between 45 and 80 °C. These last ones are more sensitive to operational conditions. However, the higher functioning temperature allows faster biogas production due to increased reaction rates (though it is an

additional cost to the process). On the other hand, mesophilic bacteria, considered to be more stable and having a wider range of species, are normally the most used in anaerobic digestion processes (Song, Kwon et al. 2004).

Regarding the process stages, the system can have a single-stage or multi-stages. In a single-stage, there is just one digester where all the steps of anaerobic digestion occur. It is less expensive due to the less equipment needed but the variables are more difficult to control. The different pH functioning of hydrolytic, acidogenic and methanogenic bacteria compromises the optimum operation during a single-stage. Multi-stage systems allow the separation of the reactions in different digesters to control precisely the reaction conditions such as pH and temperature, so, the two-stage process is considered more efficient than the one-stage (Parawira, Murto et al. 2005). The hydrolysis, acidogenesis and acetogenesis commonly take place in the first digester at low pH values due to the produced organic acids and in the second digester the methanogenesis with a higher pH.

The reactors can also work at different organic loading rates (OLR). Different OLR influence the process characteristics and parameters, leading to different biogas yields and production speeds. Residence time of the biomass inside the reactor is usually defined by the process operators to obtain an optimal biogas yield and depends of the type of substrate, thermophilic or mesophilic digestion, reactor type and number of stages.

2.3.2. Cellulosic feedstocks

Cellulosic resources are abundant biomass resources in the world. Cellulose and hemicellulose, long carbohydrate chains made of sugar units, have a big potential for energy production. The major challenge of releasing sugars from lignocellulosic materials (hydrolysis of hemicellulose and cellulose) is the crucial step for making possible a viable energy production process from these materials.

As it is demonstrated on Figure 1, biomass has three main compounds that can be used as energy sources: carbohydrates, lipids and proteins. Among carbohydrates, lignocellulosic materials (lignin, hemicellulose and cellulose) are the ones more difficult to decompose by microorganisms.

Cellulose is a polysaccharide that consists in linear polymers of n 1,4- β -D-Glucose units (starch is 1,4- α -D-Glucose) joined in fibers, that organize themselves in a mostly crystalline structure with van der Waals' attractive forces and hydrogen bonds as well (French, Bertoniere et al. 2003).

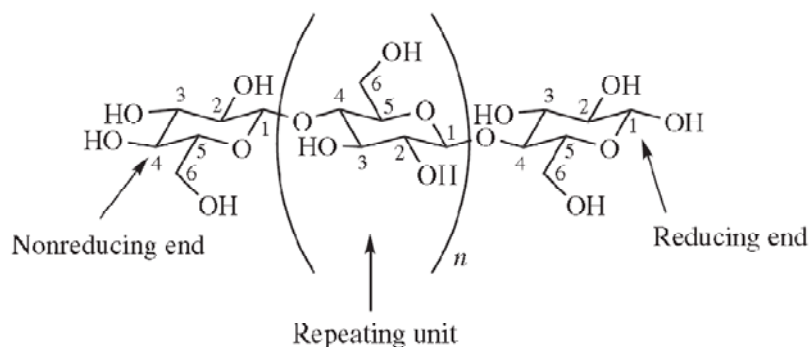


Figure 2 - Cellulose chain with 1-4- β -D-Glucose repeating unit. (adapted from (French, Bertoniere et al. 2003))

Hemicellulose is also a linear polymer of monosaccharides but besides glucose it includes other hexoses and pentoses: xylose, arabinose, galactose, manose, glucose, rhamnose and fucose. The percentage of each sugar varies depending on the substrate. Comparing to cellulose, hemicellulase have a smaller degree of polymerization and is branched. The polymers can be composed by 200 units compared to 10000 units in glucose (Thompson 1995).

Lignin on the other side, is an amorphous polyphenolic material (without sugars, unlike cellulose) composed mainly by three phenylpropanoid monomers: p-coumaryl alcohol (1), coniferyl alcohol (2) and sinapyl alcohol (3).

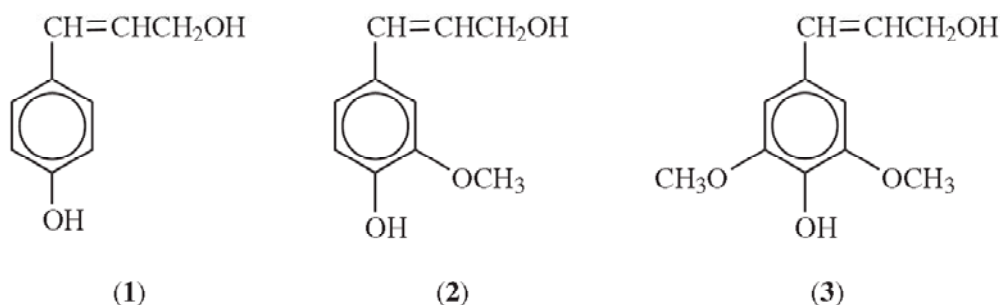


Figure 3 - Phenylpropanoid monomers from lignin: p-coumaryl alcohol (1), coniferyl alcohol (2) and sinapyl alcohol (3). (adapted from (Lebo Jr., Gargulak et al. 2001))

Among other functions, lignin provides structural support to the plants and at the same time resistance to biological degradation (Lebo Jr., Gargulak et al. 2001). The combination of these tree materials creates a very resistant mesh that provides rigidity and resistance to the tissues, as well as biological degradation resistance. On the other hand, the association of cellulose and hemicellulose molecules with lignin meshes difficult the access and removal of them to perform the hydrolysis step.

3. Enzymatic Hydrolysis: from Cellulose to Sugars

Hydrolysis is a chemical reaction that involves water molecules, where occurs the addition of water elements to a substrate. Carbohydrates, proteins and lipids can be depolymerized by hydrolysis, dilute or concentrated acid hydrolysis or enzymatic hydrolysis were specific enzymes called hydrolases catalyse the reaction (Nelson and Cox 2004). The generic reaction for cellulose is presented in equation (1).



R^1 and R^2 are the chain of cellulose and O the oxygen in the glycosidic bond that connects monomers to each other in the polymer chain. The water molecules are split in H^+ and OH^- , integrating the substrate and separating it. Long chains of cellulose can be reduced to single glucose monomers that can be metabolized by bacteria.

In biogas production, the hydrolysis optimisation can be made mostly through pre-treatment and enzymatic hydrolysis.

3.1. Enzymes

Enzymes are, not exclusively, but mostly proteins with a native specific tridimensional conformation that gives them their catalytic activity (acceleration of chemical reactions). They do not change the chemical equilibrium, they lower the activation energy of a reaction, thus increasing the reaction rate. They work in aqueous solution under mild temperatures and pH and are also sensitive to the substrate concentration. Enzymes are very sensitive to changes in pH and T. Usually, due to the protein characteristics, enzyme denaturation can occur if the conditions are too aggressive, which can make the enzyme lose the catalytic activity. Usually enzymes function optimally between 40 and 60 °C, although the higher the temperature, the higher the activity. The pH range of the enzymes depends of the enzyme type and concentration and type of substrate, but each enzyme have a specific optimal pH range (Nelson and Cox 2004).

Two main characteristics of the enzymes are their efficiency (they normally increase the reaction rate by a factor of 10^6) and specificity (often just catalyze one reaction). Hydrolytic enzymes that act on carbohydrates are mostly very specific and do not tolerate changes in the stereochemical configuration of their substrate (Kirk, Damhus et al. 2004).

Frequently enzymes work with molecules (organic or inorganic) that support the enzyme catalytic. Cofactors (inorganic) or coenzymes (organic) usually act on the active site of the enzyme and are involved in the catalytic reaction.

Enzymes are classified with the Enzyme Commission (EC) number which is based on the nature of the catalysed reaction. The EC number has four levels, each one representing specific information about the enzyme activity. An example can be β -Glucosidase EC 3.2.1.21: hydrolase (class 3: hydrolysis reactions (transfer of functional groups to water)) that acts on cellobiose (disaccharide).

3.1.1. Enzyme kinetics

Enzymatic hydrolysis occurs by a system of reactions which complexity varies depending on the substrate and enzyme. Usually single-substrate reactions can have their kinetics described by the Michaelis-Menten kinetics. The basic enzymatic reaction is defined by (2) where E represents the enzyme, S the substrate, P the product, ES the enzyme-substrate complex and EP the enzyme-product complex (Nelson and Cox 2004).



On a first step, E reacts with S to form ES. Then the enzyme catalyses the reaction (ES to EP) and release P. The most convenient equation reduces the enzymatic steps to (3).



Typically it is assumed that the $ES \rightleftharpoons EP \rightleftharpoons E + P$ step is a one step process described by a first-order rate constant k_{cat} (catalytic constant, number of molecules of substrate that can be catalyzed per active site per second). Michaelis-Menten expression can be derived from equation (3) through various assumptions: the mechanism does not involve intermediates, product inhibition, allostericity (enhancement or decrease of enzyme activity by allosteric activators or inhibitors) or cooperativity (cooperative binding, special case of allostericity); quasi-steady-state assumption that states that [ES] changes much slower compared to [S] and [P] (can be assumed that $d[ES]/dt=0$, constant concentration), as for the total enzyme concentration.

The typical Michaelis-Menten equation is (4) with $K_M = \frac{K_{cat} + K_{-1}}{K_{-1}}$ and $V_{max} = K_{cat}[E]_0$.

$$V_0 = \frac{V_{max}[S]}{K_m + [S]} \quad (4)$$

If an enzymatic reaction obeys to Michaelis-Menten kinetics, which means that the velocity of the reaction is proportional to the concentration of enzyme and depends of the substrate concentration [S], a diagram can be plotted as shown in *Figure 4*.

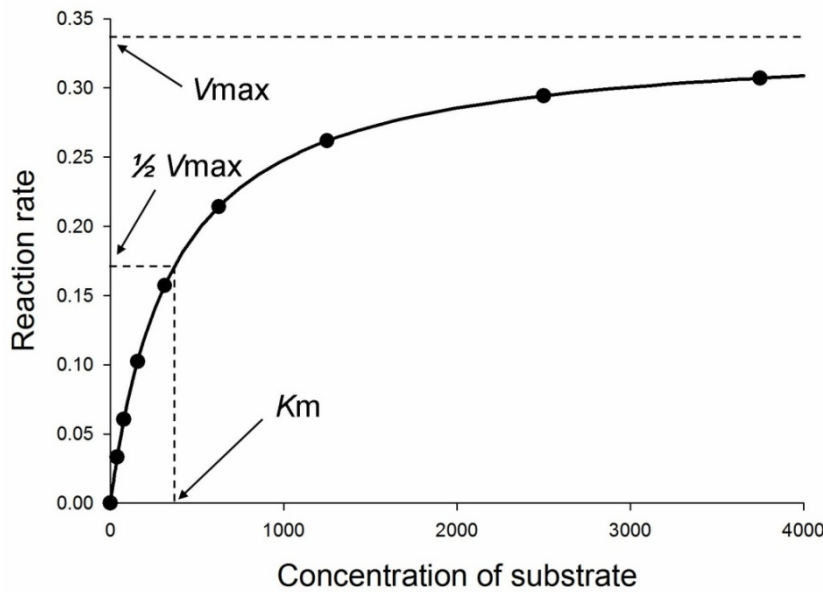


Figure 4 - Reaction rate vs Concentration of substrate: typical plot of an enzymatic reaction that obeys the first order Michaelis-Menten kinetics.

Enzyme kinetics can be studied by the use of the parameters V_{max} and K_M . The velocity V (concentration of product per unit of volume per unit of time) achieves a maximum value when $[S]$ is big enough (V_{max}). The value of $[S]$ for which $V = \frac{1}{2}V_{max}$, is called the Michaelis-Menten constant: K_M . These are important parameters for predicting the enzymes behaviour. These values can be easily obtained by the Lineweaver-Burk equation (5), a modified form (double reciprocal) of the Michaelis-Menten equation. As shown in Figure 5, V_{max} and K_M can be taken from the linear plot (Nelson and Cox 2004).

$$\frac{1}{V_0} = \frac{K_M}{V_{max}[S]} + \frac{1}{V_{max}} \quad (5)$$

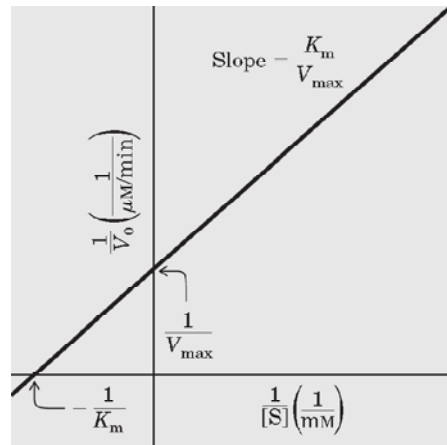


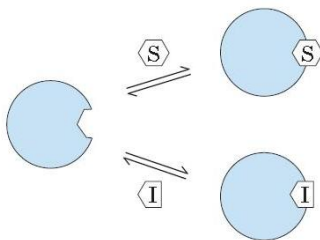
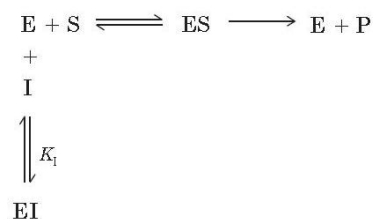
Figure 5 - Double reciprocal of Lineweaver-Burk equation. (adapted from (Nelson and Cox 2004)).

Enzymes can suffer from inhibition if certain molecules can intervene in catalysis, slowing down the reaction rate. There are two main types of inhibition: reversible or irreversible.

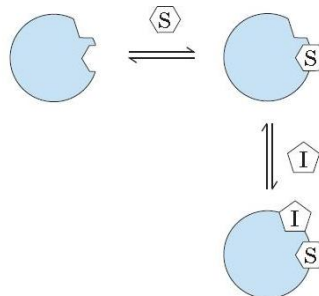
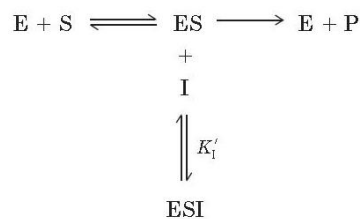
In competitive inhibition (Figure 6 - a), inhibitors bind to the enzyme active site and compete with substrate. $V_{\max}$ does not change but K_M will increase. This kind of inhibition occurs with one-substrate enzymes.

Uncompetitive (Figure 6 - b) and mixed inhibition (Figure 6 - c), in practice, are just observed with two(or more)- substrate enzymes and binding at a separate site (not on active site). Uncompetitive inhibitors bind to ES complex and mixed inhibitors binds both the ES and the S.

(a) Competitive inhibition



(b) Uncompetitive inhibition



(c) Mixed inhibition

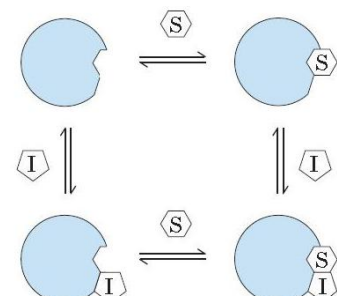
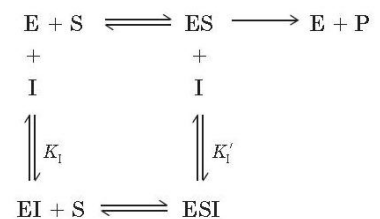


Figure 6 - Types of reversible inhibition. (adapted from (Nelson and Cox 2004)).

Irreversible inhibitors can disable enzyme activity by creating covalent bonds with the active site or inducing molecular structural modifications that cannot be reversed, thus destroying the catalytic capacity of the enzyme.

3.1.2. Glycoside hydrolases (EC 3.2.1)

To cut down long chains of cellulose and hemicellulose, cellulases and hemicellulases can be used to catalyse the hydrolysis reaction. Enzymes that can act on cellulose or hemicellulose hydrolysis are in the glycoside hydrolases group (EC 3.2.1).

Enzymes that act on hemicellulose are also important. For example xylanase, galactosidase or manosidase can cut the glycosidic bonds on hemicellulose to release xylose, galactose or manose that can be used by bacteria to produce biogas.

A cellulase system is the common name used for the groups of enzymes that can break cellulose. These enzymes act on the glycosidic linkage to reduce a bigger saccharide chain to a smaller one, or even to single sugars (Figure 7). These enzymes can act at the end (exo) or in the middle (endo) of the chains.

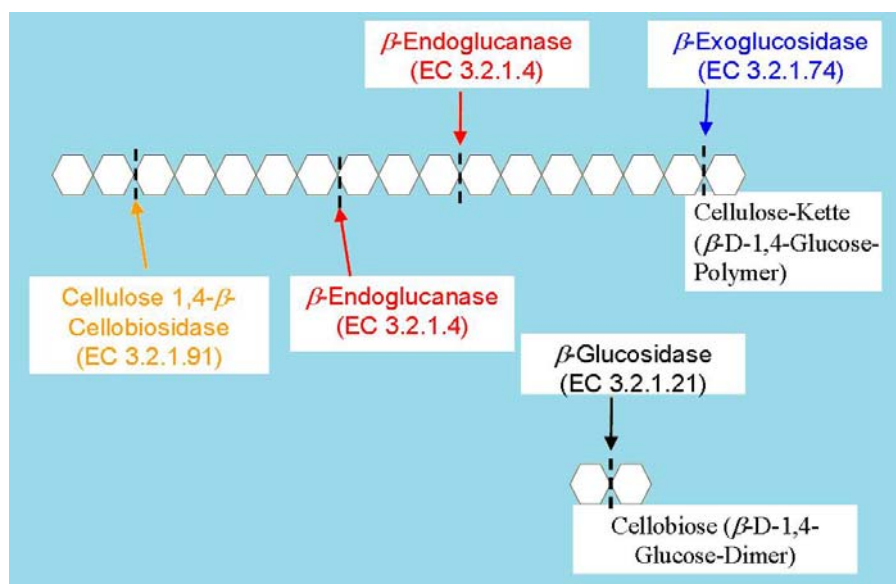


Figure 7 - Enzymes that can act on hydrolysis of cellulose.

The major enzymes that can interact on hydrolysis of cellulose, as well as the reactions that they catalyse, are presented in Table 1. Each enzyme has different possible structures (produced for example from different microorganism's strains) that can act in

different ways in the substrate or synergistically with other enzymes. New species of fungus or bacteria are always being studied, as well as the enzymes that they produce. Comparing to the number of studies using fungal cellulases, the bacterial cellulases are much less studied. *Trichoderma viride*, *Trichoderma reesei* and *Aspergillus niger* are the best known fungus that can produce cellulases (among other enzymes). Microorganisms can also be genetically engineered (mutant strains) to produce more active enzymes in higher quantities. The cost of enzymes has always been too high to consider a commercialization of enzymatic hydrolysis of cellulose. The major challenge nowadays is to decrease the price of enzymes so cost-effective technologies could be applied commercially (Badger 2002). Several approaches are being made towards this goal.

Table 1 - Enzymes that can act on hydrolysis of cellulose. (addapted from (TU-Braunschweig 2009)).

3.2.1.X	Glycoside hydrolases	
X	Name	Reaction
4	β -Endoglucanase (cellulase)	Endohydrolysis of (1 $\rightarrow$ 4)- β -D-glucosidic linkages in cellulose, lichenin and cereal beta-D-glucans
21	β -Glucosidase (cellobiase)	Hydrolysis of terminal non-reducing β -D-glucocyl residues with release of β -D-glucose
74	β -Exoglucosidase (glucan 1,4- β -glucosidase)	Hydrolysis of (1 $\rightarrow$ 4) linkages in (1 $\rightarrow$ 4)- β -D-glucans, to remove successive glucose units
91	Cellobiohydrolase (Cellulose 1,4- β -cellobiosidase)	Hydrolysis of (1 $\rightarrow$ 4)- β -D-glucosidic linkages in cellulose and cellotetraose, releasing cellobiose from the non-reducing ends of the chains

Endoglucanase and cellobiohydrolase are considered to detain the large percentage of activity in cellulase mixtures. Cellobiohydrolase releases cellobiose units from the non-reducing ends of cellulose chain. On the other hand, Endoglucanases cut randomly the cellulose chain in smaller fragments (degrades crystalline cellulose), reducing the viscosity (faster than sugar release (Walker and Wilson 1991)) and providing new chain endings for cellobiohydrolase progressive action (Zhou, Wang et al. 2009). These concerted actions promote the efficient degradation of the cellulose enhancing the hydrolysis extends. However, inhibitory effects of glucose and cellobiose (product inhibition) are widely known and cited in most of the literature. Cellobiase act to reduce this inhibitory effect, breaking down cellobiose into two glucose molecules (Walker and Wilson 1991). Unless the cellulases:cellobiose ratio is optimized, inhibition will occur and hydrolysis will lose

efficiency. As a matter of fact, an important lack on *Trichoderma reesei* enzyme preparations is the low concentration of cellobiose (Berlin, Maximenko et al. 2007).

The symbiotic behaviour between enzymes is called enzymatic synergy. The boost of the enzyme mixture activity is the most notorious effect of synergy. A recent study demonstrated that with a statistically designed multi-cellulase mixture, using seven purified enzymes from a *Trichoderma viride* T 100-14 mutant strain, the amount of glucose produced from steam-exploded corn stover was maximized (Zhou, Wang et al. 2009). The amount of glucose produced was 2.1 times higher compared to a crude cellulase mixture. Although synergy of purified enzyme mixture can improve synergistically the enzymatic hydrolysis, the immediate application to an industrial process is not possible. Conditions have to be created to produce the specific enzymes in a commercial volume. However, synergy between commercial enzyme preparations can optimize hydrolysis without the need for producing specific enzymes. A study demonstrated that a commercial cellulase preparation of Celluclast 1.5 L from *Trichoderma reesei* could see their activity improved by the addition of three types of crude commercial enzyme preparations of Multifect Xylanase, Multifect Pectinase, and Novozym 188 (xylanase, pectinase and cellobiase activities respectively) (Berlin, Maximenko et al. 2007). For the same amount of glucose and xylose produced from pretreated corn stover, just half of the enzyme amount was needed for the optimized mixture. Benefit was taken with the cellobiase addition, reducing the cellobiose inhibition in the other enzymes. Xylanases and pectinases can also remove non-cellulosic polysaccharides that restrain the cellulose fibers, stimulating the cellulose hydrolysis. In several studies it was found that the presence of cellobiase in the cellulase mixtures can significantly improve the hydrolysis ratio (Bommarius, Katona et al. 2008; Zhou, Wang et al. 2009). Several synergism reviews are analysed in (Walker and Wilson 1991)). Laccases are also interesting enzymes. Although they are not cellulases, they have an important activity on lignin degradation (Eggert, Temp et al. 1997). Although lignin does not contain any sugars that bacteria could use for biogas production, the degradation of lignin would release the cellulose and hemicellulose fibers which would facilitate cellulase and hemicellulase accessibility, enhancing the hydrolysis.

In Table 10 on Appendix B are presented enzymes used in hydrolysis experiments, as well as the substrate used.

As mentioned before, the enzyme kinetics can be described by Michaelis-Menten kinetics if some assumptions are taken into account (such as no inhibition). However the lignocellulosic network and the synergistic process are more complex than that. At least four different factors can create impact on kinetics: inhibition of cellulase by cellobiose; adsorption of cellulase on cellulose (cellobiose absorbs much less on cellulose than cellulase);

fractal kinetics limitations (tree-dimensional structure of cellulose limits cellulase diffusion, Fick's law cannot be used, fractal theory is applied successfully to kinetics of cellulose conversion to cellobiose and glucose); jamming effects (enzyme crowding interfere in enzyme action due to the difficult enzyme diffusion through cellulose crystalline fibers) (Xu and Ding 2007; Bommarius, Katona et al. 2008). Even synergy is affected by this kind of phenomena. The enzyme concentration to create optimal synergy is the concentration that does not surpass the substrate binding sites saturation. The hydrolytic efficiency will even decrease with the increase of cellulase saturation. This phenomenon is called parabolic effect and several studies describe it (Berlin, Maximenko et al. 2007; Zhou, Wang et al. 2009). The enzyme competition for binding sites and the enzyme saturation can justify this effect although the phenomenon mechanism is still unknown. However, this factor indicates that an excess of enzyme would not be advantageous for hydrolysis even leading to a reduction of hydrolysis efficiency.

The use of enzymes in biogas production is a very immature research topic. Just some literature was found about it. In a very interesting report (Gerhardt, Pelenc et al. 2007) are presented results from laboratory experiments, as well as field experiments in 30 agricultural biogas plants in Germany. Several enzymes were studied to test the hydrolysis extension and the biogas yields. In the hydrolysis step, Methaplus L100 was the most effective for all substrates. For maize silage a reducing sugar releasing activity of about 26 U/g was obtained. This enzyme preparation, composed by a *Trichoderma reesei* DSMZ 10682 enzyme complex, was used in 30 agricultural biogas plants and an average increase of 18% on biogas yield was reported. However, the enzyme quantity or the method applied (single or two stage, continuous, semi-continuous or batch, mesophilic or thermophilic) is not described, which makes it difficult to evaluate the viability of the process and also the replication of it. Another study (Pariyar and Wulf 2006) also reports the use of enzymes for biogas production in a semi-continuous process (substrates and enzymes are added daily and fermentation residues are removed). Silage materials such as maize silage are used, but, due to just having access to abstract, no biogas yield values or enzyme type are known. However, is claimed an increase of biogas yields from maize silage by enzyme addition (loading of 1g VS.l⁻¹.d⁻¹). Also some companies are trying to use enzymes to develop ethanol-from-cellulose processes, but just experimental or pilot plants have been used. The viability of the process is still under research (Badger 2002).

3.2. Pretreatment

As discussed previously, for enzymes to work efficiently, they must obtain an easy access to the molecules to be hydrolysed. For enzymatic processes to be effective, some kind of pretreatment is needed to break the crystalline structure of the lignocellulose and remove the lignin to expose the cellulose and hemicellulose molecules. Pretreatments should also be able to increase the specific surface area and, some treatments, to increase the pore volume. The crystalline regions are less susceptible to degradation than amorphous regions, so the crystalline index is considered an important factor that affects the rate and extend of enzymatic hydrolysis. Enzyme adsorption increases linearly with the increase of specific surface area. Hydrolysis extension also increases with the increase of specific surface area. Absorbed enzymes are more effective in substrates with low crystallinity. (Walker and Wilson 1991). A good pretreatment that allows cellulases to access the cellulose fibers is crucial to an efficient hydrolysis.

Either physical and/or chemical pretreatment methods may be used. Physical pretreatments usually apply high temperatures, milling or cut. Chemical treatments can use acid, alkali, ammonia, liquid hot water, etc. Combination of physical and chemical treatments is also common. Each treatment has its own advantages and disadvantages, however, everyone are able to reduce the specific surface area and the crystallinity index. Several experiments are reviewed in literature (Walker and Wilson 1991; Saha, Dien et al. 1998). Several substrate treatments are presented in Table 10 on Appendix B.

Acid pretreatment is often used as pretreatment and has also been the main hydrolysis process used in ethanol production. The process is executed at high temperatures and diluted strong acids are commonly used. High sugar yields are obtained. Acids can also catalyse cellulose hydrolysis but at the same time, they can open the lignocellulosic network. The more adverse conditions are used (higher temperature, higher acid concentration and treatment time) the higher is the sugars yield (Yáñez, Alonso et al. 2004).

Milling or cutting are size reduction techniques used already since dozens of years ago with good results (Reese 1956; Spano, Medeiros et al. 1976). The techniques allow a reduction of specific surface area and of crystallinity, increasing the susceptibility to enzyme hydrolysis, without the use of expensive and polluting chemicals. This treatment can also complement the use of other treatments such as the acid treatment.

Steam explosion is a method that is receiving plenty of attention. Cellulose is exposed to saturated steam at high pressure and high temperature, and then quickly passes to

atmospheric pressure. An increase in surface area can be observed that can be compared with dilute acid treatment at high temperature but with the advantage of not using acid. Conventional mechanical pretreatments need 70 % more energy to perform the same size reduction (Walker and Wilson 1991).

AFEX (Ammonia Fiber Explosion) is identical to steam explosion, but ammonia is used. It is a physical-chemical process that allows lignin solubilisation, reduction of crystallinity and increase on specific surface area, for a near complete enzymatic cellulose and hemicellulose hydrolysis. In Figure 8 is presented a scheme of AFEX process.

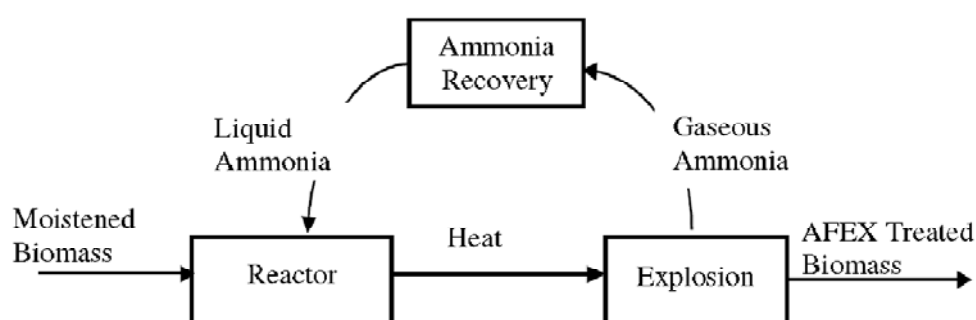


Figure 8 - Schematic diagram of AFEX process (addapted from (Teymouri, Laureano-Perez et al. 2005).

The ammonia can be almost all recovered and reused. The remaining can be used by bacteria in biogas production. It is a dry process without the need of wash step. The treated biomass is stable for long periods. No neutralization is needed before enzymatic hydrolysis (Teymouri, Laureano-Perez et al. 2005). New improvements on this technology contributed for lower operation costs. It is suggested that the minimum ethanol selling price, produced by this process, can be reduced from 1.41\$/gal to 0.81\$/gal, which is a remarkable breakthrough (Sendich, Laser et al. 2008).

4. Materials and Methods

The main objective of this research is to optimize the enzymatic hydrolysis in maize silage. To achieve that objective, different approaches were made:

- Characterisation of substrates (silage from maize and rests from biogas process) for measurement of the effects of enzyme treatment (Reducing Sugars, Total Solids, (TS), Volatile Solids (VS), Non Volatile Solids (NVS), Crude Protein (CP), Analyse of the Acids);
- Test of some enzymes on the real biogas substrate (maize silage) to describe the kinetic of cellulose degradation;
- Search for the optimum in particle size and enzyme activity;
- Study of a washing step;
- Test enzyme-treated substrate in a lab-plant for biogas production.

4.1. Enzymes

Enzymes used in the process are hydrolases, specific to break cellulose and hemicellulose into glucose. All the enzymes used are commercially available. Usually a commercial enzyme is a mixture of different enzymes in order to meet the needs of specific applications. Therefore the ratio between each enzyme component could not be optimized in order to search for a better enzyme synergy.

The chosen measuring unit was the weight, so liquid and solid enzymes could be compared in terms of quantity. Each liquid enzyme has a different specific weight. The mass of a known volume was weighted to calculate the specific weight.

Due to the viscosity of the liquid enzymes, it was difficult to measure small volumes with micro-pipettes. To avoid this problem, the enzymes were diluted in H₂O with a factor of 100, allowing a more precise pipetting.

In Table 2 are presented the enzymes used on the experiments.

Table 2 - List of enzymes used in enzymatic hydrolysis experiments.

Enzyme	Brand	Microorganism	State	Composition
Viscoflow MG	Novozymes	<i>Aspergillus oryzae</i> , <i>Trichoderma reesei</i>	Solid	β -glucanase, (endo-1,4) Xylanase
Hemicellulase	Sigma	---	Solid	undefined mixture of glycolytic enzymes usually containing xylanase, mananase and other activities
Methaplus L100	Biopract	<i>Trichoderma reesei</i> M 18.2	Liquid	β -glucanase, cellulase, xylanase
Cellulast 1.5L	Sigma/Novozymes	<i>Trichoderma reesei</i> ATCC 26921	Liquid	cellulase mixture
Novozyme 188	Sigma/Novozymes	<i>Aspergillus niger</i>	Liquid	cellobiase

4.2. Substrate characterization and pre-treatment

4.2.1. Samples and particle size reduction

Samples from maize silage were obtained from Fraunhofer Institut Keramische Technologien und Systeme (IKTS). Different samples had to be used when previous samples were out. TS, VS, NVS and CP were evaluated during the research. However, other parameters are also important and should have been evaluated, such as lipids, starch, cellulose, hemi-cellulose and lignin content. Also the crystalline index should be measured. Characteristics of the maize, such as strain type, FAO (value for the maturity of the maize), harvest conditions, duration and type of silage were not supplied by IKTS. However, these are variables that receive more and more importance due to the strong influence on biogas yields (different substrate composition) (Amon, Kryvoruchko et al. 2004; Amon, Amon et al. 2007a; Amon, Amon et al. 2007b). The same can be applied to the bioreactor residues that contained a mixture of maize and cattle manure. The variety of animal manures and the conditions that animals are exposed to, strongly influence the methane yields (Kryvoruchko, Amon et al. 2004; Amon, Amon et al. 2007a). In Table 3 are presented the samples used on the laboratory experiments.

Table 3 - Samples used in the laboratory experiments. Description of the original treatment.

Reference	Substrate	Treatment
U1667	Maize Silage	Untreated
U1667	Maize Silage	Treated - Size reduction
U1741	Maize Silage	Untreated
(1)	Maize Silage	Untreated
(1)	Maize Silage	Treated - Size reduction (10;8;6;4;2.5 mm cutting)
U1756 (2)	Bioreactor Residue	Untreated
U1803 (3)	Maize Silage	Untreated
U1803 (4)	Maize Silage	Treated - Size reduction (Extruder)

Some samples received a pre-treatment for size reduction: U1667, (1), and U1803. Sample (1) was reduced to different sizes in order to evaluate the effect of size reduction in hydrolysis. It was used a meat grinder with the possibility of changing the exit heads, each one with a different holes size (Figure 9). Sizes of 10, 8, 6, 4 and 2.5 mm were used.



Figure 9 - Meat grinder similar to the one used for size reduction on sample (1).

These samples were analysed by IKTS to characterize the particle size distribution (see Figure 32 on Appendix A).

Sample U1803 was processed in IKTS Extruder, a device present on IKTS laboratorial installation that allows the particle size reduction before feeding the digesters.

4.2.2. Nutrient Composition

To analyse the TS, VS and NVS, the common methodology was followed (Zauner and Küntzel 1986). VS tests are usually used in a big variety of solid and liquid samples (ex: wastes, soils, sea water) and the conditions of the test varies with the material.

- Ceramic pots were weighted on an analytical balance, 2 for each sample, so the weight difference could be calculated. Approximately 4 g of sample were weighted in the ceramic pots;
- After weighting, samples were taken to a MEMMERT oven at 103 °C during 4 hours to remove all the water content (Zauner and Küntzel 1986). Then samples were taken to a desiccator to cool down. After cooling down, they were weighted to determine TS and water content;
- After weighting, a pre-burning was made with a Bunsen burner in a fume hood. Special care was taken for not letting the samples burn to avoid sample loss;

- After pre-burning, samples were placed in a NABERTHERM B 170 oven at 600 °C for 4 hours, in order to remove all organic compounds from the samples (ashing). This temperature was indicated in literature as the most suitable for the determination of organic compounds by ashing (Bisutti, Hilke et al. 2004);
- After cooling in a desiccator, the samples were weighted to determine VS and NVS.

Values were calculated with equations (6), (7), (8) and (9).

$$TS (\%) = \frac{m_{dried\ sample}}{m_{sample}} * 100 \quad (6)$$

$$H_2O (\%) = (1 - TS) * 100 \quad (7)$$

$$VS(\%) = \frac{m_{dried\ sample} - m_{sample\ ash}}{m_{sample}} * 100 \quad (8)$$

$$NVS(\%) = \frac{m_{sample\ ash}}{m_{sample}} * 100 \quad or \quad NVS (\%) = (TS - VS) * 100 \quad (9)$$

TS - Total Solids (%)

H₂O - Water weight content (%)

VS - Volatile Solids (%)

NVS - Non Volatile Solids (%)

m_{sample} - initial sample mass

m_{dried sample} - dried sample mass

m_{sample ash} - sample mass after ashing

4.2.3. Kjeldahl Method (nitrogen content)

To evaluate the percentage of CP in the samples, the Kjeldahl Method was used with the methodology followed on laboratory. This method consists in the determination of nitrogen content on the samples. Since proteins have nitrogen on their composition, a relation can be made between the nitrogen and protein content.

The Kjeldahl method consists in a strong digestion of the sample by the addition of concentrated sulphuric acid, distillation step (capture of ammonia) and titration.

The following steps were executed:

For each material, 4 samples were weighted on nitrogen free papers: 2 with aprox. 1 g and other 2 with aprox. 0.5 g.

- Weighted samples were introduced, together with the nitrogen free papers, on Kjeldahl test tubes. Some glass spheres were added. Was added a tea spoon (aprox. 5 g) of $K_2SO_4/CuSO_4 \cdot 5H_2O$ (catalyst) in the mass proportion of 1/0.025 respectively. 10 ml of concentrated H_2SO_4 (1.84 g/ml) was added;
- Kjeldahl tubes were placed in a BÜCHI K-424 Digestion Unit at a moderate temperature. Here, the proteins in the samples are digested by the sulphuric acid, producing ammonium sulphate. Sample boiling was avoided to prevent sample loss. The acid vapours are extremely corrosive and special security measures were taken to ensure safety. The acid step is executed in a hotte. Vapours are bubbled in water and sent to residues;
- After 20 minutes, heating is increased to a level in which a stable bubbling occurs. This level is maintained at least one hour;
- When the samples turn to a green liquid, no evidence of original sample is detected and bubbling starts to fade, the heat is turned to the maximum for 10-15 min and then turned off;
- After samples cooling, Kjeldahl tubes are introduced in a BÜCHI B-324 Distillation Unit, one at a time. 40 ml of 0.4925g/ml NaOH solution and 50 ml of H_2O were added automatically. Ammonia is liberated from ammonium sulphate. The recovered liquid is bubbled in a 2% H_3BO_3 solution to capture the ammonia;
- The recovered liquid is titrated with HCl 0.1 mol/l (BRAND Digital Bürette) and some drops of indicator. The indicator consists of 0.2 g of methyl red (F43) and 0.1 g of methyl blue (F41) in 100 ml of 96% Vol. Ethanol.

The calculation for the CP content is made using equation (10).

$$CP (\%) = \frac{1.4(V_{sample} - V_{blind})}{10 * m_{sample}} * F \quad (10)$$

CP - Crude Protein (%)

V_{sample} - volume of sample titration (ml)

V_{blind} - volume of blind sample titration (ml)

m_{sample} - mass of the sample (g)

F - Specific sample factor

F is a specific constant that depends on the analysed material. The value used was 6.25. However, this value is a general value, not specific for maize silage. It was used for instance, to determine the CP in municipal solid waste (Biswas, Chowdhury et al. 2006).

4.2.4. Washing step

Washing steps were made to analyse the effect on hydrolysis. Maize silage is rich in VFA produced by bacteria during the silage process, thus conserving the maize. A washing step should reduce the acid content on the material. A higher pH would be better for enzymes, thus improved hydrolysis could be achieved. This washing water (rich in VFA, intermediate nutrient for methane production) would be used in the methanogenesis digester, avoiding nutrients loss.

Some different types of washing were tried. After adding the washing water to the material, the following processes were attempted:

- Filtration with a vacuum pump.
- Squeezing the material with a potato masher (Figure 10), to try to remove the absorbed water and at the same time remove more acids from inside the sample material; in order to prevent solids loss, the water recovered from the washing was centrifuged in a Heraeus Biofuge Stratos centrifuge at 15000 rpm during 5 min and the solid phase recovered.



Figure 10 - Potato masher.

The first process was abandoned due to the filtration time and the several filter substitutions needed. Some materials, specially the reduced size samples, were impossible to filtrate, due to the big quantity of small particles that clogged the filter. By example, the sample U1667 with size reduction treatment was not washed; due to the first trial was a failure.

After some experiments, the second process was the chosen one due to the better results.

Different volumes of water were tested, depending on the experiment. Some samples were able to be washed quickly while other samples were washed more slowly. On experiment A and B, due to the lack of knowledge about the material, it was used different volumes of washing water: A3, A4 - 4 ml/g; A5, A6 - 12 ml/g; B3, B4 - 20 ml/g. During the other experiments with washing step, C to F, it was used always the same value of 3 ml/g ($\text{ml}_{\text{H}_2\text{O}}/\text{g}_{\text{substrate}}$).

4.2.5. VFA detection - GC

A Gas Chromatography (GC) analysis was made to determine the content of VFA in the washing water. The acids targeted were acetic acid, propionic acid, iso-butyric acid, butyric acid, iso-valeric acid and valeric acid. The experiment was executed on samples (1) and U1752(2) (2 samples: 8.3333 g each).

3 Washing steps were made without squeezing, to represent a normal washing without mechanical intervention. The water amount was according to the other experiments: 3 ml/g. The washing water was removed on each washing step, filtrated and centrifuged. A sample of each step was taken for GC analysis. The GC was a Hewlett Packard HP 6890 with an Agilent 7683 B injector (auto-sampler), Hewlett Packard INNOWax Polyethylene Glycol column and a FID detector working at 260 °C. Detailed information about the GC working parameters and chromatograms can be found on Appendix D.

4.3. Enzymatic hydrolysis experiments

For studying the hydrolysis step, several experiments were made. Reaction units (Erlenmeyers or Eppendorf Falcon Tubes) were used to perform the hydrolysis step. Experiments from A to K were performed. Samples were approximately taken at 0, 0.5, 1, 2, 3, 4 and 21 hr; however, they may vary depending on the experiment. The samples were collected to Eppendorf 2 ml tubes and centrifuged in a Eppendorf Minispin Plus at 14100 rpm during 5 min. The supernatant was collected to Eppendorf 2 ml tubes (A and B) or to VWR 96-Deep Well (2 ml) Polypropylene plates (Figure 11; C to K) and stored in a freezer at -18 °C.

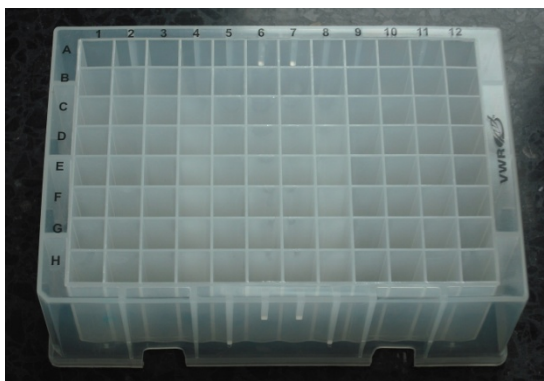


Figure 11 - VWR 96-Deep Well (2 ml) Polypropylene plate.

Deep well plates were chosen preferentially due to the possibility of storing 96 samples in just one plate and giving the possibility of micro-pipetting with multi-point Eppendorf pipettes. A disadvantage was the time necessary to defreeze the samples because there is no space between the wells. On Table 11 and Table 12 (Appendix E) is presented in detail all the experiment parameters for all enzymatic hydrolysis experiments.

4.3.1. Reaction Units

The hydrolysis reaction was executed in 500 ml Erlenmeyers or in 20 ml Falcon tubes, depending on the experiment. All experiments were conducted at room temperature; therefore, the influence of temperature in hydrolysis step was not studied. Samples were weighted in a KERN 870 analytical balance.

4.3.1.1. Erlenmeyers (A to H)

- Weighted samples (already pre-treated or not) and the correct amount of water were added to 500 ml Erlenmeyers; depending on the experiment, the number of Erlenmeyers varies and also other parameters such as the substrate/water ratio;
- The pH value was measured with a WTW inolab pH Level 2;
- A sample for $t=0$ hr is taken from each Erlenmeyer to represent the residual sugars in each sample;
- After the first sample collection (0 hr), enzymes were added according to the experiment parameters; an experiment without enzyme was always made (samples for all times);
- The Erlenmeyers were placed in a IKA-WERKE REO basic C magnetic stirrer (A) or in an EDMUND BÜHLER KS-15 Control shaker (B to H) at 175 lpm;
- Samples were usually taken at 0.5, 1, 2, 3, 4 and 21 hr (the shaker was stopped during the samples collection), were collected to Eppendorf 2 ml tubes and centrifuged in an Eppendorf Minispin Plus at 14100 rpm during 5 min. The supernatant was collected to Eppendorf 2 ml tubes (A and B) or to VWR 96-Deep Well (2 ml) Polypropylene plates (C to H) and stored in a freezer at -18°C ;
- After collecting the last sample, the pH was measured and the maize residues were stored in an appropriate residue box.

4.3.1.2. Falcon Tubes (I to K)

- For each time (0, 0.5, 1, 2, 3, 4 and 21 hr) and for each experiment parameter, a falcon tube was prepared with 0.75 g of maize silage and 6 ml of H_2O ;
- A sample for $t=0$ hr is made to represent the residual sugars; to the other falcon tubes, enzymes were added according to the experiment parameters;
- The falcon tubes were placed in an EDMUND BÜHLER KS-15 Control shaker at 175 lpm;
- Each falcon tube represents a sample for a specific time of a specific experiment number. When the time is reached, the tubes corresponding to this time are taken from the shaker (without stopping it); a dilution is made with the addition of more 6 ml of H_2O to facilitate the sampling and to promote the liberation of sugars in the material. Samples were well shaken and then filtrated with filter paper. 2 ml of the collected samples were transferred to 2 ml Eppendorf tubes to be centrifuged in a Eppendorf Minispin Plus at 14100 rpm during 5 min. The

supernatant was collected to VWR 96-Deep Well (2 ml) Polypropylene plates and stored in a freezer at -18 °C;

- After collecting the last sample, the maize residues were stored in an appropriate residue box.

4.3.2. Sugar Analysis

After the samples collection, the sugar analysis was the next step. The sugar content allowed the evaluation of the extension of the enzymatic hydrolysis. Dinitrosalicylic acid (DNS) method was always used to determine the reducing sugars content. In some experiments it was also used a glucose biosensor for determining the glucose content and a HPLC for determining mono (pentoses and hexoses) and disaccharides.

4.3.2.1. DNS (Reducing sugars)

The DNS method is widely used for reducing sugars determination. A reaction occurs between the 3,5-Dinitrosalicylic acid and reducing sugars or reducing molecules (cellulose, glucose, cellobiose, etc.), forming 3-amino-5-nitrosalicylic acid which absorbs light at 540 nm. The resulting colour is related with the reducing sugars concentration. Values of absorbance can be measured with a spectrophotometer.

For making DNS reagent, it was used:

- 10.6 g 3,5-Dinitrosalicylic acid
- 19.8 g NaOH
- 1416 ml H₂O
- 306 g NaK

The solution was stored in a brown glass flask in a dark place. A new DNS reagent was made when the previous one was ending.

For calibration, two methods were used: calibration curve (A to K) and standard addition (E to K). Glucose was the sugar used as a standard.

The DNS method was executed following the pipette scheme on Table 4.

Table 4 - Pipette scheme for DNS method: blank, standard, sample and spiked sample.

	Blank (ml)	Standard (ml)	Sample (ml)	Sample+ST1 (ml)	Sample+ST2 (ml)
Water	0.1	0	0.05	0.03	0.01
Sample	0	0	0.05	0.05	0.05
Standard (2.27 g/L)	0	0.1 (3.6 g/L)	0	0.02	0.04
DNS reagent	0.3	0.3	0.3	0.3	0.3
Total	0.4	0.4	0.4	0.4	0.4
after 5 min cook					
Water	1	1	1	1	1
Total	1.4	1.4	1.4	1.4	1.4

- Blanks were made to obtain the zero of the absorbance for the DNS;
- Standards were made to obtain the calibration curve. A glucose solution with known concentration was used. Just one concentration was used. However, the same concentration was repeated several times and the average was used;
- For standard addition method, one or two spikes were made with a glucose solution with known concentration. Each spike has different glucose concentration addition;
- Samples from the enzymatic hydrolysis step were defrozed before use (or used directly if they were not in the freezer);
- After preparing the entire scheme for DNS method, all the samples (already with DNS reagent) were cooked in a thermostatic bath with boiling water during 5 min and then cooled in cold water for another 5 min;
- A dilution was made by the addition of water;
- The cooked samples are analysed in a spectrophotometer and the absorbance measured. With the absorbance, the concentration of reducing sugars was calculated.

Two different approaches were made for the absorbance measurement. In an initial phase of the experiments, a single cell spectrophotometer detecting at 540 nm (BECKMAN DU-520 General Purpose UV/Vis Spectrum or a ELECTRON CORPORATION THERMO Helios B, depending on the availability) was used (A to D). The DNS samples were prepared and cooked in test tubes with covering caps.

However, due to the big number of samples and the need of using standard addition, a TECAN GENIOS microplates spectrophotometer detecting at 545 nm was used (E to K). With the help of Eppendorf 6 tips micropipettes and NUNC 96-Well Polypropylene microplates (Figure 12) a big number of samples could be analysed much faster (less than 2 min for absorbance read in each plate).

The DNS samples were prepared and cooked in VWR 96-Deep Well (2 ml) Polypropylene plates. The dilution after cooking was directly made in the NUNC microplates (57 μ l sample + 143 μ l H₂O = 200 μ l). The parameters used in GENIOS were: 545 nm detection; 20 s normal orbital shake; 3 flashes per well.

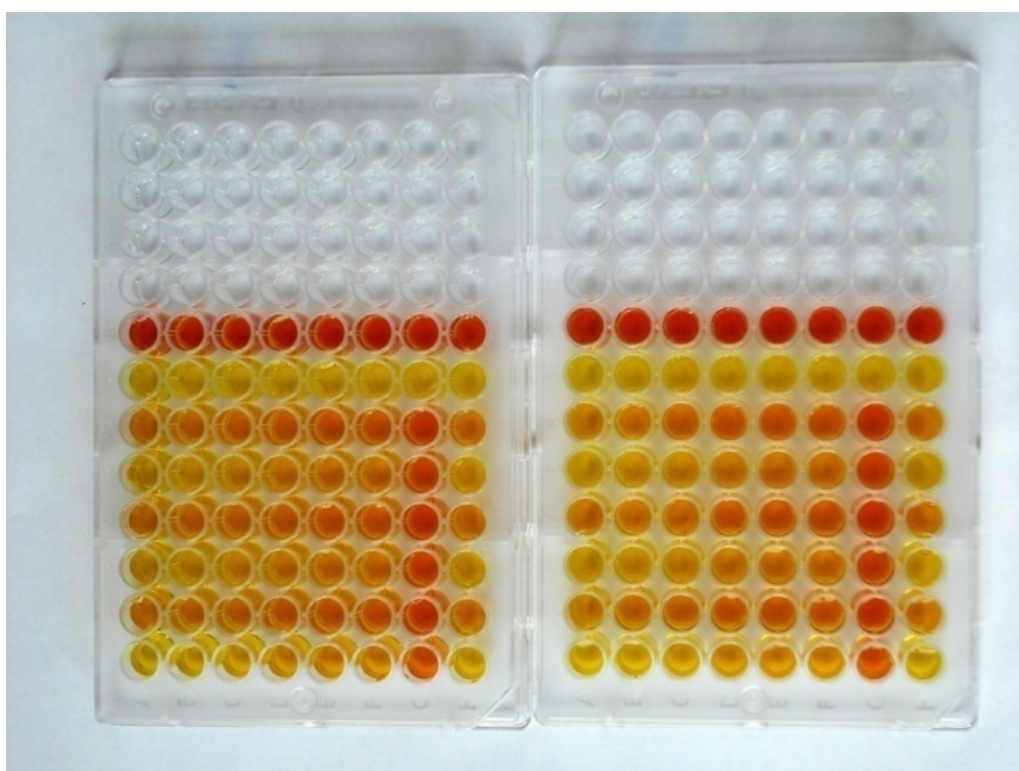


Figure 12 - Microplates with DNS method samples after cooking and dilution. Experiment J; 72 hours. Standards (1.st column), blanks (2.nd column) and samples with standard addition.

A study was made to analyse the linear range of GENIOS, as well as the minimum and maximum concentration detected. Several concentrations between 0 and 20 g/L were measured (36 concentrations, 12 per plate, 3 plates). Each column on the plate had a concentration (8 wells). The whole experiment was doubled. Each plate was read 3 times in GENIOS. Comparison was made between reads and plates. The results are presented in Appendix F.

4.3.2.2. Glucose Biosensor

An experimental glucose biosensor was used to determine the glucose concentration in some samples (G, H and J). The DNS test allows the detection of reducing sugars, which means that the obtained concentrations are not only of glucose but also of other reducing sugars or molecules. With this analysis, the concentration of glucose can be obtained. Tatyana Khaham, responsible for the operation of the biosensor, analysed the samples.

This biosensor use enzymes as receptor, a O₂ electrode as transducer and a computer for data acquisition (Figure 13). The analyte is glucose.

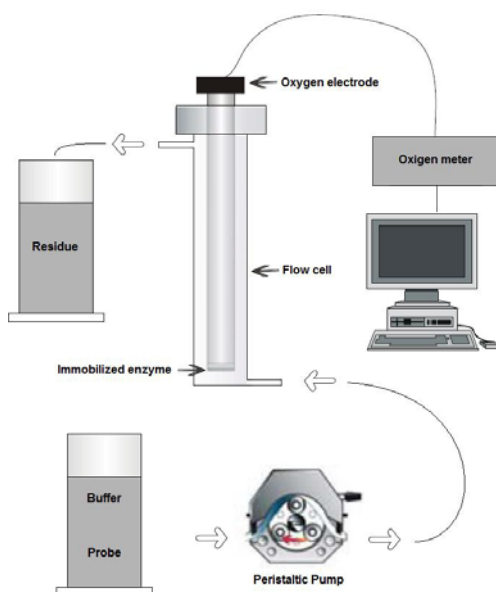
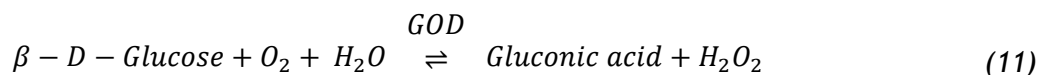
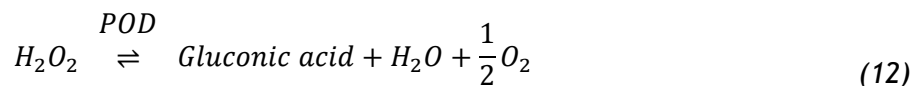


Figure 13 - Glucose biosensor apparatus.

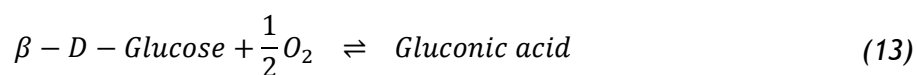
Two reactions occur with the help of two immobilized enzymes. Glucose in the presence of Glucose Oxidase (GOD), rapidly consumes oxygen and water to form gluconic acid and oxygen peroxide (11).



The oxygen peroxide formed in the first reaction inhibits the GOD activity, therefore the oxygen peroxide must be eliminated. On the second reaction, a Peroxidase (POD) acts in the oxygen peroxide, transforming it into gluconic acid, water and oxygen (12).



The sum of the reactions can be described by equation (13).



Both pH and O_2 concentration can be measured by an electrode and related to the glucose concentration. A WTW O_2 sensor was used to detect the oxygen concentration. The increase of oxygen consumption is proportional to the glucose concentration. A graphic of pO_2 vs t is made (Figure 14).

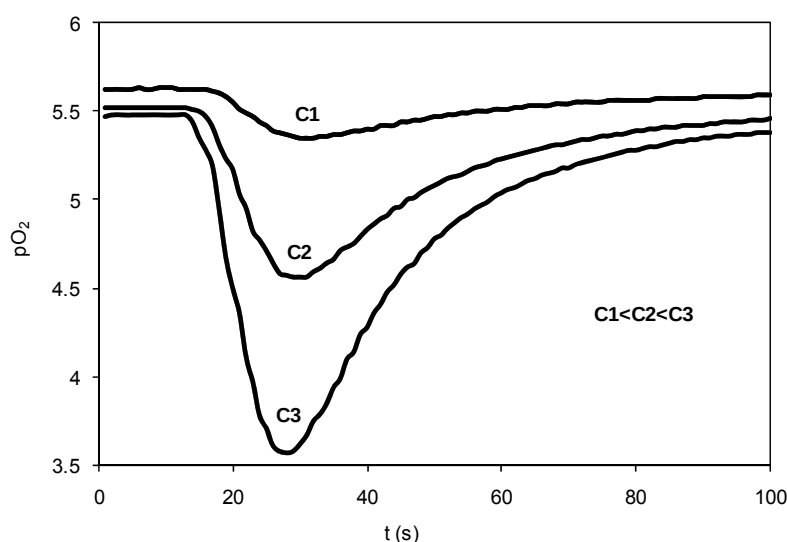


Figure 14 - Example of the response of O_2 sensor, representing pO_2 vs $t(s)$. The increase of oxygen consumption is proportional to the glucose concentration: $C1 < C2 < C3$.

The following steps were taken to prepare and analyse the samples:

- A filter membrane (Nitrocellulose) with immobilized enzymes (NOVO 771) is prepared;
- The membrane is placed in a Petri dish and exposed to the enzyme for a period of 2 min;
- The filter membranes were transferred to Erlenmeyers and were added isopropanol, water and glutaraldehyde to fix the enzymes to the membrane. The preparation was kept 1 hour under 25 °C and constant shaking;

- The membranes were washed with water and with a phosphate buffer solution (pH 7.1/15 M). Then the membrane is placed in the pO₂ electrode;
- A phosphate buffer solution (pH 7.1/15 M) is used as transport solution with a continuous flow of 0.75 ml/min, pumped by a GAT (GAMMA ANALYSEN TECHNIK GMBH) LC 1110 HPLC pump. The buffer flows through a mini-reactor surrounding the pO₂ electrode;
- The samples are previously filtered and diluted when needed. A sample of 50 µl is taken in each run. Each run took approximately 2 min.

Due to some problems in the apparatus, just the analysed samples from the experiment J were studied.

4.3.2.3. HPLC (mono and di saccharides)

Not only glucose is produced in the enzymatic hydrolysis of maize silage. The breakdown of cellulose produces smaller chains and cellobiose, this last one a disaccharide composed by two glucose units linked by a β -1,4 bond. This molecule is an important indicator of cellobiase activity. Hemicellulose is also composed by other sugars besides glucose: pentoses (xylose and arabinose) and hexoses (rhamnose, galactose, fucose, mannose and fructose).

A specific column for monosaccharides was used: PHENOMENEX REZEX RPM-Monosaccharide Pb++ (8%). The eluent was water at 0.6 ml/min and the column operated at 75 °C. For detection it was used a SEDERE SEDEX 75 ELSD (Evaporative Light Scattering Detector) at 45 °C and gain 12. The detailed equipment specifications and running conditions of the HPLC apparatus are presented in Table 14 (Appendix G).

Standard solutions of cellobiose, glucose, xylose, galactose, arabinose and fructose with different concentrations were made to calibrate the HPLC system (calibration curve). Rhamnose, fucose, mannose and fructose were not included in the calibration of the HPLC due to the retention times were too close to the other sugars. Due to lack of time, just samples without enzyme and with Novozyme 188 were studied (experiment K 1, 4, 6 and 9 with 0.75 g of substrate, equivalent to experiment J). Samples of 0, 1, 4 and 22 hr were analysed.

4.4. Biogas Production

A research partnership was made with the Fraunhofer “Institut Keramische Technologien und Systeme” (IKTS): with the help of Björn Schwartz, it was possible to prepare 24 bioreactors with biogas recovery systems in order to evaluate the biogas yields of maize silage together with cattle slurry. A one-step and a two-step process were used. In the first process, the enzymes were added directly in the bioreactor in the beginning of the experiment. On the second process, the first step of enzymatic hydrolysis was performed separated from the second step (biogas production).

For the first step, 5 Erlenmeyers were prepared equally as presented in Table 5. No samples were taken: the experience ran continuously for 21 hours and then stopped. All the Erlenmeyers were sent to Fraunhofer.

Table 5 - Conditions of enzymatic hydrolysis for the biogas production experiment.

Experiment:	Biogas yields
Substrate:	maize (1)
$m_{\text{substrate}}$ (g):	9.3
V_{water} (ml):	74.6
Enzyme:	Novozyme 188
m_{enzyme} (mg):	16.3
Size reduction:	Not Used
Washing step:	Not Used
Reaction Units:	Erlenmeyers 500 ml
Steering:	Shaker 175 lpm

In the second step, 24 bioreactors with 1 L volume at 38 °C (thermostatic bath), were prepared with 4 different conditions: blank with enzyme, pressed maize silage (PM) without enzyme, pressed maize silage (PM) with enzyme (one-step process) and enzymatic hydrolysis step content (two-step process). The experiment ran continuously over 22 days. The produced biogas volume was read daily and the gas kept in a storage bag. The methane yield was evaluated.

5. Results and discussion

In this chapter, the results of all experiments are presented and discussed. The results from the nutrient composition of the different substrates are presented first, followed by the VFA wash steps, sugar analysis in the enzymatic hydrolysis and finally the biogas production.

5.1. Substrate Characterisation

The characterization of the different samples was made by analysing the TS, VS, NVS and CP. In Table 6 the results of this analysis are presented as well as literature values for comparison.

Table 6 - Nutrient composition of maize silage and bioreactor residue. Comparison with literature.

Reference	TS (%)	VS (%)	NVS (%)	CP (% of TS)	pH
<i>Maize silage</i>					
U1667	30.4	29.2	1.2	9.54	3.8
U1741	23.7	22.5	1.2	9.56	4.0
(1)	49.4	48.0	1.4	5.83	3.9
U1803	51.6	50.0	1.6	4.82	3.9
<i>(Zauner and Kuntzel 1986)</i>					
milk ripe	20.0	18.5	1.5	8.13	3.8
dough ripe	37.2	34.4	2.8	10.00	3.9
<i>Bioreactor residue</i>					
U1756 (2)	36.0	34.4	1.6	12.52	8.9

We can observe that the TS values vary between 23.7 and 51.6%. Clearly, the maize silage characteristics (type, harvesting and silage conditions) differ between references, being the samples (1) and U1803 the most identical. As mentioned previously, there is no information about the FAO number or about the species of the maize in the different samples. However, these characteristics strongly influence the nutrient composition of the

maize, and consequently, the biogas and methane yields (Amon, Kryvoruchko et al. 2004; Amon, Amon et al. 2007a; Amon, Amon et al. 2007b). NVS percentage is very low and does not vary significantly between the samples.

Regarding the bioreactor residue, there is no information about the characteristics of the original U1756 sample, so a comparison cannot be made. However we can see that there is still a big portion of TS in the sample, showing that a complete digestion of the material is not achieved. A high percentage of CP (12.52 % of TS) also shows that a significant percentage of protein is not hydrolysed, so an enzymatic approach to protein hydrolysis should also be studied and tested.

The pH values of the samples were also presented. The values are between 3.8 and 4.0, the same as in literature. This acid value is expected since the plant material is preserved due to the formed acids on anaerobic digestion during the first two weeks of the silaging process (Encyclopaedia Britannica, 2009).

An analysis to cellulose, hemi-cellulose, lignin, starch and lipids content also should have been made. A more precise characterisation of the material would allow a better knowledge of the enzymatic hydrolysis extension. Cellulose removal should be determined (not only VS removal); the specificity of the enzymes could be tested, as well as mixtures between cellulases, hemicellulases, amylases and lipases, for a larger enzymatic hydrolysis extension.

The total carbon should also have been measured to calculate the C:N ratio, which is an important factor for bacteria stability, though this characteristic would be more important if different energy crops were used (different C:N ratios). A low C:N ratio is advantageous for digestion stability: plants with higher amounts of nitrogen had an increase formation of buffering compounds (better pH regulation on the digesters); the C₅-C₆ fatty acids synthesis is reduced, resulting in less intensive acetogenesis bypass reactions (Zauner and Küntzel 1986).

A good substrate characterization is also important if statistical models are developed to predict the biogas yield from different substrates. An example is the Methane Energy Value Model (MEVM), developed by Amon et al.; in almost all their articles, MEVM is applied by gathering information about nutrient composition. This information is crucial to calculate an approximate value for biogas yield.

5.2. VFA Detection (GC)

The results from the VFA detection by GC on the wash steps experiment are presented on this chapter. The GC method details and the chromatograms are presented on Appendix D.

The GC results for maize (1) presented in Figure 15 shows that from all the tested acids (acetic, propionic, iso-butyric, n-butyric, iso-valeric, n-valeric and caproic acid) just acetic, propionic and n-butyric acid were found (this last two in residual amounts).

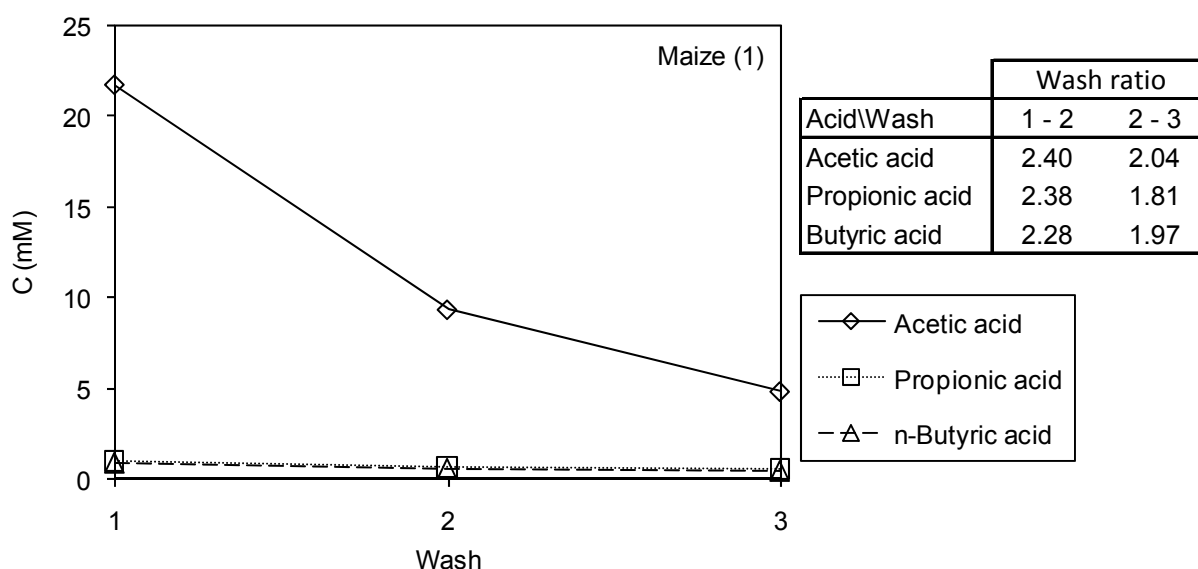


Figure 15 - VFA concentration (mM) vs Wash step. Maize (1)

A concentration of 21.69 mM of acetic acid was detected on the first wash. However the other acids were found in small amounts (propionic and b-butyric acid) or even not detected (all other acids). In the second and third wash, acids are still detected. A big percentage of water in the first washing step is absorbed by the material which avoids a better acid removal due to the low volume of water collected. However, the washing efficiency drops with the increase of washing steps. Maize silage contains around 10 %_{TS} of lactic and volatile fatty acids (Zauner and Küntzel 1986). Just 0.35 %_{TS} of VFA was removed from maize (1) on the first wash.

On the bioreactor residue U1756 (2), just acetic acid was detected in all the wash steps (Figure 16). Residual concentration of propionic acid was detected on the second wash step.

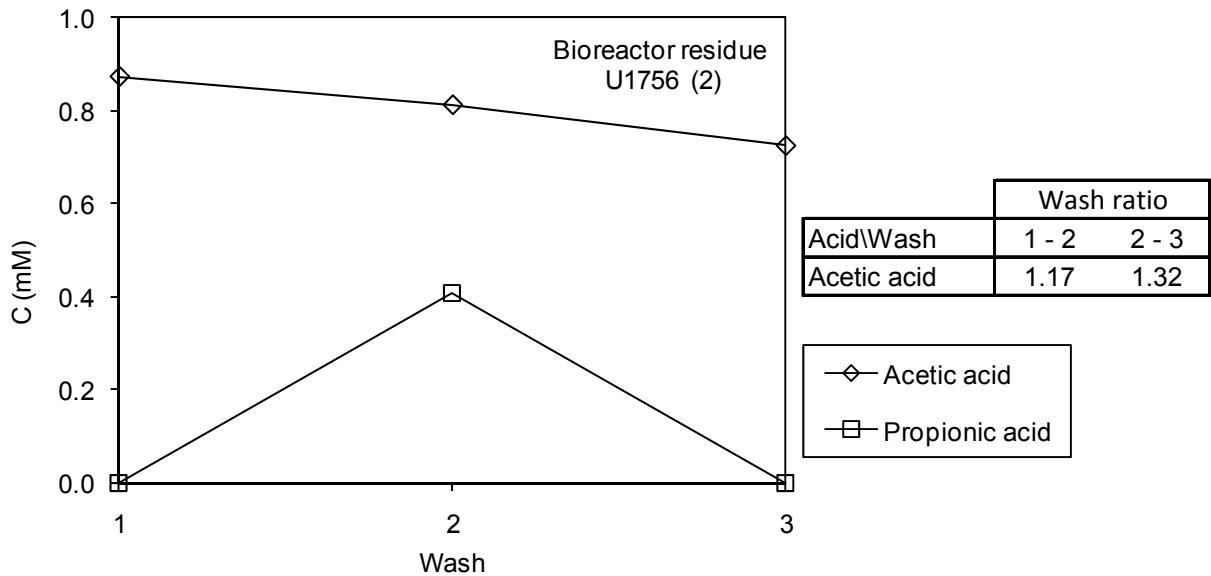


Figure 16 - VFA concentration (mM) vs Wash step. Bioreactor residue U1756 (2).

Even without being the same sample of (1), a major reduction in the acetic acid concentration (21.69 to 0.87 mM: 96% removal) and null concentration of other acids, demonstrates a good digestion stability (almost all the acids are converted into biogas). The same can be verified in the literature (Zauner and Küntzel 1986).

Figure 17 shows that in the 3 wash steps made, the pH of the washing water was constant. Acids removal was achieved but not enough to neutralize the pH. The pH value for maize (1) was 3.9 and for bioreactor residue U1756 (2) was 8.7.

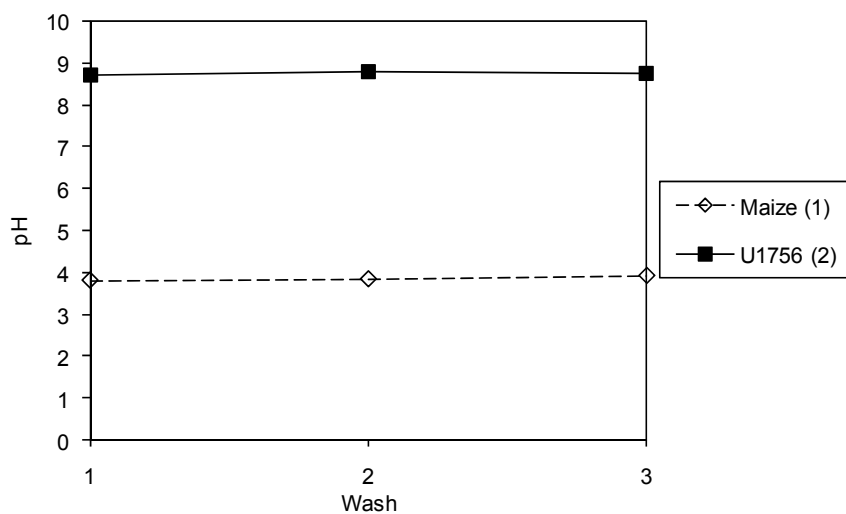


Figure 17 - Samples pH vs Wash step.

Even with 3 wash steps of 3 ml $\text{H}_2\text{O}/\text{g}_{\text{substrate}}$, the pH value remained constant, which indicates strong buffer properties of the samples. A wash step that could remove enough acid to raise the pH value of maize silage would need more than 9 ml $\text{H}_2\text{O}/\text{g}_{\text{substrate}}$, which probably is not an economically viable process step. However, the washing water, abundant in VFA and free sugars (optimal compounds to be used after hydrolysis step on anaerobic digestion), could be used on the digester, avoiding waste. A specific knowledge of the process plant is needed to try to adopt a washing step (digester loading ratio, substrate type and buffer properties).

Even if a wash step is found to be a viable process, it also demands a good control system. In the hydrolysis step, enzymes like Novozyme 188 have an optimal activity between 4 and 5 (Calsavara, De Moraes et al. 2001), which means that the wash step should increase the pH in maize silage, but not neutralize it.

5.3. Sugar Analysis

To study the extension of hydrolysis, sugar analysis was made. All the experiments were analysed by the DNS method to detect the amount of reducing sugars. Due to the large quantity of results obtained, the best ones were selected. All data was processed in Microsoft Office Excel 2007, which allowed the elimination of data considered gross errors. All experiments were made at room temperature, so, these results could be improved with a simple increase in temperature. No comparison was made with literature values due to the difference between process conditions.

In Figure 18 is made a comparison between enzymes. As we can see, Novozyme 188 is the most effective enzyme to be used in maize silage, releasing 6.41 g/L of reducing sugars, followed by Methaplus L100 with 3.52 g/L.

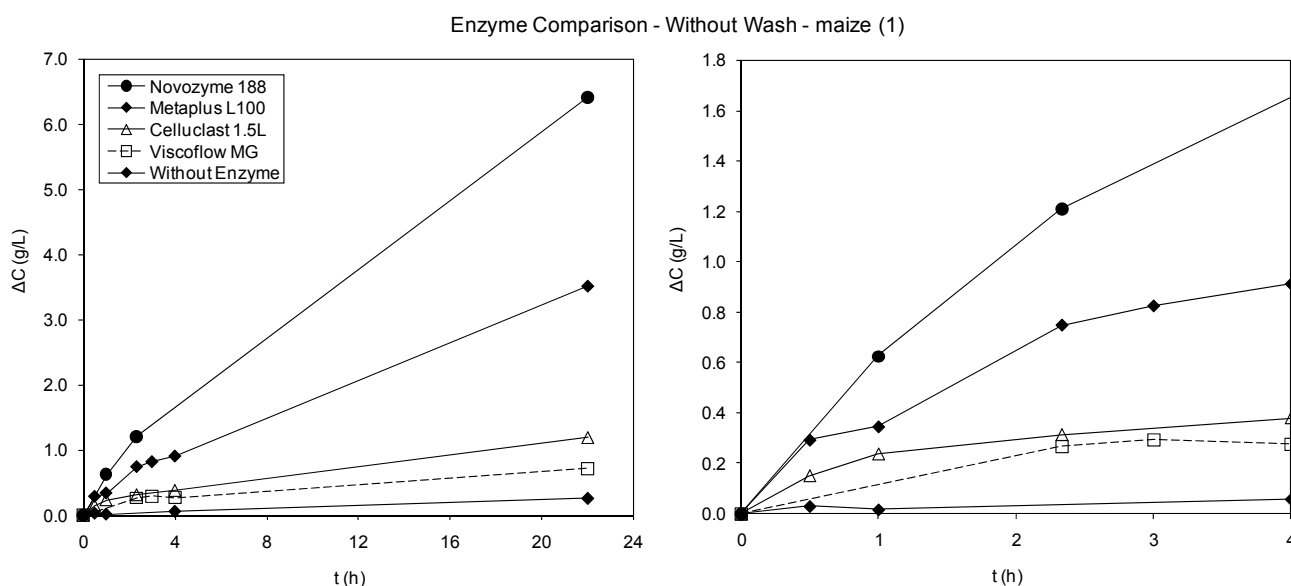


Figure 18 - Released reducing sugars concentration (g/L) vs time (h). Enzyme comparison; without wash; maize (1); Experiment E1, E3, E5, E7 and E9.

The supremacy of the cellobiose from Novozyme 188 was not expected. As discussed in Chapter 3.1.2, a synergy between different enzymes is needed to promote an efficient enzyme activity. In literature, the specific activities of Novozyme 188 are analysed (Berlin, Maximenko et al. 2007). Higher cellobiase activity is presented, comparing to low mannanase, pectinase, xylanase and cellulase activity (the value is even 0 for avicel (crystalline cellulose)) and almost no conversion is observed in pretreated corn stover. Possibly the

enzyme preparation used by us had other activities besides cellobiose that allowed this good result. The enzyme was used several times and the results were always superior comparing to the other enzymes. The specific activities of this enzyme sample should be tested to determine the source of this hydrolysis capability. Methaplus L100 that is an optimized mixture of cellulases and xylanases has better yields than Celluclast 1.5 L (mainly cellulase activity) and Viscoflow MG (cellulase and xylanase mixture not optimized for biogas production), which was expected. The addition of a cellobiase to Methaplus L100 would possibly increase the sugar yield. Further synergy studies between commercial preparations should be made.

In Figure 19 we can see a comparison between unwashed and washed maize silage for Methaplus L100 and Celluclast 1.5L. The hydrolysis extent is quite similar: the slopes of the curves are nearly the same, with a small tendency for being bigger in unwashed material, so no improvement on hydrolysis rate is observed. This can also be supported by the results obtained in Chapter 5.2, where the washing step does not cause changes in the pH level, therefore, no difference between unwashed and washed hydrolysis rate should be detected.

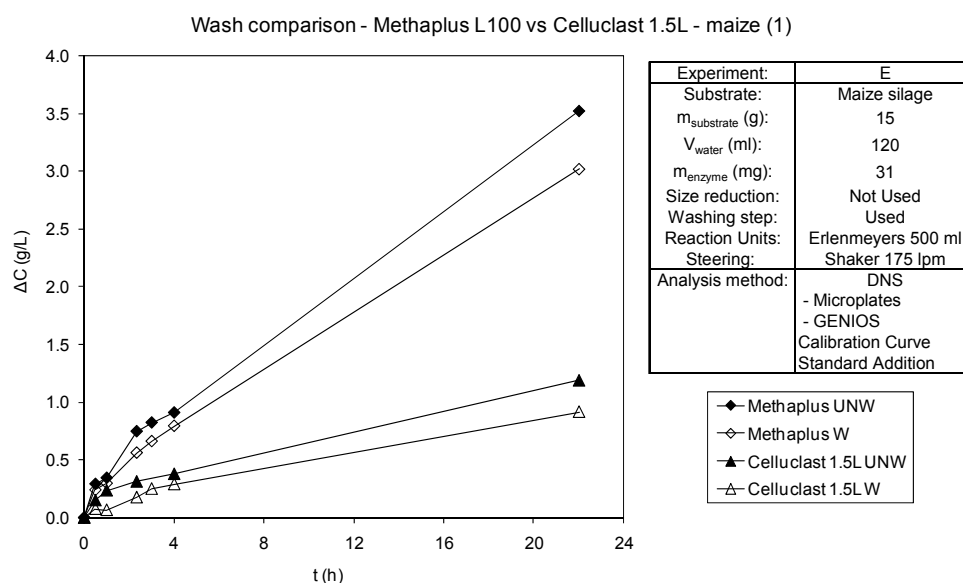


Figure 19 - Released reducing sugars concentration (g/L) vs time (h). Wash comparison; maize (1); Experiment E5 to E8.

The lag observed between unwashed and washed values is probably due to free sugars that were eventually released from the material after the experiment start. All these facts were observed in all experiments made with wash step.

The same experiment (F) was made with bioreactor residue U1756(2). For all enzymes, the released reducing sugars concentrations were similar to those without enzyme. All values

were below 0.5 g/L. Almost no hydrolysis occurs. The washing step also did not improve the results. These results were also expected due to the high pH value, the high bacteria content and the very complex environment. All these factors neutralized the enzymes work.

In experiment H, the hemicellulase activity was studied for Viscoflow MG, Novozyme 188 and a hemicellulase from Sigma (Figure 20). Locust bean gum is the standard substrate, commonly used in hemicellulase activity studies. Novozyme 188 demonstrates null activity as expected, since it is mainly a cellobiose preparation. Surprisingly, Viscoflow MG that also has xylanase activity besides cellulase, demonstrated the highest activity supplanting the Sigma hemicellulase.

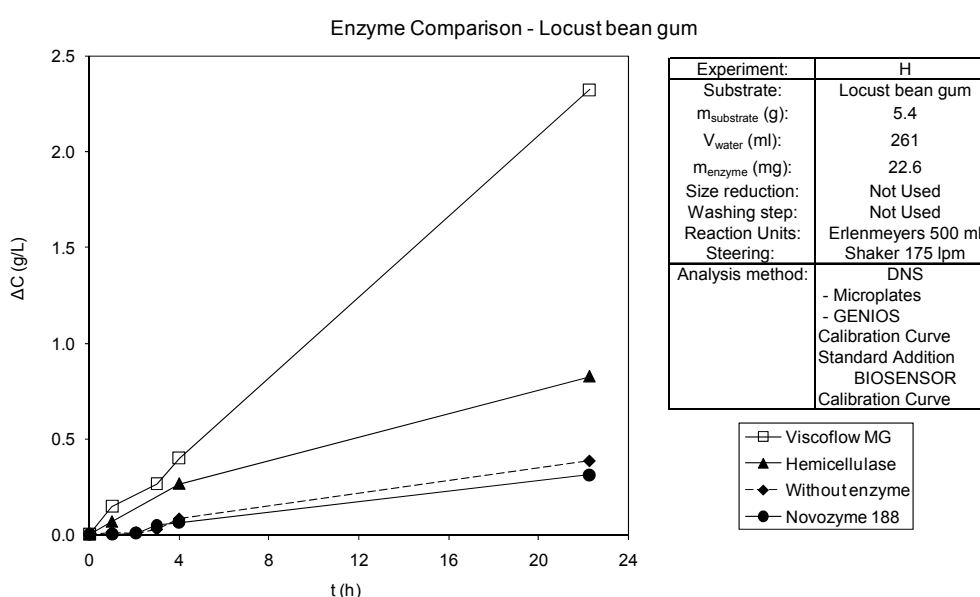


Figure 20 - Released reducing sugars concentration (g/L) vs time (h). Enzyme comparison; Locust bean gum; Experiment H1, H3, H5 and H6.

These results demonstrate the importance of the specific activities in commercial preparations. The specific hemicellulase activities of Viscoflow MG have higher affinity to the locust bean gum structure, suggesting a higher hemicellulase activity. To increase the hemicellulase activity in a cellulase mixture, Viscoflow MG should be added.

One of the most important conclusions reached is that the particle size reduction increases the hydrolysis rate. In Figure 21 the two best enzymes (Novozyme 188 and Methaplus L100) were tested in normal maize silage and in maize silage processed by the IKTS extruder. Once again, Novozyme 188 presented the best results, showing a release of 14.73 g/L of reducing sugars for extruder maize for 48 h. This value represents a release of 23.6 % of VS and was the biggest sugar release obtained. Methaplus L100 just released 9.47 g/L for

the same period of time. These sugars release values are, respectively, 2.84 and 4.08 times bigger comparing with untreated maize. The results clearly indicate a strong influence of particle size in enzymatic hydrolysis which suggests the use of this process to treat substrate.

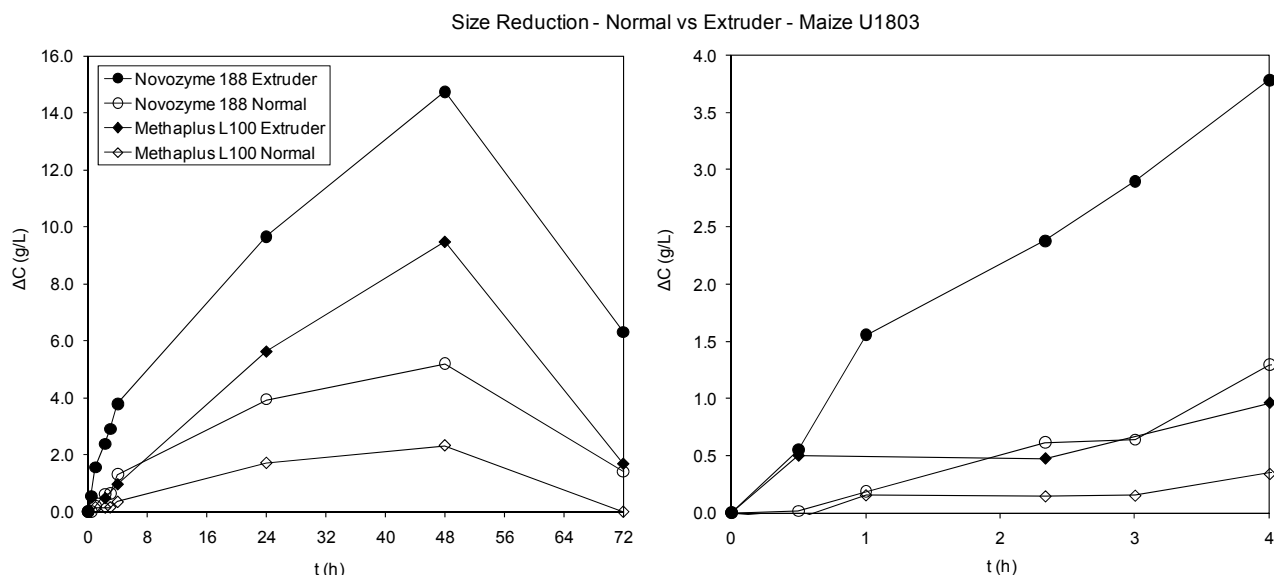


Figure 21 - Released reducing sugars concentration (g/L) vs time (h). Size reduction comparison; without wash; maize U1803; Experiment J1, J2, J3 and J4.

This influence was verified in all experiments made with maize silage with size reduction pretreatment. These results were expected due to the extensive literature that supports this fact (Spano, Medeiros et al. 1976; Walker and Wilson 1991). Other methods for particle size reduction should be explored, as well as a better ways to study the optimal particle size where the particle size could be better controlled.

This experiment ran for 72 h to verify the maximum sugar recovery possible. All 72 h samples showed a smaller reducing sugars concentration comparing to the 48 h samples. Maize silage has a big quantity of bacteria that can start to consume the produced sugars. However there are different types of bacteria that can produce different byproducts. An extensive study should be made to the samples to detect organic acids and to verify if there is already a significant production of biogas in this hydrolysis step. However, considering the obtained results, 48 h is the time window that improves the reducing sugars yield.

For the same experiment, the DNS results were compared with glucose biosensor results (Figure 22, Figure 23, Figure 24 and Figure 25). As we can observe, the glucose concentration is usually smaller than the reducing sugars concentration (with the exception for very small concentrations). This result is expected because besides glucose, all the other

reducing sugars and reducing molecules such as cellulose polymers are detected by the DNS method.

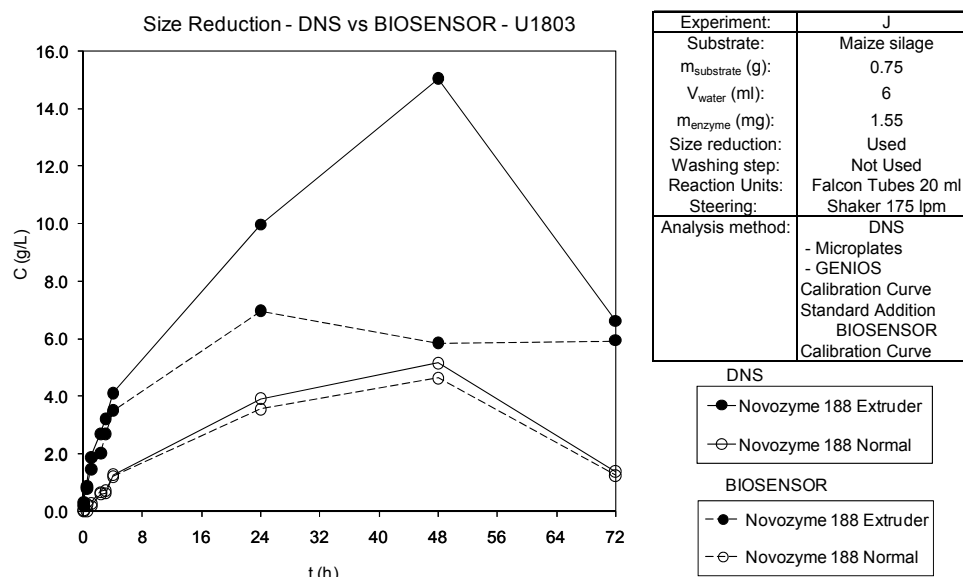


Figure 22 - DNS reducing sugars concentration and BIOSENSOR glucose concentration (g/L) vs time (h). Size reduction comparison; without wash; maize U1803; Novozyme 188. Experiment J2 and J4. 0 to 72 hours.

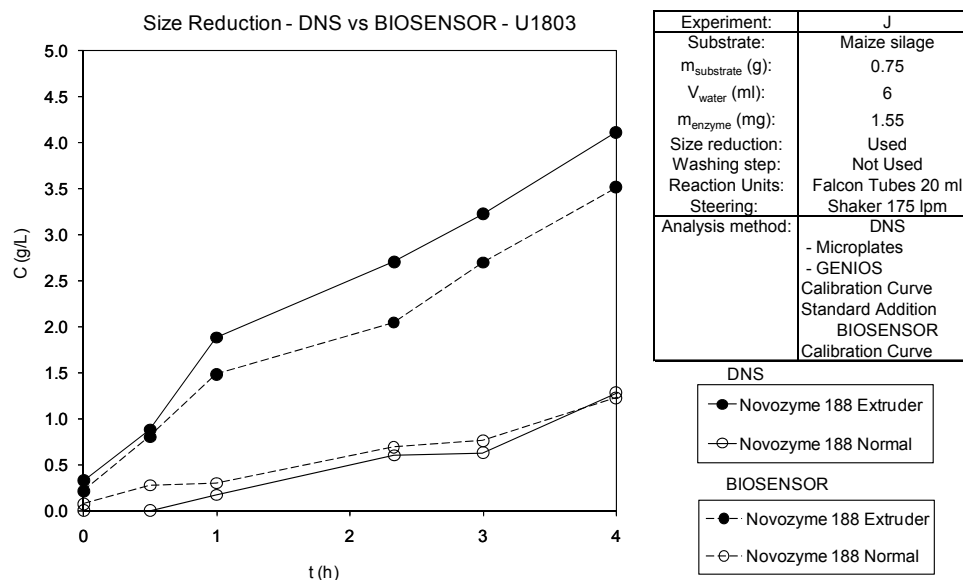


Figure 23 - DNS reducing sugars concentration and BIOSENSOR glucose concentration (g/L) vs time (h). Size reduction comparison; without wash; maize U1803; Novozyme 188. Experiment J2 and J4. 0 to 4 hours.

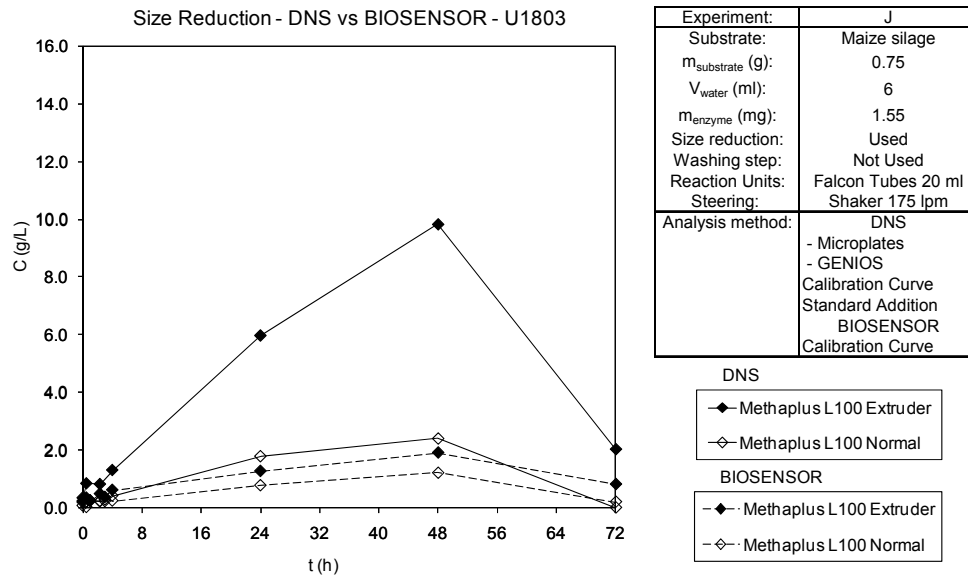


Figure 24 - DNS reducing sugars concentration and BIOSENSOR glucose concentration (g/L) vs time (h). Size reduction comparison; without wash; maize U1803; Methapplus L100. Experiment J3 and J5. 0 to 72 hours.

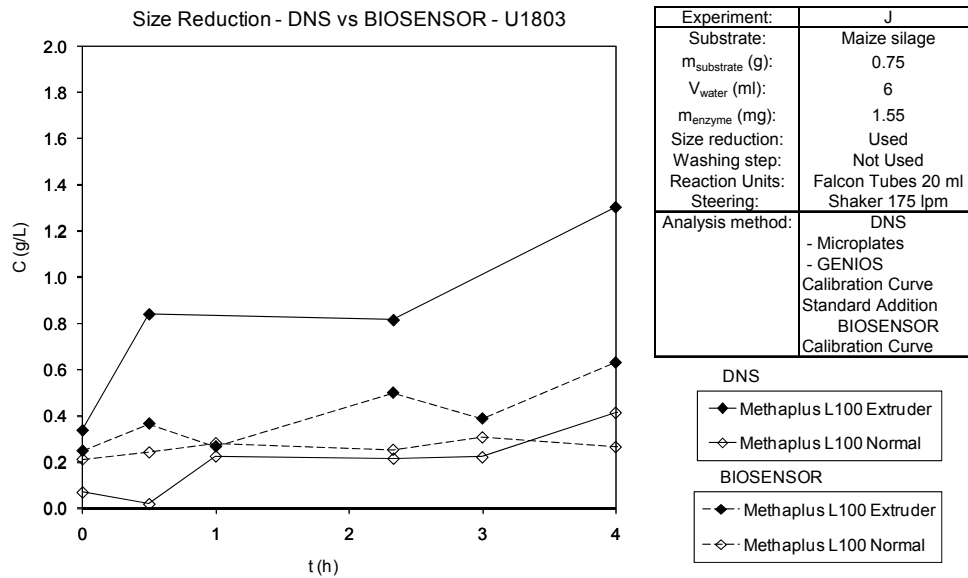


Figure 25 - DNS reducing sugars concentration and BIOSENSOR glucose concentration (g/L) vs time (h). Size reduction comparison; without wash; maize U1803; Methapplus L100. Experiment J3 and J5. 0 to 4 hours.

A good tendency can be observed in the biosensor results. The glucose biosensor is a promising technology for glucose detection that should be further studied and developed.

A kinetic study was made to analyse the best substrate concentration for the same amount of Novozyme 188 (experiment K). As we can see in Figure 26 an increase in substrate concentration is followed by an increase of released reducing sugars concentration. It can be observed again the difference between normal maize and extruder maize, with a bigger release of reducing sugars for the smaller particle size maize.

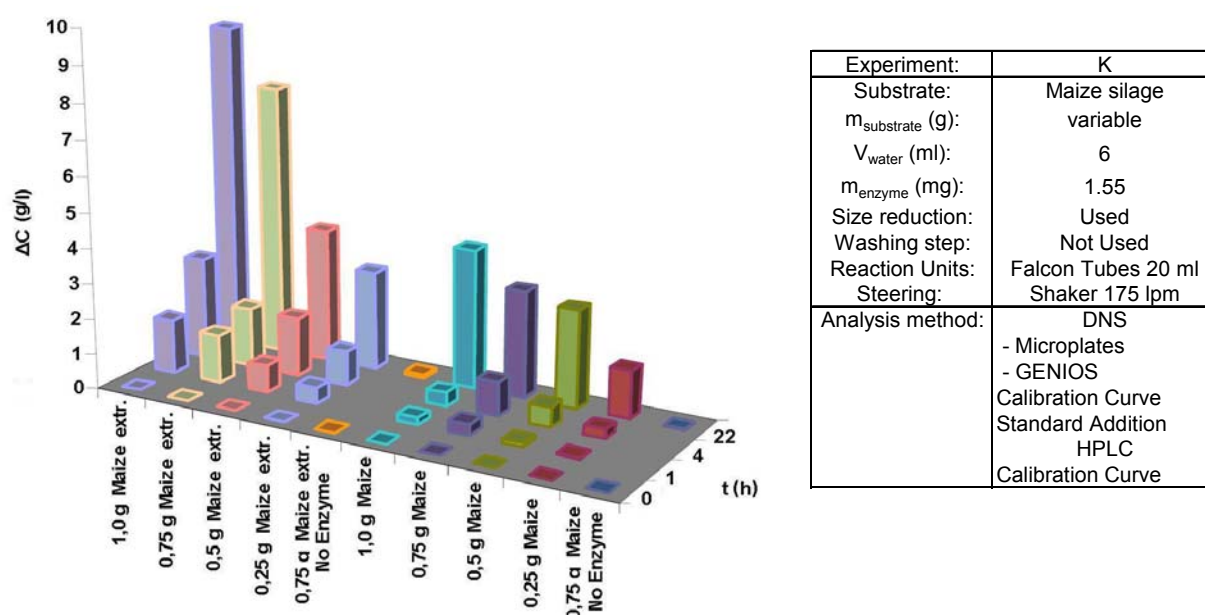


Figure 26 - DNS released reducing sugars concentration (g/L) vs maize silage content (g) vs time (h). Size reduction comparison; without wash; maize U1803; Novozyme 188. Experiment K.

The velocity of glucose production for the extruder maize was calculated in function of substrate concentration, for each time (Figure 27). The objective was to obtain a Michaelis-Menten graphic, even if the conditions are far away from being ideal; however, we can see that a value for the maximum velocity was not obtained. Two observations can be made: the hydrolysis velocity decreases in the course of time; an increase in substrate concentration is responsible for an increase in hydrolysis velocity. However, a higher velocity is not synonym of higher conversion. For the sample with 0.25 g of substrate, 21.87% of VS is released as reducing sugars after 22 h; on the other hand, for the other samples (0.5 g; 0.75 g; 1.0 g) the sugars release is inferior to 13%. The higher quantities of enzyme per gram of substance can result in a slower reaction, but usually results in a greater VS conversion. So,

although higher concentrations of substrate improve efficiency, the lower enzyme concentration promotes a lower VS conversion to reducing sugars.

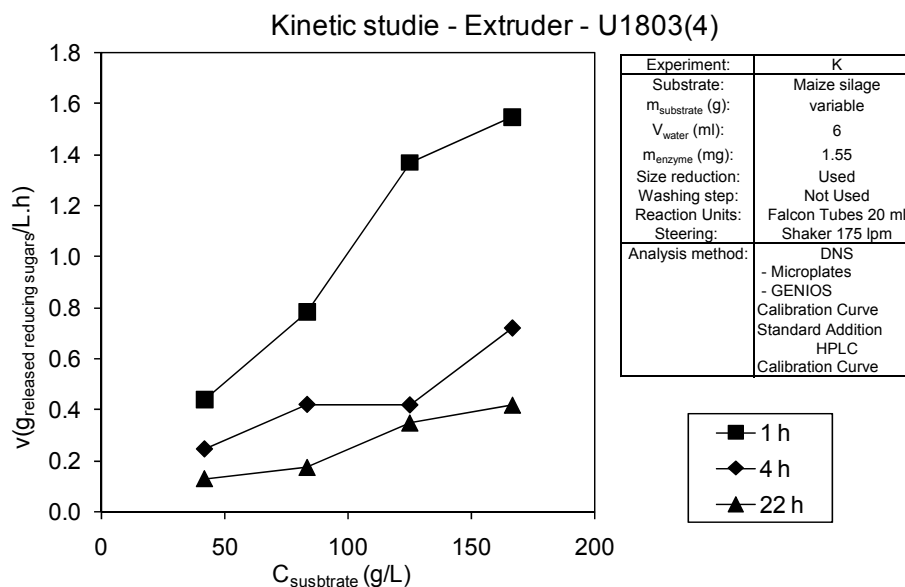


Figure 27 - Kinetic studie: velocity of reducing sugars release ($g_{\text{released reducing sugars}}/L.h$) vs substrate concentration (g/L). Time comparison; without wash; maize U1803(4); Novozyme 188. Experiment K.

The samples with 0.75 g of substrate from experiment K were also analysed in the HPLC to search for other sugars besides glucose. Unfortunately, due to lack of time, just Novozyme 188 was tested.

The first chromatogram (Figure 28) represents the 0 h for the normal maize. Several picks are detected, however there is not any compound that can be distinguished. Some galactose and cellobiose are probably in the solution, but the picks detected are wide (include other compounds), so no specific quantification can be made. In this particular sample, no free sugars were detected, which was verified also in DNS. The chromatogram for the extruder maize has a similar layout but with a glucose pick due to the free sugars. All the other picks were slightly bigger, but that does not mean that the extruder pretreatment could increase the free compounds concentration, because there are always slight differences between samples.

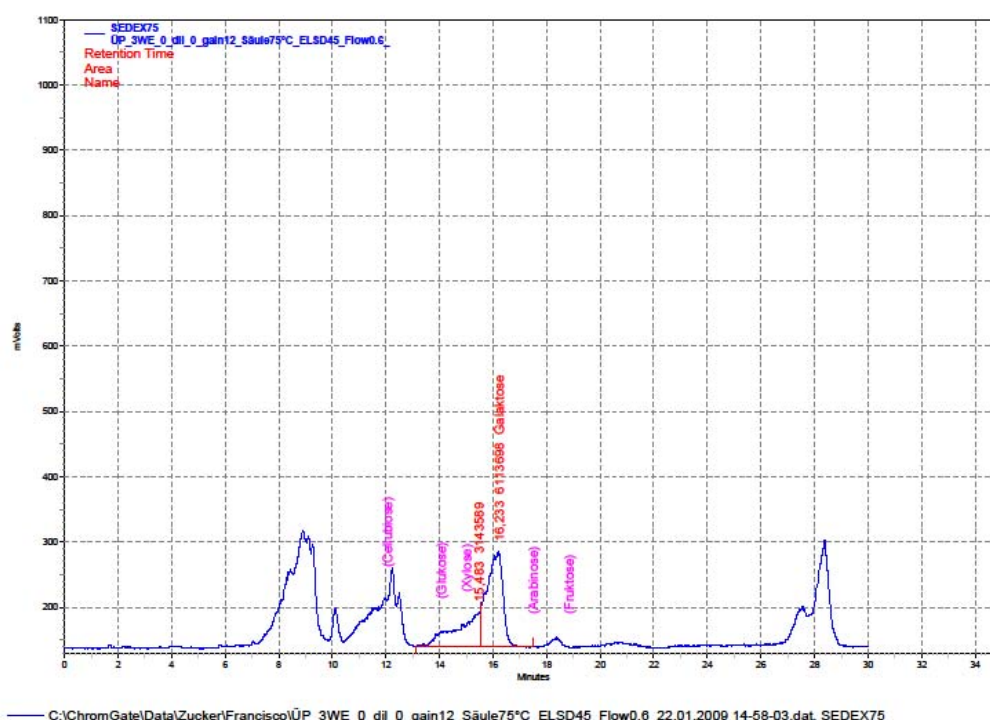


Figure 28 - HPLC chromatogram: experiment K1; maize U1803(3) normal size; Novozyme 188; 0 hr; no dilution; gain 12.

With the increase of time, the glucose produced during the hydrolysis was detected in a defined pick with a retention time of 14 min. No other sugars were detected. This result was expected due to Novozyme 188 be a cellobiose preparation: no hemicellulase activity is expected; cellobiose is quickly converted to glucose without chances to accumulate. The picks initially detected between 7 and 11 min did not disappear. They were present in all samples analysed which indicates that are some common compounds present on maize silage. A good example of a glucose pick, together with the calibration picks, is shown in Figure 29 (normal maize; 22 h; dilution 1:5).

The HPLC concentrations were compared with the DNS concentrations (Figure 30). The concentrations from HPLC were slightly higher than those from DNS. The tendency did not disappear although the values have some difference between them. This result was not expected. As previously discussed in the glucose biosensor results (Chapter 5.3), glucose concentration should be smaller than reducing sugars concentration.

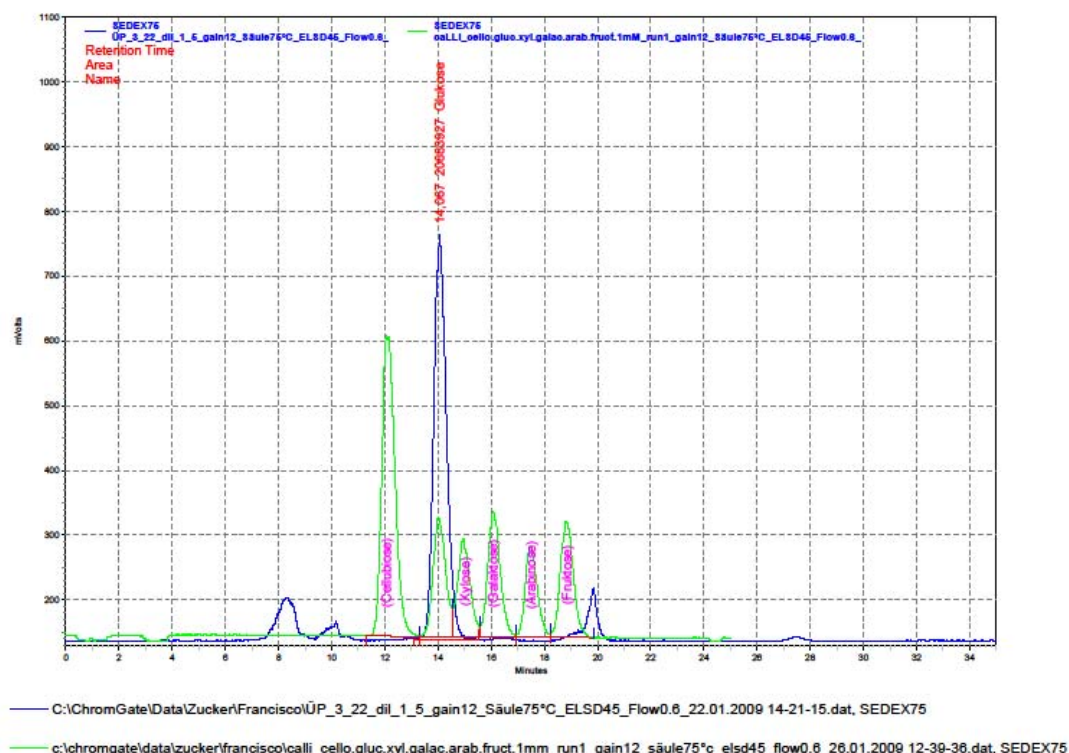


Figure 29 - HPLC chromatogram: experiment K1; maize U1803(3) normal size; Novozyme 188; 22 hr; dilution 1:5; gain 12.

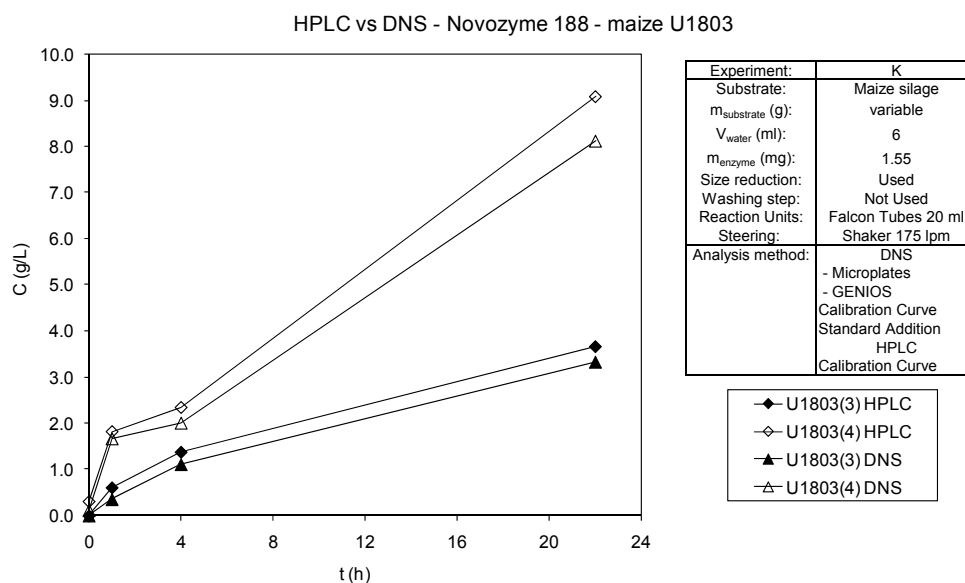


Figure 30 - HPLC released glucose concentration and DNS released reducing sugars concentration (g/L) vs time (h); experiment K; maize U1803; Novozyme 188.

5.4. Biogas Production

From the biogas experiment on Fraunhofer IKTS, just biogas yields data were supplied (there are no information regarding methane yields). As we can observe in Figure 31, the cumulative biogas yield for 22 days, with a value around 567 l/kg VS, is equal for all experiment conditions.

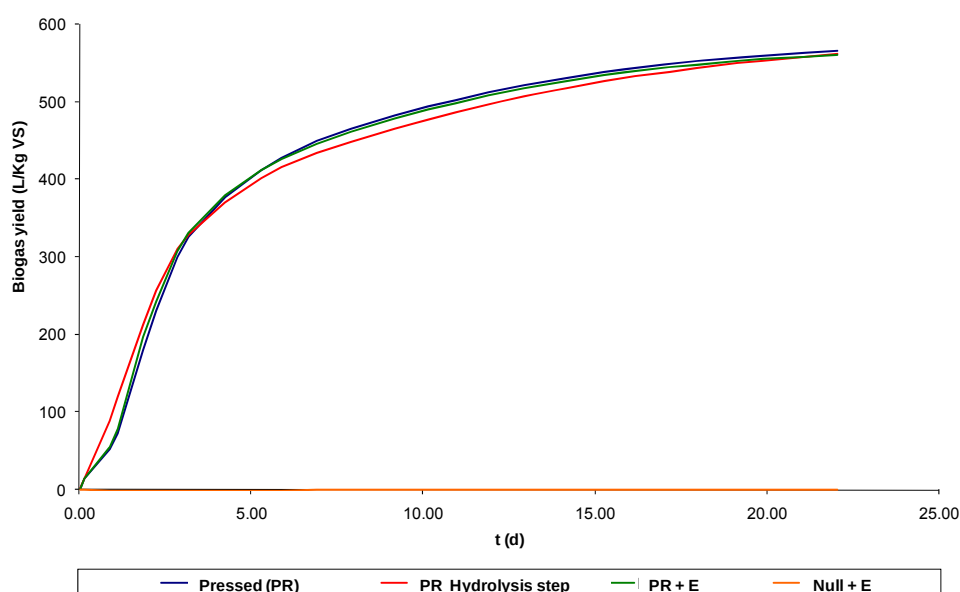


Figure 31 - Biogas yield from Fraunhofer IKTS experiments as a function of time (days): pressed maize (PR); PR hydrolysis step; PR + enzyme (E); Null + E.

A slight increase in the biogas production can be noticed in the first days when the maize from the previous enzymatic hydrolysis step was used (red line). Probably the raise is due to the sugars produced in hydrolysis step. Between day 5 and 15 the cumulative methane production is slightly lower comparing with the other two cumulative yields.

No significant difference can be seen between maize silage without enzyme (PR), and maize silage with enzyme (PR+E). The addition of Novozyme 188 directly to the reactor does not seem to have any effect on biogas production. Novozyme 188 shown good results on hydrolysis step experiments. However that does not mean that a good performance can be achieved in the bioreactor. Conditions are very different. As quoted in Chapter 3.1.2, some experiments of biogas production with enzymes addition reported increases on biogas yield. Though almost all the experiment details are unknown (especially if processes comprises one

or two stages), some different parameters are used, such as semi-continuous reactors with daily enzyme addition. Research should be made to test such different parameters.

In Table 7 are presented the methane yields. Although the biogas yield is smaller in PR + hydrolysis, the methane yield is slightly bigger. The difference is not significative enough.

Table 7 - Calculated methane yields based on biogas yield and methane percentage.

	Biogas yield l (kg VS)-1	Methane percentage	Methane yield l (kg VS)-1
PR	567	61.7	350
PR+E	562	62.4	351
PR+hydrolysis	561	63.7	357

Although the results do not favour the addition of enzymes in the biogas process, the optimal conditions were not used. The temperature used in the enzymatic hydrolysis step was 25 °C (room temperature) which clearly is not the optimal temperature for Novozyme 188 (Calsavara, De Moraes et al. 2001). A higher temperature, at least 40 °C, should be used to promote a higher sugar recovery. A pH lower than 4 is also not optimal. The pH should be between 4 and 5.

In Table 8 are presented literature values for biogas and methane yields. A direct comparison to the IKTS results cannot be made due to the difference between maize types and the different process parameters. However, the obtained values fit to batch results from literature.

Table 8 - Specific methane and biogas yields from maize silage: literature and IKTS experiment values.

Substrate	Biogas yield l (kg VS)-1	Methane yield l (kg VS)-1	Method	Reference
Maize silage	567	350	Batch	IKTS
Maize silage	-	250-360	Batch	(Kaiser, Diepolder et al. 2004)
Maize silage	-	360	Batch	(Mukengele and Oechsner 2007)
Maize silage	898-1055	359-422	Batch	(Amon, Amon et al. 2007b)
Maize silage	-	422	Calculation	(Linke, Baganz et al. 1999)
Maize silage (dough ripe)	355*	181*	Plug Flow	(Zauner and Kuntzel 1986)
Maize silage (dough ripe)		298*	Batch	(Zauner and Kuntzel 1986)
Maize silage (dough ripe)	642	-	-	(KTBL 2005)
Maize silage (dried)	-	347	Batch	(Mukengele and Oechsner 2007)
Maize silage (full ripe)	593	-	-	(KTBL 2005)
Maize silage (milk ripe)	354*	184*	Plug Flow	(Zauner and Kuntzel 1986)
Maize silage (milk ripe)	-	270*	Batch	(Zauner and Kuntzel 1986)
Maize silage (milk ripe)	578	-	-	(KTBL 2005)

* l (kg TS)-1

6. Conclusions

Enzyme addition to hydrolysis step definitely improves the hydrolysis rate, since without enzyme there is no release of reducing sugars.

Novozyme 188 presented the best results, showing a maximum release of 14.73 g/L of reducing sugars for extruder maize silage for 48 h. This value represents a release of 23.6 % of reducing sugars in VS and was the biggest sugar release obtained.

Enzymes showed null activity when tested on bioreactor residue. High pH, bacterial and complex environment contributed to this fact.

Viscoflow MG demonstrated the highest activity in locust bean gum (hemicellulase activity).

The washing step pretreatment did not improve the enzymatic hydrolysis on maize silage. No pH reduction was observed with 3 wash steps of 3 ml H₂O/g_{substrate}, although acetic acid is removed in the wash step.

Particle size reduction greatly improves the enzymatic hydrolysis. Sugars release can be improved by 1.5 to 4 times, compared with normal untreated maize silage.

Maximum hydrolysis time should be 48 h. After this period of time, sugars start to be consumed by bacteria.

Higher substrate concentration leads to a higher reaction velocity, and consequently to higher reducing sugars concentration. However, lower substrate concentration leads to a higher reducing sugars release: the higher enzyme concentration can release a higher percentage of reducing sugars from VS.

Glucose is the main sugar released from maize silage by Novozyme 188. No other sugars were detected by the HPLC, which means that no hemicellulase activity is found (no activity in locust bean gum either).

Enzyme addition, for the current project conditions, did not improve biogas yields. Without enzyme, 567 l/kg VS of biogas with 61.7% of methane was produced. Other conditions should be tested, such as higher temperatures (>45 °C) and pH around 5.

7. Achievements

7.1. Achieved Objectives

In the present study, the enzymatic hydrolysis of ensiled maize was studied.

Different enzymes were used to test the effectiveness in ensiled maize. The most effective enzyme was found. Due to technical problems was not possible to find other sugars by HPLC for other enzymes besides Novozyme 188.

Pretreatment of maize silage was performed: wash and size reduction. Conclusions were taken about the effectiveness of the pretreatment steps. However, the goal of finding the optimal particle size was not achieved due to technical problems.

Biogas and methane yield were tested for optimal enzymatic hydrolysis conditions. All expected results were obtained.

7.2. Another Works

The experimental glucose biosensor provided good results, comparable to DNS method results. The detected glucose concentrations were clearly lower than reducing sugars concentration, which was expected.

A study was made to the GENIOS spectrophotometer. GENIOS have an almost linear calibration curve for DNS method (545 nm). Errors found in statistical analysis were not significant. However, a specific study should be made to identify the source of the problem and to correct it.

7.3. Limitations and Future Work

Almost all bioethanol and biogas produced derives from starch (mostly from corn). Sources of lignocellulosics such as grasses or wood, that are available in higher quantities, inexpensive and not on the human food chain, should be the chosen carbon sources for future industrial applications. Municipal wastes, slurries and manures treatment should be

integrated with biogas production, to promote a symbiotic relationship where wastes can be treated and biofuel produced.

Besides size reduction, other pretreatments should be tested to verify their capacity to improve the hydrolysis on maize silage.

An optimization should be made with a mixture of cellulase from Methapplus L100 and cellobiose from Novozyme 188, to verify if the presence of cellobiose in the enzymatic mixture can synergistically improve the hydrolysis efficiency. Further synergy studies should be made, especially between commercial enzyme preparations.

A dialog between research institutions and enzyme producers should be made to improve the sharing of detailed information about enzymes, such as specific activities of commercial preparations (ex: cellulase, cellobiase or xylanase activity), or even the preparation of specific mixtures for specific substrates, in a policy of personalized services for the client.

Usually, enzyme activity announced by enzyme manufacturers are based on standard substrates (ex: locust bean gum for hemicellulase activity) and for determined analytes (ex: glucose release). For different substrates, enzymes exhibit different activity values, as wells as activities for other analytes (enzyme preparations usually are not pure mixtures with just one type of enzyme activity). So, the activity of an enzyme preparation will depend essentially of the composition of the substrate. Therefore, if an enzyme preparation A has an announced activity of 400 U and another one, B 800 U, that does not mean that the enzyme preparation B will be better than A if applied in the real substrate. That is why it is important to test enzymes in real substrates. Even if the activity of enzyme B is higher than A for the real substrate, it just means that enzyme B is more efficient for the same amount of enzyme used, but this does not mean that it is the best enzyme to use in the real substrate at an industrial level. The enzyme price must be taken into account. If enzyme A is much cheaper than B, an increase of enzyme A loading, for the same level of money expended, could result in a higher sugars release than B. Another example is two enzymes with the same real activity but with different prices: they have the same efficiency, but the cheaper is the better. What is suggested is to create an enzyme's price database, with different enzyme preparations. Functions could be made with price vs quantity bought. Then, real activities could be studied, not making a relation with the mass of enzyme, but with the amount of money spent on it. Also a generic analyte could be used, such as reducing sugars, because hemicellulase activity has the same importance for biogas production as cellulase activity. So, new units could be used for real substrate study: $\text{mg}_{\text{released reducing sugars}}/\text{cent}$. This is an interesting approach when the practical application of the results is the main objective.

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Appendix A - Methane and biogas yields on literature

Table 9 - Specific methane and biogas yields from energy crops, according to literature data.

Substrate	Biogas yield l (kg VS) ⁻¹	Methane yield l (kg VS) ⁻¹	Method	Reference
Alfalfa	-	340	Batch	(Pouech, Fruteau et al. 1998a)
Alfalfa	-	410	-	(Weiland 2003)
Alfalfa	-	320	-	(Brolin, Thyselius et al. 1988)
Alfalfa (Green)	-	432	Calculation	(Linke, Baganz et al. 1999)
Alfalfa silage	-	240*	Batch	(Zauner and Kuntzel 1986)
Barley	-	360	-	(Weiland 2003)
Beet leaf	400-500	-	-	(Baserga 2000)
Beet leaf silage	-	294*	Batch	(Zauner and Kuntzel 1986)
Beet leaf silage	-	174*	Plug Flow	(Zauner and Kuntzel 1986)
Cannabis silage	-	409	Calculation	(Linke, Baganz et al. 1999)
Cereal straw	250-350	-	-	(Baserga 2000)
Clover	-	350	Batch	(Pouech, Fruteau et al. 1998a)
Clover	500-650	-	-	(Baserga 2000)
Clover	264	152	Plug Flow	(Pouech, Fruteau et al. 1998b)
Clover	-	350	-	(Weiland 2003)
Fodder beet	-	411-430	Calculation	(Linke, Baganz et al. 1999)
Foliage	400	-	-	(Baserga 2000)
Forage beet	-	460	-	(Weiland 2003)
Forage beet	-	360	-	(Brolin, Thyselius et al. 1988)
Grass	-	410	-	(Weiland 2003)
Grass	-	270	-	(Brolin, Thyselius et al. 1988)
Grass	650-860	310-360	-	(Mähnert, Heiermann et al. 2005)
Grass (several species)	678-929	-	Batch	(Mähnert, Heiermann et al. 2002)
Grass silage	500-600	-	-	(Baserga 2000)
Hay	500	-	-	(Baserga 2000)
Horse bean silage	-	356*	Batch	(Zauner and Kuntzel 1986)
Horse bean silage	-	226*	Plug Flow	(Zauner and Kuntzel 1986)
Jerusalem artichoke	-	270	-	(Brolin, Thyselius et al. 1988)
Kale	-	320	-	(Brolin, Thyselius et al. 1988)
Maize	-	397	Batch	(Pouech, Fruteau et al. 1998a)
Maize	-	410	-	(Weiland 2003)
Maize (dried)	-	345	Batch	(Mukengele and Oechsner 2007)
Maize (fresh)	-	351	Batch	(Mukengele and Oechsner 2007)
Maize silage	-	250-360	Batch	(Kaiser, Diepolder et al. 2004)
Maize silage	-	360	Batch	(Mukengele and Oechsner 2007)
Maize silage	-	359-422	Batch	(Amon, Amon et al. 2007b)
Maize silage	-	422	Calculation	(Linke, Baganz et al. 1999)
Maize silage (dough ripe)	355*	181*	Plug Flow	(Zauner and Kuntzel 1986)
Maize silage (dough ripe)	-	298*	Batch	(Zauner and Kuntzel 1986)
Maize silage (dough ripe)	642	-	-	(KTBL 2005)
Maize silage (dried)	-	347	Batch	(Mukengele and Oechsner 2007)
Maize silage (full ripe)	593	-	-	(KTBL 2005)
Maize silage (milk ripe)	354*	184*	Plug Flow	(Zauner and Kuntzel 1986)
Maize silage (milk ripe)	-	270*	Batch	(Zauner and Kuntzel 1986)
Maize silage (milk ripe)	578	-	-	(KTBL 2005)
Maize straw	500	-	-	(Baserga 2000)
Marrow	-	260	-	(Weiland 2003)
Mixed-grass silage	-	199-244*	Plug Flow	(Zauner and Kuntzel 1986)

Mixed-grass silage	-	298-315*	Batch	(Zauner and Kuntzel 1986)
Oats	-	365	Batch	(Pouech, Fruteau et al. 1998a)
Parthenium	-	152±15	Batch	(Nallathambi Gunaseelan 1995)
Potato	-	280	-	(Weiland 2003)
Potato cabbage	500-600	-	-	(Baserga 2000)
Rape	-	340	-	(Weiland 2003)
Rapeseed	-	336	Batch	(Pouech, Fruteau et al. 1998a)
Rice straw	380-471	188-240	Plug Flow	(Zhang and Zhang 1999)
Rye green fodder	-	431	Calculation	(Linke, Baganz et al. 1999)
Rye straw	-	409	Calculation	(Linke, Baganz et al. 1999)
Ryegrass	-	390-409	Batch	(Pouech, Fruteau et al. 1998a)
Sorghum	-	295-372	Batch	(Pouech, Fruteau et al. 1998a)
Vetch silage	-	323*	Batch	(Zauner and Kuntzel 1986)
Wheat	-	384	Batch	(Pouech, Fruteau et al. 1998a)
Wheat	332	184	Plug Flow	(Pouech, Fruteau et al. 1998b)
Wheat	-	390	-	(Weiland 2003)
Wilting grass silage	-	433	Calculation	(Linke, Baganz et al. 1999)
Winter barley	-	420	Calculation	(Linke, Baganz et al. 1999)
Winter rye	-	419	Calculation	(Linke, Baganz et al. 1999)

* l (kg TS)⁻¹

Appendix B - Different enzymes and pretreatments used in hydrolysis experiments (literature)

Table 10 - Different enzymes and pretreatments used in hydrolysis experiments (literature).

Enzyme	Type	Company	Substrate	Pretreatments	Reference
Celucast 1.5 L	Cellulase	Novozymes	Avicel	acid alkaline organosolv	(Bommarius, Katona et al. 2008)
Celucast 1.5 L Novozym 188 Multifect Xylanase Multifect Pectinase	Cellulase Cellobiose Xylanase Pectinase	Novozymes Novozymes --- ---	pretreated corn stover	dilute acid knife mill thermal wash	(Berlin, Maximenko et al. 2007)
Novozym 188	Cellobiose	Novozymes	Cellobiose	---	(Calsavara, De Moraes et al. 2001)
T. viride mixture	---	---	steam-exploded corn stover	milling steam exposition	(Zhou, Wang et al. 2009)
Methapplus L100 Roxazym G2L Foodcell CP Cellucast 1.5 LFG	T. reesei mix Cellulase Cellulase Cellulase	Biopract DSM Dyadic Novozymes	filter paper, feed concentrate, hay fibers, grass silage, corn silage	---	(Gerhardt, Pelenc et al. 2007)
Spezyme CP Novozym 188 Multifect Xylanase PowerPulp TX200A Fibrezyme LBL Biocat	Cellulase Cellobiose Xylanase Xylanase Xylanase Xylanase	NREL Novozymes --- --- --- ---	pretreated sugar cane bagasse	AFEX	(Prior and Day 2008)
---	---	---	wheat straw	fitz-milling roller milling ball milling caustic soda peracetic acid ethylene glycol	(Walker and Wilson 1991)
Cellubrix L	---	Novozymes	Maize silage	wet oxidation thermal	(Thomsen, Holm-Nielsen et al. 2008)
Spezyme CP	Cellulase	NREL	Corn stover	AFEX	(Teymouri, Laureano-Perez et al. 2005)
commercial mixture	---	---	Corn fiber	AFEX	(Saha, Dien et al. 1998)

Appendix C - Particle size distribution for experiment G (meat grinder; maize (1))

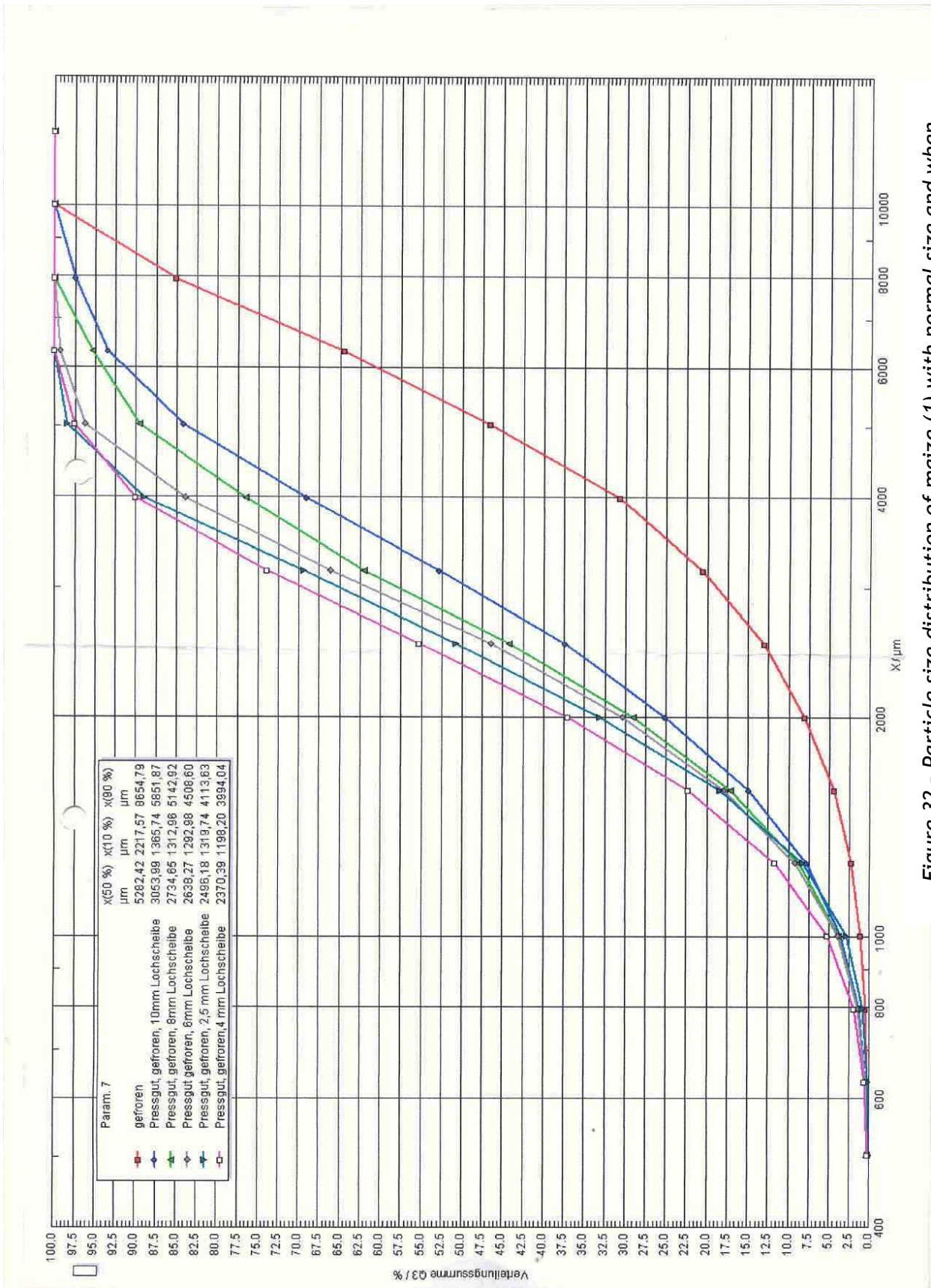


Figure 32 - Particle size distribution of maize (1) with normal size and when processed by the meat grinder with whole sizes of 10, 8, 6, 4 and 2.5 mm.

Appendix D - GC method details and chromatograms

Method: C:\HPCHEM\1\METHODS\FFA1.M of 23.09.2008 14:28:15

=====

6890 GC METHOD

=====

OVEN

Initial temp: 80 'C (On) Maximum temp: 450 'C
Initial time: 1.00 min Equilibration time: 3.00 min
Ramps:
Rate Final temp Final time
1 20.00 120 0.00
2 10.00 205 5.00
3 0.0 (Off)
Post temp: 80 'C
Post time: 0.00 min
Run time: 16.50 min

FRONT INLET (UNKNOWN)

BACK INLET ()

Mode: Split
Initial temp: 260 'C (On)
Pressure: 21.71 psi (On)
Split ratio: 20:1
Split flow: 38.0 mL/min
Total flow: 42.5 mL/min
Saver: On
Saver flow: 20.0 mL/min
Saver time: 2.00 min
Gas type: Helium

COLUMN 1

COLUMN 2
(not installed)

Capillary Column
Model Number: HP 19091N-233
HP-INNOWax Polyethylene Glycol
Max temperature: 260 'C
Nominal length: 30.0 m
Nominal diameter: 250.00 um
Nominal film thickness: 0.50 um
Mode: constant flow
Initial flow: 1.9 mL/min
Nominal init pressure: 21.72 psi
Average velocity: 42 cm/sec
Inlet: Front Inlet
Outlet: Front Detector
Outlet pressure: ambient

FRONT DETECTOR (FID)

BACK DETECTOR (NO DET)

Temperature: 260 'C (On)
Hydrogen flow: 30.0 mL/min (On)
Air flow: 350.0 mL/min (On)
Mode: Constant makeup flow
Makeup flow: 35.0 mL/min (On)
Makeup Gas Type: Helium
Flame: On
Electrometer: On
Lit offset: 2.0

Figure 33 - GC method details

Method: C:\HPCHEM\1\METHODS\FFA1.M of 23.09.2008 14:28:15		
TIME TABLE		
Time	Specifier	Parameter & Setpoint
7673 Injector		
Front Injector:		
	Sample Washes	2
	Sample Pumps	5
	Injection Volume	1.0 microliters
	Syringe Size	10.0 microliters
	PostInj Solvent A Washes	3
	PostInj Solvent B Washes	3
	Viscosity Delay	0 seconds
	Plunger Speed	Fast
	PreInjection Dwell	0.00 minutes
	PostInjection Dwell	0.00 minutes
Back Injector:		
No parameters specified		
Instrument1 17.10.2008 16:15:18 Thanet		
Page 2 of 2		

Figure 33 - GC method details (cont.)

Data File C:\HPCHEM\1\DATA\FFA_S3\1_1W.D

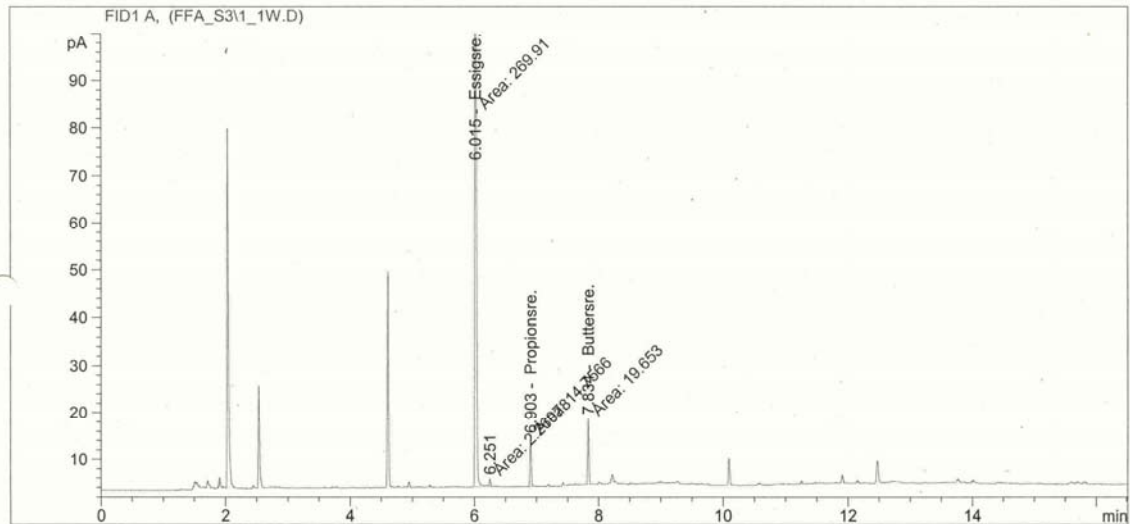
Sample Name: F.M. / 1-1w

```

=====
Injection Date   : 17.10.2008 13:04:23      Seq. Line :    1
Sample Name     : F.M. / 1-1w              Location  : Vial 102
Acq. Operator   : Thanet                   Inj       :    1
                                           Inj Volume: 1 µl

Acq. Method     : C:\HPCHEM\1\METHODS\FFA1.M
Last changed    : 23.09.2008 14:28:15 by Wunderlich
Analysis Method : C:\HPCHEM\1\METHODS\FFA7.M
Last changed    : 24.09.2008 15:29:58 by Wunderlich
Freie Fettsäuren
=====

```



=====

External Standard Report

=====

```

Sorted By      : Retention Time
Calib. Data Modified : Tuesday, 23. September 2008 14:23:08
Multiplier     : 1.0000
Dilution       : 1.0000

```

Signal 1: FID1 A,

RetTime [min]	Sig	Type	Area [pA*s]	Amt/Area	Amount [mM]	Grp	Name
6.015	1	MM	269.91010	8.03426e-2	21.68528		Essigsre.
6.903	1	MM	14.75659	6.53622e-2	9.64523e-1		Propionsre.
7.202	1		-	-	-		iso-Buttersre.
7.834	1	MM	19.65300	4.41539e-2	8.67756e-1		Buttersre.
8.277	1		-	-	-		iso-Valeriansre.
9.001	1		-	-	-		Valeriansre.

Totals : 23.51756

Results obtained with enhanced integrator!
 2 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)
 Warning : Calibrated compound(s) not found

=====

*** End of Report ***

Instrument1 17.10.2008 15:46:47 Thanet

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Figure 34 - GC chromatogram: maize (1); 1.st wash.

Data File C:\HPCHEM\1\DATA\FFA_S3\1_2W.D

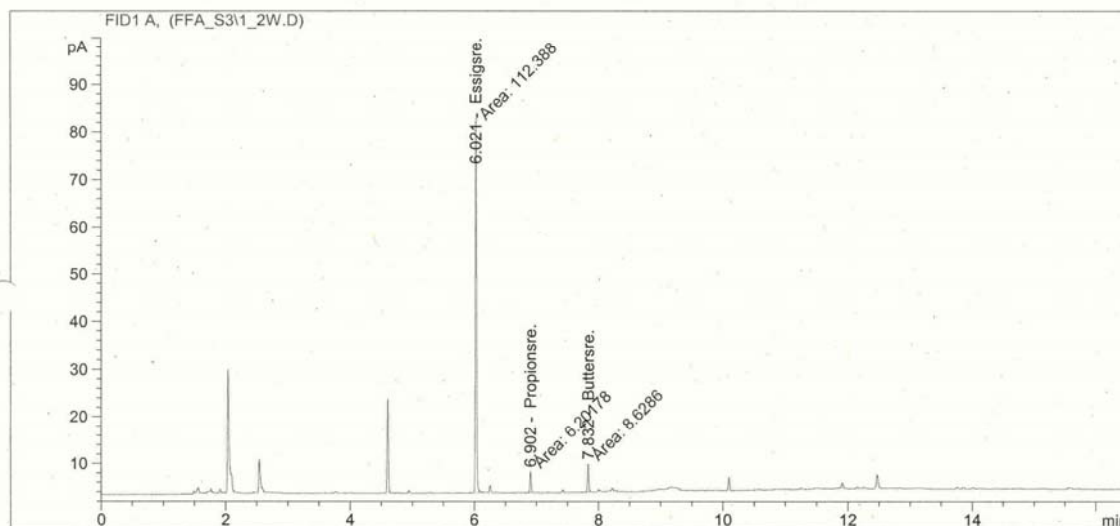
Sample Name: F.M. / 1-2w

```

=====
Injection Date   : 17.10.2008 13:27:39      Seq. Line :    2
Sample Name     : F.M. / 1-2w              Location  : Vial 103
Acq. Operator   : Thanet                   Inj       :    1
                                           Inj Volume: 1 µl

Acq. Method     : C:\HPCHEM\1\METHODS\FFA1.M
Last changed    : 23.09.2008 14:28:15 by Wunderlich
Analysis Method : C:\HPCHEM\1\METHODS\FFA7.M
Last changed    : 24.09.2008 15:29:58 by Wunderlich
Freie Fettsäuren
=====

```



=====

External Standard Report

=====

```

Sorted By      : Retention Time
Calib. Data Modified : Tuesday, 23. September 2008 14:23:08
Multiplier     : 1.0000
Dilution       : 1.0000

```

Signal 1: FID1 A,

RetTime [min]	Sig	Type	Area [pA*s]	Amt/Area	Amount [mM]	Grp	Name
6.021	1	MM	112.38751	8.27042e-2	9.29492		Essigsre.
6.902	1	MM	6.20178	1.00901e-1	6.25766e-1		Propionsre.
7.202	1		-	-	-		iso-Buttersre.
7.832	1	MM	8.62860	6.48343e-2	5.59429e-1		Buttersre.
8.277	1		-	-	-		iso-Valeriansre.
9.001	1		-	-	-		Valeriansre.

Totals : 10.48012

Results obtained with enhanced integrator!
 2 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)
 Warning : Calibrated compound(s) not found

=====

*** End of Report ***

=====

Instrument1 17.10.2008 15:48:40 Thanet

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Figure 35 - GC chromatogram: maize (1); 2.nd wash.

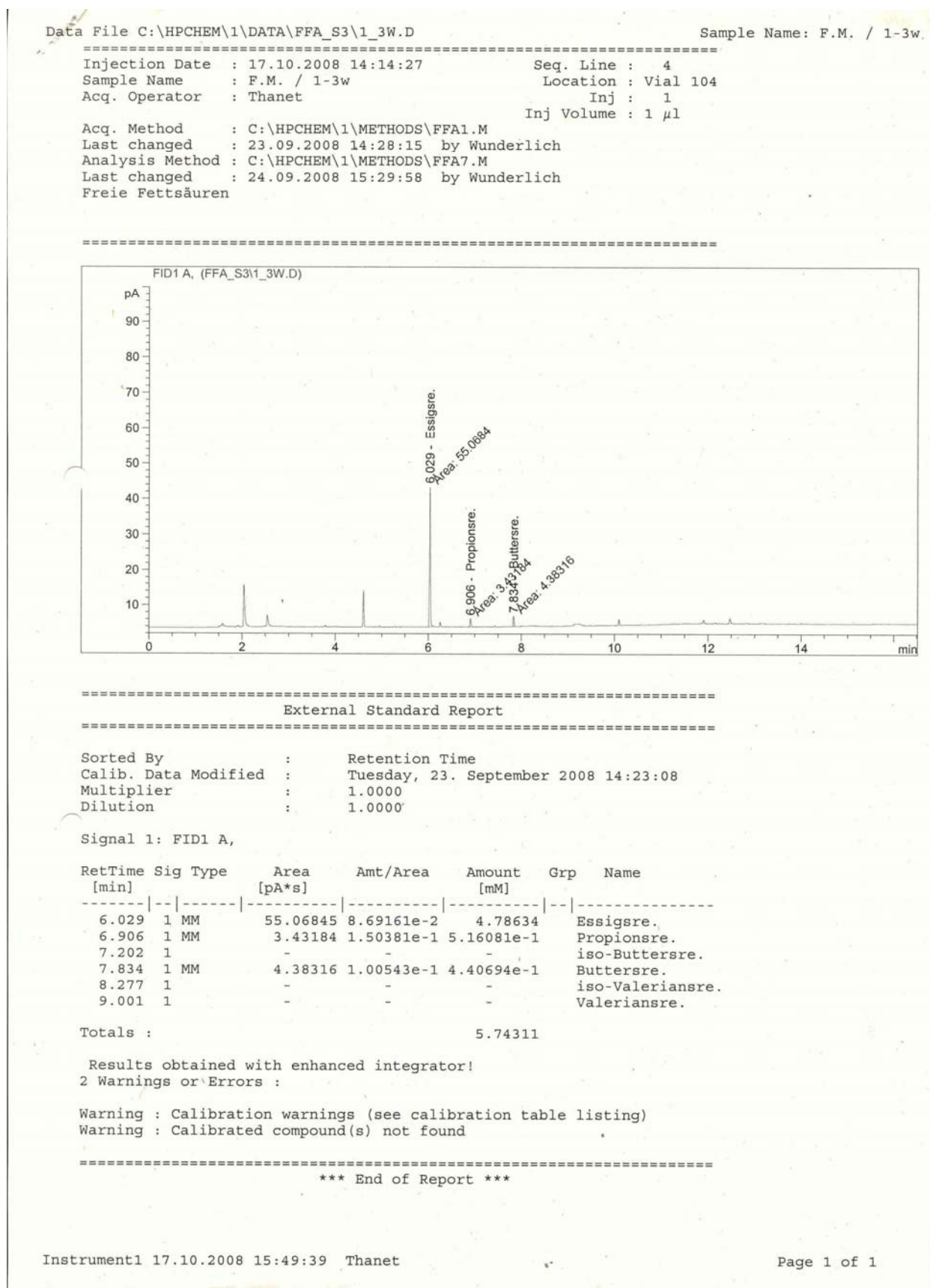


Figure 36 - GC chromatogram: maize (1); 3.rd wash.

Data File C:\HPCHEM\1\DATA\FFA_S3\2_1W.D

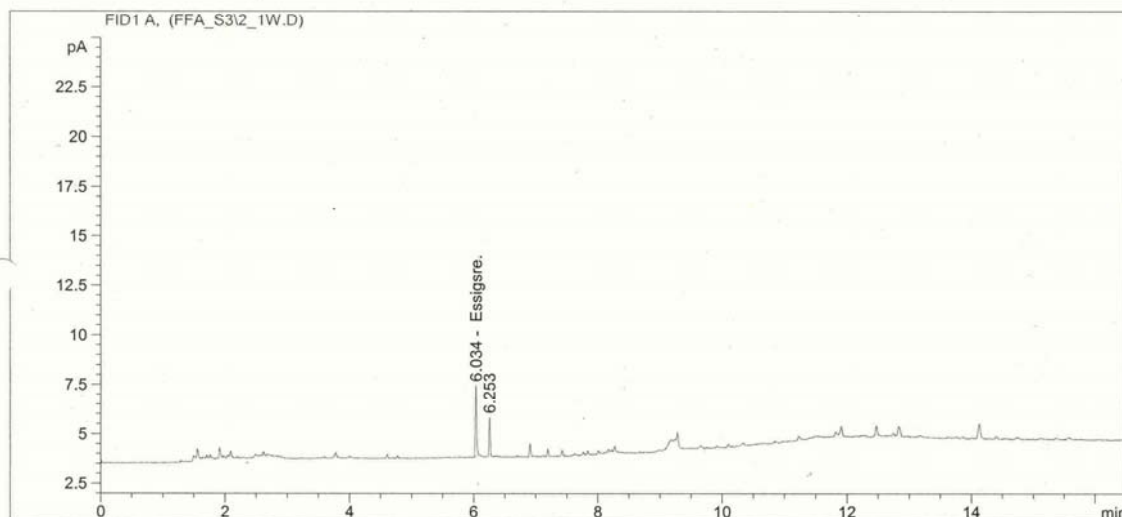
Sample Name: F.M. / 2-1w

```

=====
Injection Date   : 17.10.2008 14:37:47      Seq. Line :    5
Sample Name     : F.M. / 2-1w              Location  : Vial 105
Acq. Operator   : Thanet                   Inj       :    1
                                           Inj Volume: 1 µl

Acq. Method     : C:\HPCHEM\1\METHODS\FFA1.M
Last changed    : 23.09.2008 14:28:15 by Wunderlich
Analysis Method : C:\HPCHEM\1\METHODS\FFA7.M
Last changed    : 17.10.2008 15:51:43 by Thanet
                                           (modified after loading)
Freie Fettsäuren
=====

```



=====

External Standard Report

=====

```

Sorted By           :      Retention Time
Calib. Data Modified :      Tuesday, 23. September 2008 14:23:08
Multiplier          :      1.0000
Dilution            :      1.0000

```

Signal 1: FID1 A,

RetTime [min]	Sig	Type	Area [pA*s]	Amt/Area	Amount [mM]	Grp	Name
6.034	1	PB	5.28166	1.64763e-1	8.70223e-1		Essigsre.
6.914	1		-	-	-		Propionsre.
7.202	1		-	-	-		iso-Buttersre.
7.847	1		-	-	-		Buttersre.
8.277	1		-	-	-		iso-Valeriansre.
9.001	1		-	-	-		Valeriansre.

Totals : 8.70223e-1

Results obtained with enhanced integrator!

2 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)

Warning : Calibrated compound(s) not found

=====

*** End of Report ***

=====

Instrument1 17.10.2008 15:52:29 Thanet

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Figure 37 - GC chromatogram: bioreactor residue U1756 (2); 1.st wash.

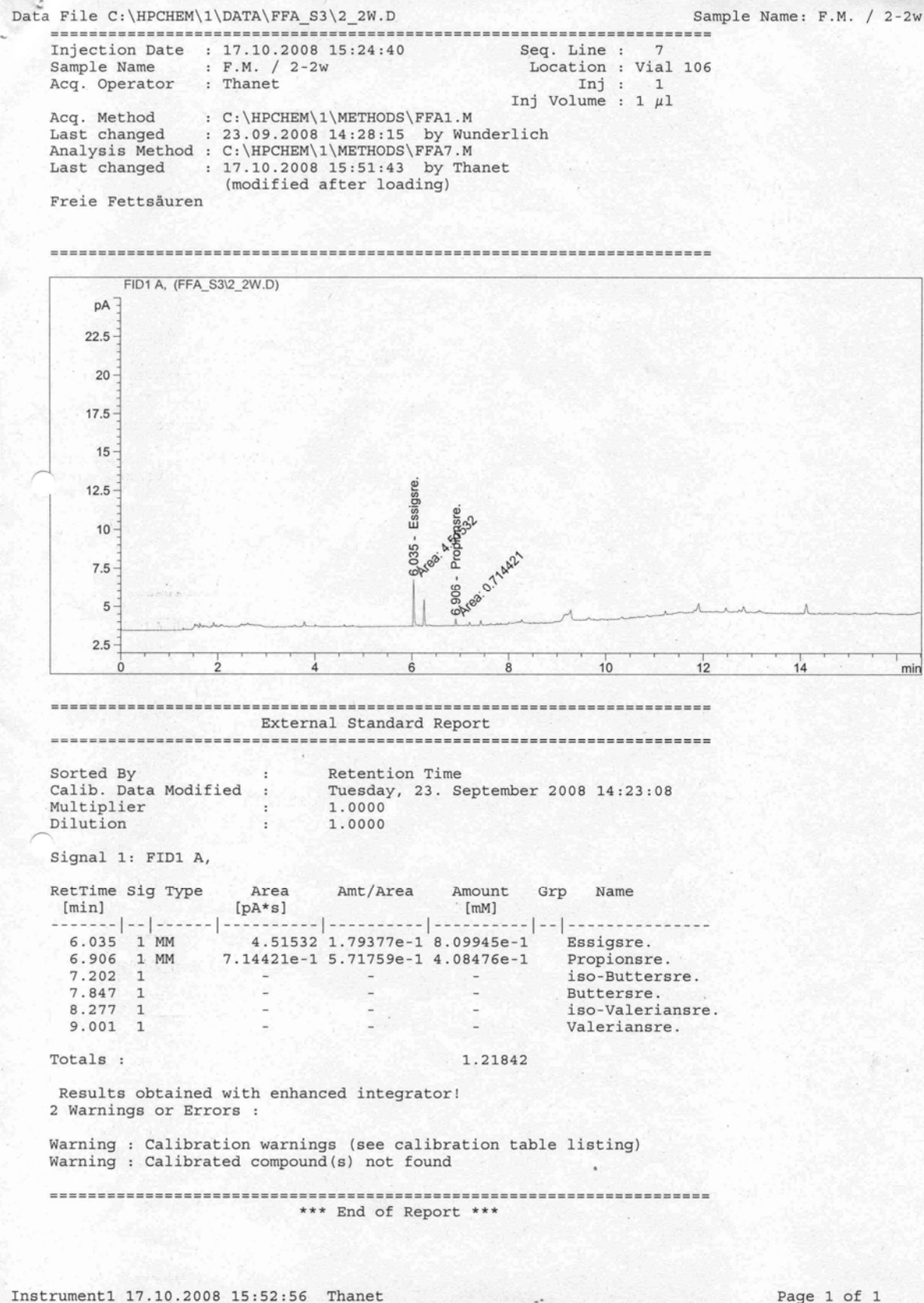


Figure 38 - GC chromatogram: bioreactor residue U1756 (2); 2.nd wash.

Data File C:\HPCHEM\1\DATA\FFA_S3\2_3W.D

Sample Name: F.M. / 2-3w

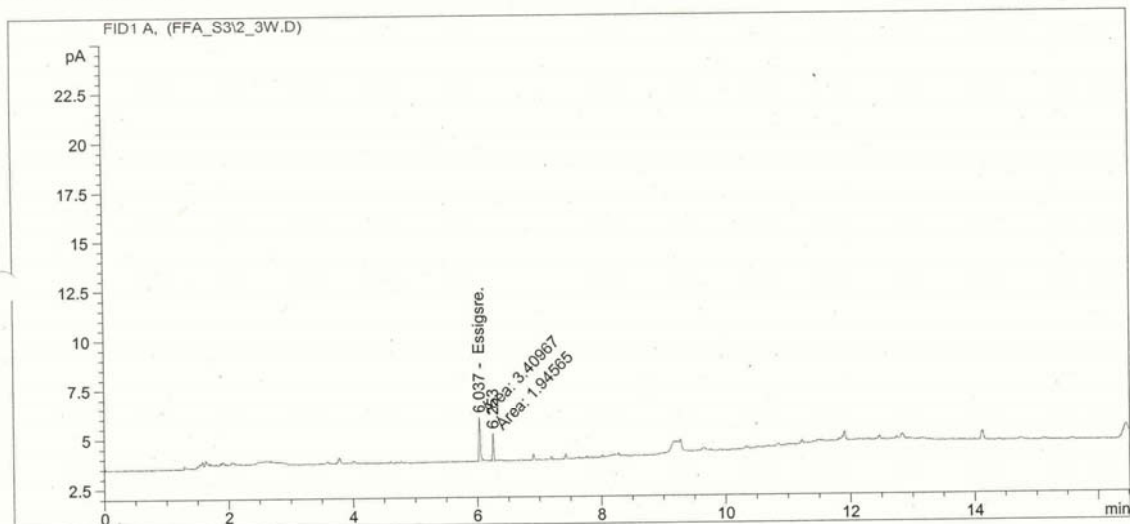
```

=====
Injection Date : 17.10.2008 15:47:59      Seq. Line : 8
Sample Name    : F.M. / 2-3w              Location  : Vial 107
Acq. Operator  : Thanet                   Inj       : 1
                                           Inj Volume: 1 µl

Acq. Method    : C:\HPCHEM\1\METHODS\FFA1.M
Last changed   : 23.09.2008 14:28:15 by Wunderlich
Analysis Method: C:\HPCHEM\1\METHODS\FFA7.M
Last changed   : 17.10.2008 15:51:43 by Thanet
                                           (modified after loading)

```

Freie Fettsäuren



=====

External Standard Report

=====

```

Sorted By           : Retention Time
Calib. Data Modified: Tuesday, 23. September 2008 14:23:08
Multiplier          : 1.0000
Dilution            : 1.0000

```

Signal 1: FID1 A,

RetTime [min]	Sig	Type	Area [pA*s]	Amt/Area	Amount [mM]	Grp	Name
6.037	1	MM	3.40967	2.12037e-1	7.22976e-1		Essigsre.
6.914	1		-	-	-		Propionsre.
7.202	1		-	-	-		iso-Buttersre.
7.847	1		-	-	-		Buttersre.
8.277	1		-	-	-		iso-Valeriansre.
9.001	1		-	-	-		Valeriansre.

Totals : 7.22976e-1

Results obtained with enhanced integrator!

2 Warnings or Errors :

Warning : Calibration warnings (see calibration table listing)

Warning : Calibrated compound(s) not found

=====

*** End of Report ***

=====

Instrument1 17.10.2008 16:05:30 Thanet

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Figure 39 - GC chromatogram: bioreactor residue U1756 (2); 3.rd wash.

Appendix E - Enzymatic hydrolysis experiments parameters

Table 11 - Enzymatic hydrolysis experiments (sugars detection):
parameters and analysis methods.

Experiment:	A	B	C	D	E	F
Substrate:	Maize silage	Maize silage	Maize silage	Maize silage	Maize silage	Bioreactor residue
$m_{\text{substrate}}$ (g):	50	50	50	50	15	15
V_{water} (ml):	215	215	215	215	120	87
Size reduction:	Used	Used	Used	Used	Not Used	Not Used
Washing step:	Used	Used	Used	Used	Used	Used
Reaction Units:	Erlenmeyers 500 ml	Erlenmeyers 500 ml	Erlenmeyers 500 ml	Erlenmeyers 500 ml	Erlenmeyers 500 ml	Erlenmeyers 500 ml
Steering:	Magnetic Stirrer	Shaker 175 lpm	Shaker 175 lpm	Shaker 175 lpm	Shaker 175 lpm	Shaker 175 lpm
Analysis method:	DNS - Test tubes - Spectrophotometer Calibration Curve	DNS - Test tubes - Spectrophotometer Calibration Curve	DNS - Test tubes - Spectrophotometer Calibration Curve	DNS - Test tubes - Spectrophotometer Calibration Curve	DNS - Microplates - GENIOS Calibration Curve Standard Addition	DNS - Microplates - GENIOS Calibration Curve Standard Addition

Experiment:	G	H	I	J	K
Substrate:	Maize silage	Locust bean gum	Maize silage	Maize silage	Maize silage
$m_{\text{substrate}}$ (g):	15	5.4	0.75	0.75	variable
V_{water} (ml):	120	261	6	6	6
Size reduction:	Used - Meat grinder	Not Used	Not Used	Used	Used
Washing step:	Not Used	Not Used	Not Used	Not Used	Not Used
Reaction Units:	Erlenmeyers 500 ml	Erlenmeyers 500 ml	Falcon Tubes 20 ml	Falcon Tubes 20 ml	Falcon Tubes 20 ml
Steering:	Shaker 175 lpm	Shaker 175 lpm	Shaker 175 lpm	Shaker 175 lpm	Shaker 175 lpm
Analysis method:	DNS - Microplates - GENIOS Calibration Curve Standard Addition BIOSENSOR Calibration Curve	DNS - Microplates - GENIOS Calibration Curve Standard Addition BIOSENSOR Calibration Curve	DNS - Microplates - GENIOS Calibration Curve Standard Addition BIOSENSOR Calibration Curve	DNS - Microplates - GENIOS Calibration Curve Standard Addition BIOSENSOR Calibration Curve	DNS - Microplates - GENIOS Calibration Curve Standard Addition HPLC Calibration Curve

Table 12 - Enzymatic hydrolysis experiments (sugars detection): substrate and enzyme parameters.

A		Substrate				
N. ^{er}	Reference	Size reduction	Washing step (ml/g)	Enzyme	m _{enzyme} (g)	
1	U1667	---	---	---	---	
2	U1667	---	---	Viscoflow MG	0.5033	
3	U1667	---	4	---	---	
4	U1667	---	4	Viscoflow MG	0.5024	
5	U1667	---	12	---	---	
6	U1667	---	12	Viscoflow MG	0.4995	
7	U1667	Yes	---	---	---	
8	U1667	Yes	---	Viscoflow MG	0.4998	

B		Substrate				
N. ^{er}	Reference	Size reduction	Washing step (ml/g)	Enzyme	m _{enzyme} (g)	
1	U1667	---	---	---	---	
2	U1667	---	---	Methapplus L100	0.5567	
3	U1667	---	20	---	---	
4	U1667	---	20	Methapplus L100	0.5567	
5	U1667	Yes	---	---	---	
6	U1667	Yes	---	Methapplus L100	0.5567	

C		Substrate				
N. ^{er}	Reference	Size reduction	Washing step (ml/g)	Enzyme	m _{enzyme} (g)	
1	U1741	---	---	---	---	
2	U1741	---	---	Viscoflow MG	0.0504	
3	U1741	---	3	---	---	
4	U1741	---	3	Viscoflow MG	0.0500	
5	U1667	Yes	---	---	---	
6	U1667	Yes	---	Viscoflow MG	0.0502	

D		Substrate				
N. ^{er}	Reference	Size reduction	Washing step (ml/g)	Enzyme	m _{enzyme} (g)	
1	U1741	---	---	---	---	
2	U1741	---	---	Methapplus L100	0.05567	
3	U1741	---	3	---	---	
4	U1741	---	3	Methapplus L100	0.05567	
5	U1667	Yes	---	---	---	
6	U1667	Yes	---	Methapplus L100	0.05567	

Table 12 - Enzymatic hydrolysis experiments (sugars detection): substrate and enzyme parameters. (cont.)

E		Substrate		Enzyme	m _{enzyme} (g)
N. ^{er}	Reference	Size reduction	Washing step (ml/g)		
1	(1)	---	---	---	---
2	(1)	---	3	---	---
3	(1)	---	---	Viscoflow MG	0.0278
4	(1)	---	3	Viscoflow MG	0.0279
5	(1)	---	---	Methapplus L100	0.0310
6	(1)	---	3	Methapplus L100	0.0310
7	(1)	---	---	Celluclast 1.5L	0.0310
8	(1)	---	3	Celluclast 1.5L	0.0310
9	(1)	---	---	Novozyme 188	0.0310
10	(1)	---	3	Novozyme 188	0.0310

F		Substrate		Enzyme	m _{enzyme} (g)
N. ^{er}	Reference	Size reduction	Washing step (ml/g)		
1	U1756 (2)	---	---	---	---
2	U1756 (2)	---	3	---	---
3	U1756 (2)	---	---	Viscoflow MG	0.0205
4	U1756 (2)	---	3	Viscoflow MG	0.0203
5	U1756 (2)	---	---	Methapplus L100	0.0226
6	U1756 (2)	---	3	Methapplus L100	0.0226
7	U1756 (2)	---	---	Celluclast 1.5L	0.0226
8	U1756 (2)	---	3	Celluclast 1.5L	0.0226
9	U1756 (2)	---	---	Novozyme 188	0.0150
10	U1756 (2)	---	3	Novozyme 188	0.0150

G		Substrate		Enzyme	m _{enzyme} (g)
N. ^{er}	Reference	Size reduction			
1	(1)	cutter 10 mm		Novozyme 188	0.0310
2	(1)	cutter 8 mm		Novozyme 188	0.0310
3	(1)	cutter 6 mm		Novozyme 188	0.0310
4	(1)	cutter 4 mm		Novozyme 188	0.0310
5	(1)	cutter 2.5 mm		Novozyme 188	0.0310
6	(1)	---		Novozyme 188	0.0310

H			
N. ^{er}	Enzyme	m _{enzyme} (g)	
1	---	---	
2	Mixture	0.0217	
3	Hemicellulase	0.0223	
4	Hemicellulase	0.2272	
5	Novozyme 188	0.0150	
6	Viscoflow MG	0.0246	

Table 12 - Enzymatic hydrolysis experiments (sugars detection): substrate and enzyme parameters. (cont.)

I	Substrate			
N. ^{er}	Reference	Size reduction	Enzyme	m _{enzyme} (g)
1	(1)	---	---	---
2	(1)	---	Novozyme 188	0.00155

J	Substrate			
N. ^{er}	Reference	Size reduction	Enzyme	m _{enzyme} (g)
1	U1803 (3)	---	Methaplus L100	0.00155
2	U1803 (3)	---	Novozyme 188	0.00155
3	U1803 (4)	Extruder	Methaplus L100	0.00155
4	U1803 (4)	Extruder	Novozyme 188	0.00155
5	U1803 (4)	Extruder	Methaplus L100	0.00155
6	U1803 (4)	Extruder	Novozyme 188	0.00155

K	Substrate				
N. ^{er}	Reference	Size reduction	m _{substrate} (g)	Enzyme	m _{enzyme} (g)
1	U1803 (3)	---	0.75	---	---
2	U1803 (3)	---	0.25	Novozyme 188	0.00155
3	U1803 (3)	---	0.50	Novozyme 188	0.00155
4	U1803 (3)	---	0.75	Novozyme 188	0.00155
5	U1803 (3)	---	1.00	Novozyme 188	0.00155
6	U1803 (4)	Extruder	0.75	---	---
7	U1803 (4)	Extruder	0.25	Novozyme 188	0.00155
8	U1803 (4)	Extruder	0.50	Novozyme 188	0.00155
9	U1803 (4)	Extruder	0.75	Novozyme 188	0.00155
10	U1803 (4)	Extruder	1.00	Novozyme 188	0.00155

Appendix F - DNS method using GENIOS Spectrophotometer

A new TECAN GENIOS microplate's spectrophotometer was used for analysis of DNS method samples. This device allows measuring the absorbance of samples in microplates with 96 wells, which allows measuring the absorbance of 96 samples in less than one minute. Absorbance filters with specific wavelengths are used depending on the samples to be analysed (545 nm on the DNS method). To understand the potential of this method, 6 microplates with a total of 576 samples, were analysed 3 times each (1728 absorbance values) in less than 40 min. The results are stored in a computer connected to GENIOS.

A study was made to analyse the linear range of GENIOS, as well as the minimum and maximum concentration of glucose that can be detected. An error analysis was made to analyse if significant errors could occur during the experiments.

The analyte on this trial was glucose. To analyse glucose concentration, the DNS method was used following the same procedures as described in Chapter 4.3.2.1 (without standard addition). Several concentrations of glucose between 0 and 20 g/L were measured (36 concentrations, 12 per plate, 3 plates) to create a calibration curve; each column (8 wells) had a concentration (Figure 40). The whole experiment was doubled, so comparison was made between plates. Each plate was read 3 times in GENIOS, so comparison was made between reads.

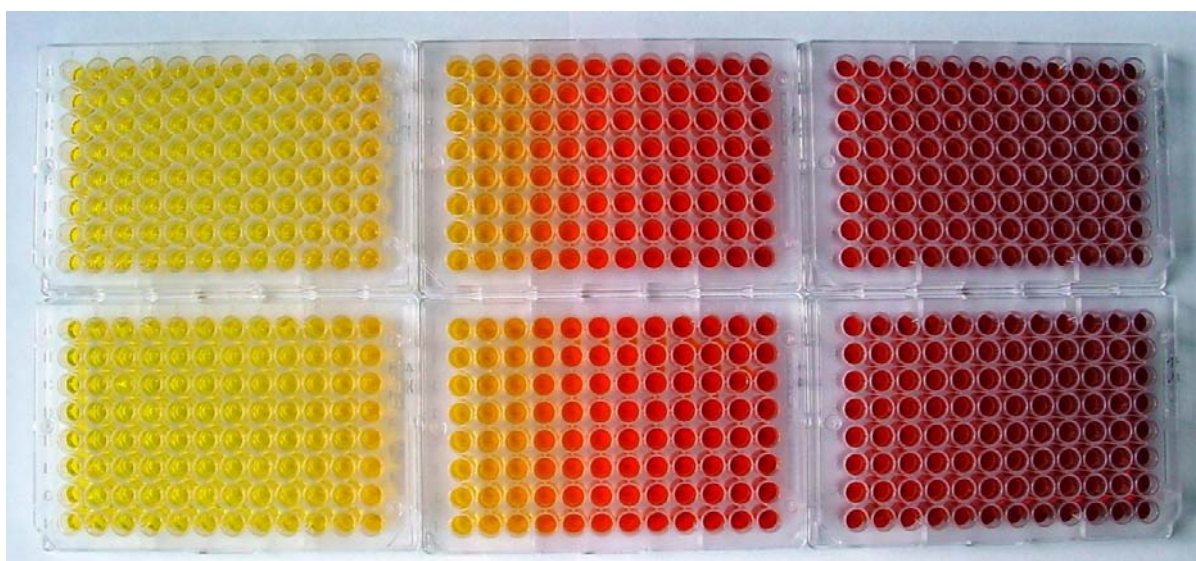


Figure 40 - 96 Well microplates with different concentrations. Concentration value increases from the left to the right.

The average zero value for the absorbance was 0.0393 ± 0.0015 . The first concentration detected was 0.1360 g/L with an absorbance of 0.0481 ± 0.0005 . The last concentration detected, before the device overflow, was 13.6448 g/L with an absorbance of 2.9143 ± 0.0366 .

A calibration curve was made for the concentrations between 0.1360 and 13.6448 g/L (Figure 41). A good linear correlation can be made with an R^2 of 0.9986.

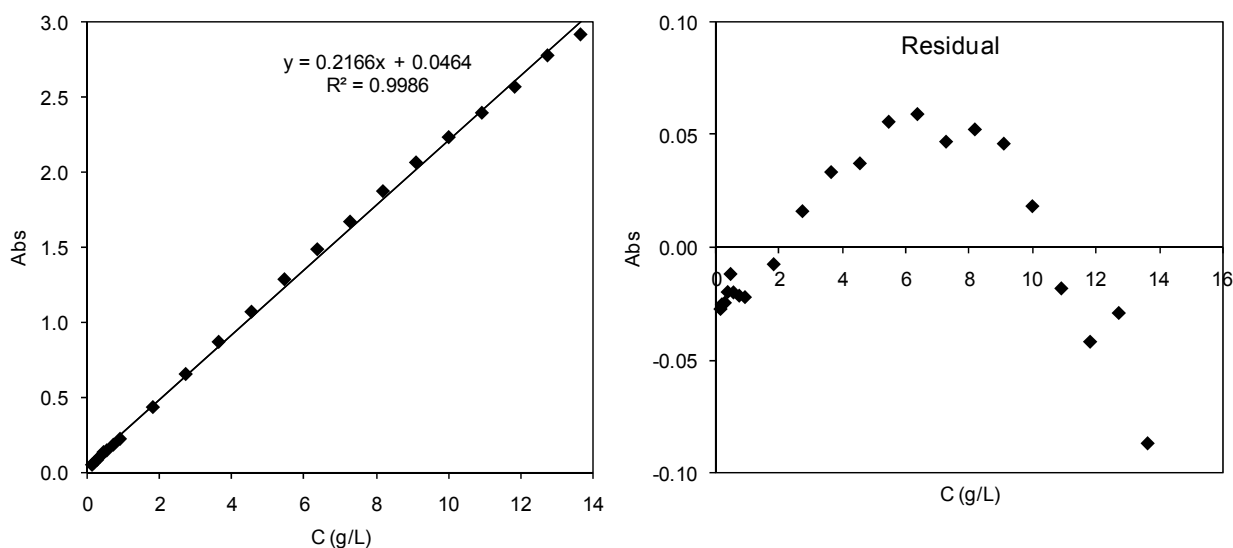


Figure 41 - Calibration curve for GENIOS spectrophotometer and residual values for the linear regression.

However the shape is not totally linear. The last concentration values have a more accentuated curvature (Figure 42). A statistical test was made in Microsoft Office Excel, with the Regression Analysis Tool (Analysis ToolPack supplement). The results are presented on Table 13. The value of $R^2 = 0.9985 \pm 0.0394$ was obtained. $F > F_{critic}$, so the regression should be accepted. The parameters for the linear regression $y = mx + b$ were obtained: $m = 0.2166 \pm 0.0038$ with $S_m = 0.0018$; $b = 0.0464 \pm 0.0262$ with a $S_b = 0.0126$.

Table 13 - Simple linear regression for GENIOS calibration curve.

Regression Statistics	
R	0.9993
R^2	0.9986
adjusted R^2	0.9985
Fit standard error	0.0394
Observations	22

ANOVA	gl	SQ	MQ	F	Fcritic
Regression	1	21.516	21.5160	13834	6.93E-30
Residual	20	0.0311	0.00156		
Total	21	21.547			

	Coefficients	Standard error	t	P	Confidence interval
Interception	0.0464	0.0126	3.69	0.001458	± 0.0262
Concentration	0.2166	0.0018	117.6	6.93E-30	± 0.0038

If a really precise regression is needed, the best approximation would be a 2.nd degree polynomial with a $R^2 = 0.9999$ or a linear regression up to 9.0957 g/L (Abs=2.0619±0.0069) with a $R^2 = 0.9997$ (Figure 42).

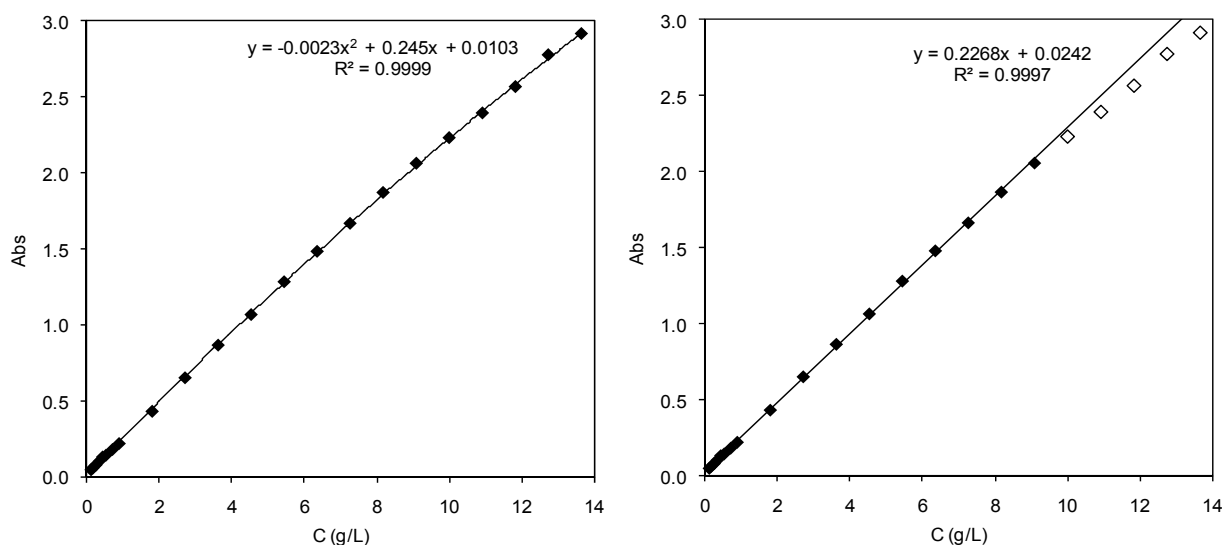


Figure 42 - Calibration curve for GENIOS spectrophotometer. Left: 2.nd order polynomial regression. Right: linear regression up to 9.0957 g/L.

Standard deviations, between readings (serie 1 and serie 2) and between plates, do not vary significantly. However a tendency of increase can be seen with the increase of concentration (Figure 43). No significant difference was detected between readings or between plates (small standard deviation values, overlapped graphics), which indicates that the method can be reproduced. Curiously, when there is an increase on absorbance values between reads, it is observed that not only in some but almost in all the wells, the increase is observed (the same happens with a decrease of values). This consistency indicates some changes in the device conditions.

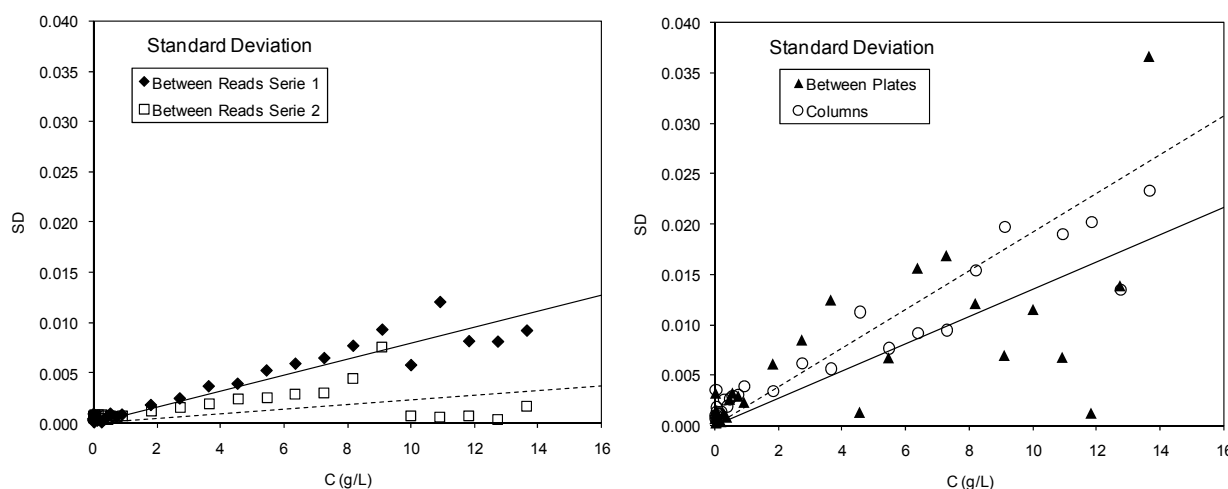


Figure 43 - Standard deviation values vs C (g/L). Left: between reads (Serie 1 and 2). Right: between plates and in each column.

On the 96 wells from a plate, a maximum of 3 wells per plate had a substantial error (big standard deviation compared with all the other wells) and were readily disposed. These errors persisted between readings and disappeared between plates, which clearly indicate that is a previous problem with the samples. The use of microplates and micropipettes demands from the user a good skill and concentration to prevent contamination between wells.

In each plate, a peculiar event occurs. When the plates are used with the same sample in an entire row (8 vertical wells), the difference in the final value is not significant because the average is calculated. However, there is a persistent difference between values of absorbance in the border wells when compared to the values in middle wells. The values of absorbance (columns) were subtracted to their average value to visualise the difference. As can be observed in Figure 44 and Figure 45, the values on the border of the microplates are generally bigger than the values in the centre.

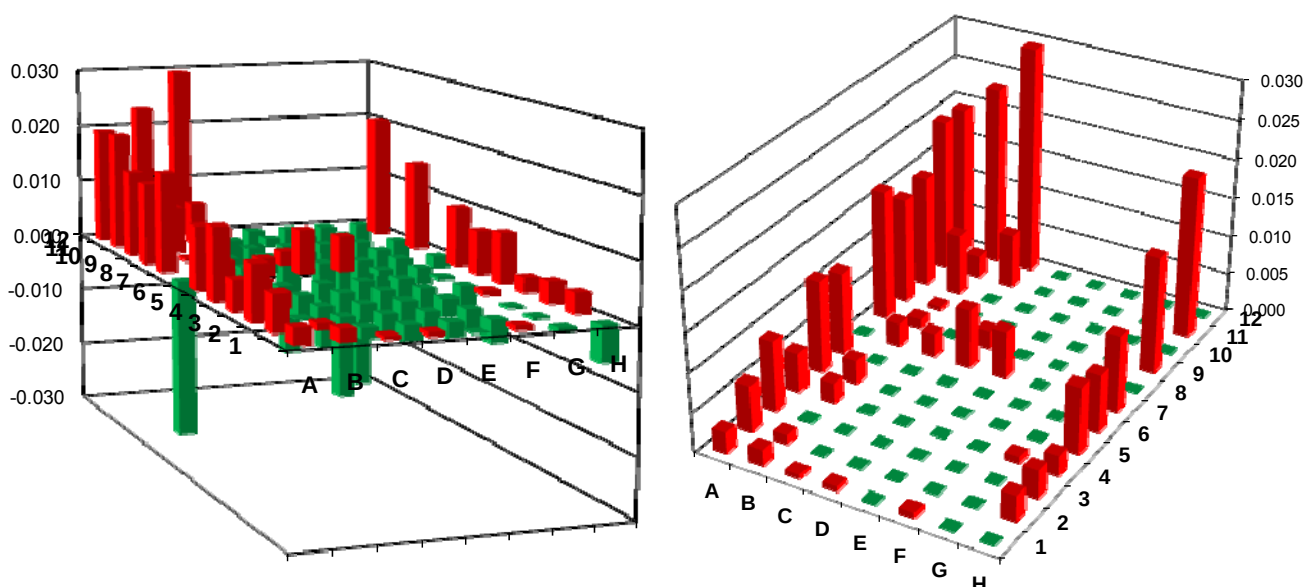


Figure 44 - Difference between absorbance and column average. Concentration between 0.5451 to 9.0957 g/L of glucose. Red: positive difference. Green: negative difference.

The bigger the concentration, the bigger the difference in absorbance can be (increase of the standard deviation), therefore an error occurs, but at the same time, is sensitive to concentration. For example, a difference of 0.1 in absorbance can introduce an error of 0,4616 g/L in the concentration values. This error can be accepted for high concentrations, however it should be analysed in more detail so it can be eliminated. This problem can be originated or by a problem on the device calibration or during the DNS method. The handling of the samples in the NUNC deep well plates can be complicated

especially during the cooking. On some experiments, it was verified loss of sample from some wells. Liquid leakage can occur due to bad sealing. The border wells are also more exposed to heat, which can be easily verified when the samples are taken from the fridge to be defrozed: middle wells take much more time to defrozed. Water can evaporate more easily on the border wells, and some water can be lost. A test should be made just with a natural colour solution so no sample preparation methods would interfere on the results. Nevertheless, microplates should be improved to meet the needs of different methods such as DNS method (better sealing, spaces between the wells to promote uniform heating).

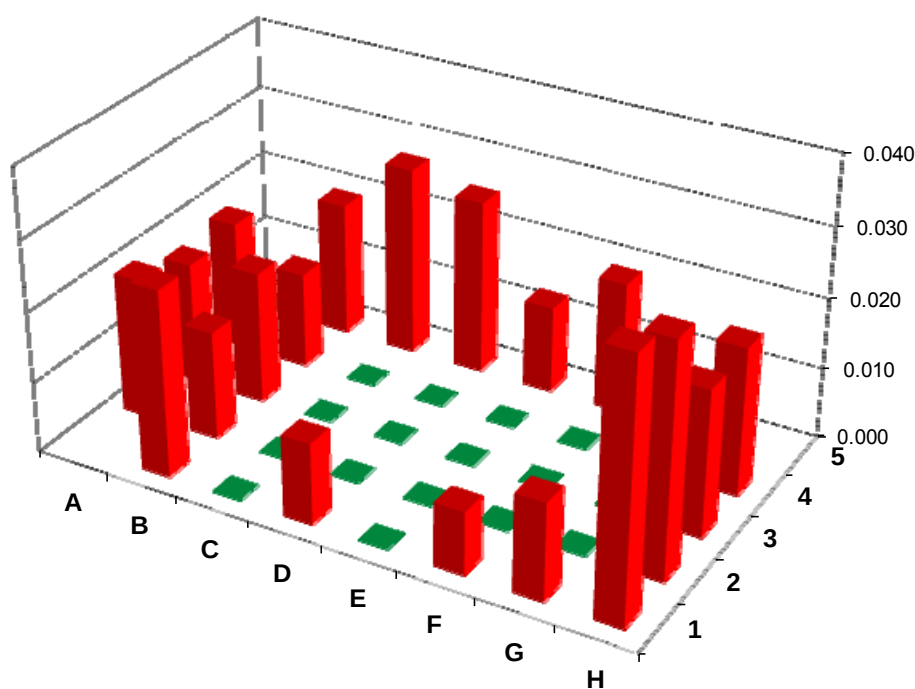


Figure 45 - Difference between absorbance and column average. Concentration between 10.0004 to 13.6448 g/L of glucose. Red: positive difference. Green: negative difference.

Appendix G - HPLC apparatus equipments and running conditions

Table 14 - HPLC apparatus equipments and running conditions.

HPLC Apparatus	
<i>Column</i>	
Brand:	PHENOMENEX
Model:	REZEX RPM-Monosaccharide Pb++ (8%)
Dimensions:	300 x 7.8 mm ID
Elution Type:	Isocratic
Eluent:	Water
Flow Rate:	0.6 mL/min
Col. Temp.:	75 °C
<i>Detector</i>	
Brand:	SEDERE France
Model:	SEDEX 75
Type:	ELSD (Evaporative Light Scattering Detector)
Temperature:	45 °C
Gain:	12
<i>Pump</i>	
Brand:	KNAUER - ADVANCED SCIENTIFIC INSTRUMENTS
Model:	SMARTLINE PUMP 1000
Type:	Low pressure mixture (mix before the pump)
<i>Controler</i>	
Brand:	KNAUER - ADVANCED SCIENTIFIC INSTRUMENTS
Model:	SMARTLINE MANAGER 5000
<i>Injector</i>	
Brand:	KNAUER - ADVANCED SCIENTIFIC INSTRUMENTS
Type:	Manual Sampler
<i>Oven</i>	
Brand:	GAT (GAMMA ANALYSEN TECHNIK GMBH)
Model:	GAT - CH 150
Type:	Column Heater