AALBORG UNIVERSITY

Aalborg University Copenhagen

Department of Biotechnology, Chemistry and Environmental Engineering

Section for Sustainable Biotechnology

MASTER THESIS

June 2016

Enhancing protein and sugar extraction from grasses in a biorefinery concept by enzymatic hydrolysis and ensiling of the press cake

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Preface

This Master thesis represents the completion of the degree Master of Science in Engineering at the Section of Sustainable Biotechnology at Aalborg University in Copenhagen. This dissertation presents the experimental work that has been performed during the second year of this program from September 2015 and June 2016.

I am very grateful to my supervisors, Maria Santamaria and Mette Lübeck for guiding me during this project, for everything they have taught me and for the encouragement they have showed to me. And of course, for their patience during the writing stage. I would also like to thank Gitte Hinz-Berg, laboratory technician, for her willingness to help, and to all the members of the Section of Sustainable Biotechnology, that have contributed to the completion of this study.

Finally I would like to acknowledge my friends, for all the support and encouragement throughout my time in Copenhagen. They are undoubtedly the foundations of this achievement. My family, and especially my parents, for offering me the opportunity of studying in Copenhagen and for their immeasurable support. My daughter, Julia my inspiration.

Abstract

The depletion of the fossil fuels claims new energies. The utilization of biofuels and bioenergy has emerged as a viable alternative. Especially, the production of energy and several products is becoming widespread in biorefineries, intended for the efficient conversion of biomass and waste through economically viable processes. More deeply, the green biorefinery refers to the utilization of grassland biomass to produce valuable products like proteins, lactic acid or silage after fractionating the biomass into a press cake and a green juice.

This study is a contribution to the Organofinery project and hypothesises the pretreatment of the press cake can yield several organic products, maximizing the profit and the utilization of grasslands, along with studying the suitability of red clover and the press cake for the production of good quality silage, that can be employed for animal feeding and as a method of preservation of the biogas potential.

Enzymatic hydrolysis of the press cake seems to be a promising alternative for the utilization of this sub-product, as several fiber-bound proteins and sugars are released. The loading of TS and the amount of enzyme included are the most important factors for the optimization of the process.

Ensilaging red clover and the press cake appears to be a viable method for maintaining the feeding value of grasses all year round. However, the ensiled biomass must achieve DM contents over 30% in order to avoid clostridia growth and become suitable for animal feeding. Finally, ensilaging the press cake increased the methane potential in comparison to non-ensiled press cake and therefore it constitutes a suitable method for preserving the methane potential.

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1 Chapter 1 - Introduction

1.1. Background

The onset of the twenty-first century has faced several economic and environmental crises at the global level that represent an unsustainable future. There is an undeniable evidence of a strong dependence on the oil industry combined with the depletion of fossil fuels that arises environmental, social and political concern (Tabak and Cajueiro 2007). The increasing population and the new markets claim the use of new energies. The sector of transport keeps growing in US and Europe and the demand for energy is accentuated by the new emerging economies in Asia and Latin America (Clark, Luque et al. 2012) with their growing industrialization and urbanization.

All these drivers request new clean, sustainable and renewable energies and the transition from the petroleum based industry towards bio-based industries, producing both energy and green products that lead to diminishing the oil industry for the production of fuel and chemicals. The climate change and the scientifically evidenced increase in greenhouse gases generated by the combustion of fossil fuels reinforce the utilization of new energies(Rodhe 1990).

Hopefully, there is an expanding global awareness and political support for the production of new clean and renewable alternatives like wind or solar energy that might be employed for the production of electricity and heat. However, the utilization of biomass is probably the most feasible alternative to fossil fuels for transport and chemical production since it is the unique carbon-rich material source accessible in this planet apart from fossil fuels (Cherubini 2010). Therefore, sustainable production of biomass is a critical concern.

Fossil fuels have a negative image socially and environmentally. They greatly contribute to the net CO_2 emission and give rise to instability in the global economy (Lotfalipour, Falahi et al. 2010). This can be eclipsed by fostering biofuels. This renewable energy is considered CO_2 neutral because they absorb CO_2 from the atmosphere through the photosynthesis and release the same amount when burnt (Clark, Luque et al. 2012).

1.2. Biofuels

First generation biofuels normally refers to those produced from biomass and raw material that are in competition with feed and food industries, and consequently, their success is limited as they are socially and environmentally unsustainable. Moreover, they have a negative effect on the biodiversity, the use of land and the usage of water. It is likely that the success of the biofuels depends on non-food feedstock (oN BioEThiCs 2011). As the first-generation biofuels compete for the

same feedstock as are required for food there is an accentuating need to employ second-generation (solid municipal waste, lignocellulosic waste) and third-generation (cyanobacteria and algae) biofuels.

Second generation biofuels include the utilization of residues from agriculture, forestry, municipal waste and the industry dedicated to lignocellulosic material and they can generate different products through several conversion routes, from enzymatic to thermochemical (Naik, Goud et al. 2010). For instance, an estimated 5 billion dry tonnes of agricultural residues are produced globally and might be converted into biofuels and bioproducts (Londo, Lensink et al. 2010). A considerable advantage is that they can rely on the whole plant for energy production and not only in the grains and seed as the first generation biofuels.

Biogas is produced by anaerobic digestion of organic waste and grasses, and therefore can be deemed as a second generation biofuel. Its use is becoming widespread and several institutions are promoting and funding projects for the implementation of biomethane throughout Europe. As a case in point, Sweden supplies 45% of its approximately 4500 vehicles with biomethane (Jönsson and Persson 2003).

However, despite the fact that the stage and accessibility of the raw material can be considered as optimal, the technology and the processes for the production of second generation biofuel is still at a precommercial stage (Clark, Luque et al. 2012) and they need further researching, implementation and investments to be a viable alternative to the fossil fuels.

In addition, many different actions and effort must be combined at a global scale in order to succeed in the transition towards a more sustainable world. Many political actions must be implemented and obeyed by all the nations, there must be changes in the behaviour of the population, reforms in the use of limited resources and modifications in means of transport (Wals 2007).

1.3. Biorefineries

A recent definition for biorefinery says “the sustainable processing of biomass into a spectrum of bio-based products (food, feed, chemicals and/or materials) and bioenergy (biofuels, power and/or heat) (de Jong, Higson et al. 2012). The biorefinery concept comprises a broad range of technologies to transform raw material and biomass (grain, lignocellulosic waste, wood…) into their pertinent building blocks (proteins, carbohydrates, triglycerides…) for the production of chemicals, value added products and biofuels. This concept is equivalent to the current concept of petroleum refineries consisting of the production of a wide range of products from petroleum. Figure 1 depicts a flow chart of the valuable products obtained in a biorefinery from a wide variety of income products.

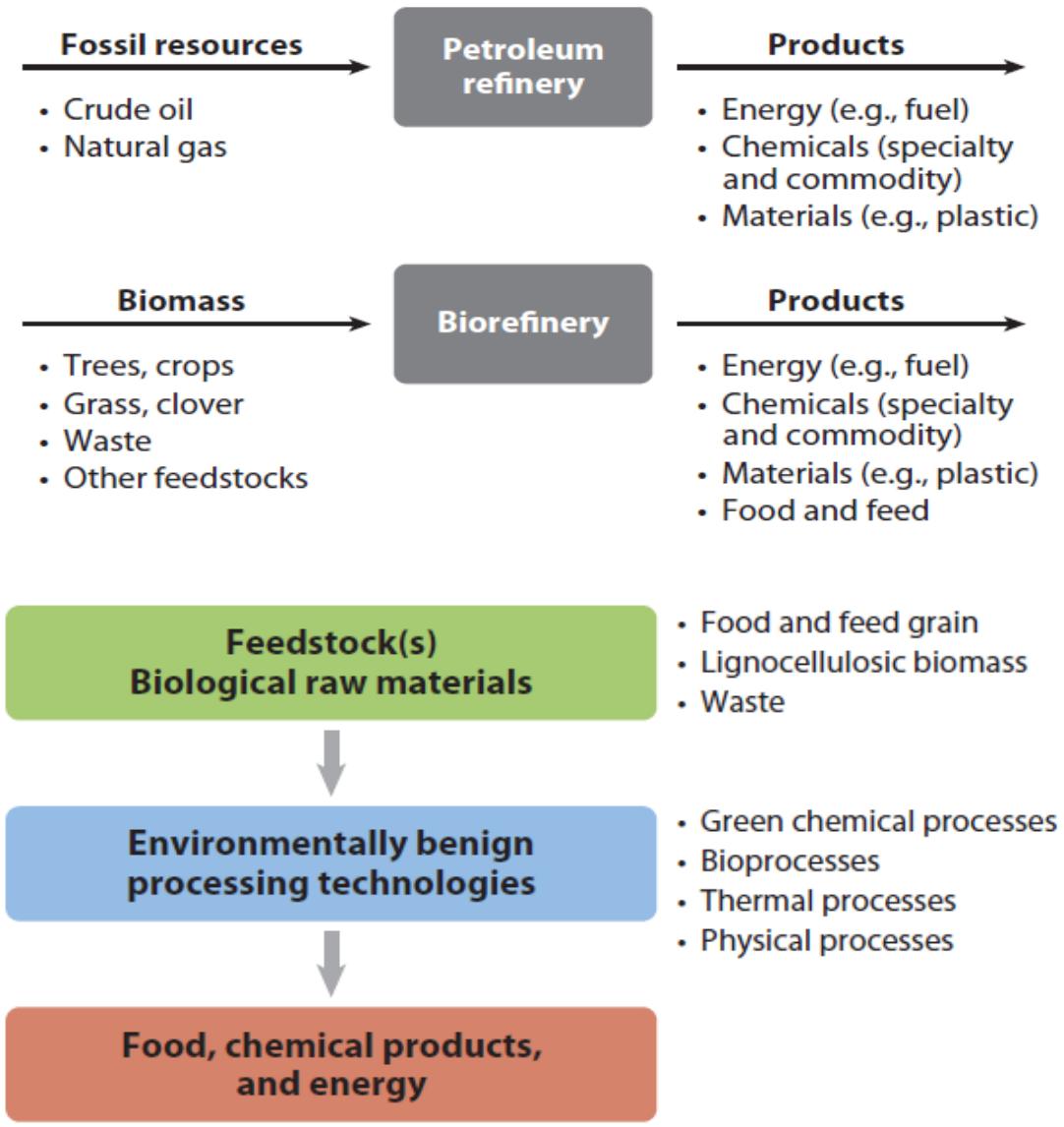

Figure 1. The biorefinery concept: from biomass to valuable products. Source: Clark, Luque et al. 2012.

Different types of biorefineries are currently developing. Some of them are limited to a single feedstock while other can make use of several biomasses and residues. The same applies to the end products and outputs, whereas some processing factories can solely yield one final product some biorefineries are able to produce many different valuable products ranging from food, chemicals and organic juices to biogas and energy.

Regardless of the type of biorefinery, the main challenge is the efficient conversion of biomass and waste through an economically viable process that might ultimately compete with the prices of the petroleum-based industry. Therefore, the total conversion of biomass must be maximized and the use of non-renewable and expensive products required during the processing of the biomass must be

minimized. To achieve this, a synergistic combination of biological, chemical and technical sciences ought to be implemented (Kamm, Gruber et al. 2007).

Biorefineries might be built up in multiple scattered areas. They can revitalize the rural areas and generate employment in the most depopulated areas, contributing to create a more balanced distribution of the population and, in the long run, to improve the forest and agricultural management as people become more aware of the available resources in their environment (Demirbas 2009). Furthermore, various bio-industries could combine their material flows targeting the total utilization and conversion of the biomass since the waste and residue of one bio-industry might be an input for another industry.

1.4. The "classic" green biorefinery

The concept is analogous to the above-mentioned biorefinery but a green biorefinery refers to the utilization of grassland biomass to produce valuable products like proteins, lactic acid, silage, fibres and energy through biomass (Kromus, Wachter et al. 2004). This concept remains at an advanced stage in some European countries like Denmark, Germany, Switzerland, Austria and the Netherlands (Grass and Hansen 1999).

While some crops dedicated to biofuels production (e.g. corn for ethanol) require an intense production system and the land available for its production is limited, grasslands and natural pastures are broadly available in many regions. It involves an appropriate amount of biomass readily available at high yields and consequently a great economical and sustainable potential.

The green biorefinery stabilizes the rural and, to some extent, natural landscapes. It preserves the landscape that has been maintained for centuries in many areas and brings about a positive impact in rural areas by increasing the rural tourism. Moreover, it improves the economic situation in such areas by creating new jobs and allowing development of multiple rural regions. In addition, the rural landscape is currently undergoing a profound transformation that will give rise to new grasslands that could be bound to green biorefineries. As a model, estimations of experts in Austria state that up to 1.000.000 tons in dry matter of grasslands biomass might be available every year (Buchgraber 2001).

On the downside, the green biorefinery faces the problem of being based on seasonal crops, as the production of grass depends on the vegetation period (generally in Europe from April to October). However, it can be surpassed by ensiling the green biomass, ensuring all year round availability of raw materials and allowing a decentralization in the production and supply of biomass (Kromus, Wachter et al. 2004).

The green biorefinery intends to exploit the grasslands in a feasible and sustainable way. The aim is to make the most of the raw material maximizing the range of end products and the production of energy (Kromus, Wachter et al. 2004). The most promising products recognized thus far are: the lactic acid that could be used for production of biogas and bioplastics or as a solvent; proteins, utilized for feed and food or for the cosmetic and pharmaceutic industry (Arlabosse, Blanc et al. 2011); fibres that might be used for bioenergy production, for the paper and pulp industry, as a packaging material and isolation material or for the production of fodder pellets (Andersen and Kiel 2000); speciality products like chlorophyll and carotenoids; and energy by transforming the juices and residues into biogas.

The green biorefinery seems to be a viable alternative for producing multiple products from green biomass, although an exhaustive research must be carried out. Key technology must be identified and processes optimized. Finally, institutions, stakeholders and people from rural areas must commit themselves to implement and ensure green biorefineries.

1.4.1 *Today's green biorefinery.*

Nowadays the green biorefinery chiefly consists of the mechanical fractionation of the fresh biomass into plant juices, green and brown juice, and a solid fraction rich in fibers known as press cake (Arlabosse, Blanc et al. 2011). The press cake can be used for the isolation of proteins (Sharma, Tilakratne et al. 2010), for the production of chemicals like levulinic acid (Kamm, Schönicke et al. 2009), for the production of silage (King, McEniry et al. 2012) or biogas. The green juice is a rich substrate that can yield considerable amounts of proteins, organic acids, free amino acids and sugars (Brown, Shi et al. 2012).

In the olden days, the juice used to be spread on the fields as a fertilizer but this practice has been restricted as a consequence of groundwater pollution (Andersen and Kiel 2000). In contrast, nowadays it is widely employed for the production of proteins for animal feed, organic acids and different biochemicals. With a more detailed outlook, the green juice can be fermented by lactic acid bacteria yielding proteins, lactic acid and vitamins. Lactic acid fermented juice could be kept under anaerobic conditions preserving vital chemical compounds during the winter period. The acidified and concentrated brown juice can be stored the whole year round (Andersen and Kiel 1997) or act as a substrate for biogas production.

Furthermore, the society claims and is attracted by organic products. Thus, the organic farming sector is growing quickly. Currently, the production of proteins for animal feed is obtained from intensive crops, like soybean which is extensively produced in only a few countries and has to be exported worldwide (Wilcox 2004). However, this pattern is bound to change if a big effort is placed in the

development of new green biorefineries in which protein-rich feed from green biomass can be organically produced.

1.5. The organofinery project.

The organofinery project is based on the previously described concept of green biorefinery and thus, a new platform for organic growth is developed. The objective is to provide solutions for the problems that hamper the organic sector. Some of the key challenges to overcome include the production of organic protein feed for monogastric animals, generation of fertilizers and energy through biogas production, better use of crops and grasslands with low yield and low value, and maximizing the efficiency of the nutrients applied in the fields. Figure 2 depicts a general scheme of the inputs and outputs in the Organofinery project. Overall, the aim is to obtain as much as possible from green biomass grown organically through sustainable, environmental-friendly, credible and economically viable processes.

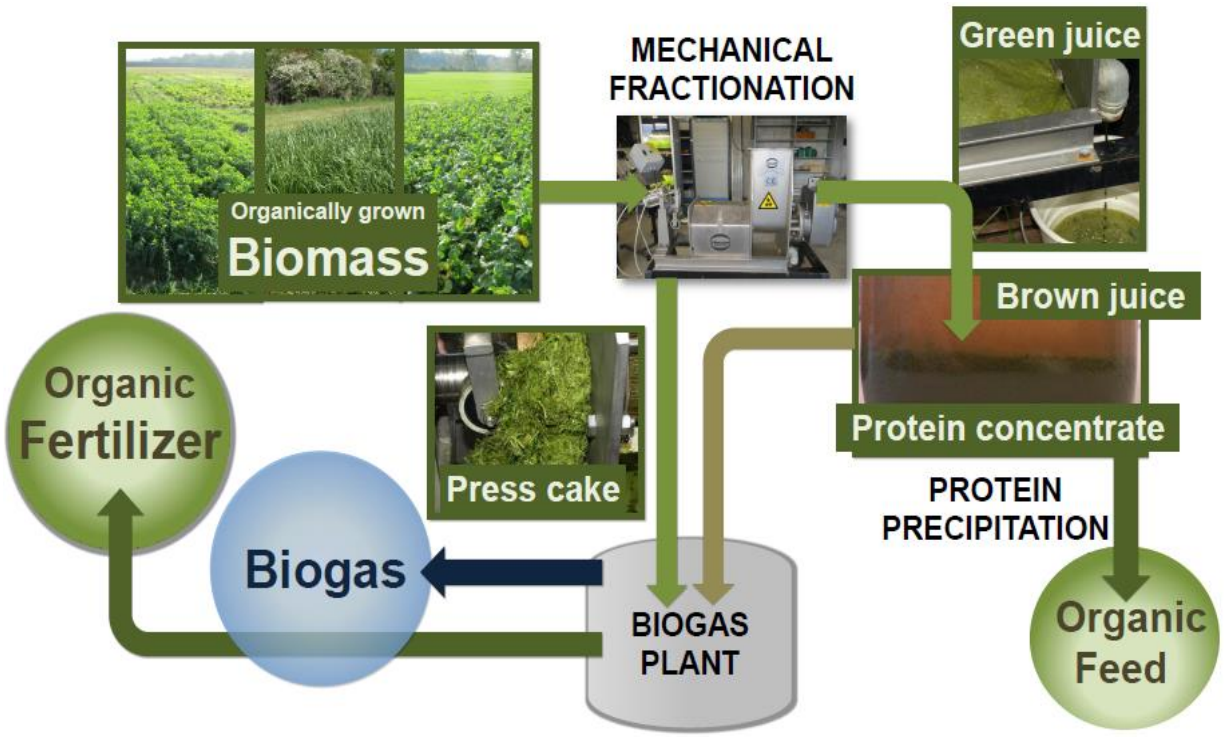

Figure 2 Outlook of the organofinery project. Source: (Molinuevo-Salces, Santamaria et al. 2015)

Several crops have been tested so far during the lifespan of this project. The utilization of plants included in the botanic family *Fabaceae* (= *Leguminosae*), broadly known as legumes, seems a feasible alternative since they are able to fix atmospheric nitrogen due to the formation of root nodules containing the endosymbiotic nitrogen-fixing bacteria *Rhizobium*. The cultivation of legumes reduces the need of incorporating nitrogen into the soil by fertilization, and allows

crop rotation, alternating legumes with other species without fertilizing with nitrogen. Some of the legumes tested so far include alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L), clover grass (*Trifolium sp*.) and the crucifer oilseed radish (*Raphanus sativus* L.)

1.5.1. Previous and current research.

The project has focused mainly in the mechanical fractionation of the green biomass to produce green juice and press cake by screw pressing. The green juice is fermented by adding specific strains of lactic acid bacteria that yield lactic acid and trigger the precipitation of proteins. After the fermentation, two fractions are collected: the protein paste that is a suitable additive for protein feed for poultry and the brown juice that becomes a substrate, along with the press cake, for biogas production by anaerobic digestion. Besides, silage as a means of preserving biomass has been investigated.

In the scope of the project, (Molinuevo-Salces, Santamaria et al. 2015) reports the protein recovered in the separation-fermentation process yield 7 kg of crude protein per ton of fresh biomass. The protein pastes contained up to 45% dry matter (DM) of crude protein. Besides, the analysis of the composition of amino acids seemed to be more favourable than the traditional soy protein feed.

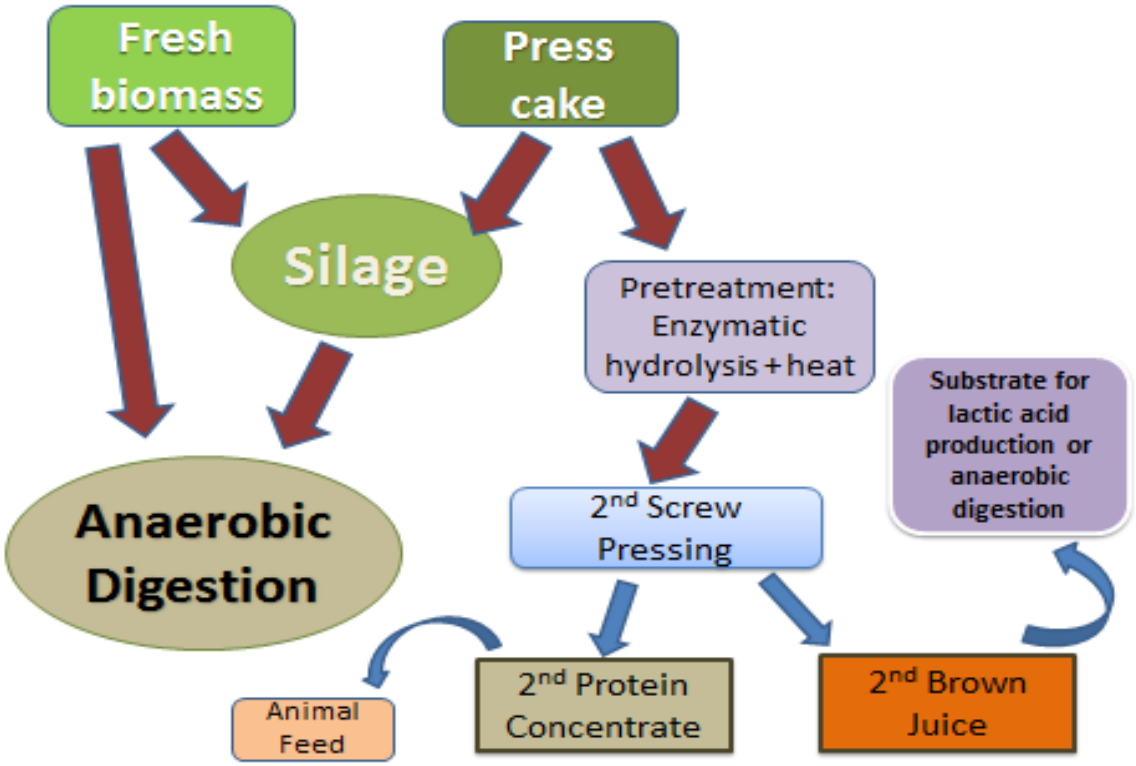

Figure 3. Scheme of research carried out at the moment within the Organofinery project.

In addition, in a previous essay with oilseed radish and alfalfa the protein recovery reached the 22% in the protein paste with a crude protein content of 50% of dry matter. However, after the fractionation, the protein (mainly fiber-associated protein) in the press cakes accounted for the 77% of crude protein in the fresh crop. These results suggest that a more exhaustive protein recovery in the press cake is a must for maximizing yield and profit. Consequently, further research is being carried out aiming to maximize the protein extraction from the press cake by different methods of pretreatment including enzymatic hydrolysis and heat treatment, followed by a second mechanical fractionation (Figure 3).

Batch test for biogas production through anaerobic digestion have been carried out. In the case of press cake, the methane yields obtained reached 240 mL CH_4 g^{-1} VS for alfalfa and 375 mL CH_4 g^{-1} VS for oilseed radish, showing a 42% and 66% recovery of the methane potential contained in the fresh alfalfa and oilseed radish, respectively. In the case of the brown juices as a substrate for anaerobic digestion, methane yields ranged between 430-558 mL CH_4 g^{-1} VS for alfalfa brown juices and between 378-526 mL CH_4 g^{-1} VS for oilseed radish, what allowed recovering up to 16% of the methane potential of the fresh biomass (Santamaria 2015). These results suggest the suitability of these crops as a sustainable way to produce bioenergy, however, further research is needed. Thus, in this project we test another crop (red clover) and the possibility of ensilaging the biomass as a method for preserving the biomass and its methane potential.

The research is also oriented towards organic acid production by conversion of brown juice into lactic acid. An overview of the research conducted in this thesis is displayed in Figure 3.

1.6. Plant material employed in this project

Red clover

It is a herbaceous species of the botanical family Leguminosae, also named Fabaceae. It is native to North Africa, Western Asia, and Europe, although it has been globally planted and become naturalized in several regions.

It is a perennial plant that can reach up to 80 cm. The leaves are trifoliated (composed of three leaflets), alternated and green with a characteristic pale crescent in the outer half of the leaf. The flowers range from pink to purple, with dense inflorescences (Figure 4).

It is usually cultivated as a fodder crop. Thanks to its root nodules, it is employed as a nitrogen-fixing crop. Besides being a suitable source of proteins, it contains a considerable proportion of carbohydrates and a great digestibility. It has also been used in medicine in many countries as antispasmodic, sedative, anti-inflammatory

and antidermatosis agent (Wang, Waltenberger et al. 2014). It has also been used for silage production due to its high content in sugars (Broderick, Walgenbach et al. 2001).

Figure 4. Red clover. Source: https://theresagreen.me/

Regarding resistance to diseases, the plant is subjected to several bacterial and fungal diseases like the black patch (*Rhizoctonia leguminicola*), the common leaf spot (*Pseudopeziza trifolii*) and the Fusarium root rot (*Fusarium oxysporum*), and it is also subjected to nematodes and viruses (Nyvall 1989).

1.7. Silage and biogas production

Ensiling is a forage preservation method based on natural lactic acid fermentation under anaerobic conditions. The epiphytic lactic acid bacteria that are present in the biomass ferment the water-soluble carbohydrates to lactic acid, and to a lesser extent, acetic acid and other organic acids. The production of these acids triggers the decrease in the pH of the ensiled material and consequently, spoilage microorganisms are inhibited (Weinberg and Muck 1996).

However, there are several spoilage organisms that deteriorate the quality of the silage. For instance, if anaerobic conditions are not maintained as a consequence of tears in the silage bags, different aerobic microorganisms like moulds or yeasts will grow decreasing the quality of the silage owing to the sugars are converted to ethanol (McDonald and Henderson 1991) and other non-intended products. Further spoilage microorganisms include Clostridia. These bacteria produce butyric acid and their spores affect the quality of the milk produced by the animals which feed on such silage (Elferink, Driehuis et al. 2000). However, the growth of these bacteria can be prevented by ensiling crops with a high content of dry matter (Wieringa 1958). Conclusively, the more desirable organisms to be present in the silage are lactic acid bacteria (LAB).

Traditionally, fresh forage crops such as grasses, maize, wheat and legumes and wheat have been preserved by ensiling. Ensiled forages are highly valued as animal

feed and provide a source of feed throughout the whole year. In European countries such as The Netherlands, Germany and Denmark more than 90%of the forages locally produced are stored as silage (Wilkinson, Wadephul et al. 1996).

Since silaging produces intermediates for methanogen fermentation, such as volatile fatty acids, and the structural polysaccharides present in the biomass can be partially degraded during storage improving the availability of nutrients and sugars for the methanogens (Amon, Amon et al. 2007), ensilaging has been promisingly studied as a substrate for biogas production (Lehtomäki 2006; Pakarinen, Lehtomäki et al. 2008). A case in point is the work of Pakarinen et al. (2008) who reported that ensiling of hemp increased methane production by more than 50% compared to fresh hemp.

1.8. Objectives and hypotheses

The mechanical fractionation of the biomass according to the current green biorefinery concept generates green juice and press cake. While the protein recovery in the green juice can yield a suitable amount of crude protein that can meet the viability of the process, a great portion still remains in the press cake. We hypothesize that the protein recovery might be considerably increased by exposing the press cake to different pretreatment and degrading methods. Therefore, we aim at increasing the protein recovery in the press cake by enzymatic hydrolysis, and heat treatment. Besides, after the enzymatic hydrolysis we endeavour to release a great content in sugars that might be used for organic acid production and to break down the press cake so that it can undergo a second screw pressing which will give rise to a second press cake and brown juice, and eventually a maximized yield.

Silage is a preservation method that enables a steady feed supply during those periods in which it is not possible to harvest. We hypothesise that green biomass could be preserved by ensilaging red clover, and employ this material for animal feed. Thus, we evaluate the quality of the silage by determining the end products of silage fermentation, comparing the suitability of ensiling the fresh biomass and the press cake. On top of that, the good silage should contain organic acids in such levels as to yield methane in great proportions that can be used in biogas plants for energy production. To that end, we investigate the specific methane yield of red clover during the ensilage process alongside its suitability as a biofuel for energy production.

1.9. Structure of the thesis

The thesis is divided into five chapters. Chapter 1 comprises an introduction to the topic, from general aspects like the need for new green energies and the concept

of biorefinery, to the organofinery project in which this thesis represents only a minor contribution.

The chapters from 2 to 5 deal with the experimental part. They topics are presented separately topic but they connected to each other. Every chapter includes a portion of the research carried out in this project, and comprise material and methods, results and discussion and conclusions with the proposal of future research.

In chapter 2, all the biological fractions employed in this study are described and characterized chemically.

The pretreatment of the biomass, in which the enzymatic hydrolysis constitutes the cornerstone, is presented in chapter 3, along with the heat pretreatment and the sugars and protein fractions obtained in these processes.

Chapter 4 encompasses the silage process, focusing on the evaluation of the products obtained and the quality of the silage, and the comparison between fresh biomass and press cake.

Finally, chapter 5 addresses the biogas production from fresh and ensiled biomass, comparing the methane production of all the substrates.

2. Chapter 2 – Chemical characterization of the biomass

2.1. Materials and methods

2.1.1. Plant material and mechanical fractionation

The three biomasses utilized in this study are clover grass press cake, red clover fresh and red clover press cake. The red clover fresh and the press cake were grown in the facilities of Aarhus University located in Foulum, Denmark (56°29'24.6"N 9°35'16.0"E) and were harvested the 20th of October 2015. The crop was planted in the spring 2015 and was supposed to contain solely red clover (*Trifoilum pratensis*), but different species were found after harvesting e.g. ryegrass (*Lolium sp.*) and fescue (*Festuca sp*). Nevertheless, we consider this crop as red clover since it was the predominant species.

The mechanical fractionation of the red clover was carried out immediately after harvesting at the same place of growing but inside of a barn, with a screw presser design by technical engineers employed by Aarhus University. It is worth mentioning that during the screw pressing technical problems aroused leading to the fractionation of less plant material than expected. The fresh crop and the press cake were frozen just after harvesting and fractionation, and then taken to the laboratory for further characterization.

The clover grass press cake was collected from the freezer at -20 ºC located in the laboratory of Alborg University (Copenhagen) and belongs to the collection of feedstock employed in the organofinery project. It was grown in the late winter 2014 and was harvested in May 2014. The mechanical fractionation was performed with a Vincent CP4 screw press rented from Runi S/A (Tarm, Denmark) in the Department of Crop Sciences of Københavns Universitet (KU), Taastrup (55°40'11.4"N, 12°18'26.1"E).

For the two fractionations the screw presser was cleaned by passing plant material through the presser in order to avoid the utilization of water. Since the project deals with organic crops, no fertilizer was applied in any of the two crops.

No green juice was utilized in this study and therefore it is not characterized. However, the brown juice obtained after the fermentation was applied as inoculum for the preparation of silage and therefore it was further characterized. The green juice obtained after the fractionation of red clover was inoculated with an overnight culture of *Lactobacilus salivarius* BS 1001 and then fermented overnight. The fermented juice (brown juice plus protein paste) was collected from

the fermentor and transferred to 50ml falcon tubes and centrifuged at 5000 rpm for 10 minutes. Finally, the protein paste was discarded and the brown juice was frozen and taken to the lab. We decided to utilize the brown juice as inoculum due to a logistic problem with a more suitable inoculum that did not arrive on time when the experimental set-up took place. However, as the brown juice contained LAB we decided to include it in our experiments.

2.1.2. Chemical characterization

The three biomasses were characterized in terms of free sugars, volatile fatty acids (VFAs), ethanol, acetic, citric, succinic and lactic acid, pH, total solids (TS), volatile solids (VS), total Kjeldahl nitrogen (TKN), and additionally for fresh red clover and its press cake the content of cellulose, hemicellulose, and Klason lignin was analysed.

To begin with, the biomass was soaked in MilliQ water in order to obtain liquid from the samples required for characterizing some parameters. According to Ambye-Jensen, Johansen et al. (2013), 0.3 – 0.4 g DM of biomass were soaked in 10 mL of MilliQ water containing Kanamycin (0.1 mg/mL) to avoid microbial activity. The samples were shaken for 2 hours at 25ºC in closed glass bottles at 150 rpm. The liquid extracted was used for measuring several parameters accounting for the dilution factor.

Total solids (TS) and volatile solids (VS) were determined as reported by APHA Standard Methods (2005). Notwithstanding the fact that during the drying process some volatile organic compounds (VOCs) are evaporated, prompting an underestimation of both TS and VS contents, we decided not to use correction coefficient as the distortion in solid samples might be interpreted as negligible (Vahlberg, Nordell et al. 2013).

The pH was estimated with a pH-meter by dipping the glass of the electrode in the liquid extracted from the biomass.

The concentrations of oligosaccharides were quantified along with the ethanol and the acids (succinic, lactic and citric) by High-Performance Liquid Chromatography (HPLC). The equipment consisted of a Dionex Ultimate 3000-LC system (Dionex Corporation, Sunnyvale, CA, USA) with an Aminex® HPX-87H column. Sulfuric acid (4mM) was utilized as a mobile phase, with a flow rate of 0.6 ml/min at 60ºC. The samples were acidified by the addition of 10% v/v of H2SO4 2M (100µL per 1mL of the sample). The samples were then centrifuged to remove solids and precipitates caused by the acid and finally filtered through 45 µm filters into chromatography vials. Chromeleon software (Dionex Corporation) was used for the integration of all the chromatograms.

Determinations of volatile fatty acids proceed similarly. The samples were acidified with 10% v/v HPO4 17%, centrifuged at 9000 rpm for 7 minutes, filtered through 45 µm filters and poured into chromatography vials. The quantification was carried out in a gas chromatograph (PerkinElmer, Clarus 400) equipped with an Agilent HP-FFAP capillary column of 30 m length and 0.53 mm i.d. ensued by a flame ionization detector (FID). The carrier gas was Nitrogen at a flow rate of 13 ml/min and the temperatures of the detector and the injector were 230 and 240°C, respectively.

The total Kjeldahl Nitrogen was determined in accordance with APHA Standards Methods 2005. The three biomasses were firstly dried overnight at 80ºC, and then grounded with a standard kitchen grinder in order to avoid heterogeneity in the samples. Furthermore, the crude protein was estimated based on the nitrogen content of the samples and a conversion factor of 6.25, grounded on the assumption that proteins contain around 16% of nitrogen. However, this assumption might bring about a 15% error, as the content of nitrogen differs among aminoacids (McClelland and Montoya 2002).

Figure 5.Titration of ammonia during TKN analysis.

Hemicellulose, cellulose and lignin composition was quantified by strong acid hydrolysis-Klason lignin procedure according to the NREL analytical method for determination of structural carbohydrates and lignin in biomass (Sluiter, Ruiz et al. 2005). Initially, samples were dried overnight at 45ºC as the content of dry matter ought to be over 90% to avoid interferences between the excess of moisture and the acid concentrations. Afterwards, the samples were grounded with a mixer to homogenise the biomass. Following the two-step acid hydrolysis procedure, the liquid extracted was analysed by HPLC. When required, H_2SO_4 2M was added to decrease the pH. The content of hemicellulose and cellulose was estimated on the

basis of the sugars released and a recovery factor according to Bjerre, Plöger et al. (1996). Finally, the Klason lignin was quantified based on the dried residue obtained after the acid hydrolysis and corrected for ash.

The brown juice utilized as inoculum for the silage was also characterized chemically. The determination of TS, VS, VFA´s, pH and oligosaccharides was performed as described above. Besides, the number of colony forming units (CFUs) was estimated using one popular method known as the technique of most-probable-number (MPN). It consists of a series of dilutions of the inoculum followed by cultivation on selective medium (LAB) and incubation conditions (37ºC and darkness) on Petri dishes. The total number was estimated by counting colonies on the different plates accounting for the dilution factor. Three replicates of each dilution were prepared to improve the accuracy of the final MPN. A picture of the plates containing different dilution factors is presented in Figure 7.

2.2. Results and discussion

The chemical composition of the different biomasses included in this project is presented in Table 1.

2.2.1. Red clover

The dry matter content of the fresh feedstock was 154.27 g/kg, with and organic content of 79,5% of the DM. After the mechanical fractionation, the dry matter content of the press cake augmented 1.6-fold in comparison to the fresh biomass. These results are fairly similar to the figures reported in previous research on red clover within the organofinery project (Molinuevo-Salces, Santamaria et al. 2015), although the organic matter resulted lower (79.5% against 88%).

The proteins in the press cake, mainly fiber-bound proteins, accounted for the 15% (dry matter basis), a bit lower than the 17% estimated in the fresh biomass, what hints at some proteins are recovered in the green juice after the fractionation. These values of proteins are smaller than those reported by Molinuevo-Salces, Santamaria et al. (2015), where the protein represented around the 18% and 20% of the dry matter content of the press cake and fresh respectively. The explanation of this divergence might be the maturity content of the biomass, as in the previous analyses the plant material was harvested in May, just before flowering when the content of protein is higher, whereas in our project the biomass was harvested in the late October. Additionally, the content of nitrogen in the fresh material was 36.7 g/kg DM and therefore it is within the range of the amounts generally reported in the literature (Spedding and Diekmahns 1972).

The pH was similar in all the biomasses and congruent with prior research. Surprisingly, the content of free sugars was the same in the press cake and the fresh material (7% dry matter basis), what disagrees with

	Units	*Red Clover Press Cake*	*Red Clover Fresh*	*Clover Grass Press Cake*	*Brown juice inoculum*
Total Soilds	g/kg	251,12 (15,4)	154,28 (13,62)	327,33 (14,75)	32,90 (0,42)
Volatile Solids	g/kg	214,48 (21,01)	122,75 (8,28)	268,40 (17,2)	22,48 (0,47)
VS over TS	%	85,4	79,6	82,0	68,3
pH		5,48 (0,04)	5,68 (0,03)	5,27 (0,04)	3,57 (0,01)
TKN	g/kg	6,08 (0,38)	4,23 (0,04)	5,96 (1,11)	n.d.
Crudo protein	g/kg	37,97 (2,41)	26,41 (0,27)	37,24 (6,98)	n.d.
Cellulose	g/kg	56,13	31,05	n.d.	n.d.
Hemicellulose	g/kg	31,06	17,26	n.d.	n.d.
Lignin	g/kg	47,47	28,97	n.d.	n.d.
Free sugars	g/kg	17,71 (1,62)	10,76 (2,97)	15,63 (1,76)	18,41
Lactic acid	g/kg	0,00	0,00	2,07 (0,15)	2,26
Citric acid	g/kg	1,72 (0,2)	3,38 (3,64)	1,34 (0,30)	2,79
Acetic acid	g/kg	1,72 (0,21)	1,16 (0,22)	1,08 (0,05)	0,46
Succinic acid	g/kg	0,00	0,00	0,85 (0,03)	0,29
VFAs	g/kg	3,11 (0,31)	2,19 (0,55)	n.d.	2,14

Table 1. Chemical Characterization of the different fractions utilized in this essay. Standard deviations are shown in brackets. "n.d." stands for "no determined".

Molinuevo-Salces, Santamaria et al. (2015) as it is reported that most of the free sugars are recovered in the green juice. It might be due to an underestimation of free sugars in the fresh biomass, since the content of sugar in this study (10.76 g/Kg fresh material) was well below the aforementioned study (23.0 g/Kg fresh material). Another possible explanation of this low content in sugar might be a low leaf-to-stem ratio or the influence of the light intensity and temperature (Smith 1973).

Regarding the composition of the fresh red clover and the press cake, the analysis displays alike results (Table 1, Figure 6). The amount of cellulose in the fresh material accounted for the 20.1% of the dry matter, the hemicellulose for the 11.2% and the lignin represented the 18.8%. These figures are comparable to those reported in other studies (Smith 1964), although some authors reported higher contents of cellulose(39%) and lignin (11%) (Parsi, Godio et al. 2001). It is worth mentioning that the content of lignin could have even been underestimated as acid-insoluble lignin was determined as the weight of the acid hydrolysis residues. However, acid-soluble lignin was not measured. Besides, only minor amounts of organic and volatile fatty acids were registered.

The green juice obtained after the mechanical fractionated was not quantified but sampled. Consequently, we cannot provide mass balances of the whole fractionation process to evaluate the yield of the process in terms of the product obtained per tonne of fresh biomass. The chemical characterization of the green juice is not showed in this Master thesis owing to it is not directly relevant for this study, although it has been characterized in the frame of the organofinery project and reported in other essays.

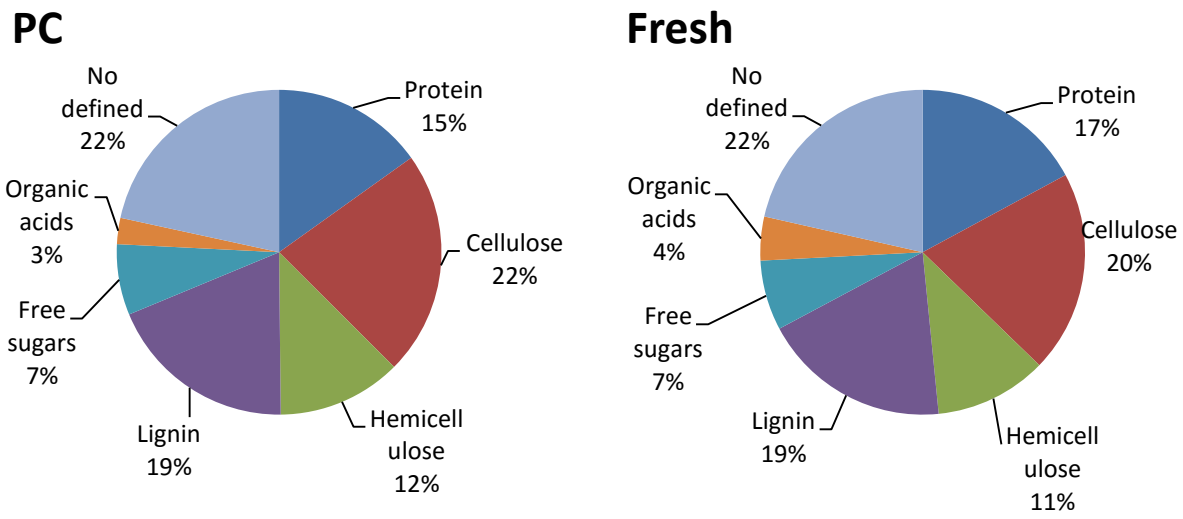

Figure 6. Composition in percentage of the dry matter of red clover PC and fresh material. It can be appreciated the similarity in the composition of the two biomasses.

2.2.2. Clover grass

Concerning clover grass solely the press cake was chemically characterized since it was the unique feedstock employed in this essay. The dry matter content rose to 327.3 g/kg of press cake, with 82% of organic matter. This value is considerably greater than the figures reported in previous essays within the organofinery project (Santamaria 2015), probably as a result of storing, freezing and thawing the plant material several times, what allowed the feedstock to dry more than the press cake characterized right away after the fractionation.

The pH (5.27) was congruent with other feedstocks, as well as the TKN, giving rise to a crude protein content of 37.24 g/Kg PC, on a par with the red clover press cake (37.97g/kg PC). However, the crude protein only accounts for 114 g/Kg DM, which is noticeably low for a leguminous plant. For instance, crude protein content of 182 g/Kg DM was previously determined for red clover and 210 g/Kg DM for alfalfa in the scope of this project (Molinuevo-Salces, Santamaria et al. 2015; Santamaria 2015). It might be due to a different maturity stage at the harvesting time, although some errors when estimating the TKN must be also considered. Finally, the content of free sugars and organic and volatile fatty acids were analogous to the red clover press cake.

2.2.3. Brown juice

The brown juice contained 32.9 g DM/kg, but only 68% were volatile solids. This value is lower than the one displayed in earlier research within the organofinery project on brown juice inoculated with the same bacteria in leguminous species. For instance, a greater content of volatile solids (around 76%) was reported by Santamaria (2015). The fact of not having corrected the values of volatile solids as suggested by Vahlberg, Nordell et al. (2013) might explain this difference.

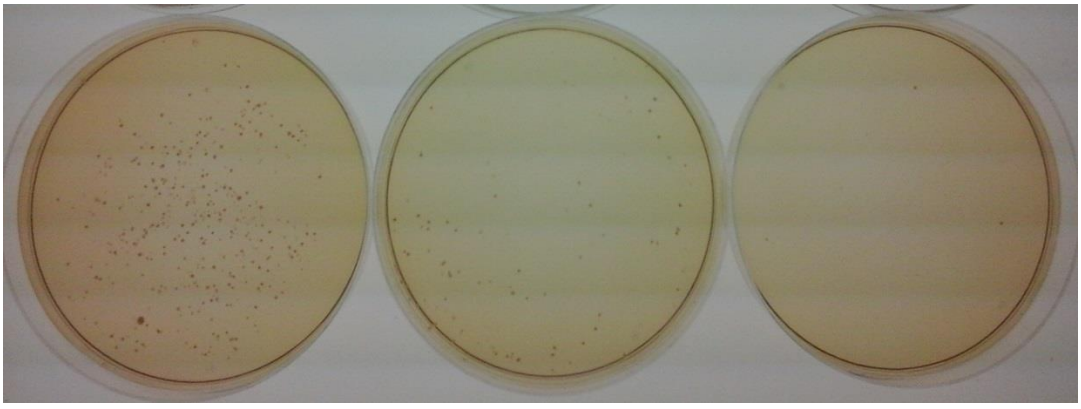

Figure 7. Plates containing CFU of *Lactobacillus salivarius* in different dilutions. From less diluted (left plate) to more diluted (right plate).

The pH resulted more acidic than previous studies (3.57 versus 4.3), but interestingly the amount of lactic acid was clearly lower than the reported by Molinuevo-Salces, Santamaria et al. (2015) and Santamaria (2015) in which 6.8 and

11.8 g/kg on red clover and alfalfa respectively were obtained. Perhaps the transformation of the sugars into lactic acid was not successful, and the low pH might be explained by the appearance of other acids, like citric acid, as its presence is more notorious than in the aforementioned studies. Another likely explanation could be an error during HPLC, attributable to that only one sample was analysed.

The amount of bacteria present was estimated to 10^7 CFU/ml of Brown juice, and the amount of free sugars (18.41g/Kg) was considerably higher than the 7,8 g/kg reported by Molinuevo-Salces, Santamaria et al. (2015).

2.3. Conclusions and future prospects

Most of the carbohydrates are recovered in the press cake (great cellulose and hemicellulose content in comparison to fresh). This large percentage in the lignocellulosic material in conjunction with the fiber-bounded protein propose the press cake as a suitable product to be used as a substrate for biogas production, feed for ruminants or even for thermal combustion or bioethanol (Richter, Fricke et al. 2011).

In addition, harvesting the crop at an earlier stage would yield a greater content of proteins. For instance, in May, most of the red clover varieties are about to flower (Frame, Charlton et al. 1998) and the amount of protein in the plant prior to flowering is maximum (McEniry and O'Kiely 2014).

It would be interesting to evaluate the yield of the mechanical fractionation providing a mass balance of the income and outcome products, as well as to provide data about tonnes of red clover harvested per hectare so that an economic evaluation can be reported based on the yield of press cake and green juice obtained per cultivated hectare.

The brown juice seems to be a suitable inoculum for biogas production since it contains considerable amounts of free sugars, organic acids and *Lactobacillus salivarius*.

3. Chapter 3 – Evaluation of pretreatment methods

The aim was to obtain a high yield of free sugars and free proteins from the press cake that will be employed for different purposes. Several pretreatment methods were applied in combination, including thermal pretreatment (80ºC), incubation in a thermoshaker at 50ºC for several time spans (4, 24 and 72 hours), addition of the enzyme Cellic® CTec2 obtained from Novozymes Denmark at different concentrations (0-5 %, equivalent to g enzyme/100 g TS), diverse loadings of dry matter (5 to 28 % TS) and inclusion of kanamycin as antibacterial treatment.

3.1. Materials and methods

In order to determine the optimal method, two batches of pretreatment were carried out as preparatory assays. It was aimed to evaluate the performance of the different pretreatment methods applied in combination on the press cake, so that the least efficient methods were ruled out and the most promising treatments were selected for a final batch of enzymatic hydrolysis on red clover press cake.

Treatment	% TS	Incubation Time (h)	Heat 80ºC	% enzyme	Kanamycin
T1	5	4	-	3	+
T2	10	4	-	3	+
T3	5	4	+	3	+
T4	10	4	+	3	+
T5	5	72	-	3	+
T6	10	72	-	3	+
T7	5	72	+	3	+
T8	10	72	+	3	+
T9	5	72	-	0	+
T10	10	72	-	0	+
T11	5	72	+	0	+
T12	10	72	+	0	+
T13	5	72	-	3	-
T14*	5	0	-	0	+
T15*	5	0	-	0	-
T16**	Cellulose	0	-	3	-

Table 2. Different treatments included in the enzymatic hydrolysis 1 (preparatory essay). *The treatment T14 and T15 are controls for no pretreatment. **The T16 is a positive control to evaluate the conversion of cellulose to sugars employing powder cellulose as a feedstock. Another treatment (not shown in the table) was added to determine the amount of sugars and proteins release by the enzyme blend as a consequence of the preservatives included in the blend that might overestimate the results.

In all the batches, the supernatant was collected and stored at -20 ºC for further characterization. However, the water recovery after the pretreatment was different in some treatments (see below). Besides, the water recovery for the controls T1 and T2 in the first enzymatic hydrolysis was carried out according to Ambye-Jensen, Johansen et al. (2013) whilst the water recovery in the control E1 of the second enzymatic hydrolysis was carried out by soaking the biomass in water overnight and simple recovery.

The analysis of sugars and acids was performed by HPLC as described in Chapter 2, and the protein quantification was estimated by the bicinchoninic acid assay (BCA assay). In the determination of both substances, the amount of sugars and proteins released by the enzyme blend Cellic® CTec2 was subtracted from the sample estimations. Pretreatment effectiveness was evaluated based on sugar and protein yield during biomass conversion. In order to uniform the results, the amount of both protein and sugar are expressed in g/Kg of Press cake.

Figure 8. 117 cm^3 Serum vials containing the biomass.

3.1.1. Enzymatic hydrolysis 1 (Preparatory assays)

In this preparatory assay, the following pretreatment methods and concentrations were analysed:

- Thermal pretreatment at 80ºC for 60 minutes.
- Enzyme loading of 3 g/100 g TS (3 %).
- Dry matter concentration of 5 and 10% (w/w) of the final volume (20 mL).
- Incubation time of 4 and 72 hours in the thermoshaker at 50 ºC.
- Addition of Kanamycin at a concentration of 0.1 mg/mL.

The different treatments tested are presented in Table 2.

Experimental set-up

Loadings of clover grass were placed in 117 cm3 serum vials according to the TS added to each treatment and filled with distilled water to a final volume of 20 mL (Figure 8). The enzyme Cellic® CTec2 with a density of 1,15 g/mL was added accordingly and accounted for filling the 20 mL of final volume. The serum vials were shortly shaken to uniform the blend.

The corresponding treatments were applied accordingly. Some vials underwent thermal pretreatment prior to incubation in the thermoshaker.

3.1.2. Enzymatic hydrolysis 2 (Preparatory assays and optimization)

In order to optimize the previous treatment, and to test new concentrations, the following pretreatment methods and concentrations were analysed:

- Thermal pretreatment at 80ºC for 60 minutes.
- Enzyme loadings of 1 and 3 g/100 g TS (1 and 3 %).
- Dry matter concentration of 10, 15 and 20% (w/w) of the final volume.
- Incubation time of 4, 24 and 72 hours in the thermoshaker at 50 ºC.
- Addition of Kanamycin at a concentration of 0.1 mg/mL.
- Volume on treatment: 20 and 40 mL.

The different treatments tested are presented in Table 3.

Treatment	% TS	Incubation Time (h)	Heat 80ºC	% enzyme	Kanamycin	Volume (mL)
E1*	10	0	-	0	-	20
E2	10	0	+	0	-	20
E3	15	4	-	0	-	20
E4	15	4	-	1	-	20
E5	15	4	-	3	-	20
E6	15	24	-	0	-	20
E7	15	24	-	1	-	20
E8	15	24	-	3	-	20
E9	15	72	-	0	-	20
E10	15	72	-	1	-	20
E11	15	72	-	3	-	20
E12	15	24	-	3	+	20
E13	20	72	-	3	-	20
E14	15	72	-	3	-	40

Table 3. Different treatments included in the enzymatic hydrolysis 2 (preparatory essay). *The treatment E1 is a control with no pretreatment. Additionally, controls of conversion of cellulose into sugars by the enzyme blend were included (data not shown).

The experimental setup was the same as the first batch. However, due to the high dry matter content, the liquid obtained after the enzymatic hydrolysis was difficult to extract and consequently, the biomass was washed with sterile water, and the sugars an proteins were estimated accounting for the dilution factor.

3.1.3. Integral enzymatic hydrolysis

The core biomass employed in this master thesis, the red clover, was tested as a feedstock for sugar and protein recovery by enzymatic hydrolysis. Considering the results obtained in the first and second enzymatic hydrolysis (preparatory essays), the most important parameters (%TS and %enzyme) were selected for performing a Central Composite Design (CCD). The preparatory essays also ruled out the inclusion of thermal pretreatment and the addition of kanamycin in all the treatments. The volume of the treatments was scaled up to 50 mL.

Central Composite Design (CCD)

The design of the experiment was performed according to a two-factor Circumscribed Central Composite Design. This kind of factorial design commonly consists of a 2^k factorial nucleus (cube points), six replications of the central point and 2*k axial points, where k is the number of factors or variables to be analysed (Box and Wilson 1951). In particular, in this test the two factors were % of TS and % of enzyme added. The Central point is duplicated (6 replications) to strengthen the model. The axial points provide extra levels to compute a quadratic model and are calculated using an α distance to the central point to ensure rotability, where α is $2^{k/4}$ and k is the number of levels tested for each factor (Esbensen, Guyot et al. 2002). The real variable values (X) were given dimensionless values (x) from -1 to 1 where 0 is the central point according to the equation (Chong, Rahim et al. 2009):

$$X = \frac{X - (Xmax + Xmin)/2}{(Xmax - Xmin)/2}$$

The combination of variables in the different experimental settings is presented in Table 4. Overall, 9 different treatments were tested in triplicates, aside from the T9 (central point) that was tested 6 times. Statistical analyses were carried out with the software STATISTICA 8.

Experimental setup

The corresponding amount of biomass was added to 1L glass bottles. The enzyme blend Cellic® CTec2 was added if applied to each treatment and the final volume of every treatment was set to 50 mL. The bottles were incubated in a thermoshaker at 50 ºC for 72 hours. After the incubation, the bottles were washed with water for recovering the liquid. The water added to each bottle for the recovery was calculated according to the formula:

$$\frac{TS\ (g) * 25}{H_2O\ (mL)}$$

Where TS are the grams of dry matter added in each treatment, and H_2O is the water added prior to the incubation. Finally, for the estimation of sugars and protein, the dilution factor was taking into account and the corresponding amount of sugar and proteins released by the preservatives present in the enzyme blend were subtracted.

		Real values		Codified values	
	nº Replicates	X1	X2	x1	x2
T1	3	8,37	4,27	-1	1
T2	3	8,37	0,73	-1	-1
T3	3	24,63	4,27	1	1
T4	3	24,63	0,73	1	-1
T5	3	16,50	5,00	0	1,4142
T6	3	16,50	0,00	0	-1,4142
T7	3	5,00	0,00	-1,4142	0
T8	3	28,00	0,00	1,4142	0
T9	6	16,50	2,50	0	0

Table 4. The combination of variables in the different experimental settings. X1 is the first variable (% of TS); X2 is the second variable (% of enzyme); x1 and x2 are the codified values.

3.2. Results and discussion

3.2.1. Pretreatment of the Press Cake of Clover grass

This feedstock was tested through two batches in which several pretreatment methods were applied. The pretreatment included thermal pretreatment (80ºC), incubation in a thermoshaker at 50ºC for several time spans (4, 24 and 72 hours), addition of the enzyme Cellic® CTec2 in different concentrations (0-3%), diverse loadings of dry matter (5 to 20% TS) and inclusion of kanamycin as antibacterial treatment.

The pretreatment of the PC researched in this project seems to be a promising technology for the production of sugars, proteins, organic acids and other valuable chemicals that might be employed for different purposes in the scope of the organofinery project. Among all the treatments presented, the highest yield registered corresponds with a 72-hour enzymatic hydrolysis of 5% TS and 3% enzyme addition were 51.1 g of sugars /Kg PC and 19.68 g of protein/Kg PC were obtained (Tables 5 and 6). The highest observed sugar yield corresponds to 14,7 g/100 g TS what is the level of similar yields achieved by treating the press cake

g/Kg PC	Cellobiose	Glucose	Xylose	Arabinose	Total sugars	Total Protein	Lactic acid	Citric acid	Acetic acid	Ethanol	% TS	Time (h)	Heat 80ºC	% enzyme	Kanamycin
T1	0,93	27,41	16,25	0,29	**44,88**	**18,51**	0,00	1,01	0,00	0,00	5	4	-	3	+
T2	0,83	17,71	11,74	0,35	**30,63**	**15,79**	0,00	1,38	0,40	0,00	10	4	-	3	+
T3	1,97	27,23	18,98	0,33	**48,51**	**17,21**	0,00	1,27	0,00	0,00	5	4	+	3	+
T4	1,69	24,10	17,32	0,48	**43,59**	**13,87**	0,00	1,42	0,39	0,00	10	4	+	3	+
T5	0,00	23,85	14,68	1,83	**40,35**	**19,68**	5,41	2,24	6,30	0,00	5	72	-	3	+
T6	0,00	27,26	11,50	1,70	**40,46**	**14,54**	4,61	1,72	3,83	0,00	10	72	-	3	+
T7	0,00	28,97	14,03	1,93	**44,93**	**16,56**	1,79	1,21	5,40	0,00	5	72	+	3	+
T8	0,00	28,57	17,60	1,49	**47,66**	**13,10**	7,34	1,74	1,68	0,00	10	72	+	3	+
T9	0,00	5,78	9,05	0,31	**15,14**	**9,16**	3,11	2,02	3,51	0,00	5	72	-	0	+
T10	0,00	3,50	11,61	0,39	**15,49**	**8,63**	9,96	1,02	0,46	0,18	10	72	-	0	+
T11	0,00	1,77	0,99	0,00	**2,77**	**8,66**	1,44	0,81	8,30	0,00	5	72	+	0	+
T12	0,00	3,16	8,31	0,00	**11,47**	**7,27**	7,76	0,83	2,54	0,00	10	72	+	0	+
T13	0,00	32,32	16,75	2,03	**51,10**	**16,18**	2,94	1,21	8,92	0,00	5	72	-	3	-
T14	0,00	12,64	0,99	3,26	**16,88**	**6,60**	1,96	1,55	1,12	0,18	5	0	-	0	+
T15	0,00	10,01	0,93	3,44	**14,38**	**6,32**	2,18	1,12	1,04	0,27	5	0	-	0	-
T16 (g/L)	0,00	0,49	0,00	0,00	**0,49**	**N.D.**	0,00	0,48	0,00	0,00	0	0	-	3	-

Table 5. Evaluation of the sugars, proteins and acids released in the different treatments analysed in the enzymatic hydrolysis 1. "N.D" stands for "no determined"

with more costly and less environmental-friendly methods. For instance, Neureiter, Danner et al. (2004) reported a sugar yield of 16,4 g/ 100 g of DM using sulfuric acid hydrolysis and a bioreactor. This maximum protein released accounted for the 52% of the total protein contained in the PC of clover grass as estimated by TKN (Table 1, Chapter 2), proposing the enzymatic hydrolysis as an important method for protein extraction.

Generally, short incubation times yield high production when the dry matter loading is low whereas long incubation periods require greater TS loadings. In both cases, the content of sugars released range between 40 and 50 g/Kg PC. The protein liberation is helped by short dry matter loadings. The results are shown in the Tables 5and 6.

The method employed to measure the protein liberation, the bicinchonic acid assay (BCA), helps to obtain an estimation of the likely amount of protein released. However, the method lacks accuracy, as it has some limitations. Hence, caution must be taken when reporting these results, as the amount of protein released must be overestimated. Therefore, in order to report more reliable results different methods to measure proteins like spectroscopic procedures, absorbance or SDS-PAGE should be applied (Rommi, Hakala et al. 2014).

In addition, there is also a noticeable amount of organic acid produced, what can be beneficial or detrimental depending on the targeted products. The positive activity of the enzyme was analysed as a control of powder cellulose conversion into sugars, showing a positive transformation (data not shown). The different pretreatments are discussed individually below.

Kanamycin

The effect of Kanamycin exhibits contrasting results. In the first batch of enzymatic hydrolysis its effect seems unnoticeable. The T5 (+ kanamycin) and T13 (- kanamycin) incubated under the same pretreatment conditions performed similarly, and the sugars released were even higher when there was no kanamycin. The amount of acids produced by microorganisms remains inconsistent. The control vials (without any kind of pretreatment) T14 (+ kanamycin) and T15 (- kanamycin) showed no differences. Likewise, in the T13 (- kanamycin) the concentration of acetic acid was double in comparison to the average for incubations at 72 hours (8.9 vs 4.5) g/Kg PC, but was half for lactic acid production (2.9 vs 4.9) g/Kg PC. Perhaps there was a fermentation of sugars into acid into lactic acid.

However, the second batch presented more consistent results, according to previous research (Joersbo, Donaldson et al. 1998). When comparing two treatments incubated under the same pretreatment conditions, but one positive

g/Kg PC	Glucose	Xylose	Arabinose	Total sugars	Total Protein	Lactic acid	Glycerol	Acetic acid	% TS	Incubation Time (h)	Heat 80ºC	% enzyme	Kanamycin	Volume (mL)
E1	0,48	0,76	0,00	**1,24**	**5,50**	0,67	0,11	0,38	10	0	-	0	-	20
E2	1,50	3,33	0,00	**4,83**	**8,68**	0,00	0,33	0,08	10	0	+	0	-	20
E3	5,14	5,66	0,42	**11,22**	**9,17**	0,00	0,35	0,31	15	4	-	0	-	20
E4	10,48	9,97	0,58	**21,03**	**9,27**	0,00	0,77	0,59	15	4	-	1	-	20
E5	14,42	10,02	0,60	**25,04**	**10,69**	0,00	0,65	0,68	15	4	-	3	-	20
E6	8,18	7,88	0,55	**16,61**	**6,70**	3,57	0,72	1,13	15	24	-	0	-	20
E7	18,81	7,77	0,29	**26,87**	**6,09**	3,52	0,63	0,70	15	24	-	1	-	20
E8	20,82	12,70	1,04	**34,55**	**12,79**	1,55	0,73	1,25	15	24	-	3	-	20
E9	2,38	8,22	0,38	**10,98**	**7,72**	5,99	0,83	1,16	15	72	-	0	-	20
E10	19,41	18,20	0,81	**38,42**	**14,20**	4,81	0,00	1,61	15	72	-	1	-	20
E11	26,42	21,57	1,20	**49,19**	**16,65**	4,41	0,28	1,79	15	72	-	3	-	20
E12	22,37	18,63	0,62	**41,62**	**14,89**	0,00	0,24	0,84	15	24	-	3	+	20
E13	16,37	14,14	0,55	**31,06**	**11,65**	3,22	0,15	0,81	20	72	-	3	-	20
E14	18,83	15,86	0,96	**35,64**	**11,09**	3,77	0,33	1,08	15	72	-	3	-	40

Table 6. Evaluation of the sugars, proteins and acids released in the different treatments analysed in the enzymatic hydrolysis 2.

for kanamycin and one negative, the treatment positive for Kanamycin increased the sugar releasement in 20%, the lactic acid production was completely inhibited and the acetic acid production 45% reduced. It might be explained by the fact that the second batch was prepared with the same biomass as the first batch, and the bags were thawed and frozen 2 times. Consequently, the activity of microorganism was more activating (probably due to fermentations took place while handling the press cake), giving rise to higher yield when Kanamycin was applied

Furthermore, the analysis of the average lactic acid production for 72-hour incubation in the first batch (+ kanamycin) against the second batch (- kanamycin) still rises doubts about the effect of the kanamycin. In the first batch the average lactic acid production was 4.93 g/Kg PC whilst in the second batch, it was 5.07 g/Kg PC. Therefore, either there is no consumption of sugars by bacteria or the amount of kanamycin was not enough to kill the bacteria. A more exhausting research increasing the concentration of kanamycin is needed in order to elucidate its real effect on this biomass. Alternatively, it might be interesting to supplement the vials with other antibiotics, like zeocin, ampicillin or X-gal (Atlas 2010).

Thermal pretreatment

The heat pretreatment seems to have a positive effect in the absence of other methods of pretreatment. When heat treatment was the only method applied, the content of sugars and proteins recovered was twice the amount obtained when no treatment was applied, although the overall amount of sugars and proteins obtained was still low.

However, when the heat was applied in conjunction with other ways of pretreatment its positive effect remains unclear. Precisely, the combination of Thermal pretreatment (80 ºC) and incubation at 50ºC for enzymatic hydrolysis under different incubation times and amount of enzyme revealed that heat pretreatment increased the number of sugars 10,7% on average, but the protein recovered diminished by 11%. It is likely that the synergistic effects of applying two sources of heat give rise to protein denaturation and thus lower protein recovery.

It seems as if the incubation at 50ºC reduces the effect of the thermal pretreatment, or at least alleviates the differences. In any case, according to these results, the application of heat treatment in conjunction with incubation for enzymatic hydrolysis might be positive if the intended products obtained are sugars, but it seems to be detrimental if protein recovery is aimed.

Anyhow, the positive effect of this pretreatment is in disagreement with other studies. For example, Oleskowicz-Popiel, Thomsen et al. (2011) reported high performance of thermal pretreatment, although applying higher temperatures.

Addition of enzyme (0, 1, 3%) and incubation.

The addition of enzyme was the most important factor for the pretreatment of the biomass. In the first batch, the enzymatic hydrolysis with 3% of enzyme resulted in a 2.7 –fold increment in sugar released and 1.9-fold for protein when the biomass is incubated at 50ºC for 72 hours (Figure 9). This difference is accentuated if we compared the enzymatic hydrolysis with the control (no incubation for 72 hours).

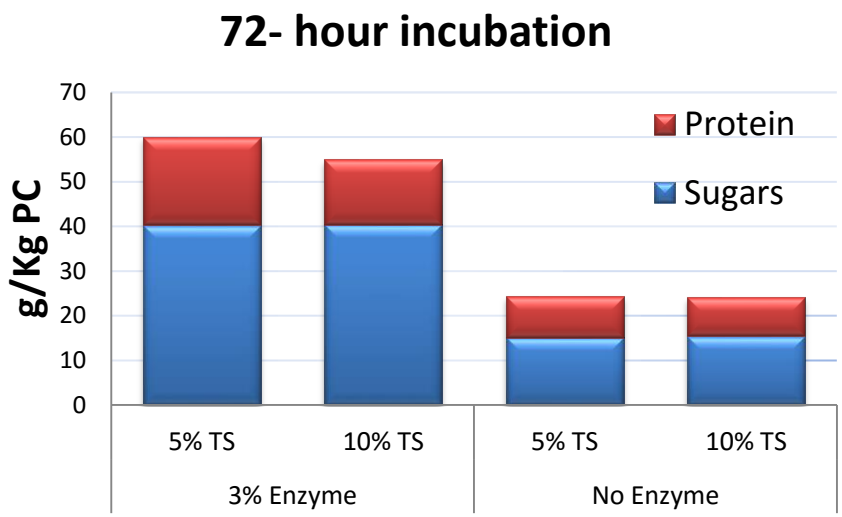

Figure 9. Protein and sugar obtained for a 72-hour incubation, with two concentration of enzyme and loadings of TS.

In the 2nd batch, when 15% of TS were tested, the result was confirmed. The addition of 3% of enzyme gave rise to an increment of 2.9-fold in sugar liberation and 1.7-fold in the event of proteins, calculated as an average of different incubation times, but always presenting the same pattern. A similar pattern is showed when only a 1% of enzyme was added, giving increments of 2.3-fold and 1.3-fold for sugars and proteins respectively (Figure 10).

Consequently, the addition of enzymes appears to be the most important factor in the pretreatment of press cake for the release of sugars and proteins. The addition of 3% of enzymes showed better results, however great amounts of sugars were released with only 1% of enzymes supplemented during the incubation. Further research should be carried out from an economical point of view in order to evaluate if the addition of 3% of enzymes is more profitable in the long run.

In addition, the enzyme employed in this essay was Cellic® CTec2, which mainly contains a blend of aggressive cellulases, high level of β-glucosidases and some hemicellulases. But there is another promising enzyme in the market, the Cellic HTec2 which contains endoxylanases with high specificity toward soluble hemicellulose what might lead to an increase of those sugars present in the hemicellulose. Since our feedstock contains an appreciable amount of hemicellulose, it would be interesting to analyze the combination of Cellic® CTec2

with the Cellic HTec2 as it has been proven that Cellic HTec2 can improve cellulose hydrolysis when combined with CTec2(Novozymes) (Megyeri, Bélafiné Bakó et al. 2014)

Duration of enzymatic hydrolysis

The impact of the duration of the enzymatic hydrolysis was evaluated in the two batches. In the first batch, the incubation was tested for 4 and 72 hours. The results were rather similar for both treatments. When the biomass utilized was 5%, there is no difference between 4 and 72-hour incubation, but the addition of 10% of TS required longer incubation periods in order to yield the same sugars and proteins as the addition of 5% of TS. The amount of sugars obtained in the incubation of 10%TS of PC for 72 hours was 1.3-fold higher than the 4-hour incubation, but the yield was still slightly below the yield obtain in the treatment of 5%TS for 4 hours (Figure 11), what suggest that low dry matter content incubated for short period might be optimal.

In the second batch, aside from the 4 and 72-hour, a 24-hour enzymatic hydrolysis was evaluated. In this case, we solely essayed the addition of 15% TS. The HPLC revealed that the treatment of high dry matter content is considerably affected by the incubation time. The 72-hour enzymatic hydrolysis exhibited a 4.5-fold increment in sugar released with a 3% enzyme addition against no enzyme addition, whilst the 4-hour enzymatic hydrolysis only showed a 2.2-fold increment in sugar released against no enzyme addition, so the yield for 72 hours incubation was twice as high as for the 4-hour incubation (Figure 10). The 24-hour enzymatic hydrolysis presented alike results to the 4-hour incubation, and consequently, it seems to be a short incubation time for obtaining economically viable yields.

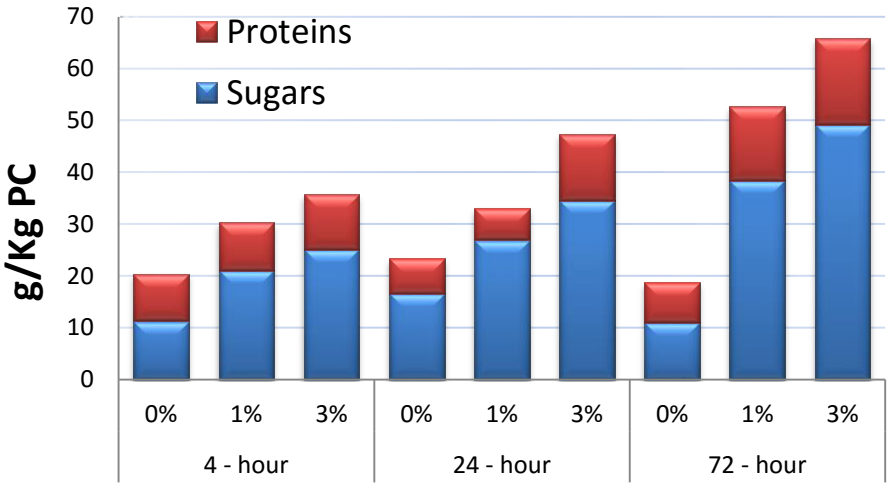

Figure 10. Protein and sugar obtained when loadings of 15% TS were added, for three different concentration of enzyme and incubation times.

Additionally, the production of acetic and lactic acid was negligible during the 4-hour incubation regardless of the inclusion of Kanamycin. Perhaps the microorganisms did not have enough time to grow in the favourable conditions encountered in the thermoshaker. However, the production of lactic and acetic acid in the 24 and 72-hour incubation ranged between 2 and 8 g/Kg PC, what hints at the consumption of sugars by microorganisms present in the biomass with the consequent reduction of intended products. Therefore, unless these acids are utilized further on for the generation of any valuable product, short incubation periods must be set.

Total Solids (%)

The dry matter included in the different treatments seems to be a key factor. There is a limit of dry matter from which the activity of enzymes cannot degrade de cellulosic material and the amount of released sugars decreased. According to the specifications of the supplier (Novozymes), high levels of dry matter inhibit or decline the positive effect of the enzyme, and theses limits depend on the kind of feedstock. In our essay, 20% TS loading resulted in the lowest recovery rates for both sugars and protein. The interpretation of this result must be cautious. Apart from the limited enzymatic activity, the lower amount of water added and recovered in those treatments might lead to errors when performing HPLC and BCA, as the dilution factor is greater.

Therefore, the optimum loading might range between 5 and 15% of dry matter. As previously mentioned, the loading of TS is highly dependent on the duration of the enzymatic hydrolysis (Figure 11). For short incubation periods, the lower the dry matter addition the higher the yield. The 4-hour enzymatic hydrolysis exhibits a 2-fold increment in both sugars and protein for a loading of 5% TS against 15% TS.

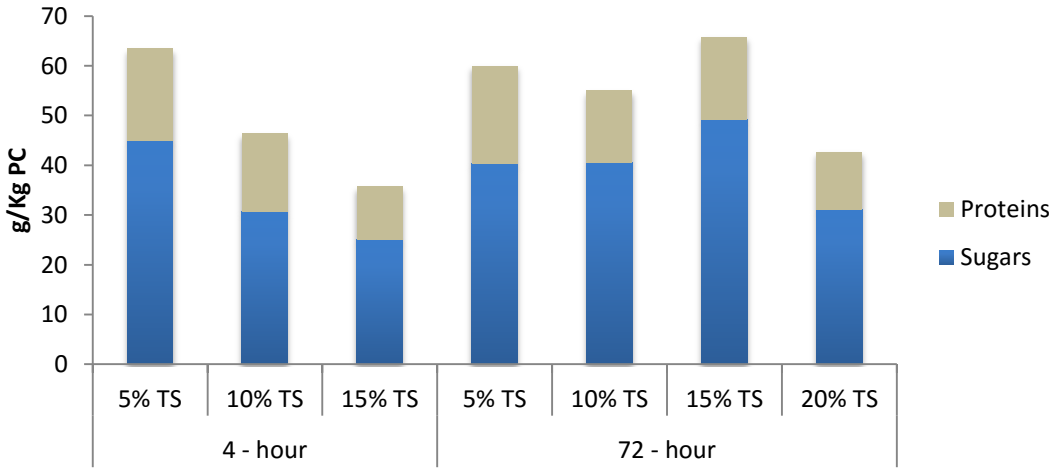

Figure 11. Comparison of the protein and sugar obtained for a 4 and 72-hour incubation, including different loadings of TS. The enzyme added was always 3%.

Conversely, the 72-hour enzymatic hydrolysis displayed different yields. The highest sugar releasement was obtained with a loading of 15% TS. Even though the concentration of dry matter is high, the 72 hours seems to be enough to allow the enzymes to break down the cellulosic material. Nevertheless, the addition of 5 and 10% TS exhibited considerable yields.

Regarding the proteins, low dry matter loadings seem to be optimal. In both incubation lengths the maximum protein obtained was at 5% TS loading. There is an important correlation between dry matter loading and protein released exhibiting that the lower the addition of TS, the higher the protein obtained, with a R^2=0.97 and R^2=0.7 for the 4-hour and 72-hour enzymatic hydrolysis, respectively (Figure 12). Conclusively, the protein release is mainly affected by the dry matter, rather than by the incubation period.

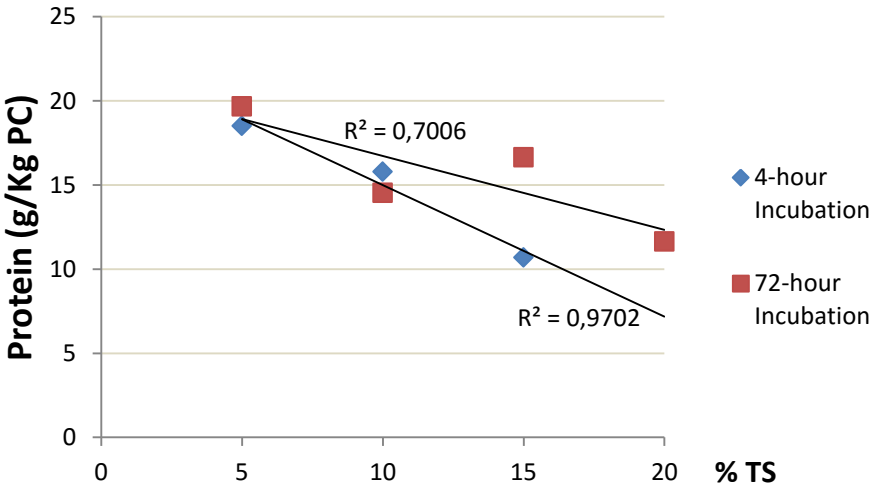

Figure 12. Correlation between protein released and loadings of TS. It can be appreciated how low loadings of TS bring about the greatest release of proteins.

Volume of treatment

Finally, an extra vial was prepared in order to test the reproducibility of the results in a lab scale using the same vials (117 cm^3) but with 40 mL of working volume instead of 20 mL. The HPLC results reflect a decline in sugar and protein production. A possible explanation might be an irregular mix during the incubation in the thermoshaker, along with likely errors with the dilution factors. As a consequence, in order to test different pretreatment treatments in a lab scale with greater working volumes, the size of the vials employed must be increased.

3.2.2 Pretreatment of the Press Cake of Red Clover

Although in the preparatory essays the 4-hour enzymatic hydrolysis presented noted results, we decided on a 72-hour hydrolysis as it was the time recommended by the supplier according to their assays. In spite of the fact that the effect of

Kanamycin remained unclear, this antibiotic was included in all the treatments in order to prevent any bacterial growth. As previously mentioned, the most important parameters were the amount of TS and the amount of enzyme added, and consequently, these were the factors selected for the central composite design.

The best results were obtained in the T3, with high dry matter loading (24,6%) and high enzyme addition (4,27%), recovering up to 22,7 and 11,9 grams of sugars and proteins respectively. The overall figures are shown in Table 7.

Generally, the results exhibited in this enzymatic hydrolysis are fairly inconsistent with the preparatory essays, and with the results reported by other authors. The main reason that might explain the inconsistency in the results is the growth of several unidentified moulds after the enzymatic hydrolysis. Unlike the previous enzymatic hydrolysis, in this batch the biomass in most of the glass bottles a mantle of fungi was found, but it remains to be investigated if the fungi were present in the biomass as endophytic or epiphytic microbes, or if some spores from common environmental moulds contaminated the biomass during the experimental setup.

The main drawback of this unexpected fungal growth is the distortion of the results. This enzymatic hydrolysis should be characterized as a failure, as the amount of released sugars was well below the expected results, as a consequence of the fungal growth. The presence of considerable amounts of some secondary metabolites derived from sugar degradation by microbes (i.e. lactic acid, acetic acid) confirms this assumption. Further research must be conducted in order to evaluate the real yield, including fungicides to prevent fungal growth.

Furthermore, it is worth mentioning that the results might be biased owing to the dilution factor applied in the water recovery. Since some treatments contained high loads of dry matter, and very little water was added for the enzymatic hydrolysis, the fact of referring the sugars and protein released to this small initial amount of water after subtracting the dilution factor might give rise to deformity in the results.

As a consequence, all the statistical analysis, including correlations with factors and optimal parameters were highly inconsistent, and thus we decided not to include these results in this master thesis.

g/Kg PC	Citric acid	Glucose	Xylose	Arabinose	Lactic acid	Glycerol	Acetic acid	1,2 Propanediol	Total sugars	Total protein	%TS	% enzyme
T1	0,54	3,28	1,58	0,00	1,48	0,00	6,75	2,72	**4,86** (1,35)	**3,65** (0,04)	8,37	4,27
T2	0,61	2,61	1,46	0,00	0,00	0,00	3,62	0,32	**4,07** (1,14)	**8,02** (0,52)	8,37	0,73
T3	1,06	13,65	8,66	0,46	1,04	0,00	3,18	0,53	**22,77** (5,26)	**11,90** (0,76)	24,63	4,27
T4	0,80	6,47	3,50	0,17	2,42	0,09	2,44	0,64	**10,15** (5,40)	**9,05** (1,41)	24,63	0,73
T5	1,40	10,83	7,50	0,41	1,83	0,23	9,11	0,55	**18,74** (10,35)	**9,12** (2,58)	16,50	5,00
T6	0,63	4,86	3,25	0,00	6,35	0,23	1,20	0,00	**8,11** (4,34)	**7,61** (0,84)	16,50	0,00
T7	0,00	2,11	0,92	0,00	0,00	0,00	0,29	0,00	**3,03** (2,20)	**10,57** (0,30)	5,00	0,00
T8	0,76	8,29	7,57	0,00	1,15	0,30	0,90	0,00	**15,86** (1,78)	**10,40** (0,60)	28,00	0,00
T9	0,93	6,69	4,33	0,21	3,44	0,12	5,56	1,00	**11,23** (7,06)	**10,04** (2,23)	16,50	2,50

Table 7. Evaluation of the sugars, proteins and acids released in the different treatments analysed in the integral enzymatic hydrolysis of red clover. The numbers in the parenthesis indicate the standard deviation.

3.3 Conclusions

The pretreatment of the PC seems to be a promising technology for the production of sugars, proteins, organic acids. However, great yields were exclusively obtained in the “preparatory assays” with clover grass.

Thermal pretreatment was the least influential method of pretreatment, while the addition of enzymes was the most decisive. The loading of TS, and the amount of enzyme included were the most important factors. However, low amounts of TS might release a considerable amount of sugars and proteins when the incubation period is short, with the pertinent economic improvement by reducing energy costs. But long incubation periods are required when processing batches with high amounts of dry matter. If the main goal is recovering proteins, low dry matter loadings seemed to be optimal.

The integral enzymatic hydrolysis of the red clover clearly failed as a consequence of the fungi growing inside the vials. The sugars and proteins recovered in this batch were considerably low as to regard this process as economically viable. Although the effect of kanamycin remained unclear, we conclude that bactericides (e.g. Kanamycin) and fungicides must be included in the experimental set-up in order to get trustworthy figures.

4 Chapter 4 – Silage: Characterization and evaluation

Ensiling is a method for preserving all year round the biomass obtained from seasonal crops. Besides, it has been studied as a method for improving biomass yield from grasses and for its capacity to increase enzymatic convertibility of cellulosic material into fermentable sugars. In this project, we aimed at studying the silage as a preservation method for the fresh red clover and its press cake, and to evaluate the biogas yield of the ensiled biogas. Precisely, in this chapter we focus on investigating the relations of two factors; the effect of biomass composition (PC or fresh), and the addition of addition of LAB inoculum for obtaining good quality silage.

4.1 Materials and methods

4.1.1 Ensiling conditions and sampling

The silage was prepared with two different materials: fresh crop and press cake (characterization in Table 1, Chapter 2). Both biomasses were collected from the same harvest that was supposed to contain solely red clover (*Trifoilum pratensis*), but different species were found after harvesting, e.g. ryegrass (*Lolium sp.)* and fescue (*Festuca sp.).* Therefore, the silage does not come from a monospecific crop. However, the other species present in the cropland are suitable for silage (Moseley, Jones et al. 1988).

Figure 13. Preparation of silage bags. Around 200 g of fresh material were included in the bag after homogenization in the box on the right.

The biomass was allowed to dry inside of a barn located in the facilities of Aarhus University in Foulum, Denmark (56°29'24.6"N 9°35'16.0"E) by spreading the biomass on the ground for two days after harvesting in order to decrease the moisture of the substrate. For the two biomasses, two treatments were selected

for ensiling, one with only biomass and another with biomass and inoculum. The inoculum consisted of the liquid fraction of brown juice freshly produced containing homofermentative LAB, *Lactobacilus salivarius* (characterization shown in Table 1, Chapter 2). The biomass containing the inoculum was properly mixed in a big plastic box at a rate of 100 ml of brown juice per kilogram of the pertinent biomass (Figure 13).

Silaging was performed in transparent plastic bags for vacuum (30 x 40 cm) containing 200 and 300 g of fresh red clover and press cake red clover respectively. Bales were vacuum packed with a vacuum packaging machine (Webomatic, Germany) (Figure 14). Overall 48 bales of silage were prepared according to Table 8.

	FRESH - RED CLOVER		**PRESS CAKE - RED CLOVER**	
Sampling dates	*Without inoculum*	*With inocula*	*Without inocula*	*With inocula*
Day 5	2 bales 2 x 200g	2 bales 2 x 200g	2 bales 2 x 300g	2 bales 2 x 300g
Day 30	2 bales 2 x 200g	2 bales 2 x 200g	2 bales 2 x 300g	2 bales 2 x 300g
Day 55	2 bales 2 x 200g	2 bales 2 x 200g	2 bales 2 x 300g	2 bales 2 x 300g
Day 80	3 bales 3 x 200g	3 bales 3 x 200g	3 bales 3 x 300g	3 bales 3 x 300g
Day 200	3 bales 3 x 200g	3 bales 3 x 200g	3 bales 3 x 300g	3 bales 3 x 300g

Table 8. The Day refers to the time-point after silage preparation in which the silage is sampled in order to monitor the changes in the composition of the silage with the passage of time.

After silage preparation, the bales were taken to the laboratory and kept in dark conditions and room temperature. Samples were analysed the days 5, 30, 55, 80 and 200 after ensiling and were frozen prior the chemical analysis. Samples of fresh red clover and press cake were included in the analysis a control of the different parameters at time=0. The samples collected after 200 days of storage are not included in this project due to time limitations, but will be considered within the organofinery project.

4.1.2 *Chemical analysis*

Total Solids (TS) and volatile solids (VS) were determined according to APHA Standard Methods (2005). It is well known that the content of true dry matter (TrDM) in silage is greater than the DM estimated by using oven drying (McDonald and Dewar 1960), and consequently, volatility coefficients for the determination of TS and VS were included as reported by Porter and Murray (2001). Thus, the true TS and VS were estimated by adding 37.5 % of the lactic acid, 97.5 % of the ethanol and 89.2 % of the VFAs measured in the corresponding bales.

Figure 14. Vacuum packing machine with the bales prior (left picture) and after (right picture) vacuum packaging.

Aliquots of roughly 1.6 g DM biomass from thawed silage were extracted in 40 ml MilliQ water containing 0.1 mg/ml of kanamycin to impede microbial activity during water extraction. The samples were shaken in closed glass bottles for 2 hours at 150 rpm and 25 ºC (Ambye-Jensen, Johansen et al. 2013). The liquid extracted was utilized to measure the pH and determine the content of soluble sugars, lactic acid, ethanol and volatile fatty acids (VFAs). Duplicate samples of each bale were prepared by adding 10% (v/v) of H_2SO_4 2M for HPLC analysis and 10 % (v/v) of HPO_4 17% for estimation of VFAs. Samples were filtered through 45 µm filters into standard chromatography vials. Analyses were carried out as described in chapter 2. Finally, the concentration was estimated considering the dilution factor.

4.2 Results and discussion

4.2.1 *Characterization and performance of the silage in terms of DM and organic matter.*

The dry matter content of the fresh red clover and the press cake were 182 and 244 g/Kg wet weight respectively (Table 9). Consequently, the dry matter content of the fresh red clover was considerably low for a good silage performance, despite the fact that it was allowed to dry for 48 hours after harvesting. According to McDonald and Henderson (1991), the dry matter content of a good quality silage ought to be in the range of 250/300 g TS/kg, and therefore the PC seems to fit

		TS (g/Kg)	VS (g/Kg)	TS corrected (g/Kg)	VS corrected (g/Kg)	pH	VS gain (%)
Fresh red clover	0	182,66 (46,18)	151,27 (34,94)	187,49 (46,18)	156,11 (34,94)	5,77 (0,12)	0,00
	5	209,58 (53,47)	172,75 (41,24)	220,87 (55,65)	184,04 (46,49)	4,76 (0,03)	17,90
	30	189,18 (37,81)	142,15 (21,51)	200,86 (49,40)	153,83 (29,05)	5,38 (0,09)	-1,45
	55	196,28 (56,46)	135,14 (24,4)	210,70 (68,01)	149,56 (26,94)	5,52 (0,13)	-4,19
	80	193,43 (14,06)	156,18 (14,15)	210,17 (12,53)	172,92 (13,1)	5,04 (0,12)	10,77
Fresh red clover + Inoculum	0	151,97	130,09	156,81	134,93	5,77 (0,12)	0,00
	5	164,60 (8,03)	132,15 (3,91)	175,18 (0,68)	142,73 (1,74)	4,64 (0,08)	5,78
	30	161,82 (21,67)	126,98 (22,00)	171,13 (17,82)	136,28 (19,67)	5,49 (0,04)	1,01
	55	156,20 (7,92)	122,85 (12,9)	171,79 (3,73)	138,44 (5,95)	5,52 (0,1)	2,60
	80	167,99 (6,95)	133,68 (12,22)	181,70 (5,11)	147,40 (11,96)	5,41 (0,02)	9,24
PC red clover	0	244,34 (5,63)	200,87 (3,12)	249,61 (5,63)	206,14 (3,12)	6,04 (0,79)	0,00
	5	262,72 (10,15)	217,06 (6,89)	273,50 (5,31)	227,84 (4,41)	4,72 (0,01)	10,53
	30	241,33 (15,94)	196,90 (13,89)	252,53 (19,25)	208,10 (16,08)	5,15 (0,26)	0,95
	55	238,14 (13,33)	194,51 (6,47)	252,55 (15,3)	208,91 (6,20)	5,30 (0,06)	1,35
	80	246,95 (7,05)	202,02 (5,54)	260,92 (6,59)	215,99 (5,98)	5,31 (0,28)	4,78
PC red clover + Inoculum	0	228,71	186,61	233,97	191,76	6,04 (0,79)	0,00
	5	245,98 (9,23)	196,23 (7,30)	256,66 (6,29)	206,91 (8,24)	4,69 (0,04)	7,90
	30	219,88 (5,96)	178,03 (6,95)	230,60 (5,20)	188,75 (7,82)	4,94 (0,02)	-1,57
	55	223,77 (9,99)	183,16 (8,20)	238,33 (7,39)	197,71 (6,14)	5,20 (0,08)	3,11
	80	232,48 (6,26)	190,86 (6,70)	247,22 (0,58)	205,60 (1,29)	5,25 (0,02)	7,22

Table 9. The figures in brackets refer to the standard deviations.

better for the preservation of the biomass. Furthermore, silage with a TS content below 200 gTS/kg is more susceptible to the growth of the undesired clostridia (Wieringa 1958).

This low dry matter content is more accentuated in the case of the treatment with inoculum. After mixing the biomass with the lactic acid bacteria the TS content of the fresh biomass barely reached 150 gTS/kg. On average, the DM content of the fresh red clover inoculated was 16,8% lower than the treatment no inoculated. Concerning the press cake, the dry matter content only decreased 6,4% after adding the inoculum.

However, when the dry matter or volatile solids are estimated by drying, some volatile compounds are lost and therefore the TS and VS content must be corrected for the loss of volatile compounds in order to avoid and underestimation of the methane yield (Huida, Väätäinen et al. 1986). After the corrections, the TS increased on average 6,3% while the VS rose 7.9%. The determinations of TS and VS for the different treatments throughout the silaging are displayed in Table 9.

In all the treatments the content of the VS increased (Table 9) especially at day 5. Afterwards, the VS content decreased, and in some cases there was a loss of VS in comparison with non-ensiled biomass (30 and 55 days of ensiling), but finally the VS content increased in the four treatments what hints at a promising preservation of the methane potential. Anyhow, in those cases in which losses in VS are found, these losses are lower than the losses in energy, as the fermentation products formed during ensiling have a higher gross energy (GE) value than the original substrates (McDonald and Henderson 1991).

4.2.2 Production of acids and evaluation of the silage performance

Ensiling entails the production of organic acids and a drop in pH that in consequence prevents the growth of yeasts bacteria and fungi, which may otherwise decompose the carbohydrate structure in the biomass (Buxton, Muck et al. 2003). According to this definition, the performance of the silage was successful for the four treatments analysed, although to some extent. Generally, the pH decreased according to organic acid production, mainly congruent with the production of lactic acid, but also due to acetic acid production.

The content of lactic acid in the ensiled fresh biomass reached more than 7% of DM, which is in the same range than several studies of ensilaging of grasses (Ambye-Jensen, Johansen et al. 2013). In the case of the PC the concentration only reached 4% of the DM, probably due to a lower content of water soluble carbohydrates (Appendix 2, Figure 15). The pH decreased to around 4.6 in every treatment, preventing thus the growth of undesired microorganisms like

enterobacteria and clostridia, at least at the beginning of the process, since these microorganisms are highly affected by a decrease in pH (McDonald and Henderson 1991), although some of them can survive as spores (Elferink, Driehuis et al. 2000).

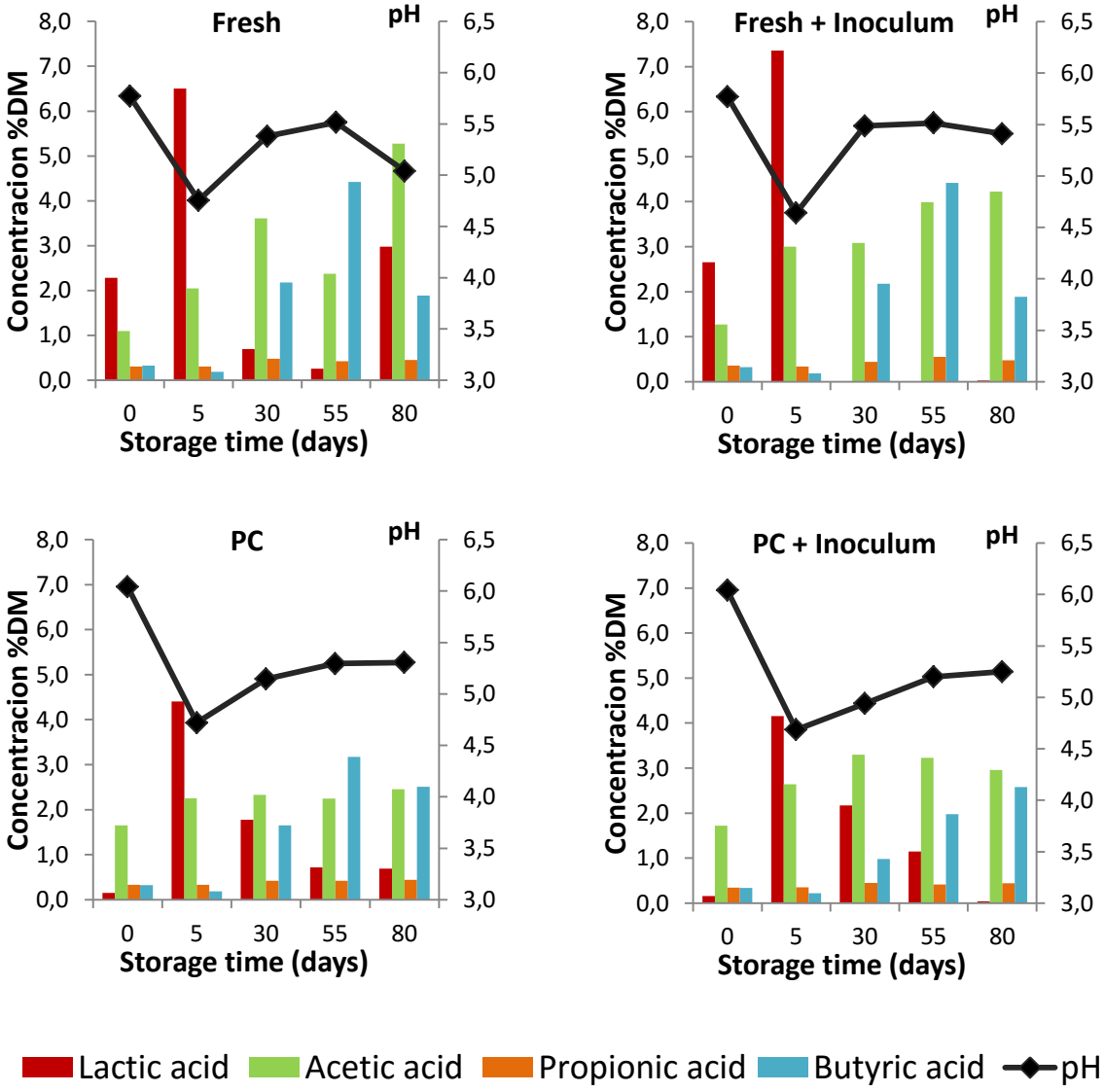

Figure 15. These charts show the concentration of the different acids produced during the storage time. Interestingly, some acids were already presents at the onset of the ensilaging, what suggest that an initial fermentation took place inside of the biomass prior to packaging.

Kung and Shaver (2001) reported that a good silage with 30 % DM content must present a lactic acid content between 6 and 10 % of DM, giving rise to a pH of around 4,3-4,7. This statement is in accordance with our results, suggesting a good performance of the process at the first stage.

Furthermore, the pH of the fresh ensiled biomass might have been a bit lower in comparison to the ensiled press cake, considering the lactic acid content in the fresh was 7% DM whereas only 4% DM was achieved in the press cake. It hints at the higher buffer capacity of the fresh biomass. This fact is in harmony with the higher amount of crude protein presented in the fresh biomass, since high protein content is known to facilitate buffer capacity towards silage fermentation (Muck, O'Kiely et al. 1991).

The lactic acid bacteria can be classified as obligate homofermenters, facultative heterofermenters and obligate heterofermenters based on their sugar metabolism (Elferink, Driehuis et al. 2000). The most desired bacteria are the obligate homofermenters, as they produce the highest lactic acid production. According to our results, it seems as if the fermentation was mainly carried out by facultative or obligate heterofermenters due to the great amount of acetic acid production that reached a concentration of 3 and 5% DM for the ensiled PC and fresh respectively. The obligate heterofermenters degrade the hexoses to equimolar amounts of lactic acid, CO_2 and acetic acid and /or ethanol (Hammes and Vogel 1995), and our results are in harmony with this definition, supported also by the fact that in the silage bags, even though they were tight packed, considerable amounts of air appear through the silage process, what might be due to the CO_2 produced by these bacteria.

After the fermentation phase that causes the decrease in pH, the silage enters a stable phase in which most of the microorganism that participated in the fermentation gradually reduce in number (Elferink, Driehuis et al. 2000) and some other microorganisms start to proliferate. After 30 days of storage, large amounts of butyric acid were generated inside the bales in all the treatments analysed. The concentration of butyric acid overcame 5% DM basis for the ensiled fresh grass and 3% of the DM content for the PC (Figure 15 and 16). These figures make the silage a typical “clostridial silage”, since this is the term coined for those silages containing more than 5 g/kg dry matter and a pH over 5 (Vos 1966).

Clostridium tyrobutyricum, aside from breaking down the carbohydrates, degrade the lactic acid to butyric acid, H_2 and CO_2 (Elferink, Driehuis et al. 2000). The lactic acid in the bales decreased consistently as it was transformed into butyric acid, until disappearing completely in some treatments. This effect is a symptom of a badly preserved silage and is greatly undesired because the lactic acid produced by the lactic acid bacteria is lost, triggering an increase in pH that might lead to the activation of more undesired microorganisms and a significant energy loss due to the release of hydrogen (Kreuger, Nges et al. 2011). In addition, clostridia reduce the feeding value of the silage and impairs milk quality.

This undesired effect was due to the low dry matter content of the biomass. Clostridia are very susceptible to low water availability content (Wieringa 1958) but the TS content of the fresh biomass was below 20% w/w. According to Pakarinen, Lehtomäki et al. (2008) total dry matter contents greater than 30% are preferred in order to restrict clostridia growth. Thus, the low dry matter content of the fresh biomass made the feedstock not suitable for ensilaging, and the biomass should have been let to wilt to a larger extent. In the case of the PC, the TS content seemed to be more appropriate for silaging, but the fact of containing low amounts of carbohydrates to ferment and a not very acidic pH allowed the growth of clostridia (Kalač 2011).

Therefore, a successful method to inhibit the growth of clostridia consists of allowing the biomass to wilt until obtaining DM contents between 30 and 50% DM. However, in high dry matter silages, the presence of lactic acid bacteria could become a limiting factor. Hence, another solution is the addition of additives. The most effective fermentation inhibitors of clostridia appear to be additives based on formic acid, hexamethylene and nitrite (van Schooten, Corporaal et al. 1989).

Effect of the inoculum

The effect of adding inoculum did not improve the performance of the silage. For the two feedstocks, the profile of acids produced after the fermentation was rather similar, as well as the pH (Figures 15 and 16). There was even a higher production of lactic acid in the ensiled non-inoculated fresh biomass at 80 days of the process in comparison to the inoculated one.

Since lactic acid bacteria ferment the sugars to lactic and acetic acid, it resulted fairly surprising that the inoculum did not affect the acid production of the ensiling regardless of the type of lactic acid bacteria within the inoculum. A likely explanation for this inconsistent effect is that the natural epiphytic organisms presented on the plant material dominated the fermentation processes to a great extent, and therefore the effect of the acids produced by the inoculated bacteria remains unnoticeable. Similarly, the inoculated amount of microorganisms may not have been enough to dominate the fermentation process and a greater amount of inoculum should be considered.

In a similar essay (Ambye-Jensen, Johansen et al. 2013), the addition of inoculum had no effect on the ensiling performance, but it was assumed that the organic acid profile after ensiling was dependent on the composition of the biomass and the DM content, instead of the inoculum. Therefore, a further essay including biomass with various DM contents and different amounts of inoculum should be carried out to in order to investigate the optimal amount of inoculum for any given DM content.

Enzymatic hydrolysis

Apart from the traditional usage of silage as animal feedstock all year round, ensilaging constitutes a way of enzymatic pretreatment of the biomass, that could become an advantage in downstream processes, for instance as a biological pretreatment for the production of biochemicals and cellulosic biofuel. More than half century ago, Dewar, McDonald et al. (1963) reported that during ensiling, hemicellulose from rye grass was hydrolysed initially by several enzymes extracted from the grass and during longer storage by means of acid hydrolysis at pH 4. Several reports have recently confirmed this finding (Chen, Sharma-Shivappa et al. 2007) suggesting that the organic acids produced during ensiling bring about a gentle hydrolysis of lignocellulosic structures, increasing thus the access of the cellulosic enzymes to the cellulose.

According to our results, the concentration of acids inside the bales did not increase throughout the storage period what propose that only water-soluble sugars already present at the onset of the silaging were utilized as fermentation substrates. A possible reason that explains the absence of biological pretreatment is the high recalcitrance of the biomass due to the maturity of the grass when harvested (Ambye-Jensen, Johansen et al. 2013). Exclusively in the ensiled fresh grass without inoculum the overall content of organic acids increased after 80 days of storage, perhaps as a consequence of an enzymatic hydrolysis that released some sugars and were converted into lactic acid. The addition of silage additives containing lactic acid bacteria along with a proper enzyme cocktail (cellulase, pectinase and xylanase) might boost the liberation of soluble carbohydrates and thereafter the lactic acid formation (Pakarinen, Lehtomäki et al. 2008).

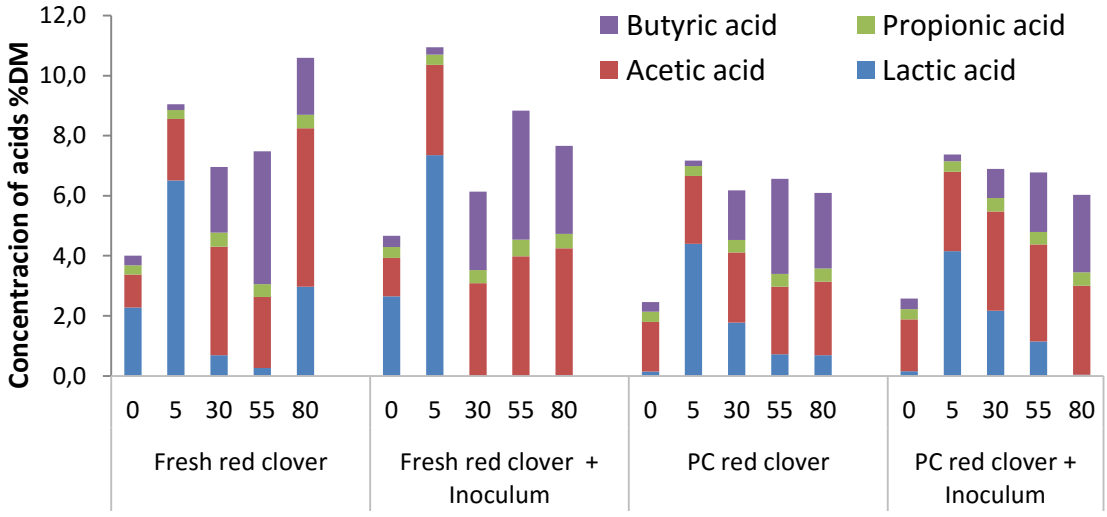

Figure 16. Acid profiles of the different biomasses collected after several days of storage.

4.3 Conclusions and future prospects.

Ensiling of fresh red clover and the press cake was only successful to some extent. No aerobic spoilage took place, and the production of lactic and acetic acid at the beginning of the process resulted in a low pH that inhibited the growth of other spoilage microorganisms. However, the low dry matter content of the feedstock in conjunction with a low concentration of water soluble carbohydrates gave rise to the proliferation of clostridia. Although the presence of these bacteria and its fermentation products impair the milk and the value of the silage as a feedstock, it can have a positive effect if the silage is intended for the production of biogas as a preservation method. The analysis of the preservation of the methane potential is shown in the next chapter.

The effect of the inoculum remained unnoticeable. Greater amounts of bacteria should be included in the inoculum to obtain higher fermentations. Besides, identifying the bacteria included in the inoculum (brown juice), as well as the initial epiphytic bacteria might help to develop a more suitable inoculum.

Further research is needed to optimize the quality of the silage. Feedstock harvested at an earlier maturity stage might improve the silage quality. Finally, the addition of additives containing sugars and enzymes would increase the sugars released and consequently the lactic and acetic acid concentration.

5 Chapter 5 – *Silage as a method of preserving the methane potential*

Besides preserving the feedstock for animal feeding all year round, and for enzymatic pretreatment of the biomass, ensilaging has been successfully studied as a substrate for biogas production (Lehtomäki 2006; Pakarinen, Lehtomäki et al. 2008). For instance, ensiling of hemp increased methane production by more than 50% compared to fresh hemp (Pakarinen, Lehtomäki et al. 2008). Consequently, ensiling it is a viable option for preserving the methane potential of the crop during the whole year. Although several authors have investigated the use of ensiled fresh biomass as a preservation method, the utilization of the ensiled press cake for biogas production have yet to be investigated. We aimed at evaluating the ensiled fresh red clover and press cake for the preservation of the methane potential, as well as the effect of adding inoculum to the silage.

5.1 Materials and methods

5.1.1 Substrates and inoculum

The experiments with anaerobic digestion were oriented towards the evaluation of silage as a preserving method for maintaining methane potential and thus, the biomass employed was the ensiled fresh and press cake red clover collected 55 and 80 days after the onset of the silaging, for both treatments with and without inoculum. In order to determine the methane potential of the silage against not preserved biomass, fresh red clover and press cake collected right away after harvesting and shredding were included in the experiments. The chemical composition of the substrates is presented in Table 1, chapter 2).

The inoculum was supplied by Hashøj biogas plant (West Sealand, Denmark). No substrate adaptation was performed. Nevertheless, prior the set-up of the batches, the inoculum was activated by adding some biomass to the inoculum and storing the inoculum for one month at 37 ªC and darkness. The content of total solids (TS), volatile solids (VS) and pH of the inoculum is shown in Table 10.

TS (g/Kg)	VS (g/Kg)	pH
37,72 (0,16)	20,07 (0,09)	8,3

Table 10. Composition of the inoculum. The number between parenthesis refers to standard deviation.

5.1.2 Experimental set-up

Overall eleven batches were prepared. Eight of them with biomass from silage, two batches with biomass collected after harvesting or processing, without allowing to

dry (notice that is not the same sample t=0 as in silage, as those samples were allowed to dry), and one batch containing solely inoculum. Experimental design of the batches is displayed in Table 11.

Batch	Day of sample collection (silage	Biomass	Biomass inoculated with LAB
1	0	PC	
2	0	F	
3	55	F	
4	55	F	*
5	55	PC	
6	55	PC	*
7	80	F	
8	80	F	*
9	80	PC	
10	80	PC	*
11		Blank	

Table 11. The asterisks refer to the experiments with silage supplied with an inoculum of bacteria. The day 0 (zero) hints at the collection of samples immediately after harvesting or shredding, but not to the bales of silage regarded as time zero (t=0), since that biomass was allowed to dry for 2 days.

The batches were tested in triplicates in 117 cm^3 vials intended to estimate the content of methane produced by digesting the biomass anaerobically following the procedure described by Biswas et al. (2012). Batch vials were filled with the pertinent amount of substrate and 30 ml of inoculum at a substrate/inoculum (So/Xo) ratio of 1 on VS basis (Figure 17). Blanks vials contained only 30 ml of inoculum and were used as a control. After filling with the corresponding biomass and inoculum, batch vials were flushed with N_2/CO_2 (80%/20%) prior to closing air tight with rubber stoppers and aluminium crimps. No adjustment of pH was considered for the batch set-up. The vials were incubated in darkness under mesophilic condition 37 ± 2 ºC for 45 days. The production of methane was monitored periodically by gas chromatography (GC).

The composition of the biogas produced in the headspace of the vials was analysed using a gas chromatograph (SRI GC model 310), equipped with a Porapak Q column of 182.88 cm length and 2.1 mm i.d. The injector and detector temperatures were 80°C. The carrier gas was Nitrogen with a pressure of 196 kPa. A mixture of 30% CH_4 and 70% N_2 was employed as a standard gas mixture for GC.

The yield of biogas production (mL CH_4 $g^{-1}VS_{added}$) was estimated by measuring the methane concentration in the headspace of the vials and applying the following equation (Biswas, Ahring et al. 2012), where the methane production in the blanks

(B) is subtracted to the methane production in the batch vials containing biomass (S):

$$CH_4 yield_S = \frac{(CH_4\,\% \times V_{headspace})_S - (CH_4\,\% \times V_{headspace})_B}{g\,V_{added,S}}$$

The excess of pressure was released in the batch vials showing overpressure and the methane content was estimated by measuring before and after gas releasement for calculation of the cumulative methane yield.

Figure 17. Batch vials containing the biomass and the inoculum.

5.2 Results and discussion

5.2.1 Performance of the anaerobic digestion

According to our results, the methane yields obtained for the two no-ensiled biomasses tested and the ensiled feedstock were rather similar, confirming the suitability of ensilaging as a preservation method of the methane potential.

Fresh red clover against press cake:

The anaerobic digestion of the fresh red clover yielded 377 mL CH_4/g VS after 45 days, what it is the maximum methane potential obtained in this essay. The methane yield of the press cake not ensiled was 1.4-fold lower than the fresh (277 mL CH_4/g VS) (Appendix 1).

The reason for this lower methane yield might be a lower amount of organic matter present in the press cake since a great portion of organic matter is recovered in the green juice after the screw pressing, and hence the decrease in the methane yield. Furthermore, lignin is a recalcitrant compound towards anaerobic degradation, impairing the enzymatic attack of the cellulose (Haug 1993). Haug (1993) proposed that lignin molecules could reduce the surface area available to enzymatic penetration and activity resulting in a lower cellulose bioavailability and convertibility to sugars. The fact that the press cake presented a

higher lignin content might also have an impact on the lower methane yield of the press cake (Molinuevo-Salces, Larsen et al. 2013).

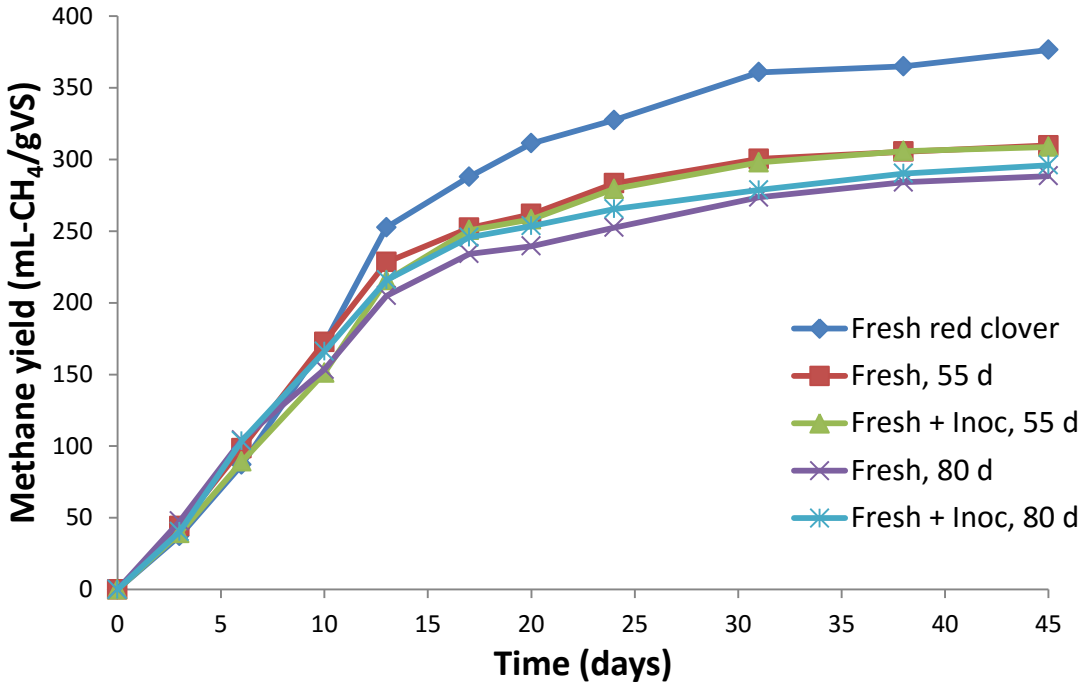

Figure 18. Evolution of the methane production for the fresh no-ensiled red clover and the ensiled red clover throughout the 45 days of anaerobic digestion. The numbers 55 and 80 on the legend refer to the amount of days that the silage bales were stored.

Effect of ensiling

Ensilaging of all the treatments with fresh red clover gave rise to lower methane yields in comparison to fresh red clover no ensiled (Figure 18). Concretely, ensiled fresh grass resulted in the range of 17-24 % lower methane production for the different treatments. This lower yield is however not surprising. In spite of the fact that ensiling is believed to improve methane yields of many commonly used crops, the efficiency depends on plant species (Pakarinen, Maijala et al. 2011). Pakarinen, Maijala et al. (2011) reported lower methane yield testing also ensiled leguminous plants in comparison to fresh material, while the yield of ensiled hemp was up to 50 % higher respect to the fresh hemp.

In the case of press cake, the 55-day ensiled biomass without inoculum increased the methane potential with respect to the no ensiled press cake. In contrast, the ensiled press cake with 80 days of storage showed no increase, but the methane yield was rather similar to the no-ensiled press cake (Figure 19). Therefore, the methane potential of the press cake was efficiently preserved by means of silage. Besides, no energy loss was observed during ensilaging of press cake despite the formation of butyric acid within the bales.

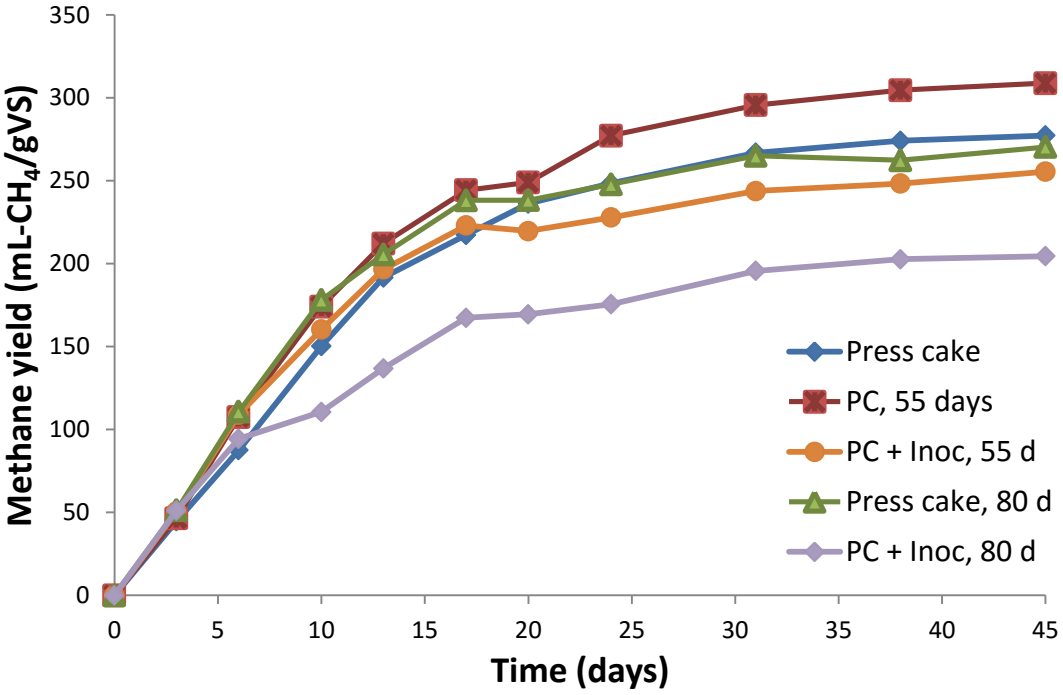

Figure 19. Evolution of the methane production for the raw no-ensiled press cake and the ensiled press cake throughout the 45 days of anaerobic digestion. The numbers 55 and 80 on the legend refer to the amount of days that the silage bales were stored.

A possible explanation for not improving the methane yield of the ensiled fresh red clover but improving a bit the methane yield of the ensiled press cake might be ground on the composition of the materials. Protein is a better substrate for methane production than carbohydrates (Pakarinen, Lehtomäki et al. 2008) and consequently, the fresh red clover crop which has a greater protein content may not benefit from loosening the structure during ensiling as much as the more cellulose-rich press cake (Pakarinen, Maijala et al. 2011).

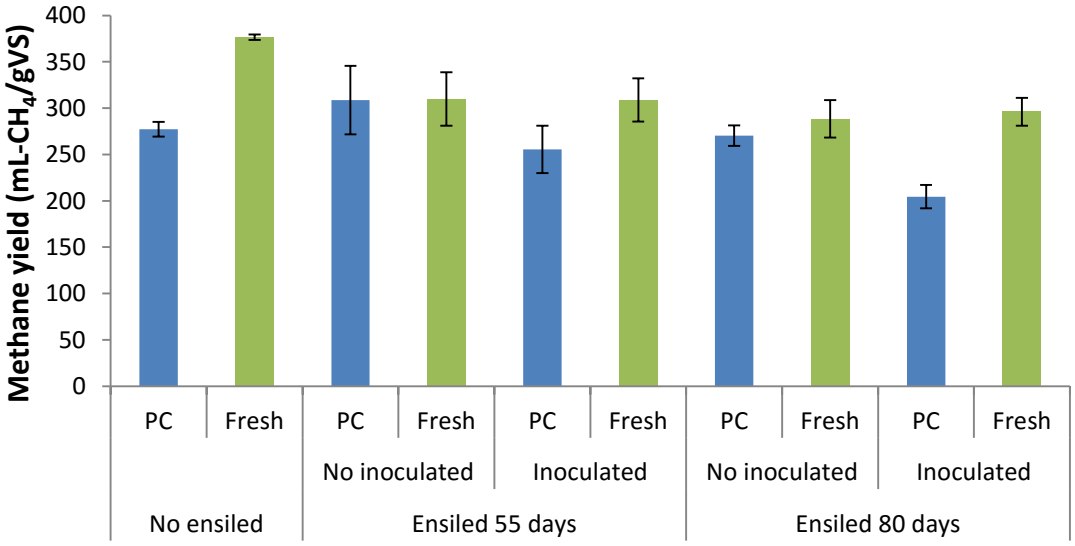

Figure 20. Methane production of all the treatments included after 45 days of anaerobic digestion.

Effect of inoculum (LAB)

Concerning the ensiled fresh red clover, the methane yields in the treatments inoculated with LAB were in the range of the no inoculated, or slightly higher (3% greater in the silage stored for 80 days)(Figure 20, Appendix 1). Thus, the effect of inoculum on the fresh grass seems to be neutral. On the other side, the addition of inoculum to the press cake was detrimental for the production of biogas. The methane yields for the inoculated treatments in comparison to the no-inoculated ones were 17 and 24% lower for the 55-day and 80-day storage silage respectively. Consequently, the addition of inoculum to the press cake must be reconsidered and further analysed. This disparity with the inclusion of biological additives have already been reported in the literature (Neureiter, dos Santos et al. 2005). As a case in point, Vervaeren, Hostyn et al. (2010) reported that biological additives, such as lactic acid bacteria or hydrolytic enzymes, had inconsistent effects on methane yields, mainly because of suboptimal ensiling methods.

Lastly, no inhibition nor lag phase took place for any of the treatments, including inoculated and no inoculated silages, and the methane yield produced within the first 17 days of anaerobic digestion for all the treatments ranged between 76 and 87% (Figures 18 and 19)

5.3 Future prospects and research

Further research should evaluate the effect of the substrate to inoculum ratio (S/X). For instance, a substrate to inoculum ratio of 0.5 rather than 1 might improve the performance of the anaerobic digestion, especially in the case of the silage containing the biological additive. Already in the scope of the organofinery project, (Santamaria 2015) reported substrate inhibition in previous batch tests with leguminous plants when the substrate to inoculum ratio was 1 instead of 0.5.

It would also be interesting to study if the methane potential of the ensiled biomass is maintained or even increased after 200 days of anaerobic digestion.(Ahn, Smith et al. 2010). Pakarinen, Lehtomäki et al. (2008) reported a consistent increase of the methane potential after 200 days of anaerobic digestion.

Finally, ensiling with additives like formic acids or urea pretreated biomass might yield greater amounts of methane. For instance, Pakarinen, Lehtomäki et al. (2008) reported that the use of formic acid in ensiling of maize enhanced the methane yields by 16%.

5.4 Conclusions

The methane potential of the press cake was efficiently preserved by means of silage, and therefore, silage might be an appropriate storage method for the press cake before its utilization as a substrate for biogas production. However, ensilaging

fresh red clover did not improve the methane yield in comparison to the no-ensiled feedstock and additives should be used to implement this preservation method in this grass.

Consequently, the effect of ensiling a crop, when compared to the pertinent fresh crop on the specific methane yield, is still not clear. Further research is needed testing different fresh crops and press cakes and including several additives to credibly regard silage as a viable method for improving biogas production.

6 Conclusions

The chemical characterization of the press cake suggests this material for biogas production, feed for ruminants or even for thermal combustion or bioethanol since it contains a large percentage of lignocellulosic material in conjunction with the fiber-bounded protein. The yield of the mechanical fractionation providing a mass balance of the income and outcome products should be assessed in order to perform an economic evaluation of the whole process.

Among all the pretreatment methods tested in this study, thermal pretreatment was the least influential method, while addition of enzymes was the most decisive. The loading of TS, and the amount of enzyme included were the most important factors. The pretreatment of the PC seems to be a promising technology for the production of sugars, proteins and organic acids.

Ensiling of fresh red clover and the press cake was only successful to some extent. No aerobic spoilage took place, and the production of lactic and acetic acid resulted in a low pH that inhibited the growth of spoilage microorganisms. However, the low dry matter content of the feedstock in conjunction with a low concentration of water soluble carbohydrates gave rise to the proliferation of clostridia. Ensiling the biomass with a DM content over 30 % should be applied in order to generate good quality silage. However, this clostridial silage can have a positive effect if the silage is intended as a preservation method for the production of biogas.

Anyhow, further research is needed to optimize the quality of the silage. Feedstock harvested at an earlier maturity stage might improve the silage quality. Finally, the addition of additives containing sugars and enzymes would increase the sugars released and consequently the lactic and acetic acid concentration.

The methane potential of the press cake was efficiently preserved by means of silage, and therefore, silage might be an appropriate storage method for the press cake before its utilization as a substrate for biogas production. However, ensilaging fresh red clover did not improve the methane yield in comparison to the no-ensiled feedstock and additives should be used to implement this preservation method in this grass. Further research is needed testing different fresh crops and press cakes and including several additives to credibly regard silage as a viable method for improving biogas production.

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8 Appendix

Days of AD	3	6	10	13	17	20	24	31	38	45
Press cake	44,8	87,6	150,2	191,7	217,3	236,2	248,4	266,8	274,0	277,3
Std	4,1	4,1	6,1	6,9	8,2	4,0	5,3	4,3	6,6	7,9
Fresh red clover	37,2	87,0	171,5	252,6	287,9	311,3	327,4	360,9	364,9	376,5
Std	4,9	13,6	25,1	24,0	22,3	20,7	19,4	4,2	2,3	2,8
Fresh, 55 d	44,2	98,5	172,6	228,4	252,3	261,9	283,3	300,4	305,4	309,8
Std	6,5	14,1	15,7	11,3	18,4	25,8	23,0	25,9	23,6	28,8
Fresh + Inoc, 55 d	39,2	89,2	151,1	215,6	250,7	258,2	279,4	297,9	305,8	308,7
Std	1,4	3,4	1,6	17,8	23,0	21,6	22,0	23,8	20,4	23,4
PC, 55 days	46,5	107,5	174,1	212,1	244,3	249,0	277,2	295,5	304,6	308,8
Std	3,9	4,5	11,5	3,1	6,8	4,8	33,6	34,3	38,4	36,9
PC + Inoc, 55 d	50,6	109,1	160,3	196,6	223,0	219,6	227,8	243,8	248,3	255,4
Std	2,3	3,4	16,4	19,9	19,0	23,7	21,1	25,2	27,2	25,5
Fresh, 80 d	47,6	104,3	153,4	204,9	234,2	239,5	252,5	273,6	284,1	288,4
Std	7,2	8,4	13,2	9,0	7,0	16,4	12,0	18,3	21,8	20,3
Fresh + Inoc, 80 d	40,4	103,6	166,1	215,9	245,7	253,6	265,5	278,8	290,3	296,1
Std	7,6	10,0	16,9	5,6	4,4	6,5	11,4	15,1	14,1	15,0
Press cake, 80 d	51,3	110,7	178,0	205,5	238,2	238,3	247,8	265,0	262,3	270,3
Std	3,2	2,8	3,5	5,8	8,7	9,6	10,5	11,4	10,4	11,1
PC + Inoc, 80 d	51,2	94,4	110,6	136,9	167,4	169,4	175,6	195,6	202,7	204,5
Std	8,6	11,7	7,6	3,0	2,9	11,8	10,5	11,4	5,7	12,6
BLANK	4,7	7,0	10,1	12,0	15,6	16,3	18,0	21,6	26,1	27,2
Std	0,3	0,6	0,3	0,4	0,4	0,9	0,4	0,7	1,0	1,9

Appendix 1: Production of methane in mL CH_4/g VS during the 45 of anaerobic digestion.

%DM	Time	Lactic acid	Std. Dev. Lactic	Acetic acid	Std. Dev. Acetic	Propionic acid	Butyric acid	Total VFAs	pH
Fresh red clover	0	2,28	0,00	1,09	0,00	0,31	0,33	2,01	5,77
	5	6,51	0,52	2,04	0,26	0,31	0,19	2,53	4,76
	30	0,69	0,52	3,61	1,69	0,48	2,18	5,62	5,38
	55	0,26	0,37	2,37	0,00	0,42	4,42	7,57	5,52
	80	2,98	4,21	5,27	0,25	0,45	1,89	7,36	5,04
Fresh red clover + Inoculum	0	2,66	0,00	1,28	0,00	0,36	0,38	2,34	5,77
	5	7,35	0,71	3,00	0,21	0,34	0,25	3,22	4,64
	30	0,00	0,00	3,08	0,00	0,44	2,61	5,46	5,49
	55	0,00	0,00	3,99	0,32	0,55	4,29	9,55	5,52
	80	0,03	0,04	4,22	0,05	0,48	2,94	7,78	5,41
PC red clover	0	0,15	0,00	1,65	0,00	0,33	0,33	2,35	6,04
	5	4,40	0,39	2,25	0,00	0,33	0,19	2,57	4,72
	30	1,78	1,95	2,33	0,32	0,42	1,65	4,02	5,15
	55	0,72	0,04	2,24	0,20	0,42	3,17	6,09	5,30
	80	0,69	0,98	2,45	0,73	0,44	2,51	5,60	5,31
PC red clover + Inoculum	0	0,16	0,00	1,72	0,00	0,35	0,34	2,46	6,04
	5	4,16	0,10	2,64	0,02	0,35	0,22	2,85	4,69
	30	2,17	0,06	3,30	0,05	0,45	0,98	3,83	4,94
	55	1,15	0,98	3,23	0,32	0,42	1,98	5,83	5,20
	80	0,04	0,06	2,96	0,08	0,45	2,58	6,10	5,25

Appendix 2. Acid composition of the different treatments of silage in %DM.