Enhancing Lactic Acid Production from Green Biomass Fractions

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Introduction

The interest of scientists and industries in utilizing different types of biomass for the production of different products is not new. The world has started to become more concerned about the utilization of fossil fuels, and since then the utilization of biomass has increased in these sectors (Corona *et al*., 2018).

Initially, energy crops were thought to be good for the production of different products, namely fuels and chemicals, but quickly concerns about competition for land started to arise (Corona et al., 2018). Furthermore, different organizations started to understand the issue, at both ethical and economical levels, of using material that could feed populations and animals, being used instead for the production of other desired products. Hence, the new tendency is to use non-edible crops, the so-called second-generation feedstock, for the production of different products in a more sustainable way than fossil fuels (Lee et al., 2009).

In a biorefinery, the biomass is upgraded to one or more primary products in a more sustainable way than petrochemical refineries, at the same time as other secondary products are obtained in order to reduce the costs and waste from the production of the primary products (Jong & Jugmeier, 2015).

Biorefineries can be classified in different ways. In the case of Green biorefineries, the name itself stems from the substrate that is utilized. In this type of biorefinery, only green biomass is uside, especially different types of legumes. The green biomass can be primarily separated into a solid part, called press cake (PC) and into a liquid part, called green juice (GJ), after which each part can be used in different processes for the production of different necessary products (Andersen & Kiel, 2000; Jong & Jugmeier, 2015; Kromus, et al., 2004).

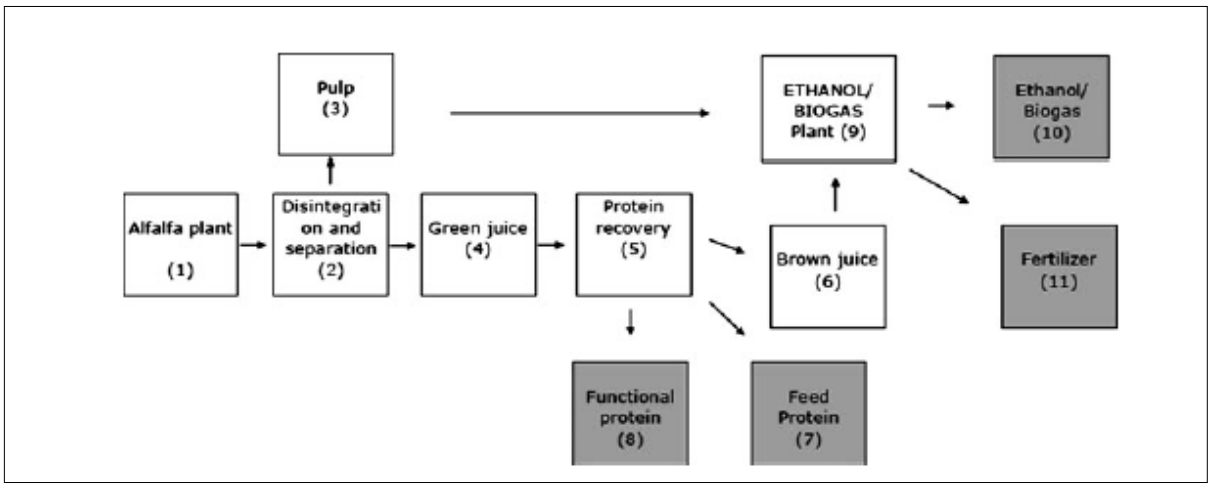

Figure 1: Alfalfa in a green biorefinery. (Source: Thomsen et al., 2015)

Figure 1 shows an example of a green biomass in a green biorefinery. Here, the main product is the production of proteins and then fertilizer and ethanol or biogas are produced as secondary products, from the waste obtained during the process (Thomsen et al., 2015).

However, other products, like lactic acid, have been produced from the green juice fraction. In this case, the green juice is combined with sugar stock solutions in order to produce high concentrations of lactic acid from fermentation, as even though green juice contains some monomer sugars, it is not enough for industrial productions of lactic acid that can compete with already applied solutions (Dietz et al., 2016).

In fact, the majority of the sugars present in different grasses are left in the press cake after pressing. However, these sugars are mainly present in the form of complex molecules that are impossible to be utilized by the microorganisms used during fermentation. The fact that the press cake contains a lot of complex molecules in its composition seems to be one of the main reasons for its lower use than the green juice fraction (Sreenath et al., 2001).

Different techniques can be applied for the degradation of these complex molecules into monomers, to then be utilized by different microorganisms for the production of different products. However, it is still a question of how sustainable these techniques are, since most of them, like enzymatic hydrolysis, can bring negative impacts to the economical side of a biorefinery (Sreenath et al., 2001; Zhao et al., 2012).

1. Green Biorefinery

As previously explained, the green biorefinery derives from the biorefinery concept, but where only grassland biomass is converted into different products, such as lactic acid, proteins and biogas; it maintains sustainability as a basis throughout the whole life cycle: Construction, operation and dismantling.

The figure below shows an overview of different processes and products that can be produced in a green biorefinery:

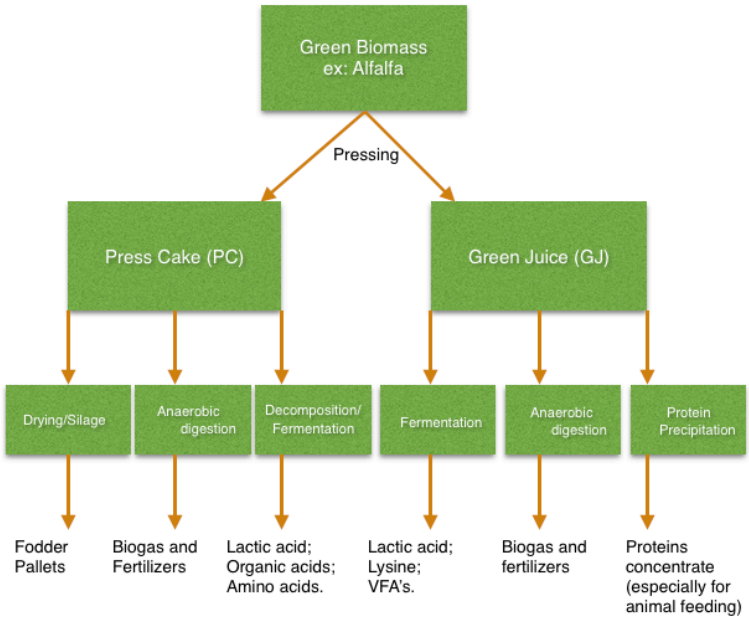

Figure 2: Overview of processes and products produced in green biorefineries

Great efforts have been taken to ensure that in the future, green biorefineries will contribute to an increase of the quality of life of the world's population by responding to the need of diverse bio-based products and renewable energy sources. However, discussions have also focused on the fact that the substrate used needs to be available in the place of production and the products need to be necessary for the local population, in order to reduce costs and transport-related environmental problems (Andersen & Kiel, 2000; Corona *et al*., 2018).

In Denmark, the green biorefineries appear to be of particular interest. First, it is easy to obtain grass in Denmark, due to the amount of land available and given that grass does not compete with food crops for land. Secondly, Denmark is a country in which a large part of the population still lives on agriculture, particularly animal farming, so if proteins are produced to feed the animals, the importation of protein-rich feed, such as soybeans will decrease. The third aspect is related to the second, insofar that Denmark is trying to switch to organic farming, and feeding animals with these kinds of proteins will increase local farming in the country. The fourth, and not least important reason, is that grasses are excellent nitrogen fixators, so rotating the soil with these grasses will increase the fertility of the soils (Corona *et al*., 2018).

Even though the potential of these green biorefineries is clearly visible, it is still necessary to optimize different productions, make most of them more sustainable and to find more ways of using the waste in order to obtain waste - free production (Sharma, 2013).

2. Alfalfa

Some plants, like corn, are already competing with fossil fuels for the production of different products, such as for example, ethanol from a fermentation process. However, besides the fact that corn can be used as food, as explained above, it also needs to be planted in large quantities for intensive productions, however the land for its cultivation is limited. Conversely, grasses are intensively available in different regions of the globe and although the green biomass is seasonal, this problem can be easily resolved by ensiling the biomass. This will guarantee its availability all year round while simultaneously providing alternatives to the constant production and supply of biomass. Moreover, green biomasses contain significant amounts of high value proteins, a considerable amount of carbohydrates and due to their green stages, less starch and lignin than when compared with other types of biomass.

In the present study, the press cake and green juice from the Alfalfa plant, a herbaceous perennial legume, will be tested. Alfalfa is considered to be cross-pollinated and its variations depend on the location of its cultivation.

Previous studies have shown that it is preferable to plant alfalfa in deep, well-drained, medium texture soil with pH=6,8. The cultivation parameters appear to be extremely important since this plant is grown for approximately three years or more before harvesting (Teuber & Graham, 2014).

Alfalfa is known to be extremely good in nitrogen fixation. In fact, nitrogen fixation is carried out by the soil bacterium *Rhizobium meliloti*, which lives in the roots of this grass. Thus, this plant is of particular benefit, not only to the biorefinery but also for soil rotation.
Plant rates vary between 16 to 45kh/ha, depending on the soil type and planting method. Contrary to the other countries, where the harvesting of these plants can be carried out up to 10 times a year, in Denmark, due to the lower temperatures, the harvest normally occurs once to three times a year (Teuber & Graham, 2014).
Alfalfa is mainly used in Denmark for animal feed, especially for cattle and horses. Most of it is grown separately or mixed with Italian ryegrass and used for production of normal silage or dried silage. When the dried biomass is carried out artificially to produce fodder pallets, it is less sustainable than letting it drying naturally, but at the same time it is a more efficient product (Thomsen *et al*., 2015).
Alfalfa fibres have been used as ruminant feed since it has compatible proteins with those present in soybeans. However, it is necessary to find more efficient ways of making these proteins available for ruminants, since when eating the fibres, only a small part is digested. Moreover, previous studies have shown that enzymatic hydrolysis of the fibres of different grasses can produce a high amount of monomer sugars, especially glucose, that can later be used for the production of different fermented products, like lactic acid (Lee *et al*., 2009).
Other types of grasses can also be used in the biorefinery, or even the usage of different grasses may be combined. It is difficult to make a precise comparison as to which type of green biomass would be the most suitable, since this would depend on the primary product to be attained and also on the cultivation and harvesting conditions. A study conducted by Santamaria-Fernandez *et al*. (2017) pointed to alfalfa presenting lower dry matter in its composition, but it was also one of the plants presenting higher amounts of nitrogen per kg of sample. In the same study, despite the presence of high nitrogen levels, it was also found that more proteins could be extracted from red clover than from alfalfa.
Even though alfalfa may not be the most desired grass for the production of certain products, it appears to have significant potential for others. Taking the example of the plant's high nitrogen levels, it makes the juice from this grass one of the most preferable nutrient media for different fermentations. Furthermore, different types of research are being carried out using this plant, in order to optimize processes and by consequence obtain higher yields of different desired products (Hermansen *et al*., 2017).

3. Enzymes and Enzymatic Hydrolysis

Cellulose is the main component of primary and secondary cell walls in plants, comprising 30-60% of its total dry matter (Balat, 2011). Even though it has a homogeneous chemical structure, namely thousands of D-glucose units, these units are linked together by ß-1,4-glucoside bonds. Due to its linear structure, intra- and inter- molecular hydrogen bonds are easily formed and together with the van der Waal's interactions, bundles are formed which, after aggregation,

results in microfibrils. Later on it is bonded into fibres, which have a high tensile strength (Bajpai, 2016).

The second most abundant polymer of the lignocellulosic structure is Hemicellulose, which can vary from 15-35% and like cellulose, is present in both primary and secondary structures of the cell wall of a plant. This fraction strengthens the plant's cell wall structure by being linked to cellulose through non-covalent interactions as well as by cross-linking with lignin. Hemicellulose has been found to be the most thermo-chemically sensitive fraction, thus, severity parameters should be considered in order to avoid by-product formation such as furfurans.

Furthermore, contrary to Cellulose, Hemicellulose is a heterogeneous polysaccharide with differently distributed short lateral chains of monomer sugars like ß-D-xylose, ß-D-glucose, ß-D-mannose. The composition of the Hemicellulose in terms of sugars, depends on the material (Balat, 2011; Horn *et al*., 2012).

Most of the microorganisms can utilize monomer sugars, especially C6 sugars, like D-glucose; however, most of them cannot utilize these sugars, when presented in the form of one of the presented polymers. Some of these microorganisms contain enzymes that can degrade these polysaccharides into monomer sugars, however, not all the microorganisms can produce the desired product at the same time.

Notwithstanding, not all the microorganisms are able to degrade these polymers. Nowadays, it is possible to apply enzymatic hydrolysis as a pretreatment of lignocellulosic material, to obtain monomer sugars. From an environmental perspective, it appears to be a viable option, however, the large-scale use of commercial enzymes should be carried out with caution. To contra-balance this problem, recent studies have even suggested the production on site or integrated production of enzymes, in order to reduce the costs associated with the utilization of this pretreatment method, and by consequence, make it more sustainable (Shallom & Shoham, 2003).

When enzymatic hydrolysis is applied, different enzymes can be used, depending on the fraction to be degraded. It is also possible to add a combination of enzymes, in order to degrade different fractions and obtain different monomer sugars at once.

Cellulose is hydrolysed into glucose via cellulases complex, which consists of: endo-1,4-ß-glucanases, exo-1,4-ß-glucanases and ß-glucosidases. These enzymes are hydrolases and cleave the glycosidic bonds by adding a water molecule. In short, endo-glucanases "attack" the regions of low crystallinity of the fibres, producing free chain ends and then further degradation of the same free-chain ends. Here the exo-glucanases remove cellobiose units. The glucose units are then produced with the action of ß-glucosidases (Horn, *et al*., 2012).

Hemicellulose is more easily hydrolysed than cellulose due to its heterogeneous and amorphous structure. At the same time, it has a far more complex system than cellulose, since it contains a variety of sugar units. Due to this fact, more enzymes need to be added if the aim is to degrade the whole polymer. Hemicellulases comprises: endo-1,4-ß-xylanases, exo-1,4-ß-xylosidases, endo-1,4-ß-mannanases, exo-1,4-ß-mannosidases, α-galactosidases, endo-1,5-α-arabinases, α-L-arabinofuranosidases, α-glucuronidases as well as some accessory enzymes that are required for

the hydrolysis of several substituted xylans, such as acetyl xylan esterases (El-Naggar, Deraz &Khalil, 2014; Shallom & Shoham, 2003).

As referred to above, enzymatic hydrolysis has been reported as an effective process, presenting various advantages and hence it is currently being intensively researched. Therefore, the impact of several factors, such as: substrate and end-products concentration, enzyme activity and reaction conditions, should be considered in each investigation. Furthermore, it has also been reported that the substrate and end-products concentration can be a limiting factor for the individual cellulases activity. The pH and temperature of the hydrolysis also appear to influence the activity of the enzymes. Novozymes commercializes cellulases and hemicellulases, indicating that optimal temperature is 50ºC and pH between 4,5-5,0.

A study conducted by del Bairrio (2016) suggests that the enzymatic hydrolysis of the press cake for example, should be performed with the lower dry matter content per total solution as possible. Additionally, the lignin content is also a factor that hinders biomass digestibility during enzymatic hydrolysis. Thus, it can cause non-productive binding of lignocellulosic enzymes to lignin and through its derivatives. Moreover, lignin can be toxic to some microorganisms (Bajpai, 2016).

It is difficult to find information on the enzymatic hydrolysis of the press cake and even if it can be treated as another plant material, some important considerations should be taken into account. One of the most important factors to bear in mind is the presence of microorganisms in the material. It is normal for different grasses to contain different types of microorganisms, some of them are not only thermo-tolerant, meaning that they can tolerate different temperatures than the desired ones, but also these microorganisms are able to perform fermentation. If enzymatic hydrolysis is applied before any other pretreatment, the microorganisms are then likely to automatically consume some of the sugars that are degraded by the enzymes. Consequently, at the end of the hydrolysis not only fewer sugars will be available in the media, but they will also be mixed with some organic acids and/or alcohols (Sreenath et al., 2001).

Some previous studies have suggested that the pretreatment should be heated before the enzymatic hydrolysis, not only to sterilize the material but also to start breaking it down, which makes the work of the enzymes far easier and effective. However, it has also been reported that this kind of pretreatment, both in the press cake and in the fresh material, can change the configuration of some of the molecules inhibiting the microorganisms during fermentation later on (Jensen *et al*., 2014). Besides this disadvantage, the heating of the material for long periods and/or at high temperatures can suggest an increase of the energy input in this type of biorefinery and by consequence, make the process less sustainable (Cerveró *et al*., 2010).

Other pretreatments could be suggested to be combined with the enzymatic hydrolysis, in order to try to extract as much monomer sugars as possible from the material. However, again, the main problem is finding a solution that will not reduce the sustainability of the process and at the same time will not change the configuration of the material, leading by consequence to a process with a lower yield than the expected one (Jensen *et al*., 2014).

It seems that even so, the enzymatic hydrolysis of the press cake may be a viable solution to obtain monomer sugars and to provide another solution for the usage of the press cake in a green biorefinery. However, many environmental, economical and technical problems also appear to call for attention before their application in order to obtain the maximum yield possible and in sufficient quantity so as to compete with the efficiency of the usage of glucose stock solution combined with green juice (Dietz et al., 2016).

4. Lactic Acid Fermentation

Fermentation is a biological process, where microorganisms can utilize different substrates as a source of energy, without the presence of oxygen, releasing organic acids and/or alcohols as final products. Lactic acid fermentation is therefore, a biological process of production of lactic acid. Lactic acid can also be produced chemically, however fermentation appears to present different advantages when compared with chemical production. In chemical production, lactic acid can be presented both in D- or L+ isomers and the industrial application of lactic acid depends on its isomeric forms, where the pure form of one or other would be more valuable. This is the main reason why nowadays, 90% of the lactic acid produced worldwide is made by bacterial fermentation and only the rest is produced synthetically, since each strain only produces one of the isomer forms of lactic acid (Dietz *et al*., 2016).

Lactic acid fermentation can be carried out using different lactic acid bacteria which are really important in the feed and food industry, since lactic acid is abundantly used as an acidulate and preservative, or in nutraceuticals, as probiotics due to their potential to inhibit pathogens and to detoxify carcinogens (Thomsen *et al*., 2015).

In the present study, *Lactobacillus salivarius* will be used, which are Gram-positive and non-spore bacilli bacteria that can produce great amounts of lactic acid in anaerobic conditions. These kinds of bacteria have been commonly used as inoculants by food fermentation industries to produce different fermented products and add other attributes to the food such as preservation. It has also been reported that the addition of this bacteria improves ensiling characteristics in wet brewers grains, alfalfa, wheat corn forage and grass-legume forage (Dietz *et al*., 2016; Yang *et al*., 2006). *Lactobacillus salivarius* is now also used to convert the fresh green juice into a stable, storable product, called acid brown juice. During the lactic acid fermentation, carbohydrates are converted into lactic acid, and by consequence, reduce the pH of the media, which leads to the precipitation of proteins. The remaining plant proteins presented in the brown juice can be hydrolysed into smaller peptides and amino acids and the brown juice is stored (Thomsen *et al*., 2015).

The juice of the grasses appears to be an extremely good nutrient media for the lactic acid bacteria, as previously explained. However, it has been reported that fermentation should occur immediately and continuously after pressing in order to prevent uncontrolled fermentation by undesirable contaminating microorganisms. However, in order to make the lactic acid fermentation using green juice a preferable process for the production of lactic acid or even only

for sterilization of the juices, the process must be simple, cost-effective and robust, and that requires low energy consumption (Thomsen *et al*., 2015).

As already mentioned, the lactic acid that is produced can then be sold in different industries. A relatively new approach is to use the lactic acid as building blocks for the production of polylactic acid (PLA), which is a biodegradable plastic. The production of plastic is 200billion kg/a and these figures need to be drastically reduced in a few years in order to reduce different environmental problems caused not only by the production of these plastics but also by its non complete degradability.

One of the main problems that researchers have faced is finding the right substrate that does not require expensive nutrient addition. Agricultural residues have been suggested as possible substrates; however, the pretreatment of this material and downstream of the broth can also be very costly. According to Andersen & Kiel (2000), the press cake can be used for the production of fodder pellets and the green juice could be utilized as nutrient source in lactic acid fermentation. Even though yeasts and bacteria appear to prefer to grow on broth, these kinds of media account for more than 35% of the total fermentation costs (Peng et al., 2014). Other most commonly used nitrogen sources in lactic acid fermentation are peptone, malt extract and corn steep liquor. However, these alternatives are still extremely costly on large scales (Dietz *et al*., 2016).

Even though the usage of green juice as a fermentation nutrient is still under development, alfalfa and clover are two examples of green crops that are rich in nitrogen and so can be intensively studied as a more sustainable nutrient source. Furthermore, since the green juice appears to present high levels of nitrogen, but not enough sugars to support a high production of lactic acid, the present study suggests the performed of hudrolisis in the press cake, and then trying to use the monomer sugars in the lactic acid fermentation. It has already been explained that the nitrogen source may be one of the most expensive parts of the process, yet using glucose stock solution or other substrates with enough sugars can also be very expensive or difficult. In an attempt to overcome this problem, a solution might be to degrade the sugars from the press cake and use them as fermentable sugars in the fermentation step. This would not only make the process more sustainable, but it would also give more utilization to the solid part of a green biomass (Andersen & Kiel, 2000) & (Dietz *et al*., 2016).

2. Materials and Methods

2.1 Material Analysis

2.1.1 Total solids and volatile solids analysis

Six crucibles were used in total for this experiment, three containing around 0,18g of press cake and three containing 0,18g of green juice.

To start the experiment, dried crucibles were weighted and the weight of each of the six empty crucibles was recorded. After each sample was added to the crucibles, the crucibles were moved again to the analytical scale and once again the weight of each was recorded.

The samples were placed in the oven at 105ºC over night (>12h). After the samples were completely dried, and cooled down to room temperatures in a desiccator, their weight was measured and recorded.
To burn each sample into ashes, the crucibles were passed from the desiccator to the muffle furnace at 550ºC for around 3 hours. After the ashes cooled down, weight measurements were taken again.
In order to find the amount of total solids and volatile solids present in each sample, some calculations were done as it is presented bellow:

TS: ((C-A)/(B-A)) x 100

VS: ((C-D)/(B-A)) x 100

A= empty crucible;
B= weight of crucible + sample;
C= weight of crucible + sample after drying;
D= weight of crucible + sample after burning.

2.1.2 Strong acid hydrolysis

To evaluate the amount of cellulose and hemicellulose present in the press cake, alfalfa stored at -18ºC was used for strong acid hydrolysis procedure.
The SAH was carried out in duplicates in a quantity of 0,1600 g per sample. Note that for this procedure was necessary to make sure that the material was dried to contain more than 80% dry matter. In the presented case, the final dry matter obtained after drying the material was 92,61%.
There were 4 samples in total, duplicate of press cake samples and two standard samples. Note that the standard composition contained 39,8g/L of D-glucose monohydrate, 34,6 g/L L-arabinose and 34,6 g/L D-xylose.
1,5 ml of H_2SO_4 was added to each substrate and then it was mixed in a vortex mixer to prevent air trapping. The samples were then incubated in a water bath at 30ºC for 60 minutes. The vortex mixer was used twice during the incubation period, once 20 minutes after the incubation started and then 20 minutes before it finished.
After the incubation period, 42ml of Millipore water were added to the two samples containing press cake to obtain an acid concentration of 4%. 41ml of Millipore water were added to the two other tubes containing press cake but here also 1ml of STAM solution was added to spike the samples.
The screw-caps were then loosened slightly before being autoclaved for 60 minutes at 121ºC. The samples were mixed once again by inversion of the tubes three times each (Kádár, 2016).
The samples were filtrated using a 0,45µl filter and then 2ml of each sample were passed to HPLC crucibles.

The samples were sent for HPLC analysis and the results were obtained based in finding first the recovery factor and based on it find the percentage of cellulose and hemicellulose present in each sample.

2.2 Enzymatic Efficiency and Activity

2.2.1 Enzymatic hydrolysis to check enzymatic efficiency

This experiment started by weighting 268mg of pretreated wheat straw into six 2ml enpendorf tubes, and, right after, 1,250ml of succinic acid was added to each tube.
The enzymes were added as it shows in the table bellow:

Tube	Tested enzyme (µl)	Buffer (µl)
1. Cellic Ctec	50µl	1250
2. Cellic Ctec	50 µl	1250
3. Cellic Htec	50µl	1250
4. Cellic Htec	50µl	1250
5. Control	-	1250
6. Control	-	1250

Figure 3: Explanation of how were prepared the different samples in this experiment.

Then the tubes were incubated in a thermo-shaker at 50 ºC and 140rpm for 48h. After the 48h heating each tube for 10 minutes at 100 ºC stopped the reaction. The pH of each sample was measured and then 45µl of 10% (w/w) succinic acid was added to each tube in order to reduce the pH of each sample.
The samples were vortexed with a vortex mixer and then 1ml of sample was filtrated using 0,45µl filter into HPLC glass tubes.
The samples were sent to HPLC analysis and the results were analysed.
The efficiency of Cellic Ctec and Cellic Htec was calculated based in the amount of cellulose and hemicellulose added and the amount of monomer sugars that were released in each sample.

2.2.2 FPase activity

For the assay sodium citrate and DNS solution needed to be prepared. To prepare the DNS solution, 10g of DNS need to be dissolved in 500ml of water and then 2M of NaOH and 300g of potassium sodium tatrate need to be added to the solution. After complete dissolution by continuous stirring, water is added until 1L of solution. The DNS however was prepared by the laboratory technician of the Aalborg University Copenhagen, in order for this solution to be shared with other groups of students during the present semester.

To prepare the sodium citrate buffer at pH=5, 2,1942g of citric acid were added to 4,281g of sodium citrate and dissolved in 500ml of water. To adjust the pH to 5, 0,5ml of NaOH was titled.
For this assay glucose solutions from 2mM to 40mM concentrations were prepared, and used as standards.
For this assay, the reaction was prepared by adding 2 times 0,6cm filter paper as substrate, 40µl of enzyme and 80µl of buffer. It was also prepared blank, where instead of enzyme was added water and controls, where the enzyme was not incubated. Commercial enzyme was also prepared in order to compare its activity with the activity of the produced enzymes necessary for the next steps.
After the preparation of the reactions, the solutions were incubated for one hour at 50ºC. The reaction was stopped in ice and then 240µl of DNS was added to the eppendorf tubes and the enzymes, used as control, were also added after the DNS addition.
All the solutions were boiled for five minutes at 100ºC and then transferred to ice again and 1,28ml of water was added. The samples were added in triplicates to the micro-plate and absorbance was read at 550nm in the micro-plate reader spectrophotometer.
Enzyme activity for each enzyme extract was calculated using absorbance and a standard curve and after removing the blanks and controls values from the enzyme extract absorbance values. Activity was defined as the amount of enzyme that catalyses the conversion of 1 micro-mole of substrate per minute.

2.2.3 Xylanase assay

Besides the sodium citrate and DNS, was necessary to prepare beechwood xylan buffer to be used as substrate. This substrate was prepared in 1% (w/v) in sodium citrate buffer.
In this case, xylose solutions with concentrations from 1mM to 10mM were prepared to use as standard.
The reaction was prepared by adding 300ul of substrate and 100ul of the appropriately dilution enzyme or standard. Blank was prepared, where instead of enzyme was added water and the controls were prepared as the reactions but the enzymes did not need incubation.
Commercial enzyme was also prepared due its activity be necessary in the next steps.
After the reactions were prepared in 2ml eppendorf tubes, were incubated at 50ºC for half an hour and then the reaction was stopped in ice. 600ul of DNS was added and the control enzymes was added.
All the reactions were then boiled for six minutes for 100ºC and then passed to ice again.
All the samples were passed to the micro-plate in triplicates and the absorbance was read at 540nm.
Enzyme activity for each enzyme extract was calculated using absorbance and a standard curve and after removing the blanks and controls values from the enzyme extract absorbance values. Activity was defined as the amount of enzyme that catalyzes the conversion of 1 micro-mole of substrate per minute.

2.3 Enzymatic Hydrolysis

Based on the DM present in the samples of alfalfa press cake, 2,81g of biomass were added to thirty falcon tubes with 50ml capacity. Then 17,19ml of water were added to each falcon tube and the pH of each sample was measured.
Add HCL or NaOH to each sample in order to obtain fifteen with pH= 5,0 and fifteen with pH=5,5.

Set up	Description
Set up number one	pH= 5,0 and temperature 50ºC. 2 samples with 27µl Cellic Ctec and 3µl Cellic Htec 2samples with 45µl Cellic Ctec and 5µl Cellic Htec 2 samples 54µl Cellic Ctec and 6µl Cellic Htec
Set up number two	pH= 5,5 and temperature 50ºC. 2 samples with 27µl Cellic Ctec and 3µl Cellic Htec 2samples with 45µl Cellic Ctec and 5µl Cellic Htec 2 samples 54µl Cellic Ctec and 6µl Cellic Htec
Cellic Ctec at 3% (reference from del Bairrio et al, 2016)	Duplicates at pH= 5,0 and temperature 50ºC. Each flask contained 30µl of Cellic Ctec.
Control	Duplicates at pH 5,0 and temperature 50ºC; Duplicates at pH 5,5 and temperature 50ºC; No enzyme addition.

Figure 4: Explanation of how were prepared the different set ups during this experiment.

After the pH was adjusted, the enzymes were added to each flask according to the table bellow:
The falcon tubes were incubated for 48hours in thermo-shakers, one at 50ºC and another at 55ºC, and at 140rpm both.
After 48h, 2ml of each sample was saved for HPLC analysis.
In the next day, after 72 hours, the reaction was stopped and 45µl of 10% (w/w) sulphuric acid were added to each sample, both after 48 hours and 72 hours of hydrolysis, to low the pH. Then 2ml of each sample were added to a syringe and then filtrated with a 0,45µl filter to HPLC crucibles.
After the crucibles were sent to HPLC analysis and calculations were made to understand how much g of monomer sugars were possible to obtain by this method in 100g of dry matter. From that, a correlation between the different set ups was made in order to choose the best set up to be used in the next step of this project.

2.4 Lactic Acid Fermentation Using Different Set-Ups, at 40ml Total Volume

This part of the experiment was based in previous studies where green juice was used as nutritional source and glucose stock solution as sugar source for the bacteria.
Here six different set ups were prepared, with different sugar sources, and all were prepared in triplicates. All the triplicates were prepared in a 40ml solution and when hydrolysis was performed, the final concentration of total solids was 5% in all.
The explanation of materials and methods of the different set ups it is presented bellow:

1) Enzymatic hydrolysis of press cake followed by fermentation: The enzymatic hydrolysis was performed at 4,8% dry matter content because the total dry matter of the green juice was calculated to be also 4,8%; so, to obtain that, 5,11g of press cake were added to each of the three 50ml sterile bottles. Right after, 34,89ml of water were added to each bottle followed by adjusting the pH to 5,5 with NaOH and HCl preparations. 6% enzyme preparation, 28µl Cellic Ctec and 2µl Cellic Htec, was added to each bottle and then the bottles were incubated in a thermo-shaker for 48 hours at 50ºC and 140rpm.
Samples for HPLC analysis were taken at 0, 24 and 48 hours in order to try to understand the difference in sugar release. All the HPLC samples were prepared by adding 45µl of 10% (w/w) sulphuric acid and then by mixing it with a vortex mixer and filtering it with 0,45µl filter to HPLC crucibles.
After the hydrolysis, the temperature of the thermo-shaker was reduced to 37ºC, the pH was adjusted in some bottles, in order to obtain the initial 5,5 pH. After the pH was adjusted, 2ml of *Lactobacillus salivarius* was added to each bottle and let it do fermentation for 24 hours. Note that the bacteria was previously cultivated in MR broth, 40ml final solution, made just one day after was added to each bottle.
The fermentation was stopped in the next day, the pH was measured, and some samples were taken for HPLC analysis following the same procedure explained above.

2) Fermentation of Green Juice with Glucose: Here 0,491g of glucose stock solution was mixed with 37,5ml of green juice and 2ml of *Lactobacillus salivarius* previously cultivated in MR broth. The fermentation was carried out at 37ºC for 24 hours and samples for HPLC analysis were taken at 0, 12 hours and 24 hours. All the HPLC samples were prepared by adding 45µl of 10% (w/w) sulphuric acid and then by mixing it with a vortex mixer and filtering it with 0,45µl filter to HPLC crucibles.

3) Short time Hydrolysis followed by fermentation: For this set up, 5,11g of press cake were added to three different sterile bottles and then 34,89ml of water was also added to each sample. The pH was adjusted to 5,5 and then 6% TS enzyme loading, 28µl Cellic Ctec and 2µl of Cellic Htec, were added to each bottle. The bottles were incubated at 50ºC for 16 hours and samples for HPLC were taken after the 16 hours to understand how much sugars were released until that period.

After, the thermo-shaker was changed to 37ºC, the pH of each bottle was adjusted again to 5,5 and then 2ml of *Lactobacillus salivarius* was also added to each of the triplicates.
Samples for HPLC were taken after 24 hours and after at the end of the experiment. All the HPLC samples were prepared by adding 45µl of 10% (w/w) sulphuric acid and then by mixing it with a vortex mixer and filtering it with 0,45µl filter to HPLC crucibles.

4) Hydrolysis for 48 hours followed by fermentation of green juice combined with press cake: Before starting this set up, calculations were made in order that the combination of green juice, press cake and water gave a total of 4,8% dry matter content in the solution. To obtain that concentration, 10,42ml of green juice was combined with 25,57ml of water and then mixed with 3,99g of press cake.
The pH of the solution was adjusted to 5,5 and then 21µl of Cellic Ctec and 2µl of Cellic Htec were added to each of the triplicates used in this set up. The hydrolysis was performed in a thermo-shaker for 48 hours, at 50ºC and 140rpm and samples for HPLC analysis were taken at the time of 0, 4, 24 and 48 hours.
After hydrolysis the temperature of the thermo-shaker was reduced to 37ºC and 2ml of *Lactobacillus salivarius* was added. Fermentation was carried out, after adjusting the pH to 5,5 again, for 24 hours. Samples for HPLC analysis were taken at the end of the fermentation. Note, that like in the previous set ups all the HPLC samples were prepared by adding 45µl of 10% (w/w) sulphuric acid and then by mixing it with a vortex mixer and filtering it with 0,45µl filter to HPLC crucibles.

5) Short time hydrolysis followed by fermentation of green juice combined with press cake: In order to maintain the 4,8% dry matter content in each sample, the amounts of press cake, green juice and water were equal to the set up four. After preparing the three triplicates, and adjusting the pH to 5,5, 21µl of Cellic Ctec and 2µl of Cellic Htec were added to each sterile bottle. Here the hydrolysis was performed for only 16 hours in a thermo-shaker at 50ºC and 140rpm and because of that, samples for HPLC analysis were taken after the 16 hours of hydrolysis.
After, 2ml of bacteria *Lactobacillus salivarius* was added to each sample, the thermo-shaker was reduced to 37ºC and the fermentation was performed for more 48 hours. In this case, samples for HPLC analysis were taken after 24 hours and 48 hours of fermentation. Note that the preparation of the crucibles for HPLC analysis was performed exactly as mentioned in the previous set ups.

6) Fermentation of Green Juice: In this last set up, fermentation of the green juice was performed in a thermo-shaker for 24 hours at 37ºC and at 140 rpm. Before starting the fermentation, the pH of the green juice was adjusted to 5,5 and then 38ml of green juice was added to each sample. 2ml of bacteria were cultivated in the green juice, the bottles were closed, and the fermentation started. Here samples were taken at 4 hours and 24 hours after the fermentation has started. Here again, all the HPLC samples were prepared by adding 45µl of 10% (w/w) sulphuric acid and then by mixing it with a vortex mixer and filtering it with 0,45µl filter to

HPLC crucibles and then sent to HPLC analysis performed by the laboratory technician of Aalborg University Copenhagen.
Note that this part of the experiment was repeated more than once, in order to get precise results. The main reason for that was that some mistakes, were performed the first time this experiment was done.

2.5 Optimization of Sugars Release

After the previous step, it was noticed that the hydrolysis of the press cake should be optimized before next fermentations are performed.
In order to try to increase the previous step, different set ups were performed where previous pretreatments are done in the press cake before fermentation:

1) Autoclaved press cake: Three samples containing a total volume of 40ml and 5% dry matter content were prepared. To achieve that, 5,32g of press cake were mixed with 34,68ml of water in bottles and the pH was adjusted to be 5,5.
The bottles were autoclaved for 20 minutes at 121ºC and then left it over night in the fridge, in order for the press cake absorb part of the water.
In the next day, the enzymes were added to the solution, due the low activity detected, 3x more Cellic Ctec was added and 2x more Cellic Htec was added, in order to try to make the activity 100%. In total, 84µl of Cellic Ctec and 4µl of Cellic Htec were added to each flask in a LAF bench in order to try to maintain the sterile conditions of the solutions, previously autoclaved.
The flasks were incubated for 48 hours at 50ºC and 140rpm. Samples were taken inside a LAF bench, at 0, 24, 36 and 48 hours after the enzymatic hydrolysis have started. In the end, samples were sent to HPLC analysis after passing through reduction of pH with HCl and filtration with 0,45µl filter and syringe.

2) Autoclaved press cake with more water addition: Theoretically 5% of the DM in solution indicates that it is necessary to account with the water present in the press cake. However, that water is not available in the solution. So three flasks were prepared containing instead 5,32g of press cake and 40ml of water.
This set up followed exactly the same procedure as the previous one.

3) Washed press cake: In this set up, the samples were not autoclaved, but the press cake was instead washed with only water. Three samples were prepared containing 5,32g of press cake and 34,68ml of MiliQ water and then the pH was adjusted to be 5,5. The samples were left closed in the fridge over night and in the next day 84µl of Cellic Ctec and 4µl of Cellic Htec were added to each flask.
The samples were added to the thermo-shaker at 50ºC and 140rpm for 48 hours. Samples were taken at 0, 24, 36 and 48 hours after the experiment started and after the end of the experiment the samples were prepared and past to HPLC crucibles and then sent to HPLC analysis.

4) Washed press cake with more water addition: This set up is equal to the set up number 3, but instead 5,32g of press cake were mixed with 40ml of MiliQ water.

5) Enzymatic hydrolysis of press cake without additional pretreatment: Here, the enzymatic hydrolysis was performed as in the previous experiment, explained in part 2.3, but due the low activity of the enzymes utilized, this time 3x more Cellic Ctec and 2x more Cellic Htec were added to each flask, to try to achieve 100% activity.
As in the previous set ups, three samples were prepared for this set up. In this particular case 5,32g of press cake were mixed directly with 34,68ml of water, adjusted the pH to be equal to 5,5 and added 84µl of Cellic Ctec and 4µl of Cellic Htec.
The samples were added to the thermo-shaker at the same temperature and shaking as the previous set-ups for 48 hours as well. Samples for HPLC were prepared at 0, 24, 36 and 48 hours of the experiment and sent to analysis.

6) Enzymatic hydrolysis of press cake without additional pretreatment but more water addition: This set up was prepared exactly as the set up number 5, but instead of 34,68ml of water, 40ml of water were added instead.

Note: *Controls were also prepared in triplicates. The controls were prepared by adding 5,32g of press cake in 34,68ml of water and by adding 5,32g of press cake with 40ml of water and put in the thermo-shaker at 50ºC and 140rpm for 48 hours without any pretreatment. As in the previous set ups, samples for HPLC analysis were prepared at 0, 24, 36 and 48 hours.*

3. Results

3.1 Material Analysis:

Before starting to work with a material, it is important to know its composition. Although it is possible to find information about the general composition of green biomass, it is also known that the composition changes not only from species to species but can also change due to cultivation, harvest and storage conditions.

Average	g dry matter/ kg sample	g organic matter/ kg sample	How much OM is in the DM (%)
Press cake	371,624 (s.d.=32,64)	346,625 (s.d.= 36,94)	93,18 (s.d.= 1,69)
Green Juice	47,880 (s.d.= 3,38)	42,000 (s.d.= 8,61)	87,390 (s.d.= 13,7)

Figure 5: Material analysis of different fractions of a green biomass: press cake and green juice.

After finding the total solids and volatile solids present both in the green juice and press cake, calculations were made to find how much is in one kg of sample. The table above indicates not only how much dry matter and organic matter exist in 1kg of each fraction, but it also indicates that 93.1% of the dry matter presented in the press cake, is organic matter; and that 87.4% of the dry matter presented in the green juice, is organic matter.

To understand how much sugar was present in each fraction, strong acid hydrolysis was performed in the press cake and HPLC was performed in the green juice. From the strong acid hydrolysis it was possible to understand that the press cake is composed by 22.3% of cellulose and 15.7% of hemicellulose. From the HPLC results, and taking into consideration how much organic matter is present in the green juice fraction, it was calculated that green juice composition contains 19.5 % glucose, 26.7% xylose and 0.8% cellobiose. In these samples 6.64g of lactic acid/kg of green juice were also presented. This corresponds to 15.8% of the organic matter presented in this fraction of the material.

3.2 Enzymatic Efficiency and Activity

As explained in the methods in materials, since enzymatic hydrolysis was performed for another subject of this semester, the efficiencies of Cellic Ctec and Cellic Htec were calculated based on the amount of cellulose and hemicellulose presented in the material and the amount of monomer sugars that were obtained at the end of the experiment. From this experiment, Cellic Ctec was found to be 86% efficient and the Cellic Htec was 84% active. However, it is important to note that the protocol mentioned a 48 hours hydrolysis incubation time, and due to problems associated with the laboratory availability, the hydrolysis was only finished 72 hours later.
Assays should have been performed at the same as the previously mentioned enzymatic hydrolysis, however, due to some internal problems, this part of the experiment was only performed after the fermentation step. The results obtained from the FPases assay and xylanases assay, showed that the activity of Cellic Ctec and Cellic Htec was lower than expected, 48U/ml and 43 U/ml, respectively. These results were then compared with those presented in the literature and Cellic Ctec was found to be 30% active and Cellic Htec was 43% active.

3.3 Enzymatic Hydrolysis of the Press Cake

The first time enzymatic hydrolysis was performed, the efficiency of the enzymes was taken into consideration, so calculations were made to add 3%, 5% and 6% enzyme loading based on the amount of cellulose and hemicellulose present in the press cake fraction and in the efficiency of Cellic Ctec and Cellic Htec. The samples were performed according to Novozymes protocol for the utilization of Cellic Ctec and Cellic Htec, pH=5,0 and temperature equal to 50ºC. Samples were also performed at pH=5,5 given that the objective of this project was to then conduct enzymatic hydrolysis and fermentation of the press cake with the green juice. This combination yielded a pH of 5,5, so that was why the enzymatic performance at pH=5,5 was also tested. Furthermore, this experiment followed the del Bairrio (2016) protocol, so in order to check if adding hemicellulases would also increase the amount of glucose released, samples followed this method and Cellic Ctec enzyme loading 3%, was also performed.

The bar charts below show an overview of the sugars released and organic acids produced during 48 hours and 72 hours hydrolysis at 50ºC and both pH=5 and 5,5. Note that calculations were made in order to present the results in g/kg of PC.

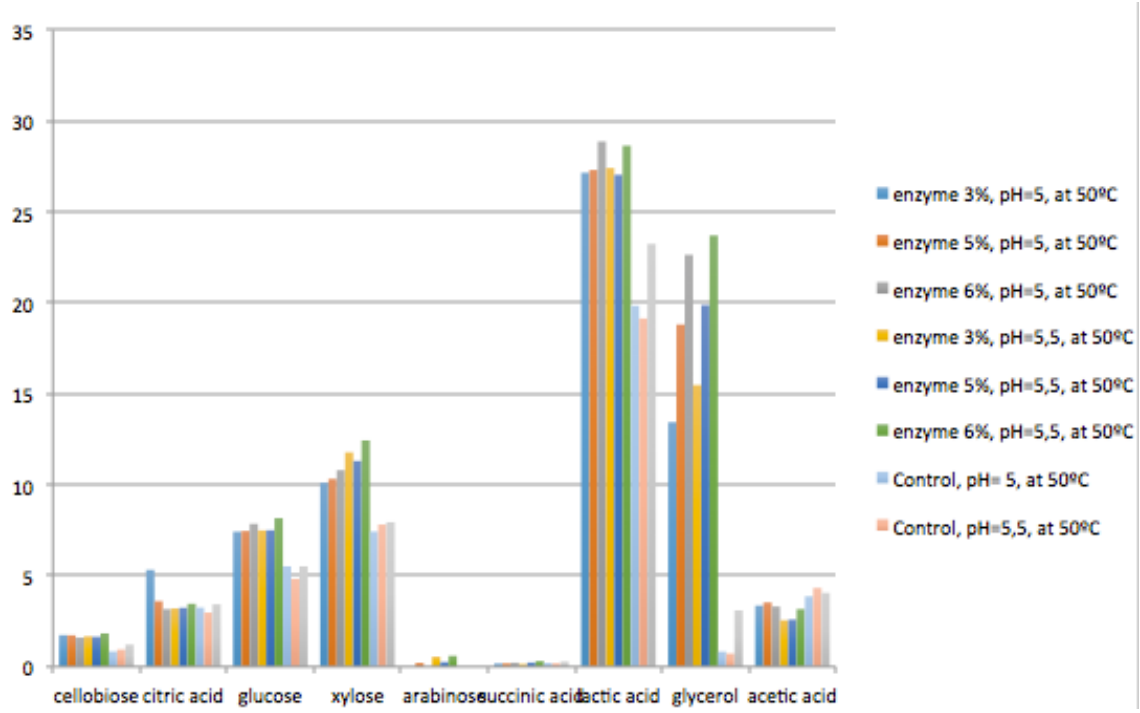

Figure 6: HPLC results in g/kg of PC of the enzymatic hydrolysis after 48 hours.

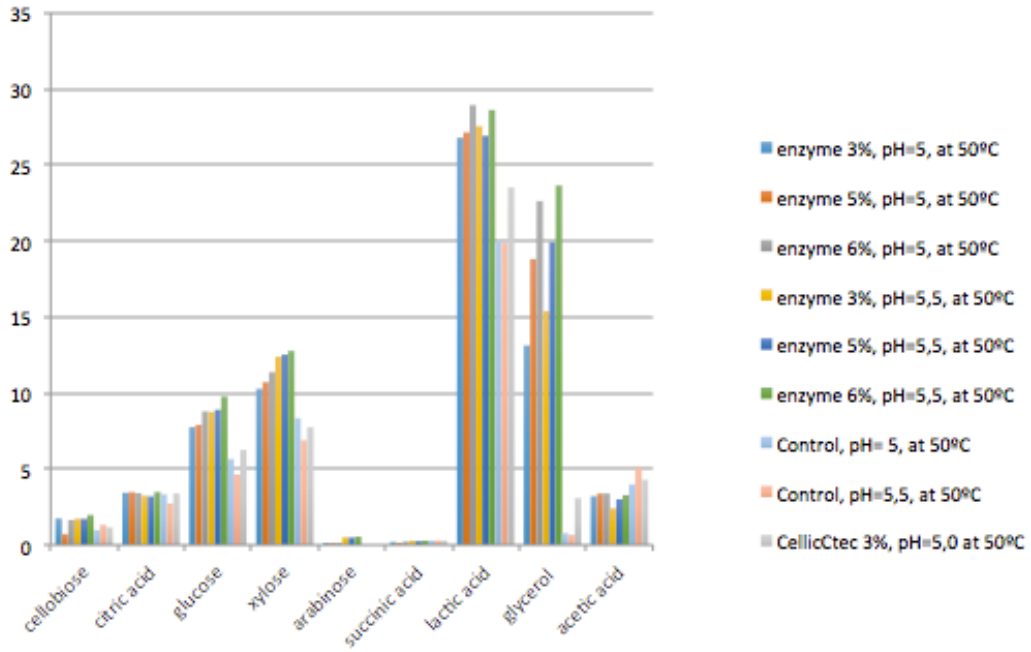

Figure 7: HPLC results in g/kg of PC of the enzymatic hydrolysis after 72 hours.

From the presented results, it appears that increasing the enzyme loading also increases the amount of monomer sugars obtained after 48 hours and 72 hours hydrolysis. The samples with enzymes loading 6% seem to present higher results than those with the same procedure but with lower enzyme loadings. In the case of glucose, for example, 6% enzyme loading at pH=5,0 after 72hours hydrolysis yielded 8,78 g glucose/kg PC, which was higher than the 7,75 and 7,90 g glucose/kg PC in the same conditions at 3% and 5% enzyme loading, respectively. When pH=5,5 and enzyme loading was 6%, 9,76g glucose/kg PC and 8,75 and 8,89 g glucose/kg PC were obtained in the samples at the same conditions and for enzyme loadings 3% and 5%, respectively. It was also noted that it does not seem so advantageous to hydrolyze the material for 72hours, since most of the sugars were obtained after 48hours hydrolysis. Only 1,6 g glucose/kg PC more were obtained after 72hours in comparison with 48hours for samples at 6% enzyme loading and pH=5,5. Moreover, only 0,32 g xylose/kg PC more was obtained after 72hours hydrolysis than when compared with 48hours.

The main problem of this experiment appears to be the production of organic compounds like glycerol and lactic acid. It seems that all the samples presented more lactic acid and glycerol than monomer sugars. For example, the sample that presented more lactic acid was enzyme loading 6%, pH=5 after 72hours, 28,94g lactic acid/kg PC. This value is much higher than the 8,79g glucose/kg of PC or 11,37 g xylose/ kg PC presented by the same sample. The higher values of glycerol were presented by the samples with pH=5,5 and enzyme loading 6% after 48 hours with, 23,7g glycerol/kg PC.

Furthermore, contrary to the literature, it was appears to have been advantageous to start the hydrolysis at pH=5,5, since when comparing the amount of sugars obtained by these samples at this pH with the samples at pH=5, more monomer sugars were obtained by the former. In Appendix 1 not only the results of monomer sugars are presented, but also the organic acids produced and the amount of monomer sugars obtained if no fermentation had occurred. From the mentioned table, it is possible to understand that the samples presenting a higher sugar release, if no fermentation had occurred, appear to be the samples at pH=5,5, regardless of the loading of the samples. 56,46g total sugars/kg PC were obtained after 72 hours hydrolysis when enzyme loading was 6% and pH=5,5.

In some cases, such as glucose and xylose, slightly more sugars were obtained by 3% and 5% enzyme loading at pH=5,5 than with enzyme loading 6% but at pH=5. However, in terms of organic compound production, the same did not happen. In fact, for the production of organic compounds, it seems that the values increased with the enzyme loading, but enzyme loading at 6% pH=5 still presented higher results than samples with enzyme loadings 3% and 5% pH=5,5.

Despite the calculation of how much sugars were obtained without fermentation, it is not 100% accurate, since the amount of glucose and other monomer sugars and organic compounds present at the beginning of the experiment was not taken into account, however it can still give an idea of how efficient the hydrolysis was. Considering that 223g cellulose/kg PC and 157g hemicellulose/ kg PC are presented, it gives us a total of 380g sugars/kg PC. At the same time, taking the best

result obtained, 56,46 g total sugars/kg PC when hydrolysis is performed for 72hours, at 6% enzyme loading and pH=5,5, it will give an efficiency of 15% of the enzyme combination. It is difficult to make assumptions on the efficiency of Cellic Ctec and Cellic Htec alone, since contamination occurred.
Additionally, due to organic acid production, it is difficult to understand how far Cellic Htec contributed to the monomer sugar release, however, when comparing the samples that only contained Cellic Ctec at 3% and pH=5 with the samples containing a combination of Cellic Ctec with Cellic Htec at 3% total enzymes loading, it appears that not only significantly more xylose and arabinose were obtained from the combination of enzymes but also a further 1,5 g glucose/kg PC and 0,58g cellobiose/kg PC were released.

3.4 Lactic Acid Fermentation of Different Set Ups

In the following experiment, even though poor efficiency of the enzymes was observed, the decision was still taken to continue with the next step where hydrolysis and fermentation of the press cake combined with water and green juice were performed. Following the best results of the enzymatic hydrolysis, during this step of the project, the enzymatic hydrolysis was performed at 50ºC, pH=5,5 and total enzyme combination loading of 6%.
During this part of the experiment, fermentation of green juice and green juice combined with glucose stock solution was also performed as control groups.
The table below presents the HPLC results of the first set up, which contained flacks where hydrolysis was performed for 48 hours at 50ºC and after fermentation was performed for a further 24 hours but at 37ºC.
The results from the first set up show an overall consumption of sugars, both C6 and C5 sugars, even before the fermentation had started. As presented in the table above, at the beginning of the experiment 0,78g cellobiose/kg biomass, 9,25g glucose/kg biomass, 7,26 g xylose/kg biomass and 0,39g arabinose/kg biomass were presented and these values got lower and lower until the end of the hydrolysis, and before the fermentation had started. As the sugars were consumed, an increase in the amount of organic acids, especially lactic acid and acetic acid could be observed. At the end of the fermentation process, it was possible to obtain 24,84g lactic acid/kg biomass and 21,07g acetic acid/kg biomass.
From this set up it became impossible to verify the efficiency of the enzymes, since it is clear that contamination of the biomass made the production of organic acids before fermentation had started. However, it is possible to notice that from the amount of total sugars added/kg sample at the beginning, only 4% was converted into lactic acid. However, it is also possible to verify that from the sugars added/kg sample, approximately 6,1% was converted into an organic acid. Furthermore, all the lactic acid appears to have been produced during the first 24 hours of hydrolysis, and contrary to what was expected, after adding the lactic acid bacteria, the amount of acetic acid increased to 21,07g/kg biomass, but the amount of lactic acid was still 24,84g/kg biomass.

In the methods and materials, the set ups are presented in the order presented in the protocol, however, for the further discussion, the only set ups that are comparable are those that present the same methodology. Since the first set up was hydrolysis for 48hours of press cake with water followed by fermentation, the hydrolysis for 48hours of green juice and press cake followed by 24hours fermentation will now be presented, in order to facilitate understanding of the discussion part of this project:

Set up 1: g/kg biomass	cellobiose	citric acid	Glucose	xylose	Arabinose	succinic acid	lactic acid	glycerol	acetic acid
Beginning experiment	0,78 (s.d.=0,04)	1,68 (s.d.=0,66)	9,25 (s.d.=0,32)	7,26 (s.d.=1,02)	0,39 (s.d.= 0,02)	0,72(s.d.=0,03)	0,00 (s.d=0,00)	4,24(s.d.=0,12)	0,36(s.d=0,04)
24h hydrolysis	0,00 (s.d.=0,00)	2,00 (s.d.= 0,25)	5,60 (s.d.= 0,05)	5,73 (s.d.= 0,55)	0,00 (s.d.=0,00)	0,00(s.d.=0,00)	25,90 (s.d=0,73)	4,10(s.d.=0,21)	3,37(s.d=0,13)
48h hydrolysis	0,00	1,33 (s.d.= 0,45)	4,01 (s.d.=0,91)	4,28 (s.d= 0,22)	0,00 (s.d.=0,00)	0,22(s.d=0,05)	21,29 (s.d=0,95)	3,67(s.d.=0,33)	15,26(s.d.=0,67)
End fermentation	0,00 (s.d.=0,00)	1,08 (s.d.=0,33)	3,76 (s.d.=0,59)	4,17 (s.d.=0,88)	0,00 (s.d.=0,00)	0,25 (s.d=0,02)	24,83 (s.d=0,67)	0,00(s.d.=0,00)	21,07(s.d=0,44)

Figure 8: HPLC results from set up one in g/kg of biomass.

As in the previous set up, in set up 4 it is possible to note a consumption of sugars across the entire length of the experiment and not only during fermentation. Here for example, 9,89g glucose/kg biomass was presented, and at the end of the experiment the amount of glucose was reduced to around half: 4,89g/kg biomass.

In this set up, most of the lactic acid also appears to have been produced in the first 24hours of the experiment, 20,04g/kg of biomass, but this production continued slowly until the end of the experiment, when 26,23 g lactic acid/kg biomass was obtained. As in the previous set up, here a production of acetic acid until the end of the experiment and a consumption of glycerol were also observed.

It is difficult to understand the efficiency of the enzymes, due to the production of organic acids even before the fermentation had started. However it is possible to note that from the initial amount of sugars added/kg sample at the beginning of the experiment, the microorganisms that produce organic acids utilized only 14% of these sugars. In terms of lactic acid, 9,4% of the sugars appear to have been converted into lactic acid in this set up.

Set up 4: g/kg biomass	cellobiose	citric acid	glucose	xylose	Arabinose	succinic acid	lactic acid	glycerol	acetic acid
Beginning experiment	0,62(s.d.=0,11)	1,62(s.d.=0,89)	9,89(s.d.=0,52)	11,16(s.d.=2,12)	0,22(s.d.=0.09)	0,46	0,37(s.d=0,11)	1,62(s.d.=0,09)	0,21(s.d=0,33)
24h hydrolysis	0,00(s.d.=0,00)	1,39(s.d=0,44)	4,60(s.d.=0,33)	4,49(s.d.=0,98)	0,08(s.d.=0,01)	0,26	20,04(s.d.=1,35)	1,35(s.d=0,45)	8,80(s.d=1,11)
48h hydrolysis	0,00(s.d.=0,00)	1,57(s.d.=0,24)	5,31(s.d.=0,19)	5,44(s.d.=0,35)	0,03(s.d=0,01)	0,14	20,75(s.d=1,45)	1,69(s.d.=0,33)	9,33(s.d=0,65)
End fermentation	0,00(s.d.=0,00)	0,94(s.d.=0,32)	4,89(s.d=1,00)	4,80(s.d=0,45)	0,00(s.d=0,00)	0,17	26,23(s.d.=0,89)	0,00(s.d.=0,14)	12,29(s.d=0,99)

Figure 9: HPLC results from set up 4 in g/kg of biomass.

Contamination was already expected to occur, since the green biomass already contains natural bacteria able to perform fermentation under oxygen inhibition. In order to try to increase the amount of sugars available for *Lactobacillus salivarius* during fermentation, it was thought to reduce the time of the hydrolysis in the set ups 3 and 5 to 16hours only and increase the time of fermentation to 48hours.

Starting with set up 3, where hydrolysis was performed in the press cake in combination with water as explained in the methods and materials section, the table below presents the HPLC results of the samples of set up 3 at different times:

Set up 3: g/kg biomass	cellobiose	citric acid	glucose	xylose	Arabinose	succinic acid	lactic acid	glycerol	acetic acid
Beginning experiment	0,00 (sd=0,00)	2,49(sd=0,22)	6,99(sd=0,34)	0,00(sd=0,00)	0,00(sd=0,00)	0,00(sd=0,00)	2,48(sd=0,22)	1,72(sd=0,15)	8,42(sd=1,13)
16h hydrolysis	0,00(sd=0,00)	2,22(sd=0,13)	12,57(sd=1,57)	9,00(sd=1,92)	1,00(sd=0,10)	0,64(sd=0,19)	9,00(sd=0,96)	4,57(sd=0,40)	1,07(sd=0,16)
24h fermentation	0,00(sd=0,00)	2,11(sd=0,35)	4,06(sd=0,68)	4,90(sd=0,22)	0,00(sd=0,00)	0,19(sd=0,03)	30,96(sd=3,84)	6,54(sd=0,83)	5,78(sd=0,63)
End fermentation	0,02(sd=0,00)	0,75(sd=0,12)	4,60(sd=0,23)	3,93(sd=0,92)	0,00(sd=0,00)	0,29(sd=0,09)	35,49(sd=0,93)	0,29(sd=0,06)	18,92(sd=1,22)

Figure 10: HPLC results from set up 3 in g/kg of biomass.

From the results presented, it is possible to understand that here the production of organic acids started before the fermentation, since after 16hours hydrolysis 9,00g lactic acid/kg of biomass were presented in comparison with the 2,48g lactic acid/kg biomass presented at the beginning of this experiment.
Contrary to set up 1 and 4, here it was possible to notice an increase in the amount of sugars from the beginning of the experiment to after 16hours of hydrolysis. A further 9,00g xylose/kg biomass, 1,00g arabinose/kg biomass and 5,58g glucose/kg biomass were presented than at the beginning of the experiment.
At the end of the experiment, not all the sugars were consumed, since 4,60g glucose/kg biomass and 3,39g xylose/kg of biomass were still presented. 35,49g lactic acid/kg of biomass and 18,92g acetic acid/kg biomass were also obtained. In terms of lactic acid alone, it corresponds to 4,8% of the initial sugars added/kg sample. If we consider the total organic acids produced, from the initial sugars added/kg sample, then it corresponds to around 12,2% of utilized sugars. Note that even though the amount of sugars left does not appear to be enough to do 100% of the total initial sugars added, that is because complex sugars are not measured using this procedure.
As explained before, a set up was also performed where the enzymatic hydrolysis of the press cake was performed for 16 hours and fermentation for 48hours but where the press cake was mixed with green juice and water instead of only water.

Set up 5: g/kg biomass	cellobiose	citric acid	glucose	xylose	Arabinose	succinic acid	lactic acid	glycerol	acetic acid
Beginning experiment	0,33 (sd=0,03)	1,78(sd=0,33)	11,61(sd=1,14)	9,65(sd=0,93)	0,00(sd=0,00)	0,10(sd=0,01)	4,30(sd=0,36)	2,55(sd=0,09)	0,67(sd=0,21)
16h hydrolysis	0,33(sd=0,01)	1,83(sd=45)	5,88(sd=1,25)	5,85(sd=1,43)	0,19(sd=0,03)	0,12(sd=0,05)	18,73(sd=2,30)	1,52(sd=0,59)	0,84(sd=0,33)
24h fermentation	0,31(sd=0,01)	1,95(sd=0,12)	4,78(sd=1,85)	2,99(sd=0,67)	0,00(sd=0,00)	0,19(sd=0,03)	20,10(sd=0,90)	2,40(sd=0,17)	2,18(sd=0,29)
End fermentation	0,00(sd=0,00)	1,12(sd=0,22)	5,95(sd=1,33)	2,85(sd=84)	0,00(sd=0,00)	0,33(sd=0,03)	22,32(sd=1,25)	2,74(sd=0,22)	5,45(sd=0,66)

Figure 11: HPLC results from set up 5 in g/kg of biomass.

Contrary to set up 3, in the HPLC results from set up 5 it is not possible to observe any degradation of cellulose or hemicellulose, since the amount of sugars presented after 16hours hydrolysis is lower than at the beginning of the experiment. In terms of glucose for example, the initial value was 11,61g/kg biomass while after 16hours it was only 5,88g/kg biomass.

In this set up, one may also observe that the highest rate of production of lactic acid was actually obtained in the first 16hours of hydrolysis, 18,73g lactic acid/kg biomass. After the addition of *Lactobacillus salivarius*, only a further 3,59g lactic acid/kg biomass was produced, yielding a final amount of 22,32g lactic acid/kg biomass. If the amount of sugars added at the beginning of the experiment to the amount of lactic acid produced is taken into consideration, it corresponds to only 13% of the sugars added/kg sample. In terms of organic acids produced from the sugars/kg sample added at the beginning of the experiment, it corresponds to 14,6%.

Set up 2: g/kg biomass	cellobiose	citric acid	glucose	xylose	Arabinose	succinic acid	lactic acid	glycerol	acetic acid
Beginning experiment	0,47(sd=0,08)	2,05(sd=0,07)	15,40(sd=1,21)	8,89(sd=0,98)	0,00 (sd=0,00)	0,17(sd=0,01)	11,63(sd=1,23)	1,62(sd=0,32)	0,21(sd=0,02)
12h fermentation	0,44(sd=0,03)	1,80(sd=0,18)	8,00(sd=1,00)	3,80(sd=0,51)	0,00(sd=0,00)	0,16(sd=0,06)	19,80(sd=1,39)	1,35(sd=0,21)	8,80(sd=1,11)
end fermentation (24h)	0,40(sd=0,03)	2,14(sd=0,21)	6,89(sd=0,76)	0,00(sd=0,00)	0,00(sd=0,00)	0,20(sd=0,05)	28,72(sd=3,45)	1,69(sd=0,53)	9,33(sd=1,38)

Figure 12: HPLC results from set up 2 in g/kg of biomass.

The table above represents the results obtained in the samples from set up 2, where green juice was added to glucose stock solution. As proposed in the literature, addition of glucose stock solution to the green juice increases the production of lactic acid, since the amount of sugars present in the green juice is normally not enough for high productions of lactic acid.
As explained in the introduction, the green juice is expected to only present monomer sugars, but contrary to such expectations, it should not present any lactic acid. However, in these results it is possible to note that 11,63g lactic acid/kg biomass was presented at the beginning of the experiment.
At the end of the experiment, a further 17,09g lactic acid/kg biomass was produced. This corresponds to 20% of the monomer sugars utilized. Note that the pH dropped to only 4 during these 24hours but it was preferable to stop the fermentation after 24hours than until the lactic acid production stopped.
Contrary to what was expected from the bacteria utilized in this experiment all the C5 sugars appear to have been consumed during the fermentation, since at the beginning 8,89g xylose/kg biomass was presented and at the end of the experiment no xylose was present.
6,89 g glucose/kg biomass seemed to be presented at the end of fermentation and the amount of cellobiose appears not to have changed significantly over time.

In order to see the difference between the production of lactic acid with glucose stock solution addition and without any other source of sugar addition, set up 6 was performed, where the fermentation of green juice alone was performed for 24hours. The table below presents the HPLC results over different times of the experiment of set up 6:

Set up 6: g/kg biomass	cellobiose	citric acid	glucose	xylose	Arabinose	succinic acid	lactic acid	glycerol	acetic acid
Beginning experiment	0,55(sd=0,11)	2,29(sd=0,25)	10,00(sd=1,23)	9,36(sd=1,23)	0,33(sd=0,03)	0,70(sd=0,13)	3,60(sd=0,63)	1,89(sd=0,22)	0,61(sd=0,09)
12h fermentation	0,42(sd=0,03)	2,34(sd=0,52)	6,70(sd=1,45)	1,07(sd=0,06)	0,00(sd=0,00)	0,20(sd=0,02)	23,44(sd=2,66)	1,45(sd=0,39)	3,88(sd=0,67)
end fermentation (24h)	0,30(sd=0,16)	1,87(sd=0,33)	6,73(sd=9,92)	0,00(sd=0,00)	0,00(sd=0,00)	0,27(sd=0,09)	23,97(sd=1,93)	1,68(sd=0,19)	5,06(sd=1,03)

Figure 13: HPLC results of set up 6 in g/kg of biomass.

From these results it is possible to note that not so much lactic acid was present at the beginning of the experiment, only 3,60g lactic acid/kg biomass, which means that around a further 20,37g lactic acid/kg of biomass was produced from the beginning of fermentation with *Lactobacillus salivarius*.

It was calculated that 48g sugars/kg biomass would be added, however, from the HPLC results obtained at the beginning of the experiment, it appears that less than 25g sugars/kg biomass was added. Utilizing this amount of sugars, calculations point to around 44% of the initial sugars/kg sample available being converted into lactic acid and 10% into acetic acid. It is important to note that the pH at the end of the experiment was found to stand at around 3,12 in the samples of this set up. It is also important to observe that more sugars were converted into lactic acid in this experiment, but less initial sugars were added here.

3.5 Optimization of Sugar Release:

Since many parameters were playing a role in the previous experiment, it was difficult to identify the main problem for the low production of lactic acid. However, the set ups where enzymatic hydrolysis had previously been performed, appeared to present less sugars converted into lactic acid than those where only green juice was utilized. Furthermore, those with hydrolysis of the press cake and green juice also presented a slightly higher conversion from initial sugars to lactic acid but not because the hydrolysis worked better, only because more monomer sugars were available, since green juice theoretically only presents simple sugars in its composition.

Even though it was really difficult to detect the reason why the previous set-ups did not work, as will be explained in the discussion section, it appears to be obvious from the efficiencies of the first enzymatic hydrolysis, as explained in topic 3.2 of this section, that hydrolysis should be improved before fermentation.

In this part of the project, the aim was to try to optimize enzymatic hydrolysis by application of autoclaving and washing, in separated flasks, as possible pre-treatments of the biomass before enzymatic hydrolysis. The decision was also taken to perform these set-ups with 5%DM per total solution and with 5%DM without accounting for the amount of water present in the biomass, so with 4,4%DM per total solution.

During this experiment, set ups without any further pre-treatment are used for comparison. An overview of the HPLC results of all these set ups is presented in the graphs below:

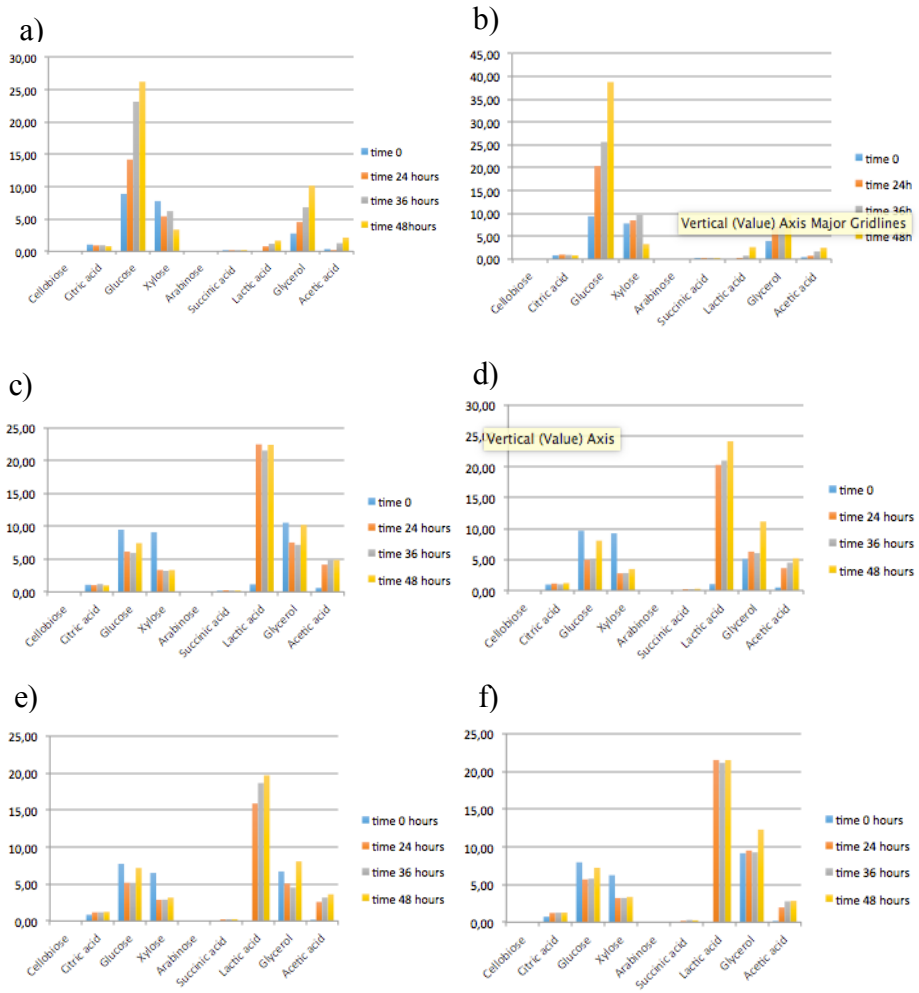

Figure 14: Results from the last enzymatic hydrolysis of press cake done in this project. Note:(a) HPLC results of autoclaved press cake 5%TS; (b) HPLC results of autoclaved press cake with lower TS concentration; (c) HPLC results from washed biomass 5% TS; (d) HPLC results from washed biomass at lower TS concentration; (e) HPLC results of solution at 5% TS without extra pretreatment; (f) HPLC results of solution with 5,32g biomass and 40ml of water without extra pretreatment.

As mentioned in the description of the tables, the first two corresponded to autoclaved press cake before enzymatic hydrolysis. In set up 1, 26,24g glucose/kg of biomass was obtained from the initial 8,89g glucose/kg biomass presented at the beginning of the experiment.
Contrary to glucose, xylose production appears to have decreased over time, since initially 7,78g/ kg of biomass was presented and at the end only 3,37g/kg of biomass was observed. This may explain why the amount of lactic acid, glycerol and acetic acid may have increased slightly over time.
In set up 2, the amount of glucose increased from 9,38g/kg biomass to 38,75g/kg biomass, becoming the highest result of glucose obtained from the different set-ups.
Similarly to set up 1, set up 2 also presented less xylose at the end of the experiment than at the beginning, 9,12 g/kg biomass compared with 3,34g/kg biomass. Here also some lactic acid, glycerol and acetic acid were produced with glycerol amounts standing at 9,75g/kg biomass after 48hours enzymatic hydrolysis.
Even though not completely accurate, it may be assumed that the Cellic Ctec performed with an efficiency of 31% in the first set up and with an efficiency of 46% in the second set up. The same assumption cannot be made for CellicHtec since under no conditions did a higher consumption of this sugar in comparison with its possible degradation by the enzyme appear to have occurred.
The results of set up 3 are represented in graph number x and show, as expected, that instead of a high amount of monomer sugars, a decrease was obtained while a constant increase was observed for organic acid production. At the beginning of the experiment, 9,5g glucose/kg biomass, 9,12g xylose/kg biomass and 1,17 g lactic acid/kg biomass were presented. At the end of the experiment, only 7,45g glucose/kg biomass and 3,34g xylose/kg biomass were presented but at the same time 22,45g lactic acid/kg biomass were presented.
Most of the monomer sugars also appear to have been consumed and converted to lactic acid and acetic acid in the first 24 hours of hydrolysis.
Here the amount of glycerol appears to have been slightly reduced at the beginning of the experiment but increased again to around 10g/kg biomass at the end of the experiment.
Set up 4 followed the same protocol as set up 3 but with lower DM content per total solution, as in set up 2. Here, it was noted that glucose and xylose were also consumed over time and lactic acid, acetic acid and glycerol were also produced in considerable quantities.
In this set up, glucose appears to have been consumed from 9,65g/kg biomass to 5,14g/kg biomass in the first 36hours, but then its accumulation increased again to 8,06g/kg biomass from 36hours to 48hours.
9,25g xylose/kg biomass was presented at the beginning of the hydrolysis in this set up and only 3,17g xylose/kg biomass at the end of the experiment. Contrary to xylose consumption, lactic acid increased from 1,04g/kg biomass to 24,13g/kg biomass, glycerol increased from 5,10g/kg biomass to 11,15g/kg biomass and acetic acid from 0,49g/kg biomass to 5,21g/kg biomass.
Set up 5 is presented in graph xxx and as in set up 3 and 4, the sugars appear to have been consumed at the same time as the production of lactic acid and other products from fermentation

were produced. Here one may note that glucose concentration was initially 7,72g/kg biomass but it was then reduced to 5,18g/kg biomass after 24 and 36hours and increased again to 7,17g/kg biomass after 48hours. The amount of xylose decreased from 6,5og/kg biomass to 3,17g/kg biomass and the amount of lactic acid increased from 0 to 19,69g/kg biomass. The glycerol amount slightly increased from 6,70g/kg biomass to 8,07g/kg biomass after 48hours but it also presented lower results during the 24 and 36 hours, as was the case with glucose. The amount of acetic acid increased from 0,14g/kg biomass to 3,61g/kg biomass.

The last set up is presented in the last graph and it shows glucose concentration of 7,95g/kg biomass and 7,22g/kg biomass at the beginning and end of the experiment, respectively, but it presents a slightly lower concentration of this sugar at 24 and 36 hours hydrolysis.

The xylose concentration was reduced from 6,24g/kg biomass to 3,35g/kg biomass and lactic acid increased from 0 to 21,52g/kg biomass. The glycerol increased from 9,15g/kg biomass to 12,29g/kg biomass and the acetic acid only from 0,10g/kg biomass to 2,84g/kg biomass at the end of the experiment.

Despite the fact that the control group, where no enzyme addition occurred, is not presented in the graphs above, it was possible to observe that 4,24g glucose/kg biomass and 2,82g xylose/kg biomass were obtained at the end of the experiment. Furthermore, only 10,53g lactic acid/kg biomass, 0,7g glycerol/kg biomass and 3,11g acetic acid/kg biomass were produced in these flasks.

4. Discussion

4.1 Material Analysis

It is difficult to find in much information in the literature on the dry matter, organic matter and the differences in the composition of the different fractions of a green biomass. In the study presented by Santarmaria-Fernandez *et al* (2017), the alfalfa press cake was found to contain 255,7 g dry matter/kg sample and 233,8 g organic matter/kg sample. In the green juice obtained from the separation of the alfalfa biomass into solid and liquid fraction, 52,6 g dry matter/kg sample and 39,6 g organic matter/kg sample were yielded in the same study. These values are different from those found in the present study, however it has been reported that the composition and quantity of dry and organic matter might change with the type of green biomass used, time and conditions of cultivation and harvesting and separation methodologies. It is important to observe that storage and handling methods may also contribute to changes in the composition of these fractions.

Furthermore, the amounts of cellulose and hemicellulose fractions presented in the present study are slightly different from other studies, especially in terms of the amount of cellulose. In some previous studies the amount of cellulose present in alfalfa fibers are reported to stand at around 28-32%, however, in the present study, this value is slightly lower, at 22,3%. The hemicellulose amount is within the parameters generally presented in other studies: 10-18% (Koegel *et al*, 1999). Again, the main reasons may explain the lower amounts of cellulose, but the most probable is that the material used for the strong acid hydrolysis analysis was left outside the fridge for a long

period of time in order to obtain a completely dry material. The material was not left in sterile conditions, so many changes may have occurred in the material during that period, for example, by contamination of the microorganisms. Another possible reason is that during the strong acid hydrolysis, some mistakes were made while adding the STAM solution, increasing the possibility of wrong results when doing the calculations.

4.2 Enzymes and Enzymatic Hydrolysis Analysis

As previously explained, during the laboratory period of this project, enzyme activity should have been checked before using the same ones in the enzymatic hydrolysis. The efficiency can give an idea of the performance of the enzymes used, under certain conditions, but it is important to notice that changing the material can make the enzymes more or less efficient. In this study, it became clear how important it is to check for the activity of the enzymes, even if using commercial enzymes, since when using wheat straw 86% of Cellic Ctec seemed to present while when performing the different hydrolysis in this study, the efficiency of this enzyme was much lower. However, it is also important to observe that the enzymatic hydrolysis of wheat straw should have been performed for only 48hours, and instead it was performed for a longer period of time, which might have increased the efficiency calculated when compared with the efficiencies when using the press cake, when hydrolysis was performed for 48hours.

Del Bairrio (2016) presented the best results when using Cellic Ctec with only 3% enzyme loading per amount of cellulose presented in the press cake. However, when repeating the same methodology in the present project, lower results were yielded than when using 3% total enzyme loading, but in a combination of 2,7%Cellic Ctec per amount of cellulose in a total solution and 0,3% of Cellic Htec per amount of hemicellulose per total solution. This is in line with the idea of previous studies that degrading hemicellulose fraction can help celluloses reach more parts of the cellulose fraction, increasing the performance of the overall enzymatic hydrolysis. Another possible reason is that some glucose is present in the hemicellulose fraction, which may correspond to the higher amounts of glucose when also using Cellic Htec, than when using only Cellic Ctec.

Due to the amount of cellulose and hemicellulose present in the press cake, than when compared with other types of material, it would be expected that such a high enzyme loading, such as 6%, would not be necessary however the fact that the activities of the enzymes were so low in this study, may have influenced the results, and slightly increasing the concentration of enzymes might have yielded higher efficiencies.

Contrary to the guidelines of how to handle the enzymes, where it is suggested that this combination of enzymes performs better at pH=5 than pH=5,5, in the present study, the pH=5,5 appears to have increased the performance of the enzymes. However, it is difficult to make any conclusions about the enzyme activity, since it is clear by the amount of organic acids, that contamination had occurred. Some microorganisms are able to produce enzymes that can degrade these complex molecules and since the kind of microorganisms that contaminated this press cake

were not analyzed, it is impossible to understand whether in fact the commercial enzymes worked and how they worked and how might the cultivation of the material by other microorganisms have also influenced the results.
As previously mentioned, optimization of the hydrolysis should have also been done before performing fermentation for lactic acid production and it would have been extremely important to take samples for HPLC analysis of the initial solution during the first hydrolysis, so a comparison between the performance of the two hydrolyses could be directly made. If the samples had been taken at the beginning of the first hydrolysis, it would have been possible to understand how many sugars and organic acids were already present at the beginning of the experiment and how many were actually degraded and produced. With access to such results, it would be possible to understand if increasing the concentration of enzymes in order to achieve a theoretical activity of 100% increased the performance of the enzymes, or if in fact, by increasing 3x Cellic Ctec concentration and 2x Cellic Htec concentration, during the second hydrolysis the turnover of some enzymes may have occurred due to the fact that enzymes can be turned off when their concentration is too high for the amount of substrate presented.

4.3 Lactic Acid Fermentation Using Different Set Ups

As previously mentioned in this project, fermentation should not have been performed in this project, instead more enzymatic hydrolysis of the press cake with water and then with green juice should have been performed before the lactic acid fermentation.
As may be observed in the results, first it is difficult to compare the different set ups as they each involved different methodologies and parameters. Furthermore, the sample taking times for HPLC analysis differed throughout the different set ups, making the comparison even more difficult.
Comparing set up 1 with set up 4, that had the same methodology but one used water while the other used green juice, respectively, more sugars were found to have been converted into lactic acid when also using green juice than when only using water. However, this does not indicate that the enzymes performed better when using a solution of green juice and press cake instead of water and press cake. In fact, green juice is composed mainly of simple sugars, meaning that the different organisms started to utilize these monomer sugars even before the enzymes started work.
When comparing set up 3 and 5, more lactic acid appears to have been produced in set up 3 than in set up 5. However, when comparing the percentages of the lactic acid produced per kg sample, more lactic acid was produced per kg of green juice and press cake than only press cake.
In the case of set up 2, the glucose stock solution was added in order to obtain the same amount of sugars added to the press cake fraction combined with the green juice. However here fermentation was only performed for 24hours, and an exponential production of lactic acid was observed in the first 12hours after which period it slowly increased until reaching 24hours. The pH of the samples was measured to be around 4 at the end of fermentation, indicating that if fermentation had continued the sugars that were left might have been converted into more organic acids, even if slowly, until the pH had achieved around 3,4-3,6 or until all the sugars had been consumed.

Set up 6 seems to have been the one to work better, since more sugars were converted into lactic acid in this set up per total amount of sugars/kg sample added. However, it is important to note that far fewer sugars were added in this set up than in the afore- mentioned ones, since only green juice was utilized.

In this experiment in general, samples were taken at time 0 in all the set ups, which helped clarify that the green juice was already contaminated and fermentation by microorganisms had already started in this fraction, before the beginning of the experiment. Nevertheless, in the samples with only press cake and water less amounts of lactic acid, acetic and glycerol seemed to present at the beginning of the experiment, contamination also seems to have occurred in all the samples, before the fermentation with cultivation of *Lactobacillus salivarius* had begun. This contamination by microorganisms made it almost impossible to understand the efficiency of the enzymes. However, based on the amount of total sugars and organic acids obtained at the end of the experiment, a large quantity of cellulose and hemicellulose was still left to be converted into monomer sugars.

Another parameter that appears to have been similar in all the set ups, is that the xylose part that was present at the beginning of all the set ups was partially or totally consumed by the microorganisms in this experiment. In theory, *Lactobacillus salivarius* cannot naturally consume C5 sugars, however, since contamination by different microorganisms occurred before fermentation started or even before the experiment started, it is difficult to understand the reason for such consumption of xylose.

4.4 Pre-Treatment of the Material Before Enzymatic Hydrolysis

Throughout this project, it is possible to notice that pre-treatment of the material should, in fact, have been performed to increase the enzymatic hydrolysis efficiency. From the results presented in section 3.5, it is possible to observe that the most efficient method for the enzymatic hydrolysis without contamination of the material is autoclaving the solution before enzyme addition. This not only appears to significantly reduce the amount of fermentable products obtained after fermentation for 48 hours but it also seems to increase the overall sugars obtained. In set up 1 and 2, it was possible to obtain a further 18,20g sugars/kg biomass and 30,18 sugars/kg sample than initially. This compared with only a further 4,77g sugars/kg biomass obtained in set up 3 or 10,75g sugars/kg biomass obtained in set up 6.

Note that the quantities of fermentable products presented during the enzymatic hydrolysis of the autoclaved samples might have occurred either due to the fact that some microorganisms can survive up to 121ºC for only 20minutes or because during the sampling, some mistake might have occurred and instead of it being performed in 100% sterile conditions, some microorganisms might have been entered the flasks.

It is difficult to know how many sugars it was possible to obtain, since contamination occurred in the samples where autoclaving was not performed. However, it is possible to calculate how many sugars were more or less possible to obtain without fermentation, based on the amount of products

obtained and the initial sugars already present at the beginning of the experiment can be subtracted in order to better understand the performance of each set up.

Contrary to what was presented in the study by Ambye-Jensen *et al*. (2014), in this study it did not seem so advantageous to wash the biomass before the enzymatic hydrolysis. In fact, washing the biomass did not increase the amount of sugars released than when compared with the set ups where no pre-treatment was performed before the hydrolysis.

Even though it is necessary to use more active enzymes in the next experiment, it was still possible to observe their efficiency in this last experiment, since the control groups presented exactly the same amount of sugars at the beginning and end of the experiment, and those containing the enzymes presented more sugars at the end of the experiment than when compared with the starting point.

Besides the pretreatment of the material, another factor appearing to have a strong influence on the amount of sugars obtained is the amount of water added. It is preferable not to consider the amount of biomass as part of the volume of the solution, since in all the cases, it was possible to obtain more total sugars at the end of a hydrolysis with 5,32g press cake and 40ml of water than in the 5,32g of press cake and 34,68ml of water.

It general, it appears to be important to find pre-treatment methods that can increase the performance of the enzymes during the enzymatic hydrolysis. In this study, autoclaving was suggested for only 20 minutes in order to kill the microorganisms present in this fraction and at the same time not to change the composition of the material. However, in order to help the degradation of the complex material into monomer sugars, it might be advantageous to repeat the autoclave at a slightly higher temperature or for a longer period of time.

Other methods of pre-treatment could also be suggested, such as acid pre-treatment, due to its efficiencies; however, one of the main goals of this project was to keep the methodologies applied as sustainable as possible, and using acid for pre-treatment does not appear to be so sustainable. Even though it may be argued that heating the material is not a sustainable procedure either, when using fossil fuels as energy for heating, however due to the short period of time of this sterilization, it did not appear to be as pollutant as using, for example, a pre-treatment with sulphuric acid.

5. Conclusion

From this project it is possible to understand that in fact it is necessary to perform another type of pre-treatment before enzymatic hydrolysis if the objective is to use the monomer sugars obtained for lactic acid fermentation afterwards.

Autoclaving appears to be a good option as pre-treatment of the press cake; however, since the results obtained were still low, it is recommended to repeat the experiment again and change some of the parameters in order to try to obtain a higher percentage of monomer sugars/kg sample.

Even though the % of dry matter/total solution lowers when not considering the amount of press cake as part of the volume of the solution, in fact this parameter appears to increase the amount of

sugars released. This shows, once again, that water is an extremely important compound used during enzymatic hydrolysis.

It is also important to mention that this project has shed light upon the fact that in order to achieve good results from a complex process, first it is important to optimize each step as much as possible.

6. Future Perspectives

One may suggest that enzymatic hydrolysis should be conducted in the future by using a combination of washing the biomass and then autoclaving it and letting the press cake absorb the water over night, since this project has identified the need for more than one pre-treatment in order to achieve the release of sugars without their further conversion into organic acids and alcohols by different microorganisms that may be present in the biomass. Furthermore, in the future the project could be carried out by utilizing enzymes with 100% activity or close to this figure, since increasing the concentration of enzymes to try to achieve 100% activity may not always work, as previously explained.

A further recommendation would be to produce enzymes from filamentous fungi in the press cake, for example, in order to try to obtain enzymes that are made to specifically degrade this type of press cake. If possible, it could also be constructed in a microorganism that is a lactic acid producer, a transformant that is able to degrade cellulose molecules, by for example, releasing extracellular enzymes that are capable of degrading these types of complex molecules.

It is also extremely important to retain from this project that the fermentation of the press cake, especially in combination with green juice, should not be performed before the enzymatic hydrolysis of this material has increased. It is also necessary to understand how the combination of the green juice with the press cake can affect the lactic acid bacteria and what the optimal parameters for its hydrolysis are and then fermentation before further conclusions. For example, it is important to understand that the same moisture content should be applied for enzymatic hydrolysis and fermentation.

Overall, the first steps should be optimized before taking further steps, in the future. This might increase the general results of the entire project.

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Appendix 1

48h hydrolysis g/kg PC	cellobiose	citric acid	glucose	xylose	arabinose	succinic acid	lactic acid	glycerol	acetic acid	total sufar if no fermentation
enzyme 3%, pH=5, at 50ºC	1,736654804	5,323843416	7,42348754	10,1067616	0	0,17437722	27,1565836	13,4412811	3,3594306	46,65658363
enzyme 5%, pH=5, at 50ºC	1,729537367	3,604982206	7,46263345	10,3274021	0,20996441	0,18505338	27,3096085	18,8113879	3,53380783	48,04448399
enzyme 6%, pH=5, at 50ºC	1,601423488	3,167259786	7,85409253	10,8042705	0	0,22064057	28,8754448	22,6441281	3,30960854	50,9519573
enzyme 3%, pH=5,5, at 50ºC	1,672597865	3,202846975	7,49466192	11,7864769	0,5480427	0,15302491	27,4163701	15,4839858	2,55516014	46,96085409
enzyme 5%, pH=5,5, at 50ºC	1,644128114	3,238434164	7,50177936	11,3096085	0,25456548	0,22775801	27,0427046	19,8790036	2,59074733	48,56405694
enzyme 6%, pH=5,5, at 50ºC	1,832740214	3,459074733	8,16725979	12,4412811	0,59786477	0,31316726	28,6441281	23,7081851	3,16725979	53,81672598
Control, pH= 5, at 50ºC	0,839857651	3,252669039	5,51601423	7,43060498	0	0,20640569	19,8434164	0,83274021	3,87188612	29,41637011
Control, pH=5,5, at 50ºC	0,946619217	2,975088968	4,82562278	7,81494662	0	0,19217082	19,1459075	0,72597865	4,32740214	28,75800712
CellicCtec 3%, pH=5,0 at 50ºC	1,217081851	3,430604982	5,51601423	7,9430605	0	0,28469751	23,2455516	3,09608541	4,0569395	33,44839858
74h hydrolysis g/kg PC	cellobiose	citric acid	glucose	xylose	arabinose	succinic acid	lactic acid	glycerol	acetic acid	
enzyme 3%, pH=5, at 50ºC	1,743772242	3,43772242	7,75088968	10,2775801	0,13523132	0,19928826	26,797153	13,1245552	3,20640569	45,0088968
enzyme 5%, pH=5, at 50ºC	0,71886121	3,473309609	7,90035587	10,7117438	0,02846975	0,14590747	27,1459075	18,8113879	3,38790036	47,57829181
enzyme 6%, pH=5, at 50ºC	1,640569395	3,412811388	8,78647687	11,3736655	0,14946619	0,22775801	28,9395018	22,6263345	3,39145907	52,95551601
enzyme 3%, pH=5,5, at 50ºC	1,733096085	3,238434164	8,75088968	12,3843416	0,51601423	0,28469751	27,5551601	15,3807829	2,41281139	49,43950178
enzyme 5%, pH=5,5, at 50ºC	1,715302491	3,195729537	8,89679715	12,519573	0,49466192	0,25622776	26,9181495	19,9181495	3,00711744	51,87188612
enzyme 6%, pH=5,5, at 50ºC	1,971530249	3,487544484	9,76156584	12,7686833	0,55516014	0,28469751	28,6227758	23,6548043	3,27758007	56,46441281
Control, pH= 5, at 50ºC	0,982206406	3,338078292	5,65124555	8,34163701	0	0,29181495	19,9786477	0,77580071	3,98576512	30,82918149
Control, pH=5,5, at 50ºC	1,330960854	2,733096085	4,6405694	6,88967972	0	0,29893238	19,8647687	0,66903915	5,13879004	28,58007117
CellicCtec 3%, pH=5,0 at 50ºC	1,160142349	3,395017794	6,25622776	7,74377224	0	0,29893238	23,5231317	3,08896797	4,29181495	34,15658363