Title of the project:

Enzyme production for subsequent biological pretreatment of clover grass press cake

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Preface

The master thesis presented represents the completion of my Master Programme in Sustainable Biotechnology at Aalborg University, Copenhagen, Denmark. The total programme had a duration of two years, from 2018-2020 and the master thesis was carried out from September 2019 to June 2020 in the section of Sustainable Biotechnology at Aalborg University, Copenhagen.

I would like to thank all my colleagues and teachers of Sustainable Biotechnology, in especial my professor and supervisor Mette Lübeck for all the knowledge, trust and supervision that she gave me, not only during the master thesis, but also previous projects that were carried out during my studies in Denmark.

Furthermore, I need to thank Maria Santamaría-Fernández and Emil Gundersen for all their support during my master thesis. From laboratory work to the writing part they were always present to teach me, support me and improve my tasks and knowledge. Until the last minute of project delivery, they were there to make some comments and to cheer me up, so I feel that they were a big part of this project.

I would like to thank my family who is always there to support me no matter what. They were the first ones supporting my travel to Denmark and who were there in the best and the worse, independently of the distance. To my mom, dad, Leonardo, Miguel, Beatriz, Rodrigo and Leonor, you are everything I could ask for in life, thank you.

Finally, I would like to acknowledge that my class of 2018-2020 was the best I ever had. We were there to help, teach and support each other in all times. No one ever kept knowledge to themselves, instead it was distributed equally to everyone, which I appreciated. To them and to Andreea Dobrescu a thank you would not be enough.

Abstract

Extreme preoccupation with climate change brought into consideration the usage of more environmentally friendly sources of food, feed, energy and materials. Green biorefineries suggest the usage of green crops for the production of different materials. However, in order to turn it into a more sustainable approach, nowadays a better utilization of the whole material is essential, but still in development.

After separation of the crops freshly harvested, it is possible to obtain a solid fraction, referred as press cake and a liquid fraction, green juice. It is known that most of the sugars and proteins are retained in the solid fraction, but due to the complexity of the material, it is difficult to take advantage of all the worth of this fraction. The current master thesis will present production of enzymes from press cake and utilization of those enzymes for biological pretreatment of the same fraction. Here, biological pretreatment is used with the aim of degrading cellulose and part of hemicellulose to obtain glucose and xylose as monomer sugars. These monomer sugars can after being utilized for the production of different other products such as lactic acid.

Production of FPases, ß-glucosidases, xylanases and ß-xylosidases was done by different fungi but *T. reesei* was the one performing better. It presented 17.73 U/ml of FPases, 17.98 U/ml of xylanases, 17.13 U/ml of ß-glucosidases and 1.33 U/ml of ß-xylosidases, when cultivation took place at 23°C for 10 days in press cake without water or nutrients addition.

Produced enzymes from *T. reesei* and *A. saccharoliticus* were used in different enzymatic hydrolysis in order to understand how efficient they are, in degrading press cake from clover grass. Commercial enzymes were also used in this part of the experiment since the protocol used was optimized for Cellic Ctec and Cellic Htec.

The best enzymatic hydrolysis results were obtained when 7% enzyme loading/DM of solution was added to a hydrolysis with 5% DM content. Cocktails from *T. reesei* and *A. saccharoliticus* presented 61.5% and 17.94% hydrolysis's yield in terms of sugar release, respectively, Cellic Ctec shown 66.37% hydrolysis yield and Cellic Htec, 69.23%. Furthermore, combination of *T. reesei* with Cellic Htec gave the best results in terms of hydrolysis's yield with 75.17%.

Altogether, this study brings a new idea for production of another high-value product, enzymes, to a green biorefinery and indicates a possible alternative for lignocellulosic pretreatment prior to monomer sugar utilization.

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Abbreviations

DM = Dry Matter content

PC = Press Cake

GJ = Green Juice

GMO = Genetic Modified Organism

SSF = Solid State Fermentation

SAH = Strong acid hydrolysis

T. reesei = Trichoderma reesei

A. carbonarius = Aspergillus carbonarius

A. saccharoliticus = Aspergillus saccharoliticus

T. asperellum= Trichoderma asperellum

ADF = Acid Detergent Fibre

ADL = Acid Detergent Lignin

NDF = Neutral Detergent Fibre

TS/VS = Total Solids/ Volatile Solids analysis

HPLC = High Performance Liquid Chromatography

PLA = Polylactic Acid

Chapter 1 – Introduction

1.1 – General Introduction

One of the main topics of the twenty first century was the substitution of fossil fuels for renewable energies. Fossil fuels are at risk of depleting if this usage continues to be extensive, but even more importantly, the consequences of utilizing these sources of energy are devastating to the environment and consequently to the society. Due to these facts, biomass has been widely studied as one of the most important sources of energy, for the near future (Garriga, Almaraz & Marchiaro, 2017).

Biomass has become an interesting source for the production of different products such as, chemicals and fuels, due to its renewable nature, versatility and low environmental impact. Furthermore, since the mid-1980s that the interest of using perennial grasses as energy crops has increased both in Europe and the US (Xiu & Shahbazi, 2015).

In fact, grasslands play a significant role in the agriculture worldwide, representing 70% of the global agricultural land area and 26% of the total land area. These lands are primarily used for animal production, well known as a principal source of feed for ruminants, for example. However, more and more different studies have been developed in order to give other purposes to grasses (McEniry, King & Kiely, 2014).

Over time, different conversion technologies have been studied to produce different products from grasses and legumes. However, considerable improvement in these technologies is necessary, especially in terms of utilizing the different fraction of the material, reducing the waste that result from the production of products and production costs. Green biorefineries can actually overcome these problems since they use a green biomass as a versatile material for the manufacture of different products. This concept is actually being exploited quite extensively in different countries in Europe, namely Denmark and Germany (Kamm & Kamm, 2007).

In a green biorefinery, the first step is to separate the raw material into a nutrient-rich material called green juice (GJ) and a fibre-rich material called press cake (PC). Both green juice and press cake have been studied as starting material for the production of different materials. However, previous studies have shown that it is easy to use the liquid fraction for such purpose, while this is not the case for press cake. Press cake has been studied for

production of biogas, soil fuel, fodder pellets and even other materials, but due to its lignocellulosic composition it is difficult to create processes with high efficiencies or with zero wastes (Xiu & Shahbazi, 2015).

Overall, the main advantage of the green biorefinery is the number of different products that can be obtained from a low-price feedstock that it can be available in large quantities. One of the major problems associated with biorefineries is how sustainable they can be from an economic point of view. Sometimes, the starting material is so complex that many costs are associated with the pretreatment of the material. Green substrates are easily pretreated into basic constituent for products processing. Thus, the costs associated with pretreatment of the material in a green biorefinery may be lower than when compared with other biorefineries (Badgujar & Bhanage, 2018).

However, further improvements in technologies, economy, complete utilization of the material and utilization of the wastes are necessary in order to make a green biorefinery concept completely sustainable. For different products that can be produced from PC or GJ, the downstream procedures seem to utilize non-green procedures, such as, extraction processes that generally involve usage of flammable volatile organic solvents or the recycling of possible acids that can be associated with protein precipitation (Van Ree, *et al*., 2011).

. These factors also reduce the sustainability of the green biorefinery, since in order to be completely sustainable, it needs to be economically feasible, environmentally friendly and the products that are produced need to be necessary for society (Badgujar & Bhanage, 2018.

1.1.1 – Green Biorefineries in Denmark

Several European countries, mainly Denmark, Germany, the Netherlands, Switzerland and Austria have taken a keen interest in green biorefineries. On the one hand, green biorefineries can help in the stabilisation of green land which is important to keep the landscapes open and attractive and to regulate water balance, besides its economic value. On the other hand, this type of biorefinery has a direct impact on rural regions by creating more jobs, thus considerably reinforcing the economic structure of these regions and increasing the educational level in these areas, since operating with the technologies used in these biorefineries, different knowledge is necessary, such as biological and chemical engineering (Kromus, *et al*., 2004).

Nowadays, the increased interest in organic farming has made the application of green biorefineries, namely in Denmark, particularly important, where organic farming areas

represents more than 7% of the total farmland area (Santamaría-Fernández, 2018). Green juice presents great potential for protein extraction that can later be used for soybean substitution in animal feeding (Ia Cour, Schjoerring & Jørgensen, 2019). Denmark is the fourth biggest pig producer in Europe, after Spain, Germany and France. However, most of the feed used in those countries is dependent on important protein-rich materials, such as, soybeans and soybean meals (Santamaría-Fernández, 2018). Most of this source of feed is imported to Europe. In order to reduce the problems associated with importation, Europe has begun to invest in soybean production, but it only represents less than 5% of world's production. Furthermore, soybean production is associated with significant environmental impacts (Sudaric, Slmic & Vrataric, 2006). As an example of those environmental impacts: soybean production and animal farming take place in different locations which disrupts the cycle of nutrients. Moreover, expansion of soybean cultivation areas creates losses in natural ones, thus destroying ecosystems (Santamaría-Fernández, 2018). Finally, all the environmental impact related with soybean transportation, even if inside the same continent, needs to be reduced (Sudaric, *et al*., 2006).

Denmark has become the main organic pig producer in Europe, representing 27% of total organic pig production in 2016 (Santamaría-Fernandez, 2018). Organic animal production demands 100% organic feed from the same farm or other places but within the same region, and the use of growth promoters or synthetic amino acids is not allowed (Hartman, Pawlowski, Herman, & Eastburn 2016). Furthermore, organic monogastric animals, require organic soybean meal consumption, which corresponds to only 0.1% of the total soybean meal production. Thus, different sources of protein-rich materials need to be exploited in Europe, especially in Denmark for pig and poultry production (Santamaría-Fernandez, *et al*., 2017).

Alternatively, to soybeans, grain legumes have been suggested as substitutes of, at least, part of the soybeans and soybean meals currently used in Europe for feeding monogastrics, fish and ruminants (Watson, *et al., 2017)*. However, grain legumes present a deficit of tryptophan and essential amino acids for animal feeding, such as methionine and cysteine. They also contain antinutritional factors such as protease inhibitors, lectins and tannins that can affect protein digestibility and amino acid accessibility. Moreover, these legumes are referred to as great source of human feeding (Santamaría-Fernández, 2018).

Instead of grain legumes, proteins from green legumes or grass, may be used to substitute soybeans, since it does not contain such inhibitors and it contains all the necessary amino acids for animal feeding (Chiesa & Gnansounou, 2011). Additionally, integrating protein extraction

to a biorefinery could reduce the costs associated protein production for animal feeding, could create a more sustainable source of proteins and it could reduce the wastes associated with protein production. Moreover, both protein extraction and protein concentrate, in a biorefinery, follow the necessary requirements implemented by the organic sector (Bals, Teachworth, Dale & Balan, 2007).

In Denmark a project called, The Organofinery Project was presented with the aim of using green biomass for the production of organic protein to feed monogastric animals, energy as biogas and organic fertilizer. The idea was to create a green biorefinery to face some challenges associated with organic farming in Denmark and at the same time create new job positions for farmers in this country. Moreover, the combination of production of animal feed with energy and fertilizers might increase the interest of local farmers to shift to organic farming (Santamária-Fernández, 2018). The scheme bellow represents overall Organofinery concept (figure 1):

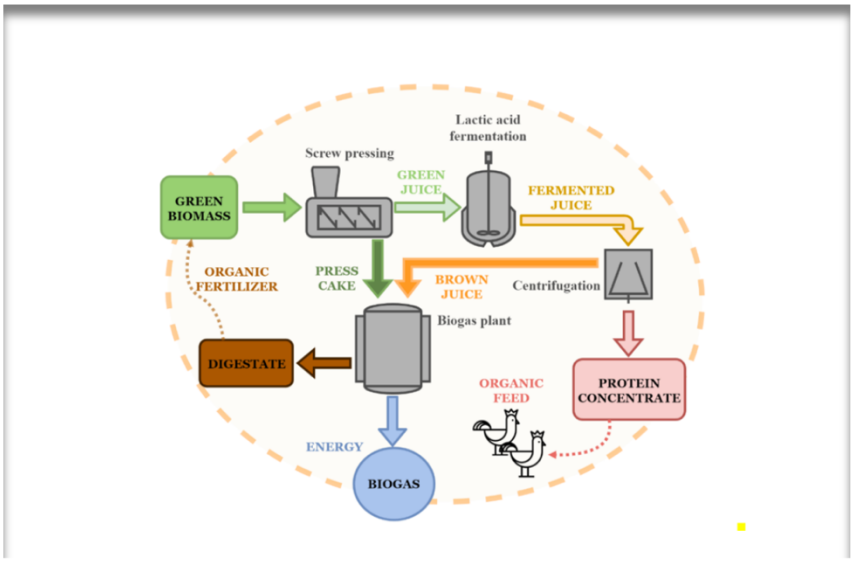


Figure 1: Organofinery Project, taken from Santamaría-Fernández, 2018.

Although, Organofinery is a project that has already finished, different projects can start based on this concept. It can also be used as an example of a green biorefinery that could improve organic farming in Denmark and help similar ones to be developed or adapted to more similar goals, to substitute soybean utilization, in Europe.

1.2 – Scope of the Thesis

In this project, the idea is to produce enzymes in PC, from filamentous fungi, and then utilize these produced enzymes in the same material, to break down cellulose and hemicellulose. The degradation of sugars will make them more available for microbial fermentation such as organic acids or ethanol. Since most of the microorganisms are not able to utilize complex sugars, its degradation into monomer sugars is crucial.

The production of enzymes and the enzymatic hydrolysis of PC could then be integrated in an existing green biorefinery, such as, one focused on protein extraction for animal feed but where sugar utilization it is still under development.

For the complete degradation of cellulose and hemicellulose, different enzymes are necessary. In this project, due to material availability, cellulases, ß-glucosidases, xylanases and ß-xylosidases activities will be calculated. However, it should be noted that other enzymes might also be produced by the filamentous fungi but are not analysed in this study.

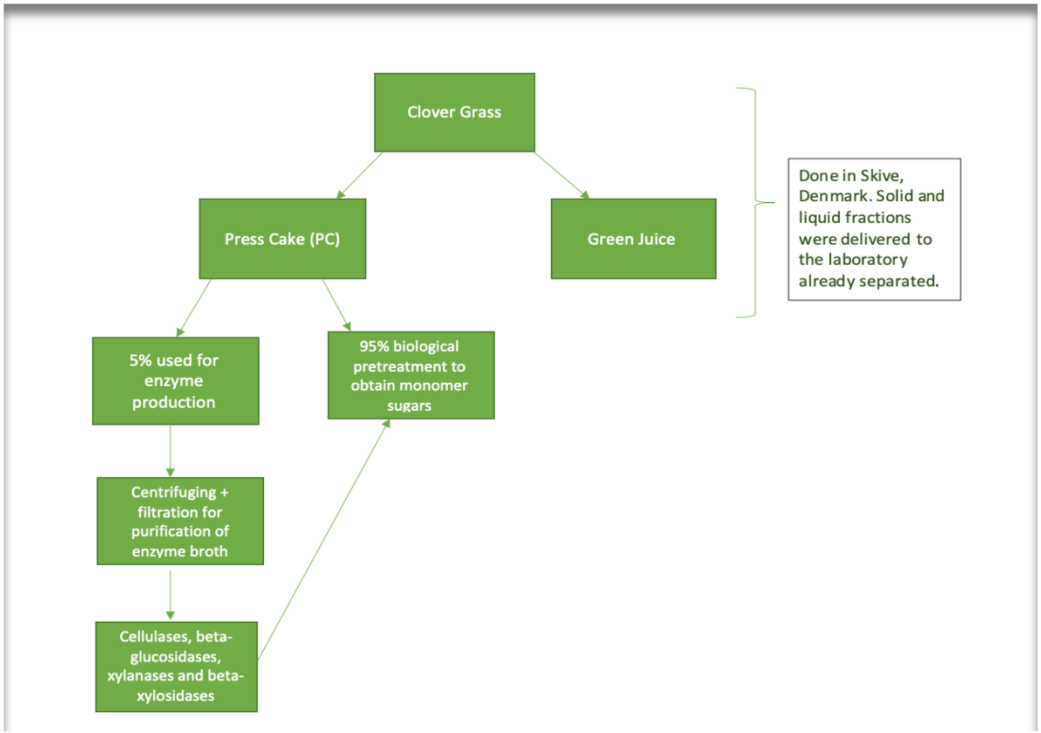


Figure 2: Scope of the project. Simplified diagram displaying the overall experimental setup with clover grass press cake (produced in Skive) used initially for fungal enzyme production and subsequent hydrolysis.

In the next chapter (Chapter 2) information will be included on what a green biorefinery is and how clover grass can be utilized in such industry. The production of cellulases, xylanases, ß-glucosidases and ß-xylosidases by filamentous fungi in solid state fermentation will be described. Finally, there will be a focus on how enzymatic hydrolysis can be used as pretreatment for the degradation of cellulose and hemicellulose.

Four strains of filamentous fungi will be cultivated in different solid media for the production of enzymes (represented in figure 2). Chapter 3 will start with materials and methods of the solid-state fermentation performed by, *Trichomderma reesei* RuT C3013 G, *Aspergillus carbonarius* ITEM 5010, *Aspergillus saccharoliticus* ML8 *and Trichoderma asperellum* PBpin 01. It follows the methods and materials of the two selected fungi based on cellulases, ß-glucosidases, xylanases and ß-xylosidases activities. At the end, in the same chapter, results and discussion of both solid-state fermentations, will be presented.

In order to understand the efficiency of the produced enzymes, Chapter 4 presents different enzymatic hydrolysis procedures (represented in figure 2). The aim of these procedures will be to identify the enzyme cocktail that will help release more monomer sugars in the PC. At the same time, it will be evaluated whether increasing the dry matter content per total solution, in the enzymatic hydrolysis, is as efficient as optimal conditions, if enzyme loading was increased to more or less the double. In sum, materials and methods, results and discussion of different enzymatic hydrolysis will be presented in chapter 4.

Due to the lockdown, the experimental part of this thesis was halted earlier than expected. In chapter 5 all the experiments that were planned to be carried out are described and two ways of further utilization of the solid fraction of clover grass are presented.

The conclusion and future perspectives of this thesis are presented in Chapter 6.

Chapter 2 – Theoretical Introduction

In this chapter, a description is provided on how clover grass can be used in a green biorefinery and the characteristics of the lignocellulosic material present in the press cake of this substrate.

The chapter also presents how *T. reesei* and *A. saccharoliticus* are able to produce enzymes that degrade at least part of the lignocellulosic material.

After enzyme production, hydrolysis can be performed in order to test the efficiency of these enzymes in degrading press cake from clover grass. Thus, enzymatic hydrolysis is also explained in this chapter.

2.1 – Clover Grass in a Green Biorefinery

Different types of green substrates have been studied to be used in Green Biorefineries. However, the choice of substrate is not only dependent on the products to be produced but it is also on the availability of that material in the place of production. In this project, clover grass press cake will be used due to its availability in Denmark and due to its utilization in a pilot scale biorefinery present in Skive, Denmark.

Clover grass is a mixture of grass and a legume, called clover. Different studies have shown the advantages of cultivating clover or other legumes with grasses. The most significant one is that clover is able to fixate enough nitrogen that the grasslands will not need fertilizers. At the same time, these studies have shown that both clover and grasses increase the transportation of carbon when cultivated together. Furthermore, legumes, such as clover, are slower growing. Cultivating these plants with grass seems to also be advantageous in the sense that grass will not allow competitors of clover to grow on the land (De Deyn, Quirk, Oakley, Ostle & Bardgett, 2012).

In terms of lignocellulosic composition, both grasses and clover contain cell walls composed of cellulose fibres surrounded by other polysaccharides. However, grasses and clover vary in the types and abundance of these non-cellulosic polymers, in the cross-linking of the polymers and in the number of phenolic compounds and proteins. Clover cell walls are type I, which consists of cellulose fibre bonds with xyloglucans, structural proteins and pectins (Xiao & Anderson, 2013). Contrary to clover, the cell walls of grasses are type II, which means

that they are cell walls composed of cellulose fibre bonds with glucuronoarabinoxylans, high levels of hydroxycinnamates and low quantities of structural proteins and pectins. Pectin is a cell wall component and is composed of acidic sugar-containing backbones with neutral sugar-containing side chains. It is a component that is responsible for wall hydration, cell adhesion and pectin crosslinking which influences plant morphogenesis and cell wall porosity (Vogel, 2008).

Grasses and legumes have become particularly interesting due to their high protein content with a good composition of amino acids, such as lysine and methionine. The demand for new sources of proteins has become particularly important, in Europe, after the importation of soybeans from Asia and South America brought economical, health and environmental concerns (Solati, Jørgensen, Eriksen & Søegaard, 2017). Furthermore, legume cultivations bring more advantages than grain legumes, such as soybean, in a way that they provide environmental benefits such as soil carbon storage and biodiversity effects. Grasses are able to utilize nutrients more efficiently at a lower cost and with lower losses of, for example, nitrate (Santamaria-Fernandez, 2018).

Different studies have shown that legumes present higher crude protein and less cell wall content, especially in terms of hemicellulose, compared with grasses. These characteristics seem to be advantageous for the extraction of protein per dry matter content. However, protein extraction is also extremely dependent on the maturity and harvest time of both grasses and legumes (Solati, *et al*., 2017).

Recently, due to the increasing interest in biorefineries, more products have been proposed to be obtained from grasses and legumes. As it will be explained bellow, the lignocellulosic material present in PC fraction can be further pretreated for conversion of cellulose and hemicellulose into monomer sugars. These sugars, especially glucose, can be utilized in different fermentation processes which can increase the overall utilization of green biomasses and might reduce the wastes of a green biorefinery (Badgujar & Bhanage, 2018).

2.1.1 – Complexity of the Fibres Present in the Press Cake

Lignocellulosic biomass is mainly composed of cellulose, hemicellulose and lignin. Together these structures create a complex assembly of polymers that are recalcitrant to enzymatic hydrolysis. This is the main reason why some pretreatment methods are generally applied prior to enzymatic conversion (Kumar & Wyman, 2009).

Factors influencing lignocellulosic recalcitrance are interconnected and sometimes really difficult to disassociate. These factors can be divided into physical and chemical factors. The former may be associated with cellulose's specific surface area, degree of polymerization and crystallinity; pore size and volume of the different molecules (Zhao, Zhang & Liu, 2012). Chemical factors are generally related to hemicellulose and lignin compositions and contents. Despite much of the research having focused on understanding how these factors can influence the recalcitrance, the conclusions obtained have often proven to be different and contradictory (Zoghlami & Paës, 2019).

Cellulose is the most abundant lignocellulosic polymer and consists of ß-D-glucopyranose units linked by ß-(1,4) glycosidic bonds, with cellobiose being the repeating unit. This polymer is made of D-glucose units that are arranged together to form microfibrils, which packed together form cellulose fibrils. These fibrils are embedded in lignocellulosic matrix making it resistant to enzymatic hydrolysis. As mentioned before, it is reported that the degree of polymerization plays a crucial role in lignocellulosic recalcitrance. However, its exact role is not clear and difficult to analyse individually with the correct information available (Ge, *et al*., 2018; Jasiukaitytè-Grojzdek, Kunaver, & Poljansek, 2012). Changes in degree of polymerization are always accompanied by changes in cellulose crystallinity and porosity. From the available information, it is assumed that long cellulose chains contain more hydrogen bonds and so are more difficult to hydrolyse than shorter chains of the same polymer. Thus, different studies suggest mechanical pretreatment priory to the enzymatic hydrolysis of cellulose (Zoghlami & Paës, 2019).

Hemicellulose contains monosaccharide subunits to form xylan, xyloglucan, mannans and other components. This polymer is amorphous and has little physical strength which makes it easier to be hydrolysed by both acids and bases or by hemicellulases (Leu & Zhu, 2013). However, hemicelluloses act as a barrier, limiting the accessibility of enzymes to cellulose. Different studies have shown that the removal of hemicellulose improves cellulose porosity and the area for enzymes to act. The influence of hemicellulose on lignocellulosic recalcitrance is still quite unclear as some lignin is often removed with hemicelluloses. Furthermore, some studies have pointed to hemicellulose removal being more important than lignin removal, for improvement of enzymatic hydrolysis efficiency, but other studies have shown exactly the opposite (Ge, *et al*., 2018; Leu & Zhu, 2013).

Acetyl groups are responsible for the acetylation of hemicelluloses which can restrict cellulose accessibility by interfering with enzyme recognition. They may also block the

bonding between cellulose and the catalytic domain of cellulases by increasing the diameter of cellulose chains or by changing their hydrophobicity (Zoghlami & Paës, 2019).

Green biomasses present less lignin than mature lignocellulosic material, 5-15% and 15-36% in terms of dry matter, respectively. Due to this fact, lignin does not seem a major inhibition factor in green substrates compared with mature ones. Grasses in general present less lignin than legumes, but their content is dependent on the harvest season (Lozano, 2015).

Lignin is a heteropolymer composed of phenylpropanoid building units and it is responsible for structural rigidity and hydrophobicity. Like hemicellulose, lignin seems to play a negative role in the degradation of cellulose and, although, it is present in a lower quantity in green lignocellulosic substrates, its composition/structure is studied as another factor that influences cellulose conversion (Kumar & Wyman, 2009). It has been reported that lignin can block the access of enzymes to cellulose and can also irreversibly absorb cellulases and other enzymes during hydrolysis due to its hydrophobic structural features such as polyaromatic structures and hydrogen bonding (Zoghlami & Paës, 2019).

Besides cellulose, hemicellulose and lignin, some studies have also focused on pectin too, due to two main reasons: pectin can affect the accessibility of other cell wall components to enzymatic hydrolysis and the sugars that compose pectin represent captured photosynthetic energy (Xiao & Anderson, 2013). Furthermore, in a study, lignocellulosic material was treated with pectinases and it increased the pore size of the material, indicating that pectin is a major influencer of wall porosity. Pectin is known to be completely degraded by the action of sixty-seven different enzymes which make it difficult to be hydrolysed in different processes, but it can be used as a complex polymer in different industries, such as the food and pharmaceutical sectors (Voragen, Coenen, Verhoef & Schols, 2009). In sum, pectin is a really complex molecule and depending on the substrate, processing regime and process's end products, it can be pointed as a hindrance to biomass degradability, a source of fermentable sugars or a potential co-product of bio-based products, such as biofuels. Understanding the pectin structure, mechanism of formation, modification and degradation will allow improvements in plant utilization as renewable sources of energy, food and materials (Xiao & Anderson, 2013).

In sum, lignocellulosic recalcitrance to enzymatic hydrolysis is a multi-variant phenomenon that is affected by several physical and chemical factors, such as hemicellulose and lignin content, degree of polymerization of cellulose and its accessible volume and surface area. However, it is important to notice that due to the complexity of lignocellulose and not

well-known interactions between its different polymers, make different studies present opposing trends in the effects of the presented factors (Zoghlami & Paës, 2019).

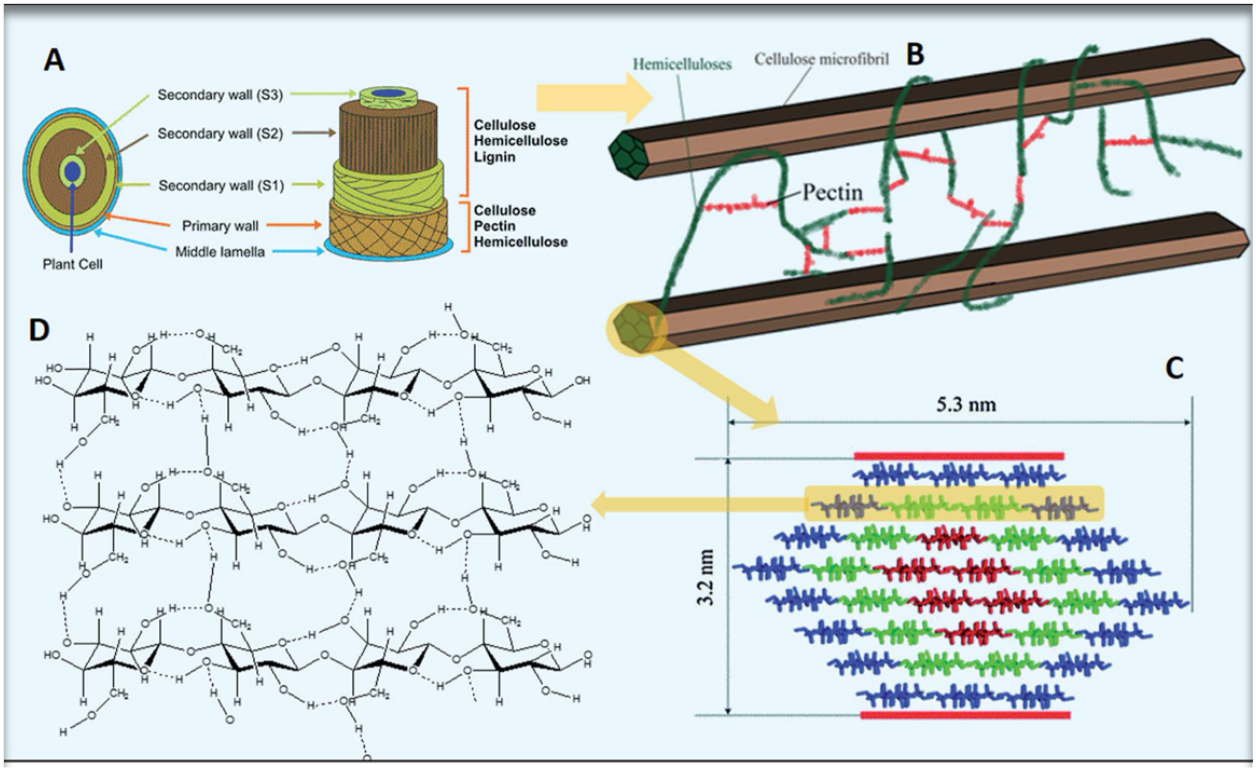


Figure 3: A) Pictorial illustration of the lignocellulosic biomass framework, B) A simplified model showing the interaction of carbohydrate polymers present in the cell wall. C) Structure of a 36- chain model for the cellulose elementary fibril. D) model of inter and intra-chain hydrogen-bonding patterns in cellulose, taken from Satari, Karimi & Kumar, 2019

Above (figure 3), lignocellulosic biomass framework is represented for a clear understanding of what was described in this section.

2.2 – Enzyme production by solid state fermentation (SSF)

Solid state fermentation is a process that occurs in solid media in absence or near absence of free water, for the production of different products such as antibiotics and enzymes. The most important advantage of SSF is the resistance of microorganisms to catabolic repression and inhibition of enzyme synthesis in the presence of high amounts of substrates such as,

glucose, glycerol or other carbon sources. Furthermore, SSF opens the possibility to use agro-industrial and biological wastes for the production of different products in an economical feasible way (Lizardi-Jaménez & Hernández-Martínez, 2017; Thomas, Larroche, & Pandey, 2013).

SSF has tremendous potential for the production of enzymes and it has become especially interesting in biorefineries, where first the enzymes can be produced from part of the crude substrate and after used as biological pretreatment of the material. Furthermore, comparing it with submerged fermentation, this system offers high volumetric productivity, less expensive fermentation equipment, a generally higher concentration of products and less nutrient requirements (Pandey, Selvakumar, Soccol & Nigman, 1999).

The selection of a substrate for enzyme production in SSF is dependent on several factors such as availability, cost and its nutrient richness. In SSF processes the substrate not only leads to microbial growth but also serves as an anchorage for the cells. The ideal substrate is the one that provides all the necessary nutrients for the microorganisms to grow and produce enzymes with high activities per gram of substrate (Lizardi-Jaménez & Hernández-Martínez, 2017). When this is not the case, some nutrients need to be added externally, which can increase the costs associated with this process. Moreover, some substrates, like lignocellulosic material, are difficult to be utilized by microorganisms. Pretreatment is then required in most of these cases. Depending on the choice of pretreatment, the costs of enzyme production by SFF can increase and make it less profitable (Pandey, *et al.,* 1999).

Besides the selection of suitable substrates and usage or not of pretreatment, other factors can influence a SSF process, such as: particle size, water and moisture content, water absorption capacity, type and size of the inoculum, temperature and pH of the fermentation, period of cultivation, maintenance of the same environmental conditions and gaseous atmosphere. It is important that all these parameters are analysed before production, in order to define the best microbial culture conditions to achieve high levels of production of enzymes. It is also important to have in consideration that the optimal conditions might vary depending on the substrate and microorganisms used (Castro & Sato, 2015).

2.2.1 – Enzyme Production by Filamentous Fungi in Solid State Fermentation (SSF)

Industrial enzymes used globally are mainly produced by fungi and bacteria. However, nowadays half of the enzyme production is done by bacteria, a wider spectrum of families from fungal kingdom have been tested for the same purposes. After different studies it was noticed that fungal enzymes are efficient, compatible and suitable for industrial processing since these enzymes have high protein stability, meet regulatory approval demands and are in general more resistant to environmental changes than bacterial ones. Filamentous fungi specifically, seem to be more resistant to environmental changes and grow under a higher variety of substrates, compared with bacteria (Lange, 2017).

Nowadays, filamentous fungi are gene donors for large-scale enzyme production, and as mentioned before, they can be used as producers of biomass-degrading enzymes, metabolites and biorefinery end products (Hansen, Lübeck, Frisvad, Lübeck, & Andersen, 2015). One of the most important characteristics of these microorganisms is their ability to secrete enzymes. Secretion occurs primarily from the tip cells of the growing hypae. Then, these secreted enzymes break down the substrate that fungi grow on. In nature, such enzymatic biocatalysis results in the conversion of polymers into smaller molecules suitable for hyphae to absorb, which leads to further fungi growth (Lange, 2017).

The majority of the commercial enzymes used in industries are secreted enzymes from fungi. The main reason for this is that these secreted enzymes are generally more stable and cheaper to produce because they can be extracted directly from the fermentation broth without the need to open the cells (Hansen, *et al*., 2015).

2.2.1.1 – Trichoderma reesei and Aspergillus saccharoliticus in enzyme production

Trichordema reesei has been widely studied for enzyme production due to its ability to produce cellulases with high activity in different types of substrates. The novel Aspergillus saccharoliticus was presented as a good producer of ß-glucosidases. Both microorganisms, belong to the Ascomycota division (Seiboth, Ivanova & Seidl-Seiboth, 2011).

T. reesei was isolated for the first-time during World War II, after it was noticed that this fungus was responsible for the degradation of cotton fabrics of US army. *A. sacchoriliticus* was isolated years later, in Denmark, from treated oak wood (Seiboth, et al., 2011; Sørensen, Lübeck, Lübeck, Teller & Ahring, 2011).

Kolasa, Ahring, Lübeck & Lübeck (2014) found that mixing the cellulases produced by *T. reesei* with ß-glucosidases from *A. saccharoliticus* gave hydrolysis efficiencies to more than 80% in steam exploded diluted-acid pretreated wheat straw. The addition of ß-glucosidades from *A. saccharoliticus* seemed to have improved the enzymatic hydrolysis efficiency to more than the double than when *T. reesei* enzymes were used alone (Kolasa, *et al.,* 2014).

Brijwani and Vadlani (2011) showed that *T. reesei* also presented great potential for the production of xylanases. In addition to these and other studies showing that *T. reesei* is a great producer of cellulases and xylanases, more recent research is now more focus in genetically modifying this organism in order to increase the production of other enzymes such as, ß-glucosidases (Keshavarz & Khalesi, 2016; Uusitalo, Nevalainen, Harkki, Knowles & Penttilä, 1991).

Since *A. sacharolyticus* is quite a novel studied organism in terms of enzyme production, much is still unknown about it. Despite different studies having shown that is a great producer of ß-glucosidades, more studies can still be conducted in order to understand if it is also a great producer of other enzymes, such as ß-xylosidases. It would be also interesting to find how this microorganism can be genetically modified in order to enhance its enzyme profile (Rana, Eckard, Teller, & Ahring, 2014).

2.2.2 – Cellulases and Xylanases Production in Solid State Fermentation (SSF)

The production of cellulases is dependent on different aspects of the substrate and characteristics of the enzyme producers. In terms of substrate, not only does its complexity play a role, but so does the presence or not of surface openings or internal voids or spaces. It seems that, from one point of view, in order to produce cellulases, the presence of cellulose is necessary to remove cell wall components in order to enhance the direct contact between these enzymes and the substrate (Brijwani, & Vadlani, 2011).

When fungi grow in lignocellulosic substrates, its propagation only happens thanks to the production of enzymes that drive hydrolytic reactions, which are responsible for the generation of monomer sugars that are essential for fungal growth. It seems that, for efficient hydrolysis of cellulose, the action of endo and exo-glucanases is necessary. Moreover, the crystalline nature of the substrate used to induce production of enzymes in fungi, significantly influences the hydrolytic efficiency of the enzyme cocktail (Hall, *et al.,* 2010).

At the same time, it is important to note that when fungi grow on cellulosic substrates become more prone to catabolite repression by glucose. The rate of repression is dependent on the rate of glucose formation, which by itself is dependent on the secretion of cellulases (Brijwani & Vadlani, 2011). Furthermore, different studies have shown that, the rate of cellulose degradation it is also dependent on the crystallinity of the cellulose. This means, that the crystallinity could not only alter the quality of the enzymes but also the quantity of enzymes produced over time (Hall, Basal, Lee, Realff, & Bommarius, 2010).

It has also been reported that, degradation of hemicellulose increases the attachment of cellulases to cellulose (Hu, Arantes, Pribowo & Saddler, 2013). When microorganisms, like fungi, grow naturally in lignocellulosic material they are able to produce not only enzymes that hydrolyse cellulose but also hemicellulose. However, at a commercial level, in order to produce a cocktail of cellulases and hemicellulases it is necessary to use more than one microorganism or make genetic modifications in one (Kumar & Sharma, 2017).

Contrary to cellulose, hemicellulose contains different sugars in its composition and the amount of each sugar that it contains varies with the type of plant. In hardwoods xylan is presented as O-acetyl-4-O-methylglucoronoxylan and in grasses it normally shows as arabionoxylans. Overcoming the complexity of these polymers requires the action of different main and side chain cleaving enzymes, such as, B-1,4-xylanases and B-D-xylosidases (Scheller & Ulvskov, 2010).

Xylanases have biotechnological potential in food and pre-bleaching of pulps in paper industries. However, due to the diversity of hemicellulose, each industry needs xylanases with distinct physico-chemical properties. Thus, integrated xylanase production might be extremely advantageous, since the xylanases produced would be specific to hydrolyse the material used in that particular industry (Sharma, *et al.,* 2015).

It is also important to bear in mind that the growth of fungi in natural substrate is usually slow. Different studies have considered the application of different pretreatment methods prior to enzyme production. However, it is known that pretreatments can alter the physicochemical properties of the lignocellulosic material which can influence enzyme production (Brijwani & Vadlani, 2011).

2.2.3 – Enzyme Production by Solid State Fermentation in a Biorefinery

At an industrial level, different studies have been made in order to optimize processes such as organic acid or biofuel production in order to make it more economically feasible. These studies normally include methods such as life-cycle assessments (LCA) that can depict the main flows in a process by analysing and comparing the used technology. During these studies, it was brought to light that commercial enzymes may be one of the most expensive parts of an entire process. Moreover, the utilization of commercial enzymes presents other issues, such as: lack of transparency on enzyme dosages, price variation from year to year and all the related greenhouse gases emissions. Thus, an effort to create more environmentally friendly and cost-effective approaches to enzyme utilization have been studied. On-site and later, integrated enzyme productions have been proposed as promising alternatives to off-site production (Olofsson, Barta, Börjesson & Wallberg, 2017).

The on-site production approach is related to enzymes that are produced in a place next to the intended production plant. Here problems related to enzymes stabilisation and transportation are eliminated. In on-site production, the enzyme cocktail can be added directly to the substrate to be hydrolysed without further expensive steps. Furthermore, in this process the enzyme production is sized up to the desired amount rather than to industrial scale production, which also reduces the costs of the enzyme production (Johson, 2016).

Recently integrated enzyme production has been suggested. This approach is similar to the one-site production since it is also located in the same place as the rest of the production plant and it is planned to fit its specific necessity requirements. The difference is that in integrated enzyme production the substrate used is the same as the one to be hydrolysed afterwards (Olofsson, *et al*., 2017).

Different studies have shown that yields are similar or even higher than those presented by on-site enzyme productions. Furthermore, these investigations have proven that integrated enzyme production lowers global warming potential by reducing the greenhouse gases emissions by eliminating transportation requirements. This factor coupled with the usage of lignocellulosic biomasses increases the sustainability of this approach compared to others (Hansen, *et al*., 2015; Johnson, 2016).

Integrated enzyme production matches biorefinery goals, such as: produce secreted, stable enzymes that are suitable for different purposes at a cheap price and always using part of the same start material or residues. Depending on purification steps, these enzymes can only be

used in the biorefinery in question or be sold to other companies or industries (Hansen, *et al.,* 2015).

Although, not much research has been conducted on enzyme production by SSF in a biorefinery, it is expected that SSF can be an effective and more sustainable method to be integrated in a biorefinery. It will be advantageous to reduce the costs of utilizing enzyme as pretreatment, it will make the enzymes specific to the material to be degraded, it will eliminate the costs associated with nutrient and water addition and it could add a high value product to this biorefinery (Dotsenko & Lange, 2016; Escamilla-Alvarado, Pérez-Pimenta, Ponce-Noyola. & Poggi-Varaldo, 2016).

2.3 – Enzymatic Hydrolysis of Press Cake

As previously mentioned, the usage of press cake it is still under development due to the complexity of its polymers. Normally, in order to utilize sugars that are present in the press cake, the degradation of these polymers is necessary. For this to occur, normally the application of one or more types of pretreatments is necessary (Dotsenko & Lange, 2016). However, it is also well known that pretreatment can be one of the most expensive and less environmentally friendly parts of the production (Agbor, Cicek, Sparling, Berlin & Levin, 2011).

The enzymatic hydrolysis of lignocellulosic material can be efficient but at the same time really expensive when enzymes are bought from another industry. Furthermore, as previously explained, the efficiency of the enzyme is related to its specificity of catalyzing a different type of material, which means that to degrade clover grass it would be advantageous to use enzymes that have been produced using, at least, a type of grass or legume as substrate (Dotsenko & Lange, 2016).

The initial parameters of enzymatic hydrolysis are extremely important for an efficient degradation of the material. Temperature and pH should be maintained constant over time and it is dependent on the optimal conditions of the enzymes utilized. The amount of DM per total solution it is also an important characteristic to be taken into consideration, since hydrolysis needs free water. The time of the experiment should also be considered due to the fact that, especially on industrial scales, the pretreatment should not take a lot of time (Batstone, Tait, & Starrenburg, 2008).

During the enzymatic hydrolysis itself, its rates reduce due to the inactivation of enzymes, inhibition of enzymes by soluble sugars and a decreased site concentration due to a reduction

in the substrate surface area. At this level, optimizing enzyme concentration to an increase of hydrolysis rate is essential for the maximum efficiency of this pretreatment while reducing enzyme loadings. In the recent years, different studies have presented different enzyme cocktails in order to enhance synergistic properties for the hydrolysis of lignocellulosic material in different substrates (Kucharska, *et al.,* 2018).

It is also important to note that, due to the complexity of lignocellulosic material, other pretreatment methods might be necessary before enzymatic hydrolysis. However, as mentioned above, some pretreatment methods can change the composition of the substrate, also reducing the efficiency of the enzymatic hydrolysis. Moreover, the addition of an extra pretreatment method can significantly increase the costs of the production. Due to these problems, it is always necessary to investigate and apply different strategies to a process before upscaling and making it commercial (Diaz Sanchez, Le Toullec, Blandino Garrido, De Ory Arriaga & Caro Pina, 2013; Hernández, et al., 2013).

Dotsenko & Lange (2017), showed that applying proteases to enzymatic hydrolysis can lead to 80% protein recovery in the press cake of white clover and ryegrass. It was also concluded in this study, that the usage of Cellic Ctec2 and Cellic Htec2 combined with proteases yielded up to 95% of protein recovery in the press cake.

In the current study, the enzymatic hydrolysis is presented as a biological pretreatment of press cake. It may be suggested that after hydrolysis, monomer sugars can be used for further fermentation processes (Dotsenko & Lange, 2017).

2.3.1 – Enzymatic Hydrolysis of Cellulose and Hemicellulose

Cellulose is resistant to enzymatic hydrolysis due to the firmly condensed structure of microfibrils which are insoluble in water. Consequently, the space where cellulolytic enzymes can act is minimised (Balat, 2011).

This polymer can be degraded into glucose by the synergetic action of three enzymes (figure 4): endo-1,4-ß-glucanases, exo-1,4-ß-glucanases and ß-glucosidases, which together are referred to as cellulases and ß-glucosidases. Endo-glucanases randomly attack regions where the fibres present low crystallinity, creating free chain ends. This is followed by further degradation of the produced free chain ends, where exo-glucanases remove cellobiose units. Later on, ß-glucosidases cleave the molecules into glucose (Horn, Vaaja-Kolstad, Westereng, & Eljisink, 2012).

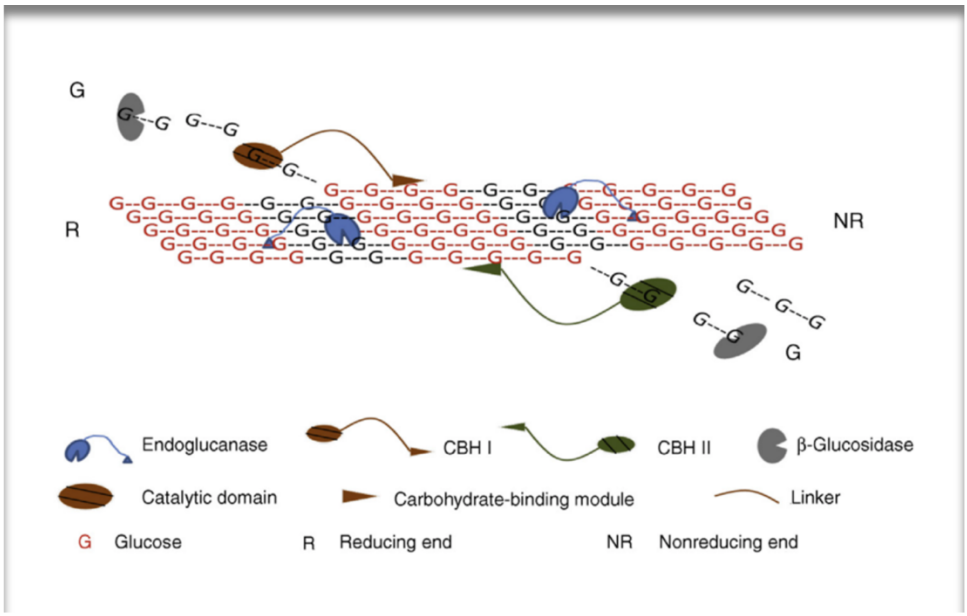


Figure 4: Enzymatic hydrolysis of cellulose. Taken from Kumar & Murthy, 2016

The degradation of hemicellulose requires more enzymes, but this polymer is easier to be hydrolysed than cellulose as a consequence of its amorphous and heterogenous structure. Hemicellulases can be divided into: endo-1,4-ß-xylanases, exo-1,4-ß-xylosidases, endo-1,4-ß-mannases, 1,4-ß-manosidases, α -galactosidases, endo-1,5- α -arabinases, α -L-arabinofuranosidases, α -glucuronidase as well as some accessory enzymes which are required for the hydrolysis of xylans substitutes, such as: acetyl xylan esterases, ferulic and p-cumaric acid esterases (El-Naggar, Deraz & Khalil, 2014).

Xylan is hydrolysed into xylose units by the action of two different enzymes (figure 5). First, endo-xylanases are able to hydrolyse ß-1,4-glycosidic bonds into shorter xylooligosaccharides, which are then degraded by ß-xylosidases into xylose. Mannan is initialy hydrolysed by endo-mannanases to yield short ß-1,4-manno-oligomers and then ß-mannosidases degrade these molecules into mannose. α -1,2-glycosidic bonds between glucuronic acid and xylan are cleaved by glucuronidases and arabinofuranosyl is degraded by arabinofuranosidases and endo-arabinases. Accessory enzymes, such as acetyl xylan esterases hydrolyse the acetyl group on xylose, whereas feruloyl esterases cleave the ester bonds in arabinose and ferulic acid (Shallom & Shoham, 2003).

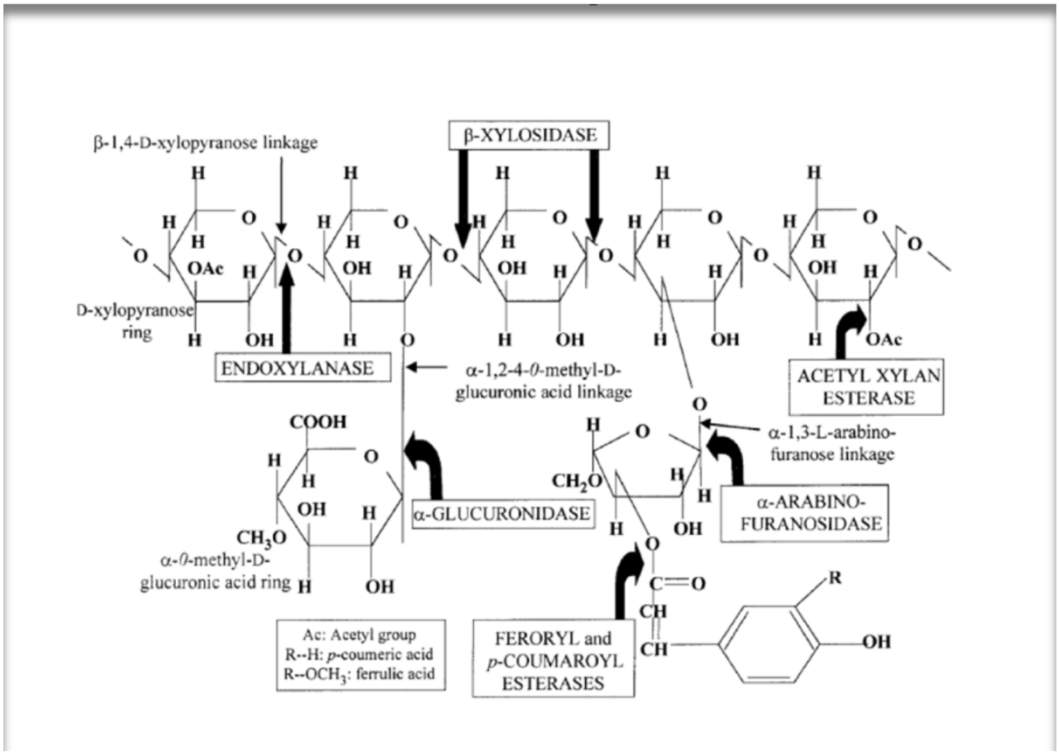


Figure 5: Xylan structure showing diferente substituent groups with sites of attack by xylanases. Taken from Khan, 2010.

2.3.2 – Inhibition of Enzymatic Hydrolysis

In recent years, increasing the yields of liberated sugars through enzymatic hydrolysis has been studied extensively. Consequently, the impact of different factors, such as: substrate and end-products concentration, reaction conditions and enzymatic activities, should be considered for further research. It has been reported that substrate and end-products concentration may be a limiting factor for cellulases or xylanases activities. Furthermore, ß-glucosidases has a substantial effect upon hydrolysing cellulose since it can be inhibited by glucose molecules (Bajpai, 2016; Jung & Kim, 2017).

Moreover, reaction conditions, such as pH and temperature can influence the efficiency of enzymatic hydrolysis. Previous reports of commercial enzymes and studies have presented pH 4-5 and temperature 45-55°C as optimal for cellulases and hemicellulases activity during hydrolysis. Additionally, it is noteworthy that the lignin content is also a factor that hinders with biomass digestibility during this process, causing a non-productive binding of the enzymes to lignin (Bajpai, 2016).

Chapter 3 – Enzyme Production by Filamentous Fungi in Solid State Fermentation Process (SSF)

Filamentous fungi are great producers of extracellular enzymes, so in this chapter different strains of filamentous fungi: *Trichomderma reesei* RuT C3013 G, *Aspergillus carbonarius* ITEM 5010, *Aspergillus saccharoliticus* ML8 and *Trichoderma asperellum* PBpin 01 will be used in order to produce enzymes from clover grass press cake. These enzymes are expected to be able to degrade cellulose and hemicellulose from press cake for further utilization of this substrate.

In a first experiment, all filamentous fungi will be cultivated in clover grass press cake for enzyme production. Here, two different types of press cake will be used: PC1, from a pilot scale in Denmark and, PC2, made in the laboratory from a juicer. From the first SSF, two strains are expected to be selected based on their cellulases, xylanases, ß-glucosidases and ß-xylosidases production. At the same time, only one of the media will be used in the following experiments based on fungi's enzyme production, when cultivated in each of the medium.

The two selected strains will be used for a second enzyme production in SSF. The cocktails obtained from the second SSF will be used in chapter 4 for potential degradation of cellulose and part of the hemicellulose.

As previously mentioned, materials and methods, results and discussion of the different SSF followed by enzymatic assays performed during this project, will be presented in this chapter.

3.1 – Materials and Methods

3.1.1 – Fermentation Using Different Fungi in Press Cake

3.1.1.1 Choice of different microorganisms

Before beginning the solid-state fermentation, *Trichomderma reesei* RuT C3013 G, *Aspergillus carbonarius* ITEM 5010, *Aspergillus saccharoliticus* ML8 and *Trichoderma asperellum* PBpin 01, were grown in PDA media, for four days at room temperature, and then harvested into falcon tubes.

3.1.1.2 – Spore counting

The spores were then counted using a Funch-Rosenthal counting chamber. The concentration of the spore suspension was calculated and adjusted, so all the spore solutions would present the same concentration, $5.5x10^6$ cells/ml.

3.1.1.3 – Media preparations

The dry matter of the press cake 1 (PC1) and press cake 2 (PC2) were analysed by preforming the TS/VS content method. PC1 was observed to present 25% DM and PC2 44% DM, so it was necessary to add water into PC2 in order to obtain the same DM content in both substrates. Upon completion of all these small pre-procedures, the samples were prepared by adding 40g of PC1 or PC2 into 250ml Erlenmeyers. The flasks were then autoclaved for 20 minutes at 121ºC and 15psi.

When the flasks had cooled down to room temperature, 0.5ml of spores were added into each flask, which corresponds to 5% of the DM content present in the solution.

The initial plan was to leave the flasks at 25ºC. However, due to problems with the incubator, the real temperature inside the incubator reached 28ºC. Therefore, the real temperature for this experiment was accounted to be 28ºC.

Note that for this experiment, a third medium was also prepared as control based in Kolasa et al., (2014). The medium contained a mixture of 25.6% (w/v) wheat bran, 15.4% (w/v) sphagnum peat and Milli-Q water in order to give total 25% DM per solution. 40 g of medium was added to 250 ml Erlenmeyer flask and also autoclaved for 20 minutes at 121ºC and 15psi. 0.5 ml of fungi were added to each flask after cooling down.

Controls without fungi addition were also prepared.

The SSF was run for seven days.

3.1.1.4 – Isolation of enzyme broth

After seven days fermentation, 100ml of Milli Q water were added to each flask and then they were let to shake at 150rpm overnight.

The next day, the solution was filtered using a muslin cloth, where the liquid was transferred into a clean and sterile falcon tube and the solid part was discarded.

Each falcon tube was centrifuged for 20 minutes at 10000 g and 4ºC, then the liquid part was transferred into a new falcon tube and the solid part left in the bottom was discarded. This procedure was repeated twice in order to ensure that there were no cells in the enzyme solution.

The enzyme solutions were stored at -20ºC until usage.

3.1.2 – Fermentation Using the Most Promising Fungi in Press Cake 1

Due to the results obtained from the experiment 3.1.1, the decision was taken to perform the next solid-state fermentation using only *T. reesei and A. saccharoliticus*.

Furthermore, it was decided to continue only with PC1, due to the results of the previous experiment.

The fermentation flasks were prepared in duplicates using 40g of PC1 in 250ml erlenmeyer with DM adjusted to 25%. Before the spore's inoculation, the material was autoclaved for half an hour at 121ºC and 15psi. After cooling down, 5% of the total DM was added in spores, with a concentration of $5.5x10^6$ cells/ml.

The flasks containing *T. reesei* were left at room temperature for 10 days, while the flasks containing *A. saccharoliticus* were left at 28ºC during the same incubation period.

In this SSF, samples were taken after 4, 7 and 10 days of fermentation.

3.1.2.1 – Isolation of enzyme broth

The second SSF sought to ascertain whether the microorganisms used produced enzymes throughout the entire ten days of fermentation or whether they stopped before. Samples were taken after 4 and 7 days of fermentation and, to this end, 1g of press cake was added to a sterile falcon tube and mixed with 2.5ml of Milli Q water. The falcon tubes were left to shake over night at 150rpm. The next day, the water was filtered using a 0.2μm filter with PES membrane into a new sterile falcon tube and then it was centrifuged for 20minutes at 10000g.

After 10 days all the enzymes produced were extracted by adding 98ml of sterile Milli Q water into each erlenmeyer and leaving it to shake over night at 150rpm. The next day, the solution was filtered using a muslin cloth first and then purified by centrifuging it twice as explained in the section 1.1.1 of Materials and Methods.

3.1.3 – Enzyme Assays

Enzymatic assays were used to measure the activity of the produced enzymes. The quantitative methods used in this project will present activities in U/ml. All samples were measured in duplicates.

3.1.3.1 – AZCL plates

Three different 100ml of AZCL solutions were prepared by adding 0.1g of insoluble substrate (cellulose, xylan or casein), 2g of Agar, 5ml of ethanol and 95ml of 0.1M sodium citrate buffer pH=5, in this order. Then, the three solutions were autoclaved for 20 minutes at 121ºC and 15psi.

After cooling down to 40ºC, the medium was passed to different plates, approximately 30ml in each, and left tosolidify.

15μl of each en zyme preparation was added to the different AZCL plates: AZCL-HE-Cellulose, AZCL-xylan and AZCL-casein. The plates were incubated over night at 30ºC.

3.1.3.2 – FPases

For the FPases assay, 40μl of enzyme was incubated in a solution containing 80μl of 0.05M sodium citrate buffer at pH=5 and two small circles of filter paper, for one hour. Then the reaction was halted in ice and 240μl of DNS was added. The samples were boiled for 5 minutes at 100C. The samples were placed in ice again and placed afterwards in a microplate. The absorbance was measured in a micro-plate reader at 540nm.

Standard solutions, prepared in the same way as previous samples, but instead of 40μl of enzyme, 40μl of glucose solutions with different concentrations were added. These standards were prepared so it was possible to make a standard curve afterwards.

In this experiment an enzyme reaction was also prepared, but without incubation and where the enzyme was added only after DNS addition. These readings were subtracted to the enzyme readings.

A blank was also prepared, where instead of enzyme, 40μl of water was added. This blank was made to be subtracted to all readings.

Calculations of enzyme activity/ml were made after calculating the glucose equivalents mM using the standard curve, following the equation below:

$$\left(\frac{glucose\ equivalents}{incubation\ time}\right) \times \left(\frac{total\ volume}{enzyme\ volume}\right)$$

3.1.3.3 – Xylanases assay

The xylanase assay was made in a similar way as the FPase but in this case the substrate used was 1% (w/v) beechwood xylan prepared in 0.05mM sodium citrate buffer pH=5. In this particular assay 100μl of enzyme solution were added to 300μl of substrate and incubated for 30minutes at 50C.

The reaction was halted in ice and then 600μl of DNS was added. The samples were boiled for 6minutes at 100C and then placed in ice again.

As in the previous assay, the absorbance was read using the microplate reader at 540nm.

Standard curve, enzyme solution without incubation and blank solution were also prepared to be used in the calculations afterwards.

The results were presented in U/ml by using the following equation after having the result in xylose equivalents mM:

$$\left(\frac{xylose\ equivalents}{incubation\ time}\right) \times \left(\frac{total\ volume}{enzyme\ volume}\right)$$

3.1.3.4 – ß-glucosidases and ß-xylosidases assay

ß-glucosidase activity was measured using 5mM pnitrophenyl b-D-glucopyranoside (pNPG) from Sigma-Aldrich as substrate. The method was carried out using the microtiter format presented by Sørensen et al., 2011, but adapted to even smaller volumes. In sum, 5ul of enzyme extract was added to 50ul of substrate previously prepared in sodium citrate buffer pH 4.8 and incubated at 50ºC for 25minutes. Then, adding 30ul of reaction to 50μl of 1M Na2CO3 stopped the enzyme reaction. The absorbance was read afterwards at 400nm.

Furthermore, a standard curve was made using different concentrations of p-nitrophenol (pNP) from Sigma Aldrich. One unit of ß-glucosidases was defined as the amount of enzyme

that liberates 1μmol pNP from pNPG per minute at pH 4.8 and 50ºC. Note that for the dilution factor, only the dilution of enzyme reaction was accounted for the calculations.

ß-xylosidase activity was measured in the same way as the ß-glucosidase assay but instead of using pNPG pNPX, pnitrophenyl b-D-xylopyranoside was used.

3.2 – Results

3.2.1 – Solid State Fermentation – First Try

As explained in the materials and methods section of this chapter, the first solid state fermentation was carried out using different fungi: *A. carbonarius, A. saccharoliticus, T. reesei and T. asperellum*. The idea was to try to acertain the fungi to be used in the posterior SSF. At the same time, the goal was also to find out whether enzyme production would differ significantly by using a PC from a pilot scale, PC1, or one pressed with a juicer in the laboratory, PC2. Note that PC1 was produced in Skive, Denmark and brought to the laboratory as PC but PC2 was brought as fresh clover grass and pressed in the laboratory.

Furthermore, in this experiment a third medium, referred as control medium, was also used. The control medium was a medium used by Kolasa *et al*., (2014), that yielded great results of FPases, xylanases and ß-glucosidases activities, from different filamentous fungi. This medium was necessary to be done for further comparisons since the incubation temperature and time used in this study were different from the previous study, but the rest of the method was the same.

The AZCL-plates were used as a qualitative method of enzyme activities, since as darker and larger are the arrows, the higher is the activity of the enzymes produced.

The images bellow present the overall results obtained after 24h enzyme incubation in AZCL plates at 30ºC. From the first image (image 1a) it is possible to understand that no activity appears to be presented in the controls, meaning samples with substrates but without fungi inoculation did not present any enzymatic activity. Overall, based on the images, no proteases appear to have been produced by the different fungi, regardless of the media used. However, here the control did not work as well, so it is difficult to understand whether the fungi did not produce proteases or if something just went wrong with the AZCL-casein production. Even so, in the further steps it was assumed that no proteases had been produced.

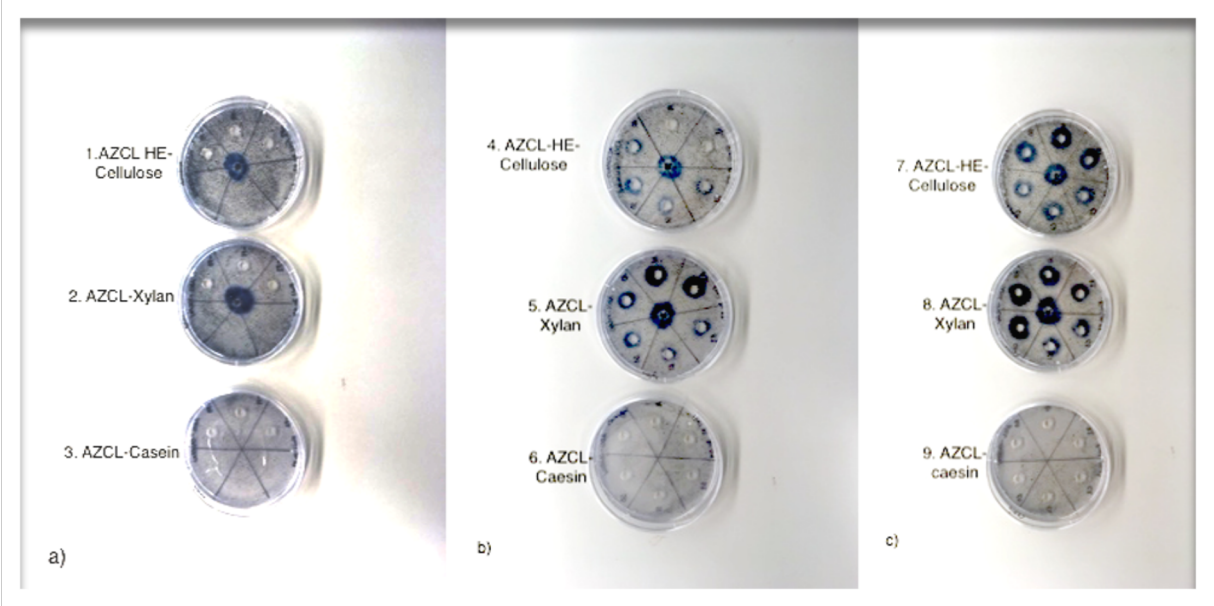


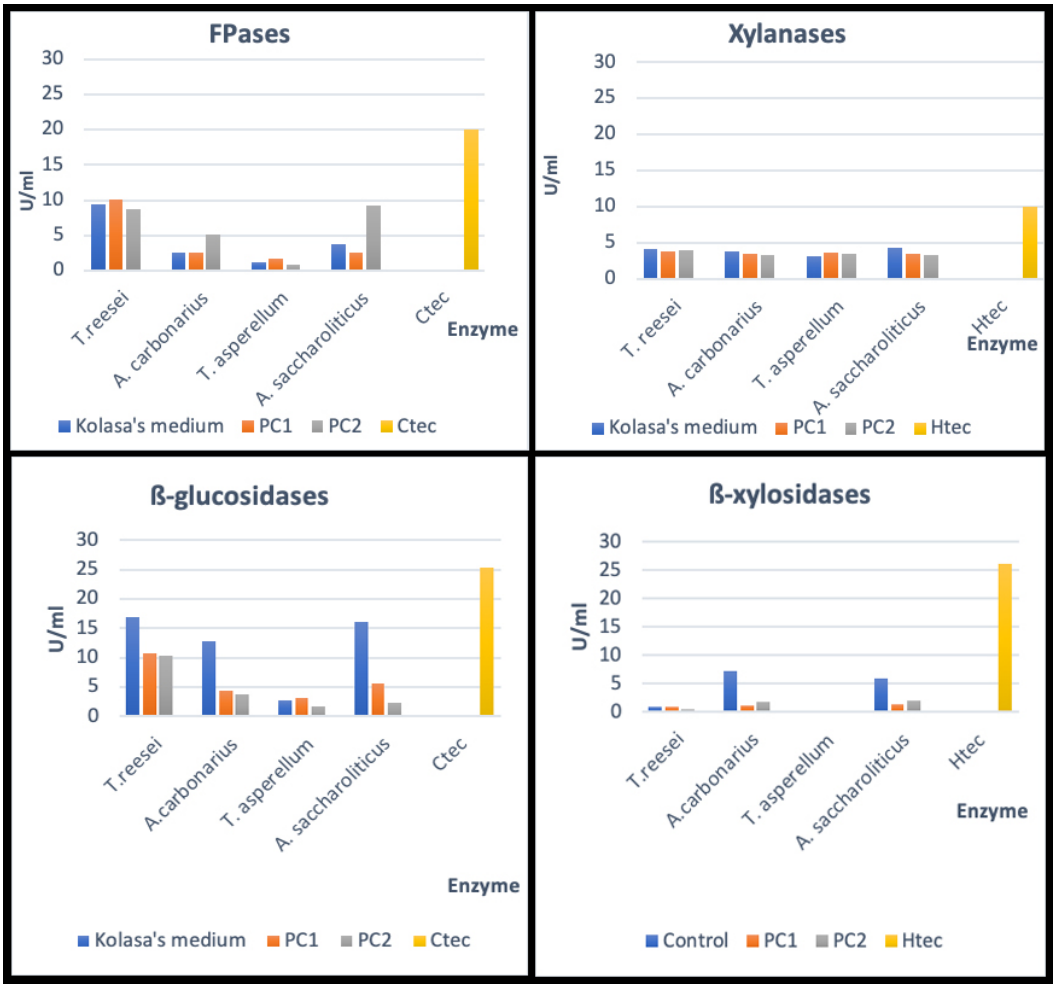
Image 1: a) Image of the controls = substrate without fungi in different 1. AZCL-HE-Cellulose, 2. AZCL-Xylan and 3. AZCL-casein (top to down); b) Image of A. sachharoliticus (three arrows in the top) and T. asperellum (three arrows in the top) in the AZCL-plates mentioned above; c)Image of A. carbonarius (three arrows in the top) and T. reesei (three arrows in the bottom) in the different AZCL-plates.

From image 1 b) it seems that *T. asperellum* appears not to have produced cellulases while *A. saccharoliticus* produced these enzymes but with low activity. When analyzing plate number 5, it seems that *T. asperellum* produced xylanases with higher activity than *A. saccharoliticus*.

The image 1 c), presents the activity of enzymes produced by *T. reesei* and *A. carbonarius*. Plate number 7 shows that *T. reesei* presented higher activity of cellulases compared with *A. carbonarius*. At the same time, it seems from plate number 8, that both *T. reesei* and *A. carbonarius* produced xylanases with high activity.

In fact, overall, cellulases and xylanases were produced by different fungi, used in this experiment. However, with a qualitative method, like this one, it is difficult to understand which enzymes presented higher activities.

In order to try to understand the activity/ml of the enzymes produced, quantitative assays were also performed. FPases, xylanases, ß-glucosidases and ß-xylosidases results are presented bellow in terms of activity/ml (graphic 1).



Graphic 1: FPases, Xylanases, ß-glucosidases and ß-xylosidases production by T. reesei, A. carbonarius, T. asperellum and A. saccharoliticus in different media.

From the graphic presented above (graphic 1), it is possible to verify the activities of some of the enzymes produced by the fungi in the different media. Overall, the control medium (Kolasa's medium) appears to have yielded higher results for ß-glucosidases and ß-xylosidases. In terms of xylanases, except for *A. saccharoliticus*, the production in different media did not yield significant differences. FPases assay seems to have been the only assay where the production in the control medium did not present better results than when using PC1 or PC2, by the different fungi.

In FPases assay, the microorganism that appears to have performed better is *T. reesei*. The highest result was obtained when *T. reesei* was cultivated in the PC1 medium, 10.13 U/ml. For the same assay, it seems that *T. asperellum* appears to be the fungus producing FPases with the lowest activity, which is in line with the results obtained in the AZCL-HE-cellulose. The most

surprising result was *A. saccharoliticus*, which contrary to what was obtained in the AZCL-HE-cellulose, appears to have produced higher FPases than *A. carbonarius*. Furthermore, the production of FPases for *A. saccharoliticus* appears to be higher in the PC2, compared to the other two media, 9.27 U/ml compared with 3.80 and 2.63 U/ml.

In terms of xylanases activity, the best result was obtained by *A. saccharoliticus* in the control medium, 7.40 U/ml. However, *A. saccharoliticus* was also the one presenting a greater difference between production in the control medium, PC1 and PC2.

T. reesei and *A. carbonarius* appear to have slightly higher results in the control medium but similar to those presented in the other media. *T. asperellum* appears to have been the only strain to perform better when cultivated in PC1 instead the control medium, in terms of xylanases activity.

In the ß-glucosidases assay, T. *reesei* presented the highest results, when cultivated in the control medium, with an activity of 16.89 U/ml, followed by *A. saccharoliticus*, when also cultivated in the same medium, with an activity of 16.01 U/ml. It is important to note, that *T. reesei* presented the highest results when also comparing the production of the different fungi in PC1 and PC2.

A. carbonarius performed well when cultivated in the control medium but not so well in the others. *T. asperellum* did not present any promising results in ß-glucosidases production.

The last assay performed was ß-xylosidases and as mentioned above, all the fungi only presented some activity of these enzymes, when cultivated in the control medium. In this particular assay, *A. carbonarius* appears to be the microorganism producing the highest activity among the fungi, 7.26 U/ml. In fact, only *A. carbonarius* and *A. saccharoliticus* appear to have produced ß-xylosidases. Overall, the production of ß-xylosidases was still really low compared with the commercial enzyme Cellic Htec, which presented an activity of 26.06 U/ml.

As may be observed in the graphs, Cellic Ctec and Cellic Htec were used for comparison. It seems that for all the tested activities, both Cellic Ctec and Cellic Htec presented higher activities/ml than the produced enzymes. However, it is important to note that the results presented by the commercial enzymes are still low in comparison with previous studies using these enzymes or with Novozymes reports.

Controls did not show any activity, and for this reason, they have not been presented in the graphs.

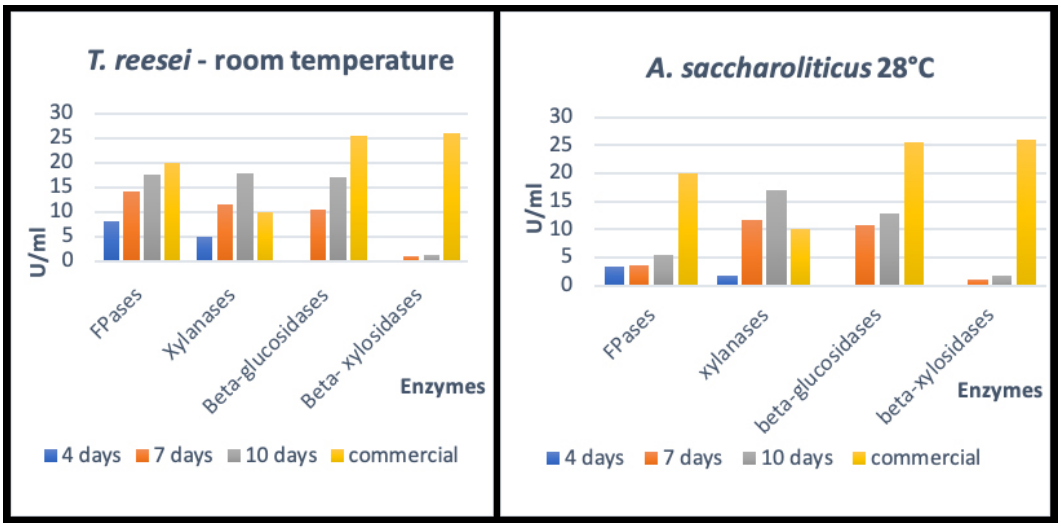
It is worth mentioning that all the samples were dry after this SFF, which represents undesired water loss during the experiment.

Note that detailed activity results are presented in Appendix 1.

3.2.2 – Solid State Fermentation in Press Cake1 Using T.reesei and A. saccharoliticus

As previously explained, due to the results presented in 3.2.1, a second SSF was performed, using two of the strains that presented higher activity/ml. It was also decided to continue only with PC1 since the differences between PC1 and PC2 were not significant and PC1 was made at a pilot scale, which is closer to the procedure used in industries.

Contrary to the first experiment, in the second SSF different temperatures were used for cultivation of *T. reesei* and *A. saccharoliticus*. Furthermore, in this experiment samples were taken after 4, 7 and 10 days of fermentation. The idea was ascertain whether it was worth performing it for 10 days or for a shorter period of time. All the results from this second SSF are presented below (graphic 2):



Graphic 2: FPases, xylanases, ß-glucosidases and ß-xylosidases productions from T. reesei at room temperature (left) and A. saccharoliticus at 28°C. The production of these enzymes was analyzed after 4, 7 and 10 days of SSF and compared with commercial enzymes.

T. reesei presented all the higher results after 10 days of SSF. It was possible to obtain FPaeses with an activity of 17.73 U/ml, xylanases with an activity of 17,98 U/ml and 17.13 U/ml and 1.33 U/ml ß-glucosidases and ß-xylosidases activities, respectively.

A. saccharoliticus also presented the best results after the last day of this experiment: 5,55 U/ml FPases, 17.03 U/ml xylanases, 12.78 U/ml ß-glucosidases and 1.91 U/ml ß-xylosidases.

Overall, it seems that the enzyme activities have increased across the days. In the case *of T. reesei*, only ß-xylosidases production appears to have increased significantly from day 7 to day 10. However, for *A. saccharoliticus*, the activity of FPases, ß-glucosidases and ß-xylosidases did not increase significantly in the same time interval.

Overall, from these results, it appears that *T. reesei* produced enzymes with higher activities than *A. saccharoliticus*. Xylanases produced by both fungi after 10 days presented similar results, *T. reesei* with an activity of 17.98 U/ml and *A. saccharoliticus* with an activity of 17.03 U/ml. In this case, due to the non-significant differences, it is difficult to understand which fungus is actually the greater producer of xylanases.

As mentioned above, commercial enzymes did not present high activities compared with what is presented by other studies. Moreover, xylanases assay presented a lower enzymatic activity of Cellic Htec than produced enzymes after seven and ten days of SSF. These results may have been related to the fact that these commercial enzymes were bought in 2017 and left in the fridge, for different students to use, throughout this period.

Even though it is not possible to make a direct comparison between *A. saccharoliticus* and *T. reesei* since different temperatures were used, depending on the microorganism, it is still possible to mention that *T. reesei* seemed to have performed better at room temperature than *A. saccharoliticus* at 28°C.

Note that detailed results of this part of the experiment are presented in Appendix 2.

3.3 – Discussion

Since quantitative assays are extremely time and material consuming, it is suggested that AZCL-plates are produced for priory analysis of enzyme activity, since it is a relatively cheap and fast method. As previously explained, AZCL plates just give a qualitative analysis, based on how dark the arrows created by the material are when enzymes degrade the substrate. It is assumed that the darker the arrow, the more substrate degraded by the enzyme cocktail added in that area. However, factors like incubation temperature and time applied to the plates might influence the darkness of the colour. Furthermore, this method only presents one colour so only one substrate can be used at a given time, which can be disadvantageous for testing multiple enzyme activities (Kracun, *et al.*, 2015). Thus, it may be suggested that AZCL plates are used as a preliminary enzymatic assay, in order to understand if the tested cocktail presents any activity of the desired enzyme. In this way, unnecessary work and material is used by

quantitative methods if the cocktails to be tested do not present any blue colour by AZCL plates.

Caseins contain a high quantity of proline residues evenly distributed throughout their amino acid sequences and present open structural features, to some extent. Due to this fact, AZCL-casein is one of the most common methods for proteases activity detection. However, as previously mentioned, in this study, not only produced enzymes but also the control sample failed to present any enzyme activity so it is difficult to understand if the produced enzyme cocktail did not contain any protease activity or if it was the plate that was made incorrectly. The control used was human saliva, which is known to present proteases able to degrade casein molecules, so it is unexpected that even the control did not show any enzymatic activity (Çelebioglu, Lee & Chronakis, 2020).

Even though it was not completely proven that the produced enzymes did not present any proteases, only quantitative assays for cellulases, ß-glucosidases, xylanases and ß-xylosidases were performed.

As mentioned above, the objectives of the first SSF were to find the best microorganisms to produce a cocktail of all the four analysed enzymes and to understand whether utilizing an industrial scale presser would reduce enzyme activity compared with a laboratory scale presser. Different enzyme productions from the PC of grass or legumes, at a laboratory scale, is generally carried out with a small-scale presser, which is basically a kitchen juicer. However, to perform enzyme production at an industrial scale it is always interesting to test using a PC of an industrial scale as starting material, like PC1.

In terms of choice of the microorganisms, it was easy to choose between the *Trichoderma* strains, since *T. reesei* seemed to have produced enzymes with a higher activity than *T. asperellum*. For *Aspergillus* it was harder to make a decision since the differences in the activities of *A. carbonarius* and *A. saccharoliticus* were not significant. In the end, it was *A. saccharoliticus* that was chosen to be used in the next experiment due to its slightly better FPases and xylanases results. Furthermore, ß-glucosidases from *A. saccharoliticus* presented higher activity when using the control medium than *A. carbonarius*. In fact, *A. carbonarius* seemed to have performed better in the production of ß-glucosidases when using PC1 and PC2, but differences in activities between these microorganisms for this enzyme were not significant.

It should be noted that *T. reesei* could be used alone in the following SSF due to the results obtained from this strain being better than when using the other microorganisms (except in

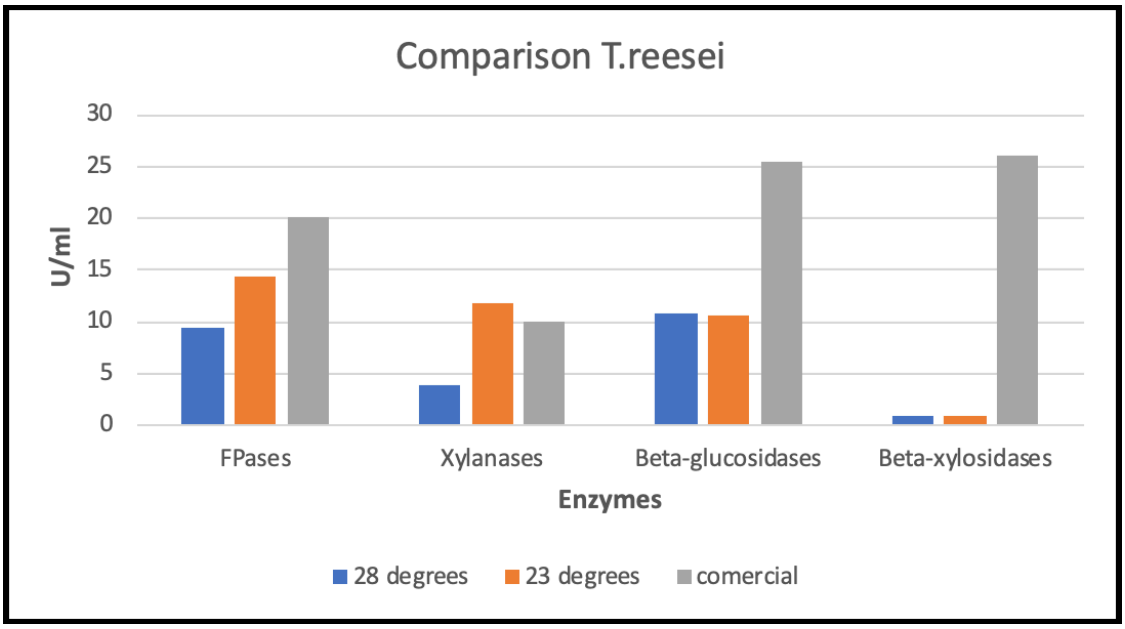
terms of ß-xylosidases). However, previous results have shown that *T. reesei* is not a great producer of ß-glucosidases. So, in this phase, it was assumed that *T. reesei* could not produce ß-glucosidases with such high activities and those results were just a consequence of a human mistake done in the laboratory, during ß-glucosidases assay performance.

Kolasa, *et al*., (2014), presented 13U/ml endoglucanases activity, 0.8U/ml exoglucanases activity and 0,5U/ml ß-glucosidases activity from *T. reesei* after ten days of SSF. For *A. saccharoliticus*, the mentioned study presented activities of 3.8 U/ml, 0,1 U/ml and 20.0 U/ml of endoglucanases, exoglucanases and ß-glucosidases, respectively. Comparing these results with the results of this study, when using the same medium as substrate for SSF, some differences are observed. In terms of overall cellulases production, *T. reesei* performed slightly better in the mentioned study and *A. saccharoliticus* performed similary in both studies. For ß-glucosidases *T. reesei* produced enzymes with a much higher activity in the current study. *A. saccharoliticus* did not perform as well as in the study of Kolasa, *et al*., (2014).

It is important to mention, that during this first SSF, the temperature increased more than expected and due to the material used, a lot of water was lost during the experiment. Since temperature and moisture content influence the enzyme production by filamentous fungi, it was thought that this factor might have negatively influenced the enzyme production in this experiment.

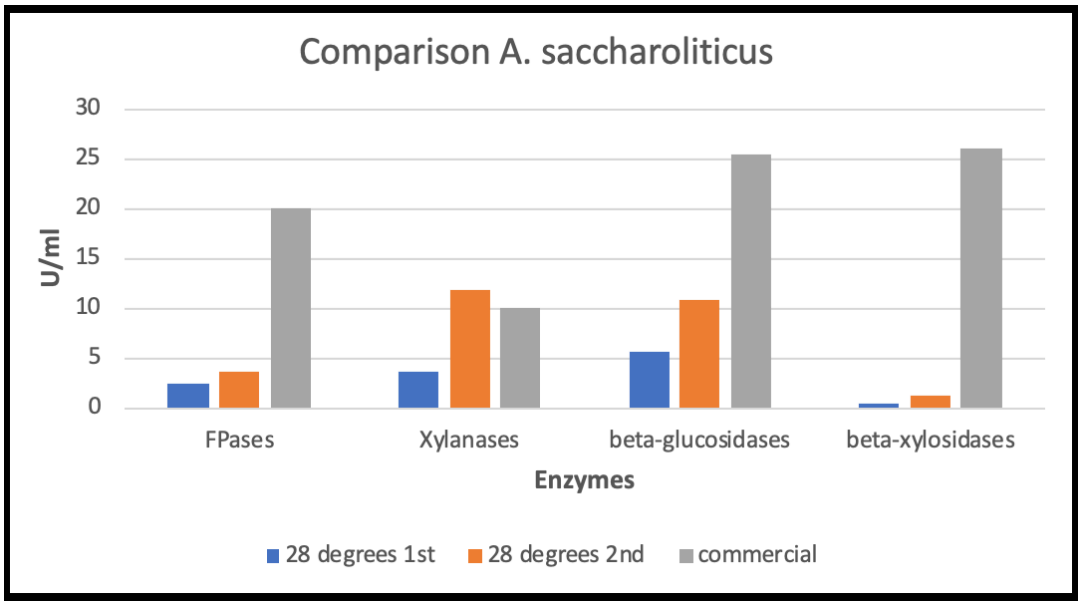
In the first experiment the temperature was set to room temperature, but due to a problem in the incubator, the temperature reached 28°C. However, it is known that *T. reesei* generally produces enzymes with a higher activity at lower temperatures than 28°C. At the same time, *A. saccharoliticus* seems to have performed better at 28°C than in previous studies where the SSF was performed at room temperature. It was due to these facts that different temperatures were used for the second SSF procedure, which was dependent on the microorganism used. Furthermore, as previously explained, it was also decided to continue only with PC1 and increase the experiment period to ten days in total. However, in order to understand whether it was worth growing the fungi for such a long time, samples were taken on days 4, 7 and 10.

Comparing *T. reesei* production, after seven days, in both the first and second experiments (graphic 3), FPases and xylanases production appears to have improved with the second experiment. ß-xylosidases also presented slightly higher activity in the second experiment. ß-glucosidases production was the only one that did not improve in the second experiment, after seven days.



Graphic 3: Comparison of enzyme production from first and second SSF experiments, after seven days of T. reesei cultivation.

Although it may be said that overall results appear to have improved with the second experiment, it cannot be concluded that temperature change was actually what influenced this change, since an unexpected loss in moisture content was observed in the first experiment, which may also have negatively influenced those results. In fact, when looking at the results of the *A. saccharoliticus*, the differences between the first and second experiments, after seven days were also significant, as it can be seen in the graphic below (graphic 4). This may actually indicate that loss in moisture content, in the first experiment, was in fact the major negative factor on enzyme production.



Graphic 4: Comparison of enzyme production from first and second experiments, after seven days of A. saccharoliticus cultivation.

Considering now, only the production of enzymes in the second experiment, it is possible to understand that all the enzymes increased their activities when cultivated for ten days instead of seven. However, it is important to mention that the costs associated with an increase of three days in enzyme production could be extremely high, especially on large scales. Further studies are necessary in order to understand whether it would be preferable to cultivate the enzymes for only seven days and increase the enzyme loading in the hydrolysis or if it would still be worth cultivating it for ten days, even if it implies an increase in the costs of this experiment.

Overall, the most interesting result of both experiments is that *T. reesei* was able to produce ß-glucosidases with high activity. *T. reesei* is known to be a good producer of cellulases but it is not a great producer of ß-glucosidases. This factor reduces efficiency in biomass degradation and compromises its industrial application. It was reported, that in an cellulases-inducing environment the production of secreted ß-glucosidases is only 1% of all cellulases produced by *T. reesei (Li, et al.*, 2016).

Genetically modified *T. reesei* has been developed in order to improve ß-glucosidases performance. It would be good to use the same microorganism for the production of a cocktail able to degrade the entire cellulose complex. However, due to the low ß-glucosidases activities presented in prior studies, different genetic approaches have been suggested in order to also make *T. reesei* a good producer of ß-glucosidases (Li, *et al*, 2016).

However, genetic modified microorganisms can not be used in different processes such as food industries and organic processes. GMOs can also involve considerable research, hours of work and costs. In this study, *T. reesei* was able to produce cellulases and ß-glucosidases, with relatively high activities. These results suggest that under certain conditions, *T. reesei* is able to produce ß-glucosidases with relatively high activities, eliminating the need to resort to GMOs for the same purpose. Juhász, Egyházi and Réczey (2005) suggested that the pH of a medium was what influenced the most ß-glucosidases production by *T. reesei* RUT 30. However, in this study no pH measurement was applied in SSF so it is unclear if in this case, it influenced ß-glucosidases production. Therefore, future SSF experiments should take pH measurement and maintenance into consideration.

Another factor that seems to influence ß-glucosidases production from *T. reesei* is the amount of D-glucose present in the medium. Using PC as start material might influenced positively the production of ß-glucosidases than previous studies where more unattached D-glucose was available in the starting material.

Overall, it would be interesting for future experiments to focus on the different parameters of enzyme production by SFF, when using PC. Moreover, other enzyme assays may also be applied for an overview of the enzyme cocktails produced by the fungi used.

Chapter 4 – Enzymatic Hydrolysis as a Pretreatment for Press Cake

After identifying fungi that produced FPases, xylanases, ß-glucosidases and ß-xylosidases with higher activity/ml their efficiency was tested by preforming different enzymatic hydrolyses of the press cake (figure 6).

The produced enzymes were compared with the commercial enzymes Cellic Ctec and Cellic Htec in order to ascertain whether these produced enzymes can substitute commercial enzymes in press cake degradation. Since the quantity of enzyme loading/total solution to be added in order to degrade press cake, is still not well known, different enzymatic loadings will be tested from both produced and commercial enzymes.

Enzymatic hydrolysis is widely considered to be normally more efficient the lower it is the DM content per total solution. However, in order to reduce the costs associated with this process, in this chapter, enzymatic hydrolysis will be tested but at a higher DM per total solution.

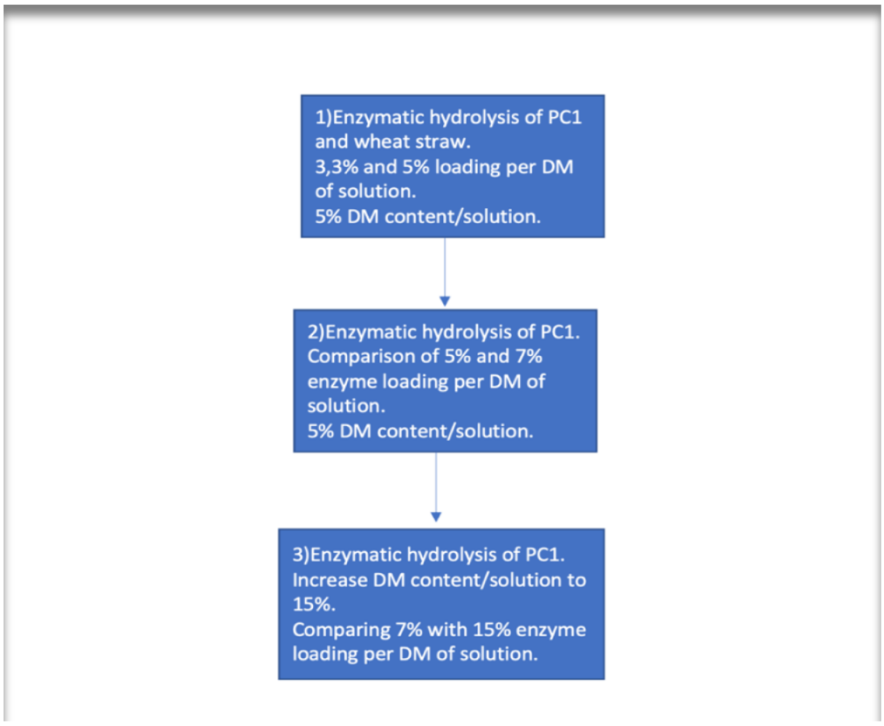


Figure 6: Enzymatic hydrolyses experimented in this project: 1) 3,3% and 5% enzyme loading in 5% DM/total solution. 2) Increasing enzyme loading to 7%. 3) Increase DM content/solution to 15%.

4.1 – Materials and Methods

4.1.1 – Strong Acid Hydrolysis

To evaluate the amount of cellulose, hemicellulose and Klason lignin present in the press cake of clover grass strong acid hydrolysis, SAH, was executed.

The SAH was carried out in duplicates and 0.1600 g of press cake was added to each sample, (>85% TS), which was weighed by using an analytical balance and then poured into 60ml Pyrex glass tubes. There were 4 samples in total, 2 experimental samples and 2 standards. Note that the standard sample composition contained 33.1 g/L of D-glucose-monohydrate, 30.0 g/L L- arabinose and 33.1 g/L D-xylose.

1.5 ml of H2SO4 (72%) were added to each substrate and were then mixed in a vortex mixer to prevent air trapping. The samples were incubated in a water bath at 30 ˚C for 60 minutes. Vortex mixing mixed the samples twice during the incubation. After incubation, 42 ml of Millipore water were added to the experimental samples in order to make a final concentration of 4% H2SO4. 41 ml of Millipore water were added to the standard samples in order to make room for the STAM solution. 1 ml of STAM was then added to the 2 standard tubes to spike the samples. All the samples were then mixed by inversion. The screwcaps were loosened slightly before being autoclaved for 60 min at 121 ˚C. The samples were mixed once again but by inversion the Pyrex glass tubes three times each. The samples for HPLC analysis were prepared and then calculations were made in order to understand how much cellulose and hemicellulose were present in the press cake of the clover grass used.

For Klason lignin measurement, the hydrolysate was filtered and then the filter was placed in the furnace at 550°C for 4 hours. The difference between the weight of the filter before and after heating gives an idea of how much Klason lignin is present in the PC used (Kádár, 2016).

4.1.2 – Enzymatic Hydrolysis of Press Cake Using Produced and Commercial Enzymes

The aim of this experiment was to verify how efficient produced enzymes were at degrading the production material, PC.

This experiment was based on a laboratory exercise from Biological Production Processes where wheat straw was used as substrate. In this project, the first hydrolysis was performed following the afore-mentioned protocol but using both acid-extruded pretreated wheat straw and press cake as substrates. It should be noted that for this experiment, the initial DM of wheat

straw was 28% and PC1 was 29%. Calculations were made in order to add the same amount of DM per total solution based on the initial dry matter. Furthermore, in the laboratory exercise, commercial enzymes were used so it was decided to also use Cellic Ctec and Cellic Htec in this experiment.

14,2 g/l succinic buffer pH=5 was added in order to make 5% DM per total solution.

0,2% kanamycin with a concentration of 50mg/ml was also added and the flasks were left at room temperature for half an hour. 3.3% of enzyme extract was then added, and the flasks were incubated for 48hours at 50ºC and 140rpm. All samples were prepared in quadriplicates. The overall preparation is presented below (table 1).

sample	Substrate	Buffer	enzyme extract	antibiotic
1.	0.89 g wheat straw	3930 μl	3.3% *T. reeesei*	10μl kanamycin
2.	0.89 g wheat straw	3930 μl	3.3% *A. saccharoliticus*	10μl kanamycin
3.	0.89 g wheat straw	3930 μl	3.3% Cellic Ctec	10μl kanamycin
4.	0.89 g wheat straw	3930 μl	3.3% Cellic Htec	10μl kanamycin
5.	0.86g PC1	3960 μl	3.3% *T. reeesei*	10μl kanamycin
6.	0.86g PC1	3960 μl	3.3% *A. saccharoliticus*	10μl kanamycin
7.	0.86g PC1	3960 μl	3.3% Cellic Ctec	10μl kanamycin
8.	0.86g PC1	3960 μl	3.3% Cellic Htec	10μl kanamycin
9.	0.89 g wheat straw	4100 μl	----------	10μl kanamycin
10.	0.86g PC1	4130 μl	----------	10μl kanamycin

Table 1: Preparation for enzymatic hydrolysis.

After this, the supernatant was put into new falcon tubes and then each was filtered using 0.25μm filters.

2ml of supernatant were put into HPLC crucibles and the rest kept in new falcon tubes. All the samples were stored in the freezer at -20°C until the HPLC analysis.

The HPLC analysis gives the concentration of monomer sugars and organic acids presented in the media after enzymatic hydrolysis. At the same time, the strong acid hydrolysis procedure made it possible to find out how much of the dry matter is actually cellulose and hemicellulose. With this knowledge, it was possible to make calculations to find the yields of the hydrolyses:

$$\frac{(monomer\ sugars + organic\ acids\ *)}{(cellulose + hemicellulose\ per\ solution)} \times 100$$

*organic acids were calculated as if they had been converted back into monomer sugars again.

Note: On the basis of previous results, the commercial enzyme Cellic Ctec appeared to present higher activity/ml than the produced enzymes, therefore it was diluted to present the same activity/ml as *T. reesei*. The dilution was based on FPases results.

4.1.2.1 – Comparison with commercial enzymes at 5% enzyme loading

Due to the results obtained from 4.1 the enzyme loading was increased to 5%, The protocol was exactly the same as presented in 4.1 but instead of 3.3%, 5% enzyme loading was added to each flask.

Note that both substrates without the enzyme addition were also prepared to serve as control.

4.1.3 – Optimization of Enzymatic Hydrolysis in Press Cake

The results of the previous hydrolysis showed that some parameters could be changed in order to try to increase the efficiency and reduce the costs of this experiment.

4.1.3.1 – Increase of Enzyme Loading per Total Solution

Due to the results obtained even with commercial enzymes; it was noted that the enzyme loading could be increased in order to try to improve the efficiency of the hydrolysis. To this end, a new experiment using 5% and 7% enzyme loading was executed.

For this experiment only press cake was used as substrate.

In this experiment, extra samples were used where T. *reesei* enzymes and Cellic Ctec cocktail were combined with Cellic Htec in order to try to improve the overall enzymatic hydrolysis of press cake without further increases in enzymatic loading.

The rest of the parameters were maintained as in 4.1.2.

4.1.3.2 – Hydrolysis with 15% Dry Matter per total solution

As previously explained, PC has a dry matter between 24-29%.

In these experiments, the DM of the solution was increased to 15% and the enzyme loadings were 7% and 15% of total DM of the solution. The rest of the parameters were maintained as explained in 4.1.2.

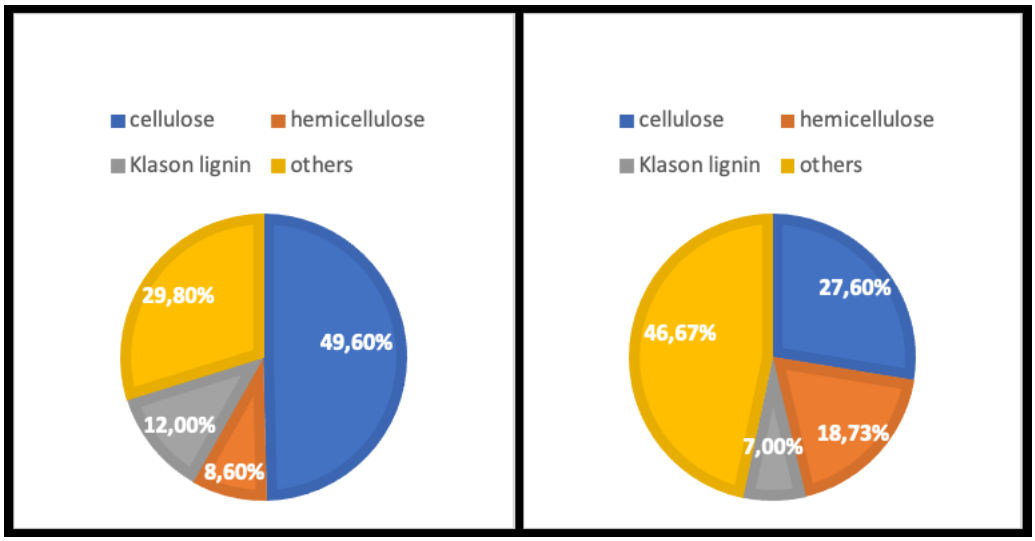
4.2 – Results

4.2.1 – First Hydrolysis of Press Cake Using Produced and Commercial Enzymes

In previous years a number of enzymatic hydrolyses were performed at Aalborg University using Cellic Ctec and Cellic Htec to efficiently degrade heat-extruded pretreated wheat straw. Due to its promising results, the produced enzymes were applied to wheat straw and to press cake. Wheat straw was used with a view to understand whether the produced enzymes work as efficiently as commercial enzymes in the material on which the protocol was based. PC was used to ascertain whether the produced enzymes were able to degrade the material efficiently where their production occurred and at the same time understand if some of the parameters should be changed in the protocol, in order to make it more suitable for the enzymatic hydrolysis of PC1.

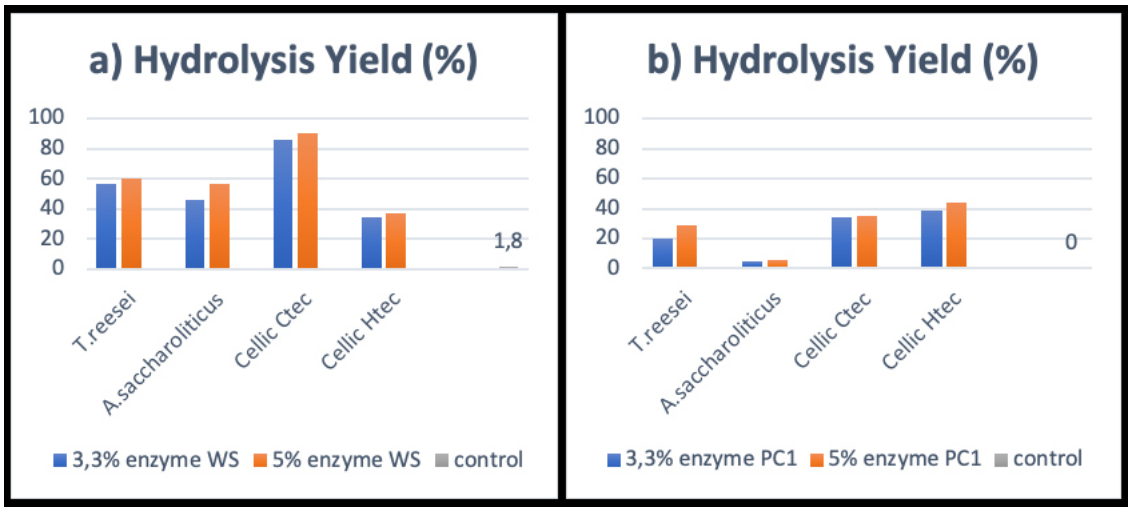
It is important to note that the yield was based on the amount of sugars that were degraded into monomer sugars, divided by the amount of cellulose and hemicellulose presented in the

dry matter of PC or wheat straw. As mentioned above, the DM of this pretreated wheat straw was 28% and PC was 29%. However, their composition varied considerably. Wheat straw presented 49.6% cellulose, 8.6% hemicellulose and 12% Klason lignin. PC presented 27.6% cellulose, 18.73% hemicellulose and 7% Klason lignin (graphic 5).



Graphic 5: Circular graph in the left represents DM composition of pretreated wheat straw. Circular graph in the right represents DM composition of PC.

The composition of wheat straw before pretreatment was not obtained. However, from previous reports it is possible to understand that wheat straw normally presents higher amounts of hemicellulose and lignin, before any pretreatment. Reducing the amount of these molecules in the substrate by acid-extrusion pretreatment might have allowed the enzymes to easily attain cellulose, increasing the overall hydrolysis yield in wheat straw compared with PC1. Although, it would be interesting to pretreat PC1 with the same method, for fair comparisons, it was not interesting to use this type of pretreatment in this study, mainly due to unsustainable aspects related to this type of pretreatment.



Graphic 6: a) Enzymatic hydrolysis yield when using T. reesei, A. saccharoliticus and commercial enzymes in pretreated wheat straw; b)Enzymatic hydrolysis yield when using T. reesei, A. saccharoliticus and commercial enzymes in pretreated PC1.

From the results presented above (graphic 6), it seems that all the enzymes performed better in wheat straw than in press cake. Even though the enzymes from *T. reesei* and *A. saccharoliticus* were produced from the same press cake that was then used for the hydrolysis, these enzymes seemed to have been more efficient in the degradation of wheat straw than press cake. These may have been related with the fact that wheat straw was pretreated and PC1 was not. As previously explained, cellulose might have become more available to enzyme degradation due to a loss of hemicellulose and lignin after pretreatment.

As expected, due to the amount of cellulose and hemicellulose presented in wheat straw, Cellic Ctec was the enzyme cocktail that performed better. However, as may be observed in the results in Appendix 3, Cellic Htec degraded most of the 8.6% hemicellulose present in this material. Cellic Htec is known to efficiently degrade hemicellulose fraction, so it was actually interesting to understand that most of hemicellulose was degraded by Cellic Htec even its lower activities of xylanases, compared with produced enzymes. This indicates that the overall hydrolysis yield of *T. reesei* and *A. saccharoliticus* cocktails were only higher due to the presence of cellulases and ß-glucosidases. It is known that Cellic Htec may present some residues of these enzymes, but not with high activities.

In PC1, the enzyme cocktail that seemed to have performed better was Cellic Htec, followed by Cellic Ctec. This indicates that even though the enzymes were produced in PC1,

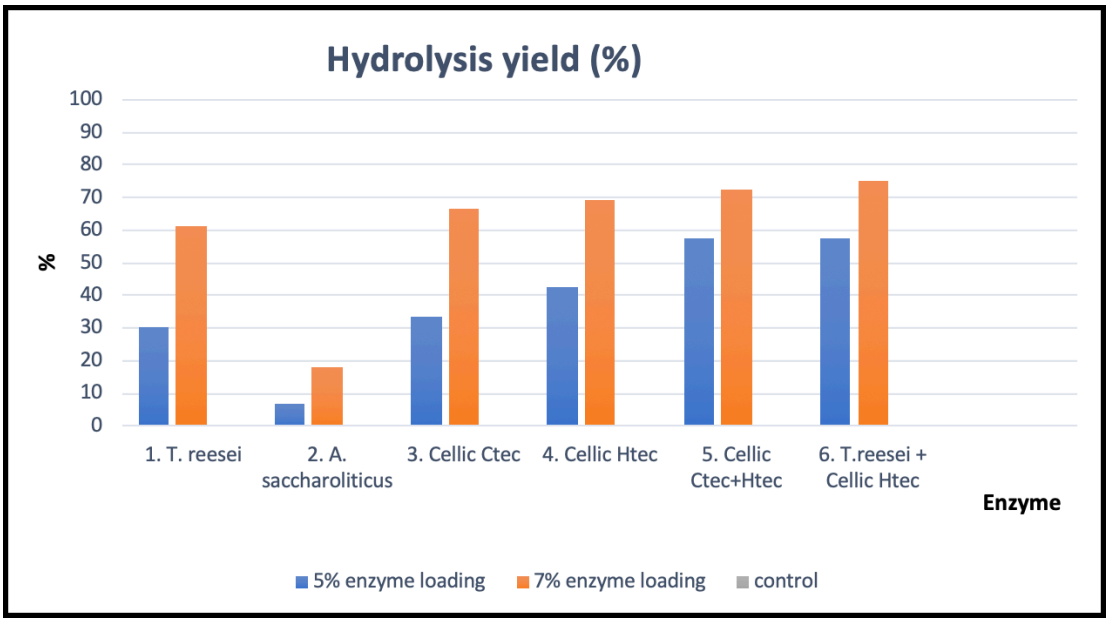
other factors might have influenced the commercial enzymes to perform better. A possible explanation is that even though Cellic Ctec was diluted to present the same FPases activity as *T. reesei* and Cellic Htec presented low xylanases activity, the commercial enzymes might have higher activities of other enzymes such as ß-glucosidases and ß-xylosidases. Another explanation is that even if commercial enzymes presented the same or lower activities, their specific activity was still higher. Commercial enzymes present higher purification than the produced ones presented in this study, which means that in a same amount of cocktail, commercial enzymes presented higher concentration than produced enzymes, which besides different enzymes also present other residues from the SSF, such as other proteins. At the same time, it may also be suggested that commercial enzymes are generally more stable than produced ones. Produced enzymes might have lost some activity between the day when the assays were performed to later hydrolysis period. This loss might also have occurred during the time of the experiment.

Due to differences in activities between the different enzymes presented, it is not possible to make a fair comparison between them. However, it can be understood from these results, that some of the parameters of this method needed to be adapted when using these produced enzymes in PC1.

4.2.2 – Increasing the Enzyme Loading in Hydrolysis of Press Cake

It was clear from the previous results that it was necessary to change some of the parameters of the enzymatic hydrolysis of press cake. In order to try to improve the enzymatic hydrolysis of press cake a second hydrolysis was performed with a higher enzymatic loading than in the previous experiment, 7%. A combination of *T. reesei* or Cellic Ctec with Cellic Htec was also used in order to understand whether a combination of both cocktails would lead to a higher amount of degraded sugars.

The results of this experiment are presented in the following graphic (graphic 7):



Graphic 7: Enzymatic hydrolysis yield in PC1 when 5% and 7% enzyme loading was applied.

As expected, 5% enzyme loading yield similar results to the ones presented in section 4.2.1. The only difference is that here, Cellic Htec was also combined with Cellic Ctec or *T. reesei*, improving the overall yield of hydrolysis. It may be seen from Appendix 4 that not only the amount of glucose increased but arabinose became present in these hydrolysates compared with those from *T. reesei* and Cellic Ctec alone.

On the basis of these results it also appears that by increasing the enzymatic loading to 7% the amount of sugars that were degraded into monomers increased considerably. In fact, when using 7% enzyme loading from *T. reesei, A. saccharoliticus* or Cellic Ctec cocktails, the yield more or less duplicated. Furthermore, increasing the enzyme loading to 7% yield slightly better results than combining *T. reesei* or Cellic Ctec with Cellic Htec at 5% total enzyme loading.

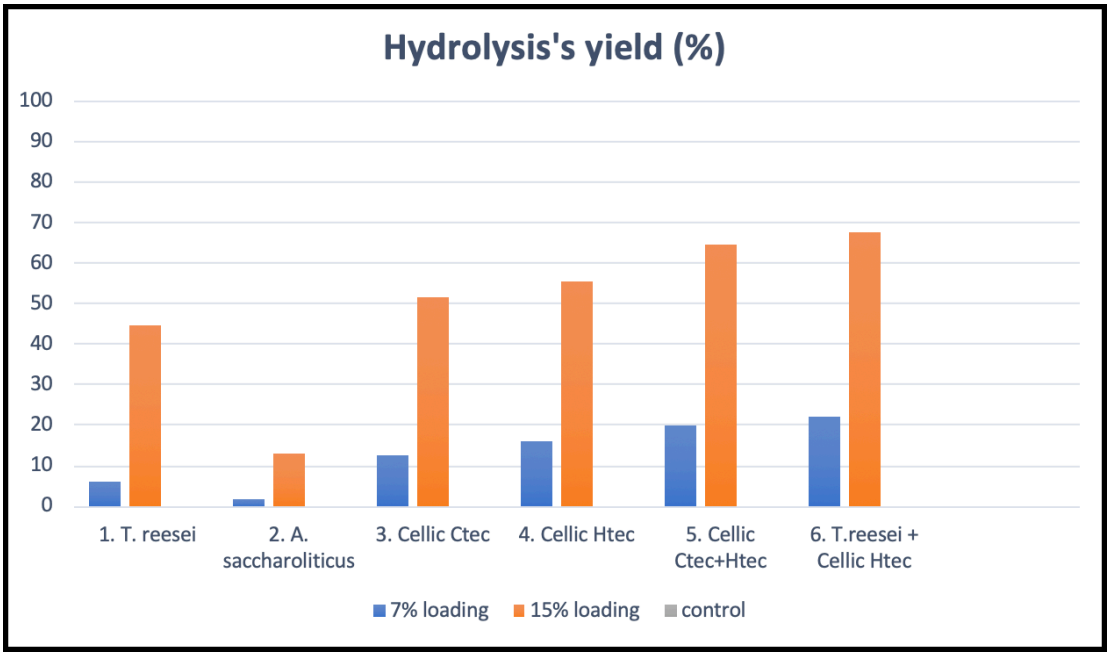
The best results of these experiments were obtained when *T. reesei* was combined with Cellic Htec at 7% total enzyme loading with a 75.15% hydrolysis yield. Here the main difference compared with *T. reesei* alone was the degradation of hemicellulose fractions giving arabinose as a monomer sugar present in the hydrolysate. However, as it may be seen from Appendix 4, the amount of glucose did not increase that much from *T. reesei* or *T. reesei* + Cellic Htec. Cellic Htec did not appear to help cellulases from *T. reesei* to reach more cellulose, it only degraded the hemicellulose fraction further.

4.2.3 – Hydrolysis of Press Cake 1 with a Total of 15% Dry Matter Per Total Solution

From the previous results it is possible to notice than none of the enzyme cocktails reached 100% hydrolysis yield. However, it is known that the addition of water to the hydrolysis can increase the costs associated with this process, especially when applied at large scales. It would be preferable to use the enzymes directly in the press cake with less or without water addition.

It was expected that no addition of water would give really low yields so, in this experiment, the amount of water added was three times less than that used in previous experiments. It was expected that, with this, effects in the enzyme cocktails performance, would be observed.

Due to the good results presented previously with 7% enzyme loading, the same amount was added to a solution containing 15%DM content. However, since water it is really important for enzymatic hydrolysis it was already expected that by reducing the water content of the solution the performance of the enzyme cocktails would decrease. Therefore, the enzyme loading was increased to 15% and then compared with the results at 7%.



Graphic 8: Enzymatic hydrolysis's yield in PC1 with enzyme loadings of 7% and 15%. Note that here solution presented 15% DM.

As expected, the results presented above (graphic 8) show that the enzymatic hydrolysis yield was reduced by reducing the amount of water presented in the total solution. The *T. reesei*

cocktail, for example, presented 6% hydrolysis yield when added to a solution with 15% DM content and 61.5% hydrolysis yield when used in a solution with 5% DM content.

Furthermore, increasing the enzymatic loading to 15% significantly improved the yields from the different enzymatic cocktails. However, these results were still lower than those obtained when hydrolysis was performed using 7% enzyme loading to a solution with 5% DM content.

In line with the results of the previous section, the combination of *T. reesei* or Cellic Ctec with Cellic Htec yield the best results with 67.58% and 64.61% respectively. Once again, the main difference was that the combination of these enzymes made arabinose available in the hydrolysate.

These last results indicate that increasing the DM content in an enzymatic hydrolysis solution significantly reduces the performance of the different enzymes, regardless of their origin or whether their concentration is increased to 15%. Thus, further increase in the DM content by no addition of water can be disadvantageous for the hydrolysis, reducing even more the amount of lignocellulosic material degraded.

4.3 – Discussion

Enzymatic hydrolysis is dependent on different factors and in order to understand which one influences the degradation of lignocellulosic material the most, only one parameter at the time should be changed. Furthermore, it seems that a cocktail of enzymes can present different types of enzymes with different activities, making it difficult to adjust the different cocktails to present the same enzymatic activities. In this study, for example, even if the activities of FPases, of different cocktails, were adjusted to present the same activity, their activities of ß-glucosidases, xylanses or other enzymes, would still differ. Such problem makes a comparison between the different cocktails used, difficult.

Although, the comparison between the different enzymes was difficult, it was still possible to compare whether both commercial and produced enzymes performed better in wheat straw or PC, if increasing the enzyme loading would be advantageous for the overall material degradation and if the combination of different cocktails would also improve the hydrolyses yields. It was also possible to understand that in order to increase the DM content of enzymatic hydrolysis, other parameters than enzyme loading need to be taken into account.

As mentioned in section 2.3, in order to increase the monomer sugars obtained with enzymatic hydrolysis, it may be advantageous to add a priori pretreatment to the lignocellulosic material. Different pretreatment methods may be used, such as milling, alkali, acid, steam explosion, hydrothermal or even other types of biological pretreatment. However, most of these pretreatments are related to high energy consumptions, pollution and/or long treatment periods (Sun, Sun, Cao, & Sun, 2016).

Among the different pretreatments that can be used priori to enzymatic hydrolysis, chemical and physicochemical pretreatments appear to be the most effective and promising processes for industrial applications. Normally, alkali and acid pretreatments are able to remove hemicellulose and lignin fractions with low cost, but the environmental problems and high costs associated with chemical recovery are generally associated with these methods. Physicochemical pretreatments can solubilize hemicelluloses, disorder the lignocellulosic structure and it may increase the surface of the material, making it more available to the enzymes with lower environmental impact than alkali or acid pretreatments. The main disadvantages of physicochemical pretreatments are in general associated with equipment and chemical costs. In fact, the combination of different pretreatment methods showed a beneficial effect on improving enzymatic hydrolysis, however costs and environmental problems are normally associated with it, reducing the sustainability of an entire biorefinery (Silveira, Venelli, Corazza, & Ramos, 2015; Sun *et al*., 2016).

To avoid the utilization of different pretreatment methods due to their disadvantages, different enzymes were tested in this project. It was expected that by using a cocktail that also contained hemicellulases, this would help degrade the hemicellulose making cellulose more available to cellulases and ß-glucosidases. Since glucose is present in both cellulose and hemicellulose, it is difficult to draw conclusions as to how much of each fraction was actually degraded, only from the HPLC analysis. When looking at the best results, 7% enzyme loading at 5%DM/solution, *T. reesei* presented 12.3g/l of glucose and *T. reesei* + Cellic Htec 12.7g/l of glucose, after enzymatic hydrolysis. This could represent 81% and 84% of degradation of cellulose, respectively, if all glucose came from cellulose fraction. It is important to mention that calculations from strong acid hydrolysis assume that all glucose units come from cellulose fraction and only other monomer sugars are counted for the hemicellulose fraction. Future research should apply a method such as material analysis via acid detergent fibre (ADF), neutral detergent fibre (NDF) and acid detergent lignin (ADL). Omer, Ali & Gad, (2012), suggested that cellulose, hemicellulose and lignin fractions could be calculated by ADF-ADL,

NDF-ADF and ADL results, respectively. ADF value represents cellulose and lignin, ADL the lignin fraction present in ADF and NDF represents all the cell wall constituents. The presented calculations could be applied for understanding concentration of each of these polymers. Such results could be compared with the ones obtain from SAH in order to try to improve material characterization.

In fact, calculations of enzymatic hydrolysis were difficult to make by using SAH, TS/VS, HPLC for material analysis. Frist, as explained, it was difficult to understand how much cellulose and hemicellulose was actually degraded. Secondly, due to material availability, not all sugars were analysed in HPLC and so, some monomer sugars, such as galactose could also be present. In the future, all hemicellulose components should be analysed not only to understand how much of this fraction was actually degraded by enzymes but also to correct the percentage of hemicellulose fraction per DM content.

Cellulose fraction received particular attention in this project, since most of the microorganisms are not able to utilize C5 sugars naturally, and when they are, it is normally done at a much slower rate than glucose. That said, one may question the mixing of glucose, xylose and other monomers in the hydrolysate, when some methods can "wash" hemicellulose fraction. Despite the related costs and environmental problems, it was thought that glucose could first be used as a monomer sugar for organic acid production and the residues of those experiments, rich in C5 sugars, could be used for certain microorganisms that are able to utilize C5 sugars. *Pachysolen tannophilus* is a yeast that is able to utilize xylose for the production of xylitol and ethanol, for example (Runquist, Parachin & Hahn-Hägerdal, 2010).

Furthermore, GMOs may also be used in order to produce organic acids, for example, from both C6 and C5 sugars, but different processes do not allow the utilization of GMOs, such as, organic industries. Moreover, when considering protein precipitation at the same time as lactic acid production, for example, the utilization of GMOs is not allowed since the end of these proteins is for organic animal feed. In organic farming processes, this type of microorganisms can not be used.

If GMO products can not be used in organic farming, then the utilization of Cellic Ctec and Cellic Htec should not be used in this project. However, as previously explained, the usage of those enzymes was just for comparison with the produced ones in terms of hydrolysis yields, since the protocol is based on the utilization of commercial enzymes. The combination of produced enzymes from *T. reesei* with Cellic Htec was related to the fact that *T. reesei* alone did not seem to present all the necessary enzymes to degrade hemicellulose. Even if it presented

xylanases, the ß-xylosidases activity was really low and the absence of arabinose indicated that the cocktail from *T. reesei* probably did not present enzymes such as α-L-arabinofuranosidase. Therefore, by using Cellic Htec combined with *T. reesei* more hemicellulose could be degraded. Later, with time, some microorganism could be used for the production of hemicellulases with higher activities than *T. reesei*, to substitute the commercial enzyme.

Chapter 5 – Future Researches

The previous chapters presented the laboratory research that was possible to perform during this thesis. However, additional experiments have been planned and may be suggested in order to utilize further, better and with more precision PC from clover grass.

As explained above, hydrolyses could be performed in different conditions, sugars could be fermented for organic acid production and/or further protein extraction could be tested after fermentation.

The following ideas are merely suggestions, based on what has stemmed from this project, but it does not mean that other products can not be evaluated to be produced by PC from clover grass.

5.1 – Enzymatic Hydrolysis's Optimization

During the laboratory work, some press cake was first autoclaved at 121°C for 40 minutes and 15psi and then enzymatic hydrolysis with 15% DM/solution was performed. However, due to the lockdown it was not possible to analyse the results by HPLC or any other method. It was thought that autoclavation of PC would act as a pretreatment of the material and help enzymes to attain cellulose and hemicellulose.

It is well known that both hemicellulose and lignin are more sensitive to heat than cellulose and it is reported that sometimes these types of methods can create inhibitory compounds for enzymatic hydrolysis. However, those inhibitory compounds are generally formed at higher temperature and pressures (Aftab, Iqbal, Riaz, Karadag & Tabatabaei, 2019).

In fact, different heating devices have been applied in the pretreatment of lignocellulosic material. Obeng, Premjet and Premjet (2019) suggest that autoclave is one of the most effective heating processes for that purpose. Compared with other heating processes, the supply of relatively low heat combined with pressure by the autoclave is very efficient in rendering the recalcitrant structure of lignocellulosic biomass. Furthermore, it is reported that autoclaving lignocellulosic material may be beneficial priory to biological pretreatment, making the material more available to the enzymes (Gabhane, Vaidya, Mahapatra & Chakrabati, 2011; Obeng, Premjet & Premjet 2019).

Since it was expected that by applying another pretreatment method, the enzyme loading could be lowered, the experiment presented in section 4.1.3.2 was performed again but in PC that passed through the autoclave, before it was enzymatically hydrolysed. The results of this enzymatic hydrolysis were frozen at -20°C, so those results could be run for further conclusions or that in the future a similar experiment could be applied in order to increase the yield of monomer sugars obtained from the hydrolysis.

It is important to note that the usage of an autoclave, especially on large scales, increases the costs of the overall production. The costs associated with this procedure are related to electricity cost, equipment capital cost, human resource cost, depreciation cost of equipment and maintenance and cleaning costs of the autoclave. Furthermore, if water needs to be added priory to the hydrolysis to make 15% DM/solution, water costs also need to be taken in consideration (Basu, *et al*., 2016).

In fact, a number of different studies need to be applied before any conclusions as to what the best pretreatment method of PC for further utilization of sugars and other components of this material may be drawn. This was only a suggestion based on what was executed during the experimental work of this project. However, it is not excluded that other pretreatment methods could be tested priory to the enzymatic hydrolysis, and then compared with this method based on efficiency and sustainability.

5.2 – Lactic Acid Fermentation of Hydrolysed Press Cake

Lactic acid has a wide range of applications in feed, food and cosmetics. It has also become of considerable interest since it was noted that this organic acid can be used as a building block for the synthesis of polymers such as polylactic acid, PLA, a renewable and biodegradable plastic. This organic acid can be presented in L(+)- lactic acid and D(-)- lactic acid or racemic DL-lactic acid by bacterial fermentation, depending on the inoculum used. At the same time, the chemical production of lactic acid is in the DL- lactic acid form, but for the production of bioplastics, for example, the pure L(+) or D(-) form is required. Hence, fermentation with a selected lactic acid bacteria presents an advantage over chemical production (Singhvi, Zend & Sonomoto, 2018).

Lactic acid fermentation is also more sustainable, and even if lactic acid bacteria need a lot of nutrients for efficient lactic acid production, green biomasses could be a suitable substrate as this material contains high amounts of proteins, carbohydrates but less lignin than mature

biomasses. It is considered a medium that could substitute a synthetic medium since it also contains a lot of vitamins, minerals and nitrogen-containing compounds. In fact, different studies have shown that green juice can substitute MR broth, which is a synthetic medium that can be used by lactic acid bacteria for lactic acid production (Lübeck & Lübeck, 2019).

Furthermore, different studies have been conducted in order to obtain proteins from plant juices. Different procedures such as the precipitation of those proteins by addition of strong acids, heat coagulation, ultrafiltration and utilization of both cationic anionic flocculants and lactic acid fermentation have been used for such purpose. Lactic acid fermentation has been proposed since it is a method that can originate protein concentrates from an organic alternative. Furthermore, different nutrients stay in the brown juice, which is the name given to the green juice after fermentation. Thus, brown juice is considered a stable and rich substrate that can be used as feedstock for biogas production or for the extraction of high value products, such as lactic acid (Dietz, Schneider, Papendiek & Venus, 2016; Lübeck & Lübeck, 2019).

Lactic acid fermentation of green juice fraction lowers the pH due to the conversion of sugars, mainly glucose, into lactic acid. Like strong acid protein precipitation, the decrease of pH by lactic acid production facilitates protein precipitation. In fact, green juice contains indigenous lactic acid bacteria and other microorganisms that will naturally undergo fermentation. Fermentation will then end when pH drops below 4 since most of the microorganisms, including lactic acid bacteria cannot survive under these low pH values. However, to undergo that problem, a method was developed by Santamaria-Fernandez *et al*., (2017), where *Lactobacilus salivarius,* a lactic acid bacteria, was used as start culture in the green juice, right after pressing, to control the fermentation process and at the same time, making it possible to obtain protein concentrates with high quality for animal feed. The usage of this organism was based on its ability to facilitate a fast drop of pH and its high growth.

The amount of proteins in the green juice is really dependent on mechanical pretreatment applied to the fresh material. However, it seems that, regardless of the method, a lot of proteins are still retained in the press cake. Due to the high complexity of lignocellulosic material, these proteins are difficult to extract, unlike what occurs in GJ. In order to extract these proteins, enzymes could be applied to the press cake to degrade lignocellulosic material and then press the hydrolysed PC in order to obtain a new green juice that could be used for lactic acid fermentation and verify if proteins could be extracted in this way.

This fermentation could also be compared with the fermentation of press cake directly after enzymatic hydrolysis or combined with enzymatic hydrolysis, in order to reduce the costs

associated with another mechanical pretreatment. These comparisons would make it possible to understand whether any of these methods could be suitable for the extraction of more proteins.

Dotsenko and Lange (2017) reported that not only the cell wall is responsible for protein retention in PC since most of the pulp proteins are present in the chloroplast and this organelle is not affected by carbohydrolases since its membrane includes mostly proteins and lipids. In this study, it was also concluded that without the addition of proteases, no significant amino acids can be extracted from PC. However, in order to recover proteins for animal feeding proteases should not be used in press cake, since the usage of these type of enzymes, would degrade proteins into its monomers. Instead, could be suggested to use lipases to act in the chloroplast membrane. This might help the accessibility of free proteins present in the chloroplasts.

It is important to have in consideration that even though different proteins can be extracted from press cake, composition analysis is also required. For animal feeding the protein concentrate should not contain fibres and it needs to contain high concentrations of essential amino acids.

As explained above, lactic acid fermentation is not only used for protein precipitation but also for the production of another low volume and high value product, lactic acid. However, the costs associated with separating enzymatic hydrolysis and fermentation can be high, making the overall biorefinery unsustainable. To overcome this problem, lactic acid fermentation could be combined with enzymatic hydrolysis in a process called, simultaneous saccharification and fermentation. It is well known, that a lot of parameters play a role in both hydrolysis and fermentation and that these parameters are harder to adjust when both processes are combined. One of the main problems is that optimal pH and temperature used in enzymatic hydrolysis can inhibit the microorganisms used during fermentation. At the same time, fermentation parameters can slow down the enzymatic hydrolysis, reducing enzyme efficiencies.

Overall, it is possible to understand that enzymatic hydrolysis and fermentation for both lactic acid production and protein extraction it is still under development and it would be interesting to conduct different studies to draw further conclusions.

Chapter 6 – Conclusion and Future Perspectives

In this chapter conclusions about this project and future researches and analyses will be presented, as options to improve the presented study. This chapter gives an overall idea of what was investigated during this project. At the same time presents strategies for possible improvements on enzyme production and enzymatic hydrolysis and further utilizations of the hydrolysate rich in monomer sugars.

6.1 Conclusion

Based in chapters 3 and 4 it is possible to understand that the project was divided into two main experiments and analysis. The first was enzyme production by different filamentous fungi and the second was the performance of produced enzymes in press cake degradation by enzymatic hydrolysis process.

Although, degradation of lignocellulosic material requires utilization of more than the analysed enzymes, in this study FPases, ß-glucosidases and xylanases activities were detected in all enzyme cocktails produced by different filamentous fungi. ß-xylosidases was also analysed with an enzymatic assay and it was found that *T. asperellum* was unable to produce those enzymes. Overall ß-glucosidases activities presented by the other filamentous fungi were still low, compared with other studies.

Overall, from different enzyme productions, *T. reesei* was the microorganism used that produced FPases, ß-glucosidases and xylanases with higher activities and thus, the most promising microorganism to be used in following experiments of enzyme production from PC. *A. saccharoliticus* was initially chosen due to ß-glucosidases activities presented by this microorganism in previous studies. However, *T. reesei* presented higher ß-glucosidases activities in the present study.

In fact, the most promising result from this first part was the production of ß-glucosidases by *T. reesei*, after other studies have shown that the same strain did not produce ß-glucosidases if not genetically modified first.

After enzyme production, followed enzymatic hydrolysis utilizing produced enzymes from *T. reesei* and *A. saccharoliticus* and commercial enzymes Cellic Ctec and Cellic Htec.

Different procedures were applied in order to find the most promising one to degrade PC at lower costs.

From the first experiment, it is possible to conclude that enzymes performed better when utilizing pretreated wheat straw, even if produced enzymes were produced using a different substrate, PC. The only enzyme that seemed to have performed better in PC was actually the commercial enzyme Cellic Htec, which might have happened due to the higher amount of hemicellulose present in PC. These results projects for the idea of possible pretreatment prior to enzymatic hydrolysis of PC.

Increasing enzymatic loading from 3.3%, as in the original protocol, to 7% improved the overall results of enzymatic hydrolysis of PC, using different enzymes. Hydrolysis yield of *T. reesei* increased to 61.5% from 29% and when combined with Cellic Htec these values increased to 75.2%.

Increasing DM content of total solution reduced the efficiency of the enzymes degrading PC, as expected. Increasing the enzyme loading to 15% when utilizing DM 15% improved significantly the efficiency of the enzymes at degrading PC. However, those results were still lower than the results obtained when the hydrolysis of PC is performed at 5% DM/total solution and 7% enzyme loading.

Overall, in order to efficiently degrade PC fraction at a lower cost and utilizing as less inputs as possible, other methods were planned to be executed during this year. However, since it was not possible, it was suggested in Chapter 5 as following experiments to this project. However, autoclaving the material was expected to improve enzymatic hydrolysis without further increase of enzyme loading, in a solution with less water addition, it needs to be analysed experimentally for further conclusions.

6.2 Future Perspectives

Due to the initial stage of this project, different future perspectives can be presented here. In chapter 5 it is discussed the use of autoclave for pretreatment of PC prior to enzymatic hydrolysis and lactic acid fermentation for utilization of sugars and possible further protein precipitation, so these procedures will not be considered in this chapter.

In chapter 3 it was referred that different other assays should be applied for the overall analysis of enzyme cocktail presented by filamentous fungi. For degradation of cellulose, FPases and ß-glucosidases seemed to be enough, but hemicellulose needs the action of more

enzymes for its full degradation (Shallom & Shoham, 2003). As an example, it seems that from strong acid hydrolysis results, arabinose is present in the hemicellulose fraction. However, it was not investigated if any arabinanases were produced by the utilized filamentous fungi. It would be interesting to apply more assays to the produced enzyme cocktails to understand if other important enzymes, for PC degradation, were also produced.

Furthermore, it would be interesting to create a full analysis of the carbohydrates present in the PC to understand which enzymes are necessary for full degradation of this material. Understanding this might lead to utilization of other microorganisms, such as, other filamentous fungi for production of necessary enzymes that were not produced by the utilized ones.

Banassi, Lucas, Jorge and Polizeli (2014), presented *A. niger* and *A. thermomutatus* as great producers of ß-xylosidases and arabinanases. Thus, it can be suggested that enzyme production in PC could be studied utilizing one of these microorganisms. It could also be suggested, that those cocktails are then combined with the ones from *T. reesei*, if necessary, for a complete degradation of cellulose and hemicellulose fractions.

Lignin fraction was not taken into consideration in the present study. Although, it is a green biomass and contains less lignin than mature lignocellulosic materials, it is still present, and it can be pointed out as one of the major factors that reduces cellulases and hemicellulases efficiencies. From the total DM of press cake, 7% was concluded to be Klason lignin but Klason lignin does not account for the total lignin content. Furthermore, even if it is a small amount it would be interesting to remove it or degrade it, at least.

Degradation of lignin could be done by using enzymes from filamentous fungi, for example. *Pharnerochaete chrysosporium*, *Pleurotus ostreatus* and *Chaetomium cochiodes* have been presented as great lignin degraders, but extremely dependent of which type of material is used. Enzymes responsible for lignin degradation are called ligninases and the major lignin-degrading enzymes are assisted by accessory enzymes, such as, oxidases. However, due to the complexity and heterogeneity of lignin, different studies suggested genetic modification of fungi and bacteria (Schoenherr, Ebrahimi & Czemak, 2018). However, due to organic purposes of this project, genetic modification is not considered.

Other pretreatment methods could be suggested for lignin removal or degradation. Although, as mentioned before, the choice of pretreatment might increase the costs and reduce the sustainability of the process. Furthermore, some pretreatment methods are known to create

inhibitors that can reduce enzyme's performance during enzymatic hydrolysis (Jung & Kim, 2017).

In the present study, it was suggested that produced enzymes were utilized as biological pretreatment of PC, in the green biorefinery where were produced. Enzymes are known to be low-volume and high-value products, but in order to gain commercial market, further purification steps and better maintenance conditions are necessary to be applied (Bajpai, 2016). Further studies could focus on understanding how advantageous the production of enzymes could be, with commercial purposes, and how much it will influence the sustainability of the entire green biorefinery. Furthermore, the increase utilization of lignocellulosic material in organic processes might influence the interest on utilizing non-GMO commercial enzymes as pretreatment of the material.

Enzymatic hydrolysis could also be optimized in different ways. An intensive work can be applied on this process, where methods and materials can be optimized. First of all, it is necessary to understand which parameters influenced the most hydrolysis of PC, such as pH, DM/ solution and enzymatic loading and make necessary changes to the presented ones, having always in consideration how these changes can affect the sustainability of the process. Secondly, since enzymes are produced in the same biorefinery, it would be interesting to understand, from an economical and environmental perspective, if it would be better to increase water or enzyme content per total solution. On one hand, free water is essential for efficient enzymatic hydrolysis, but it can add a cost and a waste to the biorefinery. On the other hand, enzymes are produced in the biorefinery, so the enzyme concentration/total solution might not increase a lot the costs associated with it. However, previous studies have shown that increasing the enzyme loading might influence negatively the hydrolysis, by enzyme deactivation (Visser, Leal, Almeida & Guimarães, 2015).

Weiss, Felby and Thygesen, (2019), recently suggested that free water content is only essential until a certain level and depending of the enzyme cocktail used. In the mentioned study, when used Cellic Ctec2, increasing the DM content from 5%-15% did not reduce the efficiency of the enzyme. At the same time, it was noticed that pretreatment of the material can reduce the effects of high DM content/total solution. Once again, this suggests that it might be necessary the application of pretreatment of PC before enzymatic hydrolysis. Instead of autoclaving the material, it can be suggested that knife-milling is applied to PC in order to reduce particle sizes. Knife mill as the advantages of creating ultrafine particles, it is easy to install and use and the maintenance is inexpensive. However, it is not easy to clean and can not

be used in extremely wet materials due to clogging of the materials (Barakat *et al*., 2014). Comparisons between autoclaving and knife milling could be made in terms of costs, environment and how efficient each one is after upscaling.

It is important to mention that other types of organic acids or even other kinds of fermentations could be suggested for further utilization of monomer sugars obtained from enzymatic hydrolysis. The choice of lactic acid was in order to try to also extract more proteins, as explained before. However, depending on the objectives and necessities of each project, different organic acid productions can be easily suggested, such as citric or succinic acids.

Future projects that can utilize GMOs could invest in the production of ethanol from both C6 and C5 sugars obtained after enzymatic hydrolysis. Moreover, it is important to mention that some bacteria are able to naturally produce butanol from C5 sugars, which gives a possible alternative for utilization of C5 sugars for projects where GMOs are not allowed.

Although, different improvements are necessary before upscaling the enzyme production and hydrolysis of PC, it is important to mention that future studies should focus in the economic, environmental and social impacts that each one of these processes would create. In order to utilize these processes in a biorefinery, it is extremely important to have into consideration all sustainable aspects. To understand the sustainability of these processes, it can be suggested that, in the future, mass balances and life cycle assessments are created and analysed.

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Appendix 1 – Detail information of enzyme activity, first experiment

Enzyme activities 1st SFF	Kolasa's medium	PC1	PC2
FPases from *T. reesei*	9.46 (0.22)	10.13 (0.41)	8.74 (0.15)
Xylanases from *T. reesei*	4.11 (0.09)	3.80 (0.11)	3.91 (0.11)
ß-glucosidases from *T. reesei*	16.89 (0.12)	10.81 (0.09)	10.26 (0.15)
ß-xylosidases from *T. reesei*	0.94 (0.02)	0.83 (0.07)	0.54 (0.05)
FPases from *A. carbonarius*	2.50 (0.11)	2.60 (0.16)	5.05 (0.14)
Xylanases from *A. carbonarius*	3.86 (0.15)	3.53 (0.12)	3.40 (0.10)
ß-glucosidases from *A. carbonarius*	12.91 (0.13)	4.31 (0.07)	3.87 (0.12)
ß-xylosidases from *A. carbonarius*	7.26 (0.11)	1.05 (0.02)	1.80 (0.02)
FPases from *T. asperellum*	1.28 (0.21)	1.64 (0.25)	0,84 (0.10)
Xylanases from *T. asperellum*	3.15 (0.13)	3.65 (0.22)	3.55 (0.12)
ß-glucosidases from *T. asperellum*	2.82 (0.09)	3.11 (0.12)	1.68 (0.10)
ß-xylosidases from *T. asperellum*	0.35 (0.01)	0.34 (0.01)	0.36 (0.03)
FPases from *A. saccharoliticus*	3.80 (0.12)	2.63 (0.23)	9.28 (0.21)
Xylanases from *A. saccharoliticus*	4.40 (0.11)	3.54 (0.17)	3.27 (0.11)
ß-glucosidases from *A. saccharoliticus*	16.01 (0.32)	5.7 (0.11)	2.44 (0.05)
ß-xylosidases from *A. saccharoliticus*	5.95 (0.12)	1.38 (0.05)	2.00 (0.03)

Note: Cellic Ctec presented 20.05 U/ml in FPases assay and 25.44 U/ml in ß-glucosidases assay.
Cellic Htec showed 10.00 U/ml in xylanases assay and 26.06 U/ml in ß-xylosidases assay.

Appendix 2: Detailed enzyme activity from the second experiment

Assays from *T. reesei* enzymes - 2nd SSF	FPases	Xylanases	ß-glucosidases	Ss-xylanases
4 days	8.18 (0.11)	5.08 (0.01)	0.00 (0.00)	0.015 (0.00)
7 days	14.13 (0.15)	11.7 (0.22)	10.55 (0.14)	1.00 (0.02)
10 days	17.73 (0.07)	17.98 (0.18)	17.13 (0.12)	1.33 (0.02)
commercial	20.04 (0.16)	10.00 (0.01)	25.44 (0.23)	26.06 (0.15)

Assays from *A. saccharoliticus* enzymes - 2nd SSF	FPases	Xylanases	ß-glucosidases	ß-xylosidases
4 days	3.43 (0.14)	1.70 (0.11)	0.00 (0.00)	0.00 (0.00)
7 days	3.55 (0.09)	11.75 (0.14)	10.75 (0.11)	1.13 (0.02)
10 days	5.60 (0.24)	17.03 (0.12)	12.78 (0.17)	1.91 (0.08)
commercial	20.04 (0.16)	10.00 (0.01)	25.44 (0.23)	26.06 (0.15)

Appendix 3: Detailed results of the enzymatic hydrolysis of WS and PC

3.3% WS	Citirc acid g/l	Glucose g/l	Xylose g/l	Arabinose g/l	Acetic acid g/l	Ethanol g/l	Yield
T. reesei	3.95 (1.10)	6.40 (2.10)	1.81 (0.56)	0.00 (0.00)	1.09 (0.34)	0.64 (0.53)	57%
A. saccharoliticus	2.10 (0.98)	3.40 (0.96)	4.40 (1.12)	0.00 (0.00)	1.19 (0.43)	1.51 (0.00)	46%
Cellic Ctec	0.00 (0.00)	8.01 (0.19)	2.99 (0.87)	0.00 (0.00)	0.72 (0.06)	0.13 (0.09)	86%
Cellic Htec	0.00 (0.00)	3.44 (0.05)	2.37 (0.09)	1.66 (0,36)	0.9 (0.04)	0.13 (0.01)	34%
3.3% PC	Citric acid	Glucose g/l	Xylose g/l	Arabinose g/l	Acetic acid g/l	Ethanol g/l	Yield
T. reesei	0.00 (0.00)	3.55 (0.05)	0.37 (0.02)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	20%
A. saccharoliticus	0.15 (0.09)	0.58 (0.12)	0.28 (0.01)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	5%
Cellic Ctec	0.62 (0.02)	5.35 (0.39)	0.55 (0.13)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	34%
Cellic Htec	0.33 (0.09)	0.48 (0.05)	0.12 (0.06)	6.68 (0.15)	0.00 (0.00)	0.00 (0.00)	39%
5% WS	Citric acid	Glucose g/l	Xylose g/l	Arabinose g/l	Acetic acid g/l	Ethanol g/l	Yield
T. reesei	3.95 (0.32)	8.25 (0.25)	2.43 (0.12)	0.00 (0.00)	1.11 (0.09)	0.00	60%
A. saccharoliticus	2.52 (0.24)	4.33 (0.05)	5.26 (0.13)	0.58 (0.09)	0.00 (0.00)	0.05 (0.01)	57%
Cellic Ctec	0.36 (0.01)	8.35 (0.00)	4.30 (0.53)	0.00 (0.00)	0.45 (0.08)	0.00 (0.00)	90%
Cellic Htec	4.00 (0.57)	4.00 (0.32)	3.65 (0.23)	2.33 (0.04)	0.83 (0.09)	0.00 (0.00)	37%
5% PC	Citric acid	Glucose g/l	Xylose g/l	Arabinose g/l	Acetic acid g/l	Ethanol g/l	Yield
T. reesei	1.77 (0.05)	7.54 (0.07)	1.64 (0.09)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	29%
A. saccharoliticus	1.22 (0.08)	3.40 (0.10)	1.26 (0.03)	0.00 (0.00)	0.00 (0.00)	0.00	6%
Cellic Ctec	1.68 (0.11)	7.8 (0.09)	2.18 (0.03)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	34%
Cellic Htec	1.50 (0.12)	2.63 (0.15)	1.13 (0.01)	7.80 (0.33)	0.00 (0.00)	0.00	44%

Note 1: Cellobiose, Lactic acid, succinct acid and glycerol were also measured in HPLC. However, no concentration of these products were found, so it is not presented in the tables.

Note 2: For calculation of yield it was taken into consideration maximum theoretical yield of products formed, citric acid, acetic acid and ethanol. It was also taken into consideration the maximum cellulose and hemicellulose that was present in the DM.
Material analysis before hydrolysis was also taken into account to verify how much of each compound was present before the hydrolysis. These values were subtracted to the

results, they could not be considered as part of the hydrolysis degradation process since they were present before hydrolysis started.

Appendix 4: Detailed results of enzymatic hydrolysis of PC

5% enzyme in 5% DM	Citirc acid g/l	Glucose g/l	Xylose g/l	Arabinose g/l	Acetic acid g/l	Ethanol g/l	Yield
T. reesei	0.00 (0.00)	7.35 (0.53)	1.74 (0.22)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	30%
A. saccharoliticus	0.00 (0.00)	3.65 (0.23)	1.13 (0.02)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	7%
Cellic Ctec	0.33 (0.02)	7.1 (0.33)	1.85 (0.12)	0.33 (0.02)	0.00 (0.00)	0.00 (0.00)	33%
Cellic Htec	0.00 (0.00)	2.70 (0.45)	1.76 (0.01)	6.87 (0.76)	0.00 (0.00)	0.00 (0.00)	43%
Cellc Ctec + Cellic H tec	0.75 (0.00)	9.19 (0.48)	2.15 (0.12)	1.93 (0.13)	0.00 (0.00)	0.00 (0.00)	58%
T. reesei + Cellic H tec	1.31 (0.09)	9.25 (0.46)	1.50 (0.02)	1.98 (0.13)	0.00 (0.00)	0.00 (0.00)	58%
7% enzyme in 5% DM	Citirc acid g/l	Glucose g/l	Xylose g/l	Arabinose g/l	Acetic acid g/l	Ethanol g/l	Yield
T. reesei	1.14 (0.06)	12.3 (0.34)	1.31 (0.11)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	62%
A. saccharoliticus	1.13 (0.03)	4.3 (0.07)	1.39 (0.12)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	18%
Cellic Ctec	1.55 (0.09)	11.86 (0.56)	2.21 (0.21)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	66%
Cellic Htec	1.23 (0.02)	4.09 (0.27)	1.52 (0.33)	9.30 (0.89)	0.00 (0.00)	0.00 (0.00)	69%
Cellc Ctec + Cellic H tec	0.98 (0.00)	12.00 (0.64)	2.14 (0.05)	1.62 (0.09)	0.00 (0.00)	0.00 (0.00)	73%
T. reesei + Cellic H tec	1.22 (0.12)	12.7 (0.44)	1.62 (0.29)	1.68 (0.01)	0.00 (0.00)	0.00 (0.00)	75%
7% enzyme in 15% DM	Citirc acid g/l	Glucose g/l	Xylose g/l	Arabinose g/l	Acetic acid g/l	Ethanol g/l	Yield
T. reesei	0.00 (0.00)	3.25 (0.24)	1.17 (0.23)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	6%
A. saccharoliticus	0.00 (0.00)	2.70 (0.33)	0.90 (0.11)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	2%
Cellic Ctec	0.00 (0.00)	3.85 (0.09)	1.48 (0.45)	0.27 (0.09)	0.00 (0.00)	0.00 (0.00)	13%
Cellic Htec	0.00 (0.00)	0.65 (0.03)	1.40 (0.19)	4.15 (1.13)	0.00 (0.00)	0.00 (0.00)	16%
Cellc Ctec + Cellic H tec	0.00 (0.00)	4.05 (0.43)	1.72 (0.23)	1.19 (0.38)	0.00 (0.00)	0.00 (0.00)	20%
T. reesei + Cellic H tec	0.00 (0.00)	4.21 (0.34)	1.20 (0.07)	1.96 (0.22)	0.00 (0.00)	0.00 (0.00)	22%

15% enzyme in 15% DM	Citirc acid g/l	Glucose g/l	Xylose g/l	Arabinose g/l	Acetic acid g/l	Ethanol g/l	Yield
T. reesei	0.00 (0.00)	5.05 (1.33)	0.65 (0.09)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	45%
A. saccharoliticus	0.00 (0.00)	2.15 (0.45)	0.69 (0.12)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	13%
Cellic Ctec	0.00 (0.00)	5.25 (0.98)	1.10 (0.08)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	52%
Cellic Htec	0.00 (0.00)	1.30 (0.24)	0.76 (0.01)	4.65 (1.43)	0.00 (0.00)	0.00 (0.00)	56%
Cellc Ctec + Cellic H tec	0.00 (0.00)	5.65 (1.94)	1.07 (0.22)	0.81 (0.04)	0.00 (0.00)	0.00 (0.00)	65%
T. reesei + Cellic H tec	0.00 (0.00)	5.15 (1.34)	0.81 (0.14)	1.84 (0.17)	0.00 (0.00)	0.00 (0.00)	68%

Note: Here only PC was used as substrate