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Sustainable biotechnology

Master thesis

Optimizing of in situ enzyme production and hydrolysis to enhance protein extraction from fresh clover grass

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Abstract

More than a million tons of soybean meal is imported from South America to Denmark every year to use as feed. To reduce the import and to reduce transportation, alternative locally grown crops are researched. A potential protein source is perennial legumes. Green biorefinery concept is a concept that converts different green substrates into multiple products including a protein concentrate. While this concept extracts some of the protein present in the substrate, there are still structure bound proteins left. This project focus on optimizing enzyme production and enzymatic hydrolysis, which is used to break the lignocellulosic structure and extract the proteins. Four different experimental parts are performed. Alfalfa press cake is used as the main substrate in three of the experimental parts and fresh clover grass is used in the last part. The first experimental part is the substrate composition, where moisture, ash, protein, sugar, and lignin content are measured. The second experimental part is the optimization of enzyme production, where the parameters time of incubation, light availability, inoculation temperature, initial moisture content, and inoculum size are analyzed together with testing four different substrates. The third experimental part is the optimization of enzymatic hydrolysis, where the parameters incubation time, enzyme units per substrate, pH of inoculum, and incubation temperature are tested together with analyzing the potential of using diluted brown juice as a buffer. The last experimental part is the effect of enzymatic hydrolysis, where the created enzyme cocktail is used on fresh substrate and the effect of the enzymatic hydrolysis is tested by analyzing the supernatant and solids using different methods. Alfalfa press cake contains a dry matter on 34.6 ± 0.7 g/100g substrate, with a protein content on 13.8 ± 0.08 g/100g-DM substrate and a cellulose concentration on 31.7 ± 0.4 g/100g substrate. The optimal parameters for enzyme production are incubation in darkness for 9 days at 25-30°C with a moisture content on 75% and at least $5*10^6$ spores/g-DM substrate. Of the tested substrates, alfalfa press cake is the substrate producing the least amount of FPA activity. The optimal parameters for enzymatic hydrolysis are 7.5 FPU/g-DM substrate at 50°C and pH in the range of 4-5.5. Incubation at 24 hours yields 8% of total released sugars. It is possible to use diluted brown juice as buffer. In the effect of enzymatic hydrolysis, substrate is degraded. Unfortunately, the protein concentration results do not indicate a higher protein extraction after an enzymatic hydrolysis. It is therefore unclear if the hydrolysis affects the extracted protein concentration.

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1 Introduction

In Denmark, the annual feed consumption of soybean meal is approximate 1.5 million tons, which corresponds to 64% of the imported feed containing proteins (Hansen, 2018, The Danish National Bioeconomy Panel, 2018). Denmark is too cold to grow soybeans and thus it has to be imported. Most of the soybean meal is imported from South America and thus needs to be transported. The transportation produces a lot of greenhouse gasses, which contributes to the global climate footprint. To reduce the climate footprint, alternative feed sources are needed to be used, which can be grown/produced locally (Hansen, 2018). Some of the potential protein sources that can be cultivated/used in Denmark are broad beans, secondary flow from slaughterhouses, perennial grasses, and proteins from insects (The Danish National Bioeconomy Panel, 2018). The broad beans are new varieties, that can be used directly as feed. The secondary flow from slaughterhouses is mainly used for food applications. The insect proteins are mainly used for food applications and feeding fish. The perennial grasses can be used directly for ruminants, but the proteins have to be extracted before they can be used as feed for monogastric animals. An advantage of using perennial grasses is that they yield around twice the amount of dry matter per hectare compared with other crops (The Danish National Bioeconomy Panel, 2018). A method of extracting the proteins from perennial grasses is to use the green biorefinery concept, which converts the grass into three products: protein concentrate, biogas, and organic fertilizer (Santamaría-Fernández et al., 2018). While a part of the proteins is extracted and turned into protein concentrate, another part of the proteins is bound to the lignocellulosic structure of the grasses (Santamaría-Fernández et al., 2019). To potentially extract these proteins, a disruption of the lignocellulosic structure needs to happen. This can happen using different methods such as pretreatment and/or hydrolysis of the grasses. A potential hydrolysis method is enzymatic hydrolysis. A disadvantage of using enzymatic hydrolysis is the price for the enzymes themselves. To overcome this disadvantage, it is possible to produce the enzymes on-site. This report will focus on optimizing the production of the three common cellulases, optimize the enzymatic hydrolysis, and use this knowledge to hydrolyze fresh perennial grass. The end goal is to use the produced enzyme cocktail to increase the number of proteins extracted into the liquid fraction, which can be used for the protein concentrate. A side goal is to analyze in which activities the three main cellulases are produced and check if *T. reesei* produces more β-glucosidase when grown on perennial grass.

In the introduction, different topics are introduced and described in the following order: green biorefinery concept, alfalfa, lignocellulosic material, enzymatic cellulase degradation, cellulase producing fungi, different fermentation techniques, potential inhibition and induction of cellulase, *Trichoderma reesei* (*T. reesei*), *T. reesei* cellulase genes, optimization of different parameters in enzyme production and enzymatic hydrolysis in literature. At last, some of the analysis procedures used in this report are introduced and compared.

1.1 Green biorefinery

Biorefinery concepts aim to utilize sustainable substrates as efficiently as possible, produce high valued products, use minimal power consumption and not generate any waste (Andersen et al., 2018). While many definitions of biorefinery exist, one of the most widely used is from the "*International Energy Agency*", which is: "*Biorefining is the sustainable processing of biomass into a spectrum of marketable products and energy*" (Morais and Bogel-Lukasik, 2013). A biorefinery can be classified in relation to its feedstock utilized, the products produced, or the processes used. In a biorefinery, some of the key features normally used are multifunctional concepts, usage of

bio-based material (for innovative concepts, non-primary feedstock is used), usage of whole crops with no or minimal waste streams, toxic and/or hazardous solvents and reagents are avoided if possible, the concept is flexible in relation to marked and has as low environmental impact as possible (Andersen et al., 2018). One of the biorefinery concepts created is the green biorefinery concept. In the green biorefinery concept, green biomass is utilized to produce protein concentrate, biogas, and fertilizer. This concept can utilize forage crops such as Alfalfa and clover grass, which are nitrogen fixating plants (Santamaría-Fernández et al., 2019). The green biorefinery consists of several steps: first is a screw pressing process, where fresh biomass is separated into two fractions: a solid fraction called press cake, and a liquid fraction called green juice. To precipitate the proteins, different steps can be used such as fermenting the green juice with lactic acid bacteria to drop the pH or heat coagulating the green juice at 75-85°C. Afterward, the liquid is centrifuged to separate the precipitated proteins and the liquid fraction, which now is called brown juice. The press cake and brown juice can then be mixed and fermented by anaerobic digestion to produce biogas. The fraction left after biogas production can be used as fertilizer (figure 1). While this process may seem straightforward, it is possible to use the different fractions for multiple applications depending on the desired end products (figure 2 (Santamaría-Fernández et al., 2020))

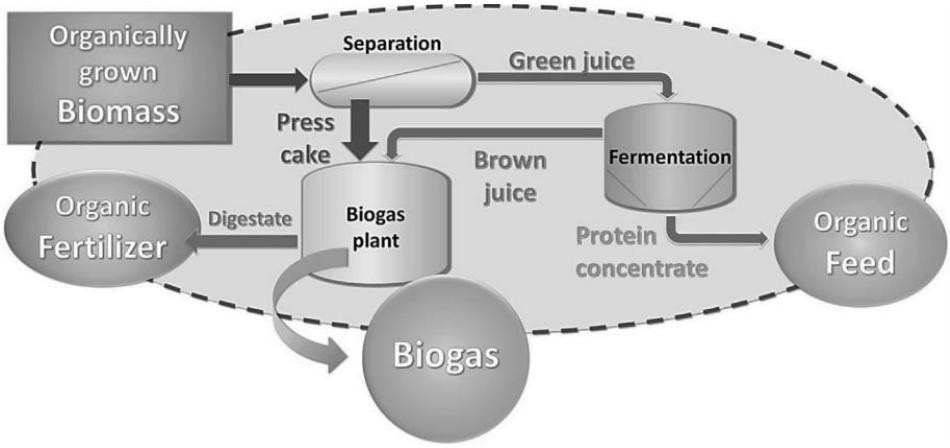


Figure 1: Overview of "green biorefinery concept" (Santamaría-Fernández et al., 2018).

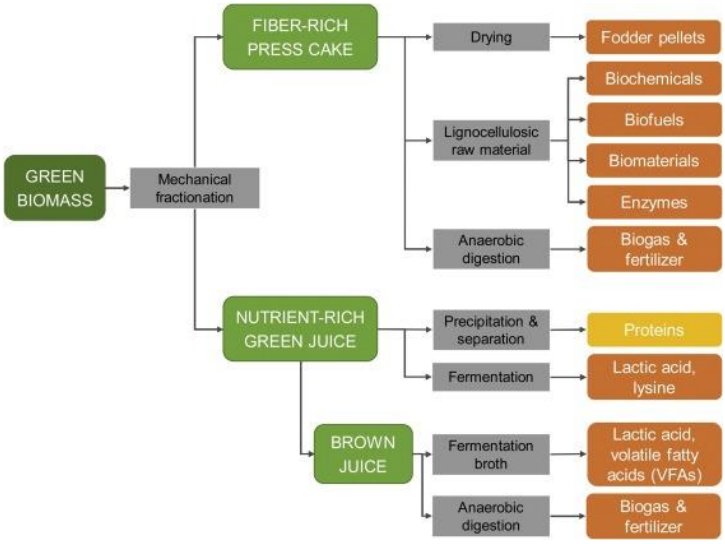


Figure 2: Schematic diagram for potential uses for the three fractions: green juice, brown juice, and press cake (Santamaría-Fernández et al., 2020).

1.2 Alfalfa

Lucerne, Alfalfa, or *Medicago sativa* is a herbaceous perennial forage legume that is the most extensively grown worldwide. More than 32 million hectares are used for alfalfa cultivation, which corresponds to around 2.5% of the world's total cultivated area (Teuber and Graham 2019, Sengupta-Gopalan et al., 2007). Alfalfa has been adapted to grow in continental and temperate climates and has a harvest yield between 16 and 45 kg/hectare. (Moot et al., 2011, Teuber and Graham 2019). Alfalfa cultivators recognize 3 subspecies (ssp) of alfalfa: ssp *medicago* or purple alfalfa, ssp *falcata* or yellow alfalfa, and ssp *varia*, which is a hybrid of the two other subspecies. Ssp *medicago* has a higher yield and early maturation but is a less hardy plant compared to ssp *falcata* (OECD 2015). Alfalfa has symbiotic interaction with rhizobia bacteria, and it can be used for both fuel and feed (Sengupta-Gopalan et al., 2007).

1.2.1 Alfalfa composition

The exact composition of alfalfa changes depending on the subspecies, the condition it grows in (temperature, sunlight, water availability, nutrient, and more), and its stage of growth. In colder climates, the lignin and protein content are lower, while the cell wall content is higher. Alfalfa grown in colder environments tend to be more digestible (NRC, 1982). The approximate composition of alfalfa is given in table 1. The numbers derive from various databases, but all data derive from the United States. All alfalfa is harvested in the late vegetative or early reproduction phase (around the time flower buds appear on the plants (OECD 2015)).

Table 1: Alfalfa composition. Alfalfa is in the late vegetative or early blooming phase (OECD 2015).

Dry matter (DM)	17.9 – 91.0 %
Crude protein	15.3 – 25.8 % DM
Crude fat	1.3 – 3.2 % DM
Crude fiber	25.0 – 25.8 % DM
Neutral detergent fiber (NDF)	26.5 – 40.0 % DM
Acid detergent fiber (ADF)	23.1 – 33.4 % DM
Lignin	3.9 – 9.7 % DM
Ash	8.4 – 15.3 % DM

The composition of the fresh alfalfa crops, press cake, and brown juice (fermented green juice) are analyzed by Santamaría-Fernández et al., (2018). The alfalfa press cake used in this study derives from the same area (table 2).

Table 2: Alfalfa fresh crop, press cake, and brown juice composition. TS is dry matter, VS is organic matter, TKN is the total nitrogen present, free sugars are the sum of different sugars (glucose, xylose-fructose, cellobiose, and arabinose), TVFA is the sum of different acids (lactic, acetic, propionic, butyric, iso-butyric, valeric and iso-valeric acid) (Santamaría-Fernández et al., 2018).

Alfalfa	Fresh crop (g/kg)	Press cake (g/kg)	Brown juice (g/kg)
TS	153.8 (0.2)	255.7 (0.6)	52.6 (0.0)
VS	136.4 (0.7)	233.8 (2.1)	39.6 (1.9)
TKN	5.0 (0.1)	8.6 (0.8)	3.1 (0.0)
Free sugars	0.0 (0.0)	4.0 (0.3)	6.8 (0.1)
TVFA	0.5 (0.1)	0.8 (0.1)	14.1 (0.1)
pH	6.03	5.6	4.4

1.3 Lignocellulosic material

Plant stems, stalks, leaves, and other components consist of lignocellulosic material. The primary metabolites of lignocellulose consist of cellulose, hemicellulose, and lignin. Up to 10% of the dry matter consists of secondary metabolites, which contain waxes, proteins, terpenoids, and phenolic compounds (Morais et al., 2014). There are three main types of lignocellulosic material: hardwood, softwood, and grasses (Bhowmick et al., 2018). The structure of lignocellulosic material consists of long cellulose fiber with hemicellulose wound around the cellulose fibers, binding them together. Lignin is attached to hemicellulose through covalent linkage, which forms a rigid matrix (Bhowmick et al., 2018). On average, the dry matter of lignocellulosic material consists of, 30-50% cellulose, 20-40% hemicellulose, and 15-25% lignin (Morais et al., 2014).

Cellulose consists of glucose monomers bound in β-1,4-glucosidic bonds. This forms a linear structure without sidechains. Multiple linear cellulose structures are placed parallel with each other and bound with hydrogen bonds, creating a structure called cellulose microfibrils (Marriott et al., 2015). At irregular intervals in the microfibrils, there are areas with no/few hydrogen bonds present. These are called amorphous regions, which are much easier to degrade than crystalline regions. The Crystallinity, linearity, and fibrous structure are what give the plant cells their mechanical strength (Bhowmick et al., 2018).

Hemicellulose is a heteropolymer, which can consist of various monosaccharides. The hemicellulose structure consists of a backbone with many side branches. Hemicellulose has a lower degree of polymerization, which makes it more susceptible to hydrolysis than cellulose (Morais et al., 2014). In grasses, the backbone mostly consists of xylose and the branches of arabinose monomers, creating an arabinoxylan structure (Bhowmick et al., 2018).

Lignin consists of a complex matrix that contains mainly three different phenylpropane alcohols. These alcohols are coniferyl, p-coumaryl, and sinapyl. The exact structure of the lignin changes from plant to plant, the plant's stage of growth, and where in the plant it is located. In grasses, the lignin content is observed to increase, as the plant matures (Monlau et al., 2013). The main functions of lignin are to make the plant resistant to microbial attack and providing the structural rigidity and impermeable properties (Monlau et al., 2013).

1.3.1 Cellulase

To achieve an effective enzymatic hydrolysis of cellulose, the actions of at least three different enzymes classes are necessary: endoglucanase, which randomly cleaves cellulose in the amorphous regions, exoglucanase or exo-cellobiohydrolase, which removes glucose or cellobiose from the end of the chains, and β-glucosidase, which hydrolyze cellobiose into glucose (figure 3 (Malherbe and Cloete, 2002)).

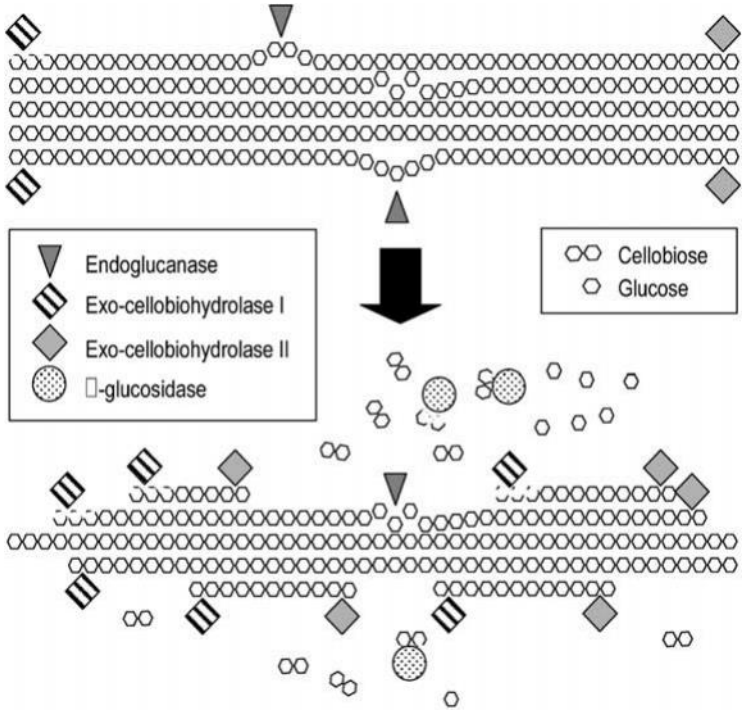


Figure 3: Hydrolysis of cellulose using the different cellulases (Malherbe and Cloete, 2002).

1.3.2 Fungi as cellulase producers

Both bacteria and fungi can produce cellulase, but for efficient cellulase production, fungi are preferred over bacteria due to their versatile substrate utilization and their ability to penetrate the substrate (Srivastava et al., 2018). While fungi can produce cellulase, having one fungus produce all three cellulases at once in the optimal concentrations for efficient hydrolysis is rare. On an industrial scale, cellulase cocktails are made from different

fungal strains (Srivastava et al., 2018). Some of the fungi that can grow on cellulose are *Alternaria*, *Aspergillus*, *Cladosporium*, *Penicillium*, and *Trichoderma* (Sukumaran et al., 2021). Only a few strains of fungi are used for enzyme production. Out of these, in around 80% of cellulosic ethanol production, cellulosic enzymes derived from *T. reesei* are used (Sukumaran et al., 2021). *T. reesei* is known to produce large quantities of cellulase in specific substrates but has low β-glucosidase activity (Bischof et al., 2016). The other fungi mostly used for industrial cellulase production are *Aspergillus* and *Penicillium* (Sukumaran et al., 2021). The production of industrial cellulolytic enzymes is carried out in submerged fermentation, which is a cost-intensive process (Srivastava et al., 2018).

1.4 Solid state fermentation

Solid state fermentation is a fermentation technology that has been used since ancient times. In the west, however, the usage of solid state fermentation was almost abandoned in the 1940th, when the discovery of submerged fermentation was made in the pursuit of producing a high amount of penicillin. Thereafter, submerged fermentation became the role model technology for producing fermented products (Pandey, 2002). Isolated pocked research continued the usage of solid state fermentation, but the development of the technology was very slow. In the 50s-60s, steroids transformation was achieved using fungal cultures. In the 60s-70s, the production of mycotoxins was achieved. The next major discovery of solid state fermentation involved protein enriched cattle feed using agro-industrialized residues, which upgraded the value of the residue. This discovery helped increase the global interest for solid state fermentation again (Pandey, 2002).

Solid state fermentation is defined as "*fermentation involving solids in absence (or near absence) of free water; however, substrate must possess enough moisture to support growth and metabolism of micro-organism*" (Pandey, 2002). When using solid state fermentation in a bioprocess, several parameters should be considered. Some of these parameters are the type of microorganism, substrate, pretreatment of substrate, isolation method, and purification of the products. Compared to bacteria, yeast and fungi has much lower water activity requirement and is thus considered to be more suitable for solid state fermentation (Yazid et al., 2017). In the solid state fermentation itself, process parameters and optimization of them need to be considered. Some of the psychochemical and biochemical parameters that can be optimized are the particle size, pretreatment of the substrate, initial moisture content, incubation temperature, agitation, aeration, and supplement of nutrient to the substrate, such as a C, N, and/or P source (Yazid et al., 2017). While conditions before fermentation can be homogenous, under fermentation they become heterogeneous, and control of the fermentation process is almost impossible. Therefore, it is also much harder to measure and model a solid state fermentation (Pandey, 2002).

1.5 Submerged fermentation enzyme production

In the 1970s, commercialized production of cellulases began, using submerged fermentation and *T. reesei*. Since then, submerged fermentation is used in industries for cellulase production (Catalán and Sánchez, 2020). In 2012, more than 75% of all the commercialized produced cellulases was produced using the submerged fermentation (Subramaniyam and Vimala, 2012). Submerged fermentation is defined as having an excess of water in the fermentation process (Singhania et al., 2010). Some of the parameters that influence enzyme production are the cellulose structure and nature, pH, temperature, and nutrient availability. The specific optimization condition for fermentation changes for each microorganism and substrate combination used and thus is not defined. Most submerged fermentation of cellulases is performed in batch fermentation but fed-batch and continuous

fermentation are attempted to decrease the repression of the cellulase genes caused by the accumulation of reduced sugars (Singhania et al., 2010). Most filamentous fungi cultivated in submerged fermentation tend to form spherical pellets or have dispersed mycelium. The formation of typical mycelium is impossible in this type of fermentation. Agitation of the fermentation media has a large impact on the morphology of the filamentous fungi. At high agitation speed, the created shear stress damage the hyphae and thus the fungi themselves. At low agitation speed, different sizes of pellets are formed. The optimal agitation forms small pellets or dispersed mycelium without damaging the hyphae (Hansen et al., 2015). There are some challenges using submerged fermentation. Due to the different sizes of pellets or dispersed mycelium throughout the reactor, the reproducibility of experiments is affected. The amount of oxygen present must never become limited. Otherwise, the production of endoglucanases and β-glucosidase can be affected. The accumulation of reduced sugars can activate the carbon catalytic repression and thus decrease cellulase production (Hansen et al., 2015).

1.6 Solid state enzyme production

At small scale, it is possible to produce cellulase in comparative high titers in a solid state fermentation compared with submerged fermentation. The microorganisms best suited for solid state fermentation is fungal strain since the fermentation conditions are similar to their natural environment. Potential fungal strains that are used for cellulase production are *T. reesei*, *Aspergillus niger (A. niger)*, *Penicillium citrinum (P. citrinum)*, and *Thermoascus auranticus* (Singhania et al., 2010). Enzymes produced by solid state fermentation tend to have remarkable stability towards different environmental conditions such as temperature, pH, metal ions and in the case of *P. citrinum* produced enzymes, shows tolerance towards alkali conditions (Dutta et al., 2008). When fungi are fermented in a solid state, the amount of inhibited product is lower and does not build up in the environment. Thereby, some enzymes are only expressed when the fungi are fermented at solid state (Hansen et al., 2015).

1.7 Comparison of the two fermentation

Both solid state and submerged fermentation are used for cellulase production, and both have advantages compared to the other. For submerged fermentation, one of the main advantages is that it is easy to control various process parameters under fermentation, such as agitation, temperature, aeration, and pH. In comparison, it is almost impossible to influence concrete parameters under solid state fermentation. The other main advantage is the scale-up process. Submerged fermentation is used a lot in industries today and thus all necessary equipment needed for large scale production is already invented. Development of equipment to use for solid state fermentation needs to be developed before large scale production is possible (table 3 (Dasari et al., 2019)). For solid state fermentation, there are many advantages. One of the advantages is that the water availability in the fermentation is limited and thus the chance of contamination is much lower compared with submerged fermentation (Dasari et al., 2019). Another advantage of solid state fermentation is the low effluent created after fermentation, whereas large volumes are generated and need to be discarded in submerged fermentation. The energy consumed in solid state fermentation is much lower than in submerged fermentation. The productivity is also much higher, and the substrate is utilized much better in solid state fermentation. Solid state fermentation has a productivity of 100-300 g/L and a substrate utilization of 20-30%, while productivity and substrate utilization of submerged fermentation is 30 g/L and 5% (table 3 (Dasari et al., 2019)). The substrate utilized for solid state fermentation can be a waste stream such as bagasse, bran, and paper pulp. For submerged fermentation, a liquid substrate with free flowing nutrients is used, such as molasses and broths (Subramaniyam and Vimala, 2012). The production cost of solid state fermentation is also much lower than submerged

fermentation. In two similar systems, the enzyme production cost for solid state fermentation system is around 10-fold lower compared with the submerged fermentation system (Singhania et al., 2010).

Table 3: Comparison of solid state fermentation and submerged fermentation (Dasari et al., 2019).

Parameter	Solid state fermentation	Submerged fermentation
Aseptic condition	Can be avoided when fermentation is under water limited condition	Is required under high moisture content to avoid contamination
Downstream processing	Dried enzymes can be used directly, which lowers downstream process drastically	Is required
Moisture requirement	Performed in the absence of any free-flowing water	Large volumes of water required
Effluent generated	No generated effluent	Need to discard effluent in large volumes
Energy consumption	Low	High
Productivity	100–300 g/L	30–80 g/L
Process parameter	Generally, under static conditions. Almost impossible to control and monitor.	Generally, involves aeration, mixing, control and monitoring of pH, temperature, dissolved oxygen, and gas flow rates. Process is highly controlled and monitored
Homogenesis	Very heterogenous	Almost completely homogenous
Scale-up	Problematic due to needing new design equipment	Easy since industrial equipment is available
Substrate utilization	Can utilize 20–30% of the substrate	Maximum 5% utilization

1.8 Inhibition and induction of enzymes

There are two areas in inhibition and induction of enzymes in a system. The first is inhibition and induction of the enzyme production inside the cells and the second is inhibition and induction of the enzymes themselves.

Inhibition and induction of enzyme production: There are lots of regulatory mechanisms that affect the transcription of cellulosic enzymes. Some of these regulatory mechanisms are the genes Cre I, Ace I, Rce I, Ace II, and Ace III (Jiang et al., 2018). Cre I has a function in the carbon catabolite regulation, Ace I and Rce I are repressors for the cellulase expression, and Ace II and Ace III are cellulase transcriptional activators. Cre I is a gene, that is phosphorylated by CKII (casein kinase) in position S241 when a surplus of glucose is available. A phosphorylated Cre I is active and binds to the DNA in the regions coding for different cellulases such as a region around 700 bp from the transcription initiation site of Cbh I (Cellobiohydrolase I). While Cre I regulates multiple different cellulase genes, it does not inhibit all, such as Cbh II (Cellobiohydrolase II) and Bgl I (β-glucosidase I) (Zhao et al., 2018). In *T. reesei Rut-C30*, a change in the Cre I gene has happened, which shortened the gene, and it lost the ability to bind to its binding site, thus does not work as a repressor anymore (Zhao et al., 2018). Ace I is a transcription factor in *T. reesei*, that has at least one binding site in all of the main cellulase genes. For Cbh I specifically, 8 binding sites are present in the promoter. It also has a binding site in two xylanase genes (Jiang et al., 2018). Rce I is a regulatory gene, which regulates Cbh I, Eg 1 (Endoglucanase 1), Bgl I, and Bgl II (β-glucosidase

II) by repressing the transcription. It does not affect the growth of the cell and is not affected by the Cre I gene (Cao et al., 2017). Ace II gene increases cellulose expression. In strains with deleted Ace II gene, downregulation of Cbh I, Cbh II, Eg I, and Eg II (Endoglucanase 2) happens (Jiang et al., 2018). Ace III is essential to the production of cellulase. In Ace III negative strains, almost no expression of Cbh I and Cbh II is detected. The expression of Bgl 1 is also lower than the parent strain (Jiang et al., 2018). Different inducers can be added to the fermentation process to increase cellulase production. The only economically feasible among these inducers is lactose (Singhania et al., 2010).

Inhibition and induction of enzymes themselves: Glucose is a non-competitive inhibitor, where at 55% glucose concentration, 37% of the cellulase activity is present. Cellobiose is also a non-competitive inhibitor, but its ability to inhibit cellulase is significantly greater than glucose (Holtzapple et al., 1990). Ethanol is a non-competitive inhibitor. At ethanol concentration between 0-20%, the activity is not affected that much. At ethanol concentration higher than 20%, the cellulases begin to denature and the hydrolysis becomes cloudy with denatured enzymes. At an ethanol concentration of 40%, cellulase activity is reduced to 2%. Acetone at a concentration between 0-158 g/L works as an inducer for cellulase activity, while higher concentrations function as an inhibitor (Holtzapple et al., 1990). While the results for glucose show non-competitive inhibition, a hypothesis is created about potential competitive inhibition instead. This is because glucose and cellobiose are insoluble in water and soluble cellulose molecules (carboxyethyl- and carboxymethyl-cellulose) have competitive inhibition (Holtzapple et al., 1990).

1.9 Trichoderma reesei

T. reesei is an ascomycete filamentous fungus growing in mesophilic conditions. It is known for its high extracellular cellulase production (Peterson and Nevalainen, 2012, Carpa et al., 2018). It was originally isolated in the Second World War from Solomon Island, where it was degrading fibrous equipment from the US army (Bischof et al., 2016). Under the oil crisis in the 1970s, a demand for alternative fuel sources created a great interest in the potential use of *T. reesei* to convert cellulose rich fractions into fermentable sugars using the extracellular enzymes. Unfortunately, the production of fungal enzymes in the wild type is low and thus cannot be used on an industrial scale directly. Therefore, random mutagenesis was performed to increase the cellulase production of *T. reesei* over the decades. The result is a several strain pedigree of *T. reesei* that produces a much larger quantity of cellulolytic enzymes, some of which have more than 15-20 times increased cellulase production compared with the wild type (Paloheimo et al., 2016, Carpa et al., 2018). *T. reesei* is the asexual form of *Hypocrea jecorina*. *T. reesei* reproduce through the production of conidia, which are created on top of conidiophores. The mycelium of the *T. reesei* is yellow when grown on PDA plates and the spores are green (Schuster and Schmoll, 2010). In the *Trichoderma* genus, a mycotoxin called trichodermin can be produced, but it has not been observed in *T. reesei*. Another potential mycotoxin in the *Trichoderma* genus is gliotoxin, which gene cluster are identified in the genome, but the production of gliotoxin has not been observed. Therefore, it seems unlikely that *T. reesei* produce any kind of mycotoxins (Paloheimo et al., 2016, Frisvad et al., 2018).

1.9.1 Endoglucanase in T. reesei QM6a

T. reesei has multiple potential genes coding for endoglucanase. Some of these are located in a sequenced T. reesei QM6a genome found on NCBI (NCBI, 2021, 1). These endoglucanase nucleotide sequences are analyzed using an online software called TMHMM. This software can predict if an enzyme ends up inside or outside of a

cell after transcription and translation. The software also detects potential transmembrane domains in the sequence (figure 4).

Table 4: Potential endoglucanase genes located in T. reesei QM6a.

Locus tag Specific name	Glycoside hydrolysate family	Number of exons in gene	Amino acid length	Predicted location	Transmembrane domains	Reference
TRIREDRAFT_120312 cel5a	5	3 (2 translated)	418	Outside cell membrane	Around 0.2 probability at the start of the enzyme	(NCBI, 2021, 2)
TRIREDRAFT_82616 cel5b	5	3	438	Outside cell membrane	Around 0.7 probability at the end of the enzyme	(NCBI, 2021, 3)
TRIREDRAFT_122081 Cel7b	7	3	459	Outside cell membrane	No	(NCBI, 2021, 4)
TRIREDRAFT_39942	17	2	413	Outside cell membrane	No	(NCBI, 2021, 5)
TRIREDRAFT_49976	45	3	242	Outside cell membrane	No	(NCBI, 2021, 6)
TRIREDRAFT_73643	61	2	344	Outside cell membrane	Around 0.2 probability at the start of the enzyme	(NCBI, 2021, 7)

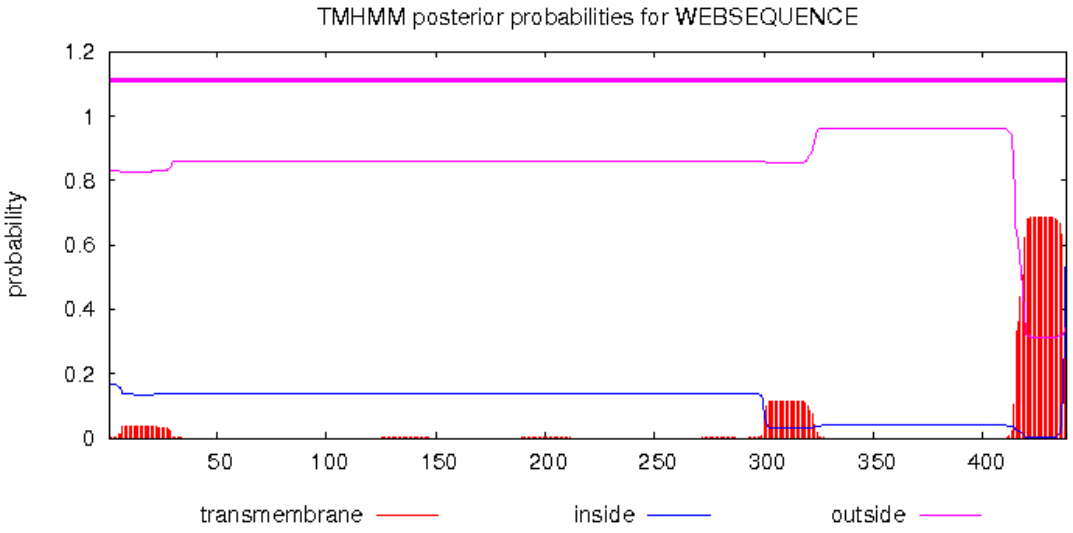


Figure 4: Transmembrane domains (NCBI, 2021 4, TMHMM, 2021).

1.9.2 Exoglucanase/cellobiohydrolase in T. reesei QM6a

T. reesei has at least two different exoglucanases: one that cuts in the N-terminal and one that cuts in the C-terminal of the cellulose chain. Candidates for these two exoglucanase genes are found on NCBI (table 5 (NCBI, 2021, 8)). The sequences are analyzed using the same tool as in (1.9.1).

Table 5: Potential exoglucanase genes located in T. reesei QM6a.

Locus tag Specific name Cut end	Glycoside hydrolysate family	Number of exons in gene	Amino acid length	Predicted location	Transmembrane domains	Reference
TRIREDRAFT_72567 N-terminal	6	4	471	Outside cell membrane	No	(NCBI, 2021, 9)
TRIREDRAFT_123989 Cel7a C-terminal	7	3	514	Outside cell membrane	no	(NCBI, 2021, 10)

1.9.3 β-glycosidase in T. reesei QM6a

Using the same procedure as previous (1.9.1 and 1.9.2), potential β-glycosidase genes are found (table 6 (NCBI, 2021, 11)).

Table 6: Potential β-glycosidase genes located in T. reesei QM6a.

Locus tag Specific name	Glycoside hydrolysate family	Number of exons in gene	Amino acid length	Predicted location	Transmembrane domains	Reference
TRIREDRAFT_76672 Cel3a	3	3	744	Outside cell membrane	No	(NCBI, 2021, 12)
TRIREDRAFT_121735 Cel3b	3	3	874	Outside cell membrane	No	(NCBI, 2021, 13)
TRIREDRAFT_82227 Cel3c	3	2	834	Outside cell membrane	No	(NCBI, 2021, 14)
TRIREDRAFT_79669	3	6	932	Outside cell membrane	No	(NCBI, 2021, 15)
TRIREDRAFT_69557	3	2	489	Outside cell membrane	No	(NCBI, 2021, 16)
TRIREDRAFT_47268	3	4	814	Outside cell membrane	No	(NCBI, 2021, 17)
TRIREDRAFT_46816	3	3 (2 translated)	700	Outside cell membrane	No	(NCBI, 2021, 18)
TRIREDRAFT_108671	3	5	927	Outside cell membrane	No	(NCBI, 2021, 19)
TRIREDRAFT_104797	3	8	781	Outside cell membrane	No	(NCBI, 2021, 20)

1.10 Statistical design of experiment

When testing multiple variables for a specific production, the number of samples quickly grows if all interactions between multiple parameters are researched. If each variable only has two possible settings, then at 7 different variables, the number of samples will exceed 100 and will only continue to increase exponentially with each new variable tested. If each variable has more than 2 settings, then the number of samples needed for each new variable increases. If it is assumed that no interaction/influence happens between the different variables, then testing is easier, but not realistic. Therefore, the development/usage of a multifactorial experiment, that can identify the most important variables while still decrease the number of samples necessary (Placket and Burman, 1946).

Placket and Burman (1946) made a Design matrix, where it is possible to determine which variable has the greatest effect on the reaction. The Design matrix works by assigning each variable two possible settings: one that is average and one that is extreme. The average value is denoted "-" and the extreme one is denoted "+". If it is assumed that the variable tested is 7, the number of settings is 2 and the number of samples is 10 ("number of samples ≥ variables tested + 1"), then a possible Design matrix may look similar to table 7.

Table 7: Potential Design equation for Plackett and Burman multifactorial experiment.

+	+	-	-	+	+	-	-	+
+	+	+	-	-	+	+	-	-
-	+	+	+	-	-	+	+	-
-	-	+	+	+	-	-	+	+
+	-	-	+	+	+	-	-	+
+	+	-	-	+	+	+	-	-
-	+	+	-	-	+	+	+	-
-	-	+	+	-	-	+	+	+
+	-	-	+	+	-	-	+	+
-	-	-	-	-	-	-	-	-

The design matrix is built up of a row consisting of "number of samples - 1" columns, containing 1 extra "+" then "-". For each new row, the row is shifted one space to the right, until the number of rows and the number of columns are equal. One last row is added, which consists only of "-". For each variable tested, a column is chosen, such as the 7 first columns. Each row symbols the number of samples. The more samples, the more precise the matrix is. The Design matrix for this example is in table 8.

Table 8: Potential Design matrix for 7 variables with 2 settings and 10 samples.

		Variable						
		1	2	3	4	5	6	7
Sample	1	+	+	-	-	+	+	-
	2	+	+	+	-	-	+	+
	3	-	+	+	+	-	-	+
	4	-	-	+	+	+	-	-
	5	+	-	-	+	+	+	-
	6	+	+	-	-	+	+	+
	7	-	+	+	-	-	+	+
	8	-	-	+	+	-	-	+
	9	+	-	-	+	+	-	-
	10	-	-	-	-	-	-	-

When all the samples have been tested in the specific settings, the influence of each variable can be calculated (formula 1). Each sample, where a "+" sign is given is added and a "-" sign is subtracted, whereafter it is divided with the number of samples (samples is denoted S):

$$Formula\ 1: Variable\ 3 = (S2 + S3 + S4 + S7 + S8 - S1 - S5 - S6 - S9 - S10)/10$$

The farther away a number is from 0, the more influence does the variable has on the specific production (Plackett and Burman, 1946).

1.11 Optimization of enzyme production

Many different parameters influence enzyme production. To reach optimal enzyme production, these parameters need to be optimized. In Idris et al. (2017), enzymes are produced using *T. reesei RUT C-30* and a synthetic substrate. A solid state fermentation is used. The 10 parameters cellulose concentration, peptone, yeast extract, moisture, ammonia sulfate, lactose, Tween 80, inoculation size, time, and pH are analyzed using a Plackett and Burman design matrix to identify which parameter had the most influence. The three parameters with the most influence are moisture, cellulose concentration, and inoculation size. Further testing using these parameters shows that the optimal cellulose concentration is 1g cellulose/5g substrate, the optimal inoculation size is $3*10^6$ spores/g substrate, and the optimal moisture content of the substrate is 57.5 % (Idris et al., 2017).
In Ortiz et al. (2015), enzymes are produced using *T. reesei CBS 836.91* and different enriched acro-industrial products, which are fermented at solid state. The 9 parameters urea concentration, $(NH_4)_2SO_4$, KH_2PO_4, $CaCl_2 * 2H_2O$, $MgSO_4 * 7H_2O$, pH, inoculation size, moisture, lactose concentration, and temperature are analyzed using a Plackett and Burman design matrix. The samples are both measured using filter paper assay (FPA) activity and endoglucanase activity. The three parameters with the most influence on endoglucanase activity are $MgSO_4 * 7H_2O$, lactose concentration, and $CaCl_2 * 2H_2O$. The three parameters with the most influence on FPA activity are $MgSO_4 * 7H_2O$, urea concentration, and $(NH_4)_2SO_4$. It should be noted that the effect of $CaCl_2 * 2H_2O$ and lactose is not measured for FPA activity (Ortiz et al. 2015).
In Abdullah et al. (2016), enzymes are produced using *T. reesei QM6a* and municipal solid waste under solid state fermentation. Different parameters are analyzed 1-2 at a time. The parameters analyzed are moisture content, temperature, inoculation size, mineral supplements (cellulose powder, peptone, cellulose powder + peptone, clay), and time. Moisture content and temperature are measured at the same time, where the optimal condition is at 30°C and 60% moisture content. The optimal inoculation size is $5*10^5$ spores/g-DM. The addition of nutrients

did not have a significant positive effect on enzyme activity. While 7 days of growth increased the cellulase production compared with 5 days, the longer production time may not be viable for industrial production (Abdullah et al., 2016).

In Singhania et al. (2006), enzymes are produced using *T. reesei RUT C-30* and wheat bran enhanced with a basal mineral salt solution. It is fermented using solid state fermentation. The 11 parameters analyzed are moisture content, substrate particle size, pH, temperature, inoculation size, Age of inoculum, NH_4NO_3, peptone, cellobiose, Tween 80, and incubation time. All parameters are analyzed using a Plackett and Burman design matrix. The two parameters with the largest effect are moisture content and temperature of the incubation. Other parameters with high estimated effects are NH_4NO_3, Tween 80, and incubation time. The optimal temperature is 30°C and the optimal moisture content is 37.5% (Singhania et al. 2006).

In Maurya et al. (2011), enzymes are produced through solid state fermentation using *T. reesei NCIM 992* and wheat bran. The 6 parameters fermentation time, moisturizing agent (acetate buffer, Phosphate buffer, distilled water, mineral salt solution), moisture, substrate size, pH, and temperature are tested one after the other. The optimal conditions are 6 days fermentation, 30°C, 70% moisture content, 500 µm substrate, pH 5, and using mineral salt solution as moisturizing agent (Maurya et al., 2011).

1.12 Optimization of enzymatic hydrolysis

Many different parameters influence enzymatic hydrolysis. In Chen et al. (2008), different parameters are tested to hydrolyze maize straw polysaccharides. The tested parameters are the addition of β-glucosidase, units of enzymes added per g substrate, substrate concentration, and addition of surfactants. With the addition of β-glucosidase, the hydrolysis yield increased from 25% to 60% after 12 hours of incubation and from 65-70% to 80-85% after 48 hours of incubation. At 7, 10, and 20 FPU/g substrate, 10%, 30%, and 40% hydrolysis yield is reached after 12 hours, and 50%, 65%, and 80% hydrolysis yield is reached after 48 hours. Increasing the substrate concentration from 30 g/l to 80 g/l decreases the hydrolysis yield from 65% to 55% after 12 hours incubation but has a similar yield after 48 hours (Chen et al., 2008).

In Fang et al. (2010), a Plackett-Burman Design matrix is used to test 6 different parameters enzymatic hydrolysis effect on steam-exploded corn stover. The 6 variables tested are the substrate concentration, agitation, cellulose dosage, β-glucosidase dosage, hydrolysis time, and Triton X-100. The parameter with the highest effect is the cellulase dosage, then time, and the β-glucosidase dosage.

In Vásquez et al. (2007), the enzymatic hydrolysis parameters temperature, enzyme loading, pH, and percentage solids are tested using acid pretreated sugarcane bagasse as substrate. pH has the lowest effect on enzymatic hydrolysis. By using a statistical model, the optimal conditions for hydrolyzing cellulose to glucose are a temperature of 43°C, a solid fraction of 2%, and an enzyme loading of 24.4 FPU/g. The optimal conditions for reaching the highest glucose concentration are the temperature of 47°C, a solid fraction of 10%, and an enzyme loading of 25.6 FPU/g (Vásquez et al., 2007).

In Haldar et al. (2018), the parameters of solid loading, enzyme dosage, pH, and temperature are tested for enzymatic hydrolysis, using the substrate banana stems. For solid loading, the total sugars are increased by more than 100% by increasing the loading from 1:20 to 1:10. For enzyme dosage, 10 FPU releases 9.4 g/L of total released sugars, 20 FPU releases 14.6 g/L, and 30 FPU releases 19.6 g/L. in pH range 4.45 to 5.48, the total released sugars changed very little and is optimal in this range. The temperature releasing the highest number of total released sugars is 50°C (Haldar et al., 2018).

1.13 Analysis procedures

This section contains information on four different analysis methods used in this report: two for analysis of sugars and two for analysis of protein/nitrogen. Each analysis is described. The sugar analysis is compared (table 9) and the protein/nitrogen analysis are compared (table 10).

1.13.1 DNS

DNS (3.5-dinitrosalicyclic acid) is a colorimetric analysis method, which is the most widely used method to estimate reduced sugars in a sample (Deshavath et al., 2020). The method is easy to perform, and it is easy to analyze a great number of samples in a short amount of time. It works by oxidizing the free carbonyl group in reduced sugars into a (COOH) group. This reduces 3.5-dinitrosalicyclic acid (yellow) into 3-amino-5-nitrosalicyclic acid (orange/red (figure 5)). DNS is especially great at estimating the concentration of a single reduced sugar, but in the presence of other reduced sugars, the results are less precise. Even so, the method has been adopted to analyze the reduced sugars present in lignocellulosic material (Deshavath et al., 2020). 1 liter of DNS reagent costs around 110 DKK to make, where a minimum of 200 µl is used per sample (Chemical book 2021, Sigma-Aldrich 2021).

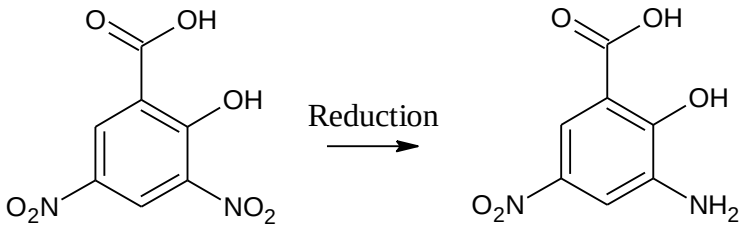


Figure 5: DNS reaction: 3.5-dinitrosalicyclic acid is reduced to 3-amino-5-nitrisalicyclic acid in the present of sugars with free carbonyl groups.

1.13.2 HPLC

HPLC (High performance liquid chromatography) is a method to separate components in a sample. A mobile phase is pumped into the system and the sample is added. The mobile phase passes through a column. This column is packed with a material functioning as a stationary phase. The molecules in the sample bind to the phase it has a higher affinity for. The faster a molecule passes through the column, the less affinity it has for the stationary phase. After passing through the column, the liquid passes through a detector, which detects all molecules passing through. The detector transforms it into a signal, which can be used to create a 1D chromatogram. With the use of integration and a standard curve, it is possible to translate the signal into a concentration of the specific molecule (Figure 6 (Agilent Technologies 2016)).

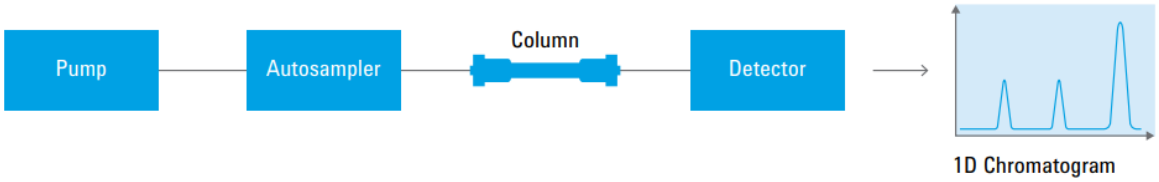


Figure 6: Simple box diagram of a HPLC procedure (Agilent Technologies 2016).

Table 9: Advantage and disadvantage of HPLC and DNS (Deshavath et al., 2020, Agilent Technologies, 2016).

Parameter	HPLC	DNS
Precision	Can reliably detect multiple components at once.	Only detects reduced ends on components and can thus only reliably detect samples with one type of reduced sugar molecules.
Time	Each sample takes multiple minutes to detect.	Whole reaction can be performed in hours.
Price	Around 10 DKK per sample.	Less than 0.1 DKK per sample.
Samples analyzed at once	Only one sample can be detected at once.	Multiple samples can be detected at once.
Standard curve	Necessary.	Necessary.
Detection	Through a detector after liquid chromatography	Through color change with a spectrophotometer.

1.13.3 Elemental analysis

The most common form of elemental analysis is the CHNS analysis. The analysis is performed by adding a weighted sample (2-3 mg) into the machine together with oxygen, which is then combusted at 950°C. The combusted samples are thus converted into CO_2, H_2O, and NC. Afterward, the combusted samples are transferred by helium through a gas chromatograph, which separates the gasses. A detector measures the amount of gas passing through the outlet and converts this into a signal, which is transformed into a 1D chromatogram. With the use of integration and a standard curve, it is possible to translate the chromatogram into a concentration of the gasses (AAU EA protocol, 2020).

1.13.4 BCA

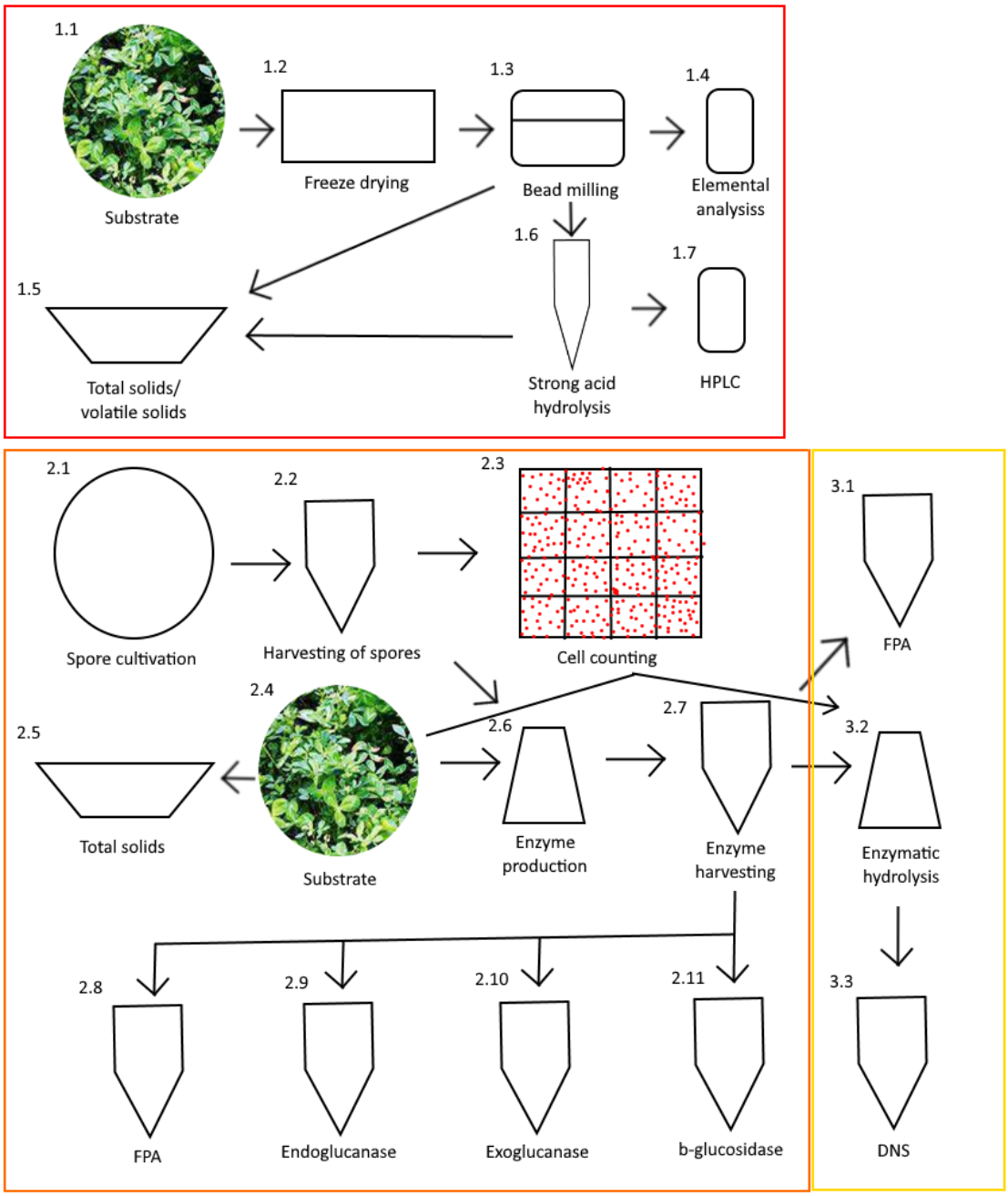
To measure the protein concentration of a liquid sample, it is possible to use The Thermo Scientific ™ Pierce ™ BCA Protein Assay Kit. The kit is based on bicinchoninic acid (BCA), which gains a purple color when Cu^{+1} ion is present in an alkali reaction. The Cu^{+1} ion is formed from the Cu^{+2} ion when it is in the presence of proteins. To form a standard curve, known concentrations of bovine serum albumin (BSA) is normally used. There is a problem with the precision of using this protein for a standard curve. Different proteins react differently to the Cu^{+1} ions and thus other protein standards may be needed to measure the exact concentration of the protein in the unknown sample. Specifically, the structure, number of peptide bonds, and the presence of four specific amino acids (cysteine, cystine, tryptophan, and tyrosine) have the most influence on the BCA reaction (Pierce ™ BCA Protein Assay Kit user guide, 2015).

Table 10: Advantage and disadvantage of Elemental analysis and BCA (AAU EA protocol, 2020, Pierce ^{TM} BCA Protein Assay Kit user guide, 2015).

Parameter	Elemental analysis	BCA
Measuring of protein	Measure N in sample, the form of the proteins does not matter. Assume that all N detected derives from proteins.	Only measure protein, not amino acids.
Form of sample	Solid or liquid.	Liquid.
Reliability	If sample is homogeneous, then it is reliable.	Depending on the proteins themselves and the state of degradation of the proteins, then the results may not be reliable.
Time	Each sample takes 5-10 min to analyze.	Whole reaction can be performed in hours.
Samples analyzed at once	One sample is analyzed at a time.	Multiple samples can be analyzed at once.
Standard curve	Necessary.	Necessary.
Detection	Through a detector after gas chromatography.	Through color change with a spectrophotometer.

2 Material and method

Material and method are divided into multiple sections. First are block diagrams of the experiments and their order performed. Next is a section of the different equipment and buffers/solutions used in the different experiments (table 11 and 12). Lastly, is the experimental procedure performed, divided into four sections: “substrate analysis”, “optimization of enzyme production”, “optimization of enzymatic hydrolysis”, and “effect of enzymatic hydrolysis”.



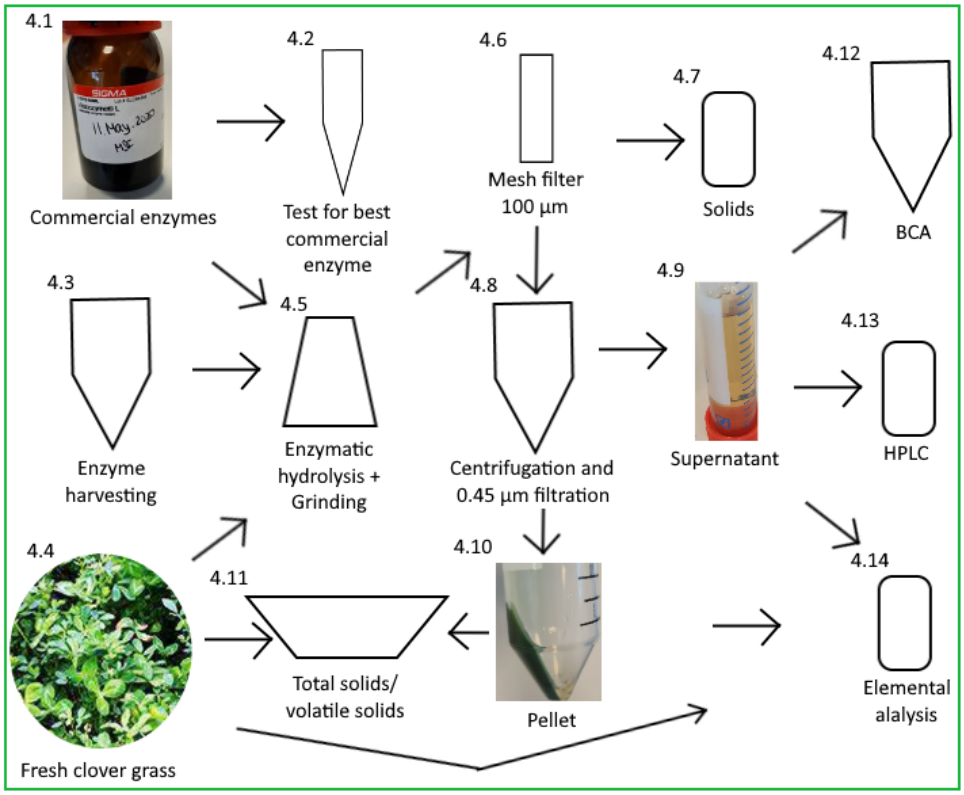


Figure 7: The four different experimental sections. Figure 7.1 (red) is the substrate analysis, figure 7.2 (orange) is the enzyme production, figure 7.3 (yellow) is the enzymatic hydrolysis, and figure 7.4 (green) is the effect of enzymatic hydrolysis.

1.1: usage of frozen alfalfa press cake, clover press cake, brewers spent grains, and wheat bran/sphagnum peat. 1.2: freeze drying around 20 g of each substrate. 1.3: pulverizing the freeze dried substrates. 1.4: using elemental analysis to determine the amount of nitrogen in the different substrates. 1.5: TS determines total solids and VS determines the amount of organic matter in a sample. Only performed TS on the powder from 1.3. 1.6: performing strong acid hydrolysis to degrade all sugar molecules and acid degrading lignin. 1.7: determine the sugar content of the different substrates.

2.1: spores are cultivated on PDA plates. 2.2: spores are harvested from the plates to make a spore suspension. 2.3: number of spores in the spore suspension is counted in a microscope using a counting chamber. 2.4: usage of frozen alfalfa press cake (same as in 1.1). 2.5: determine total solids in the press cake. 2.6: producing enzymes in a solid state fermentation. Different parameters in the fermentation are tested. 2.7: enzymes are extracted/separated from the substrate, creating supernatant. 2.8: FPA is used on the supernatant to determine the optimal parameters for solid state fermentation. 2.9: endoglucanase assay is used to determine endoglucanase activity in the supernatant. 2.10: exoglucanase assay is used to determine exoglucanase activity in the supernatant. 2.11: β-glucosidase assay is used to determine β-glucosidase activity in the supernatant.

3.1: after determining the optimal condition of the enzyme production (2.6), a large batch of enzymes is produced, and FPA is used to determine the activity in the supernatant. 3.2: non-processed press cake is enzymatically hydrolyzed using the supernatant. Different parameters in the enzymatic hydrolysis are tested. 3.3: degradation efficiency of enzymes is determined by measuring the released sugars in the broth.

4.1: different cellulase degrading commercial enzymes present in the lab (Celluclast, Viscozyme, Novozyme 188, CelliHtec, CelliCtec, and CelliCtec 2). 4.2: testing the different commercial enzymes to locate the most efficient for substrate

degradation. 4.3: supernatant from an enzymatic production step utilizing the optimal conditions (2.7). 4.4: fresh clover grass. 4.5: grinding of substrate and enzymatic hydrolysis. 4.6: mesh filtration with 100 µm pore size. Large solids are removed from the rest. 4.7: large solids. 4.8: centrifugation of filtrate and afterward filtration of supernatant with a 0.45 µm pore size filter while leaving the pellet in the centrifuged tube. 4.9: supernatant. 4.10: pellet, which is freeze dried. 4.11: TS is performed on the pellet and fresh substrate. TS/VS is performed on freeze dried and milled fresh substrate. 4.12: BCA analysis is performed on the supernatant. 4.13: HPLC is performed on the supernatant. 4.14: elemental analysis is performed on the freeze dried pellet, freeze dried and milled fresh substrate, and on the supernatant.

2.1 Equipment

Table 11: list of the equipment used and in which experiment.

Equipment used	In which analysis/experiment it is used
-80°C freezer	2.3.1.1 Freeze drying 2.3.4.4 Filtration and centrifugation
Telstar freeze drier model LYOQUEST -55 PLUS	2.3.1.1 Freeze drying 2.3.4.4 Filtration and centrifugation
Desiccator	2.3.1.1 Freeze drying 2.3.1.4 TS/VS 2.3.2.3 TS 2.3.4.4 Filtration and centrifugation 2.3.4.5 TS/VS
Coffee grinder	2.3.1.2 Bead milling 2.3.4.4 Filtration and centrifugation
FRITSCH pulverisette bead miller	2.3.1.2 Bead milling 2.3.4.4 Filtration and centrifugation
Incubator, 30°C	2.3.1.3 Strong acid hydrolysis 2.3.2.4 Enzyme production
Autoclave	2.3.1.3 Strong acid hydrolysis 2.3.2.1 Cultivation of spores 2.3.2.4 Enzyme production
Vacuum filtration setup	2.3.1.3 Strong acid hydrolysis
105°C oven	2.3.1.4 TS/VS 2.3.2.3 TS 2.3.4.5 TS/VS
Muffle furnace	2.3.1.4 TS/VS 2.3.4.5 TS/VS
Elemental analyzer (need model/serial number)	2.3.1.5 Elemental analysis 2.3.4.7 Elemental analysis
HPLC machine	2.3.1.6 HPLC 2.3.4.8 HPLC
Centrifuge, small (Eppendorf tubes), 14,500 rpm	2.3.1.6.2 HPLC, pH 5-7 2.3.2.6.2 Endoglucanase assay 2.3.2.6.3 Exoglucanase assay 2.3.3.3 DNS 2.3.4.1 Test for best commercial enzyme 2.3.4.8 HPLC

Flow bench	2.3.2.1 Cultivation of spores 2.3.2.2 Harvest of spores + counting 2.3.2.4 Enzyme production 2.3.2.5 Enzyme harvesting
Microscope	2.3.2.2 Harvest of spores + counting
55°C oven	2.3.2.4 Enzyme production
Incubator, room temperature, 200 rpm	2.3.2.5 Enzyme harvesting
Centrifuge, large (falcon tubes 15 + 50 ml), 13,880 g (9000 rpm)	2.3.2.5 Enzyme harvesting 2.3.4.4 Filtration and centrifugation
Thermoshaker (incubator 40-99°C)	2.3.2.6.1 Filter Paper Assay (FPA) 2.3.2.6.2 Endoglucanase assay 2.3.2.6.3 Exoglucanase assay 2.3.2.6.4 β-glucosidase assay 2.3.3.1 FPA 2.3.3.2 Enzymatic hydrolysis 2.3.3.3 DNS 2.3.4.1 Test for best commercial enzyme
Plate reader	2.3.2.6.1 Filter Paper Assay (FPA) 2.3.2.6.2 Endoglucanase assay 2.3.2.6.3 Exoglucanase assay 2.3.2.6.4 β-glucosidase assay 2.3.3.1 FPA 2.3.3.3 DNS 2.3.4.1 Test for best commercial enzyme 2.3.4.6 BCA
Vortex mixer	2.3.2.6.2 Endoglucanase assay 2.3.2.6.3 Exoglucanase assay
Incubator, 50°C (in temperature experiment, the incubator at 40°C and 55°C is also used)	2.3.3.2 Enzymatic hydrolysis 2.3.4.3 Enzymatic hydrolysis and grinding
Ultra-Turrax mill	2.3.4.3 Enzymatic hydrolysis and grinding
100 ml syringe with a 100 µm mesh filter	2.3.4.4 Filtration and centrifugation
Incubator, 37°C	2.3.4.6 BCA
Incubator, 40°C	2.3.4.7 Elemental analysis

2.2 Buffer/solution

Table 12: list of the buffers and solutions used and in which experiment.

Solution/buffer used	In which analysis/experiment it is used
72% H_2SO_4	2.3.1.3 Strong acid hydrolysis
STAM solution: 33 g/L glucose monohydrate, 30 g/L xylose, 30 g/L arabinose	2.3.1.3 Strong acid hydrolysis
PDA-agar: 39 g/l PDA-pulver	2.3.2.1 Cultivation of spores
0.05 M sodium citrate buffer, pH 4.5	2.3.2.6.1 Filter Paper Assay (FPA) 2.3.3.1 FPA

10 g/L glucose stock (standard curve)	2.3.2.6.1 Filter Paper Assay (FPA) 2.3.2.6.3 Exoglucanase assay 2.3.3.1 FPA 2.3.3.3 DNS
DNS reagent. In 500 ml: 5 g DNS, 100 ml 2M NaOH, 150 g Rochelle salt (potassium sodium tartrate tetrahydrate)	2.3.2.6.1 Filter Paper Assay (FPA) 2.3.2.6.3 Exoglucanase assay 2.3.3.1 FPA 2.3.3.3 DNS 2.3.4.1 Test for best commercial enzyme
7600 mU/ml Cellulase from *A. niger* (standard curve)	2.3.2.6.2 Endoglucanase assay
0.1 M sodium acetate buffer, pH 4.5	2.3.2.6.2 Endoglucanase assay
4M CM-cellulose, pH 4.5 (substrate).	2.3.2.6.2 Endoglucanase assay
Precipitant solution (stop reagent). (In 500 ml: 20 g sodium acetate trihydrate, 2g zinc acetate, 6 M HCl (to adjust pH to 5.0), 400 ml 96% ethanol)	2.3.2.6.2 Endoglucanase assay
0.01 M sodium acetate buffer, pH 5	2.3.2.6.3 Exoglucanase assay
1 % Avicel solution in 0.01 M sodium acetate buffer, pH 5 (substrate)	2.3.2.6.3 Exoglucanase assay
5 x diluted Novozyme 188	2.3.2.6.3 Exoglucanase assay
0.01 M pNP (p-nitrophenol), pH 5 (standard curve)	2.3.2.6.4 β-glucosidase assay
0.05 M sodium acetate buffer, pH 5	2.3.2.6.4 β-glucosidase assay
1M Na_2CO_3 (stop reagent)	2.3.2.6.4 β-glucosidase assay
0.004 M pNPG (p-nitrophenyl-β-D-glucopyranoside) (substrate)	2.3.2.6.4 β-glucosidase assay
0.05 M sodium citrate buffer, pH 5 (in pH experiment, the pH is adjusted to 4, 4.5 and 5.5 for this buffer)	2.3.3.2 Enzymatic hydrolysis 2.3.3.3 DNS 2.3.4.3 Enzymatic hydrolysis and grinding
0.05 M sodium citrate buffer, pH 5.5	2.3.3.2 Enzymatic hydrolysis 2.3.3.3 DNS
Brown juice “buffer”, pH 5.5. Brown juice is created by heat precipitation	2.3.3.2 Enzymatic hydrolysis 2.3.3.3 DNS
30 g/L glucose stock (standard curve)	2.3.4.1 Test for best commercial enzyme
0.12 M succinic acid buffer, pH 5	2.3.4.1 Test for best commercial enzyme
10% sulfuric acid	2.3.4.1 Test for best commercial enzyme
Novozyme 188	2.3.4.1 Test for best commercial enzyme
CelliCtec	2.3.4.1 Test for best commercial enzyme
CelliHtec	2.3.4.1 Test for best commercial enzyme
CelliCtec II	2.3.4.1 Test for best commercial enzyme
Celluclast	2.3.4.1 Test for best commercial enzyme
Viscozymes	2.3.4.1 Test for best commercial enzyme 2.3.4.3 Enzymatic hydrolysis and grinding
BCA working reagent	2.3.4.6 BCA
BSA (standard)	2.3.4.6 BCA
0.9% saline solution (dilution)	2.3.4.6 BCA

2.3 Experimental procedures

2.3.1 Substrate analysis

2.3.1.1 Freeze drying

Around 20 g of each substrate (alfalfa press cake, clover grass press cake, brewers spent grains, wheat bran/sphagnum peat (25.62 g wheat bran, 15.44 g sphagnum peat, 51.01 g MilliQ water (Kolasa et al., 2014))) are frozen in trays at -80°C for at least 1 day. Frozen substrate is added to the freeze drying chamber and closed. Substrates are freeze dried for around 1 day with the settings: freezing chamber -50°C, tray temperature: 20 °C, pressure 0.5 mbar. After freeze drying, substrates are stored in a desiccator.

2.3.1.2 Bead milling

Freeze dried alfalfa press cake and clover grass press cake are ground in a coffee grinder for 25 seconds to reduce their size. Each substrate is added to different milling chambers together with 25 metal beads. All substrates are milled with the following settings: 600 rpm, 30 sec. milling, 30 sec. break, 10 rounds. Alfalfa and clover grass press cake is milled further to reach the same consistency as the other substrates, using the settings: 600 rpm, 30 sec. milling, 1 min break, 5 rounds.

2.3.1.3 Strong acid hydrolysis

0.1600 g milled substrate and 1.5 ml 72% H_2SO_4 are added to Pyrex tubes, four tubes for each substrate. Tubes are incubated at 30°C for 1 hour before transferring them to an ice bath. 41 ml distilled water and 1 ml STAM solution are added to two tubes of each substrate, and 42 ml distilled water is added to the rest. Tubes are mixed and autoclave at 121°C for 1 hour. 6 ml supernatant is extracted from each tube. The rest is filtered through a filter (VWR European Cat. No. 516-0270. Quantitative filter papers, 434) using vacuum filtration. Solids are washed with distilled water until neutral pH is reached in the effluent. Perform TS/VS is performed on the solids/filters (2.3.1.4), acid soluble lignin is measured in the supernatant in a plate reader at wavelength 240 and 320, and sugar monomers in the supernatant are measured using HPLC (2.3.1.6).

2.3.1.4 TS/VS

Crucibles filled with filters from the strong acid hydrolysis (2.3.1.3) and crucibles containing at least 3 g of milled substrate are numbered and weighted. All crucibles are dried overnight in a 105°C oven. Crucibles are cooled in a desiccator and weighed. All crucibles are burned in a 550°C muffle furnace for 3 hours. They are cooled in a desiccator and weighed again. This experiment is performed in triplicates

2.3.1.5 Elemental analysis

Between 2-3 mg milled substrate is added to specific aluminum foil containers which are folded closed. Aluminum foil containers containing: empty, 2-3 mg unknown sample, 3 x 2-3 mg methionine, and 2 x 2-3 g urea are also prepared and folded. All folded containers are added to the elemental analyzer machine in the following order: empty, bypass (unknown sample), standards (3 x methionine), urea, milled substrate, urea. The settings used are reactor temperature: 950°C, carrier flow: 140 ml/min, reference flow: 100 ml/min, oxygen flow: 250 ml/min, run time: 720 sec, polarity: positive. This experiment is performed in duplicates.

2.3.1.6 HPLC

Two versions of HPLC are made. One, where pH is below 3, and one, where pH is between 5 and 7. One analysis is performed for each supernatant at each version of HPLC.

2.3.1.6.1 HPLC, pH<3

1 ml from supernatant from strong acid hydrolysis (2.3.1.3) is filtered through a 0.45 µm HPLC filter into HPLC vials. The vials are closed and analyzed on an HPLC machine, with the settings: column: BioRad Aminex HPX-87H, flow: 0,6 ml/min, eluent: 4 mM H2SO4, column temp.: 60°C.

2.3.1.6.2 HPLC, pH 5-7

Calcium carbonate is added to the supernatant from strong acid hydrolysis (2.3.1.3) until a pH between 5 and 7 is reached. Between 1-2 ml of the supernatant is added to Eppendorf tubes and cooled in the fridge overnight. The tubes are centrifuged at 14500 rpm for 10 min. Supernatant is filtered through a 0.45 µm HPLC filter into HPLC vials. The vials are closed and analyzed on an HPLC machine, with the settings: column: BioRad Aminex HPX-87P, flow: 0,5 ml/min, eluent: MilliQ-H_2O, column temp.: 70°C.

2.3.2 Optimization of enzyme production

2.3.2.1 Cultivation of spores

Cultivation of spores is performed 4 times, where the spores used for the cultivation have different origins. The first time, an old spore suspension (around 1 year old, that is stored at 5°C) is used. The second time, part of the spore suspension made the first time is used. The third time, spores originate from a cryostock. The fourth time, part of the spore suspension made the second time is used.

Every time a new cultivation of spores is made, 2 plates containing autoclaved PDA agar are made. The spores are added either by adding 500 µl spore suspension using spread plate technique (first time), by adding 100 µl spore suspension using spread plate technique (second time), by adding spores to the plate with an inoculation loop using streak plate technique (third time), or by adding spore suspension using an inoculation loop and using streak plate technique (fourth time). This is performed in a flow bench to prevent contamination. The plates are incubated on a windowsill at room temperature for 11-14 days depending on the spore production.

2.3.2.2 Harvest of spores + counting

The cultivated spores (2.3.2.1) are harvested in a flow bench by adding 8 ml sterile distilled water to the plates and scrape the spores loose with an L-spatula until all visible spores are released from the plate. As much liquid/spore suspension as possible is collected into 15 ml falcon tubes. Part of the spore suspension is diluted 10, 100, and 1000 times. The spore suspension is stored at 5°C. 10 µl of the diluted spore suspension is added to a Fuchs-Rosenthal counting chamber, 0.2 mm dept. The spores present in the counting chamber are counted using a microscope (10x lens, Ph2), where a total area of 16mm^2 is counted. One spore suspension is made from each cultivation of spores (2.3.2.1).

2.3.2.3 TS

The dry matter of alfalfa press cake used for enzyme production (2.3.2.4) is measured before each optimization experiment. 3 folie containers are numbered and dried overnight in a 105°C oven. The weight of each container is measured. Between 2-6 g of substrate is added to each container and the weight is measured. The containers and substrates are dried overnight in a 105°C oven and the weight is measured. This experiment is performed 3 times on alfalfa press cake in triplicates. For the “substrate” optimization experiment, TS/VS is used (2.3.1.4).

2.3.2.4 Enzyme production

Four different experiments analyzing the optimal condition of 1-2 parameters are performed. The experiment tested are “time and light/dark” experiment (time for incubation and the effect of light availability), “temperature/moisture” experiment (influence of the temperature and the moisture content of the substrate), “spore-substrate ratio” experiment (inoculum size needed for optimal enzyme production), and “substrate” experiment (test enzyme production in *T. reesei*, when grown on alfalfa press cake, clover grass press cake, brewers spent grains and wheat bran/sphagnum peat). Each optimization experiment is performed in duplicates. Supernatant used in enzymatic hydrolysis (2.3.3.2) is produced in 2 times 3 flasks. Supernatant used in enzymatic hydrolysis and grinding (2.3.4.3) is produced in 4 flasks.

In “temperature/moisture” experiment, the substrate is dried overnight in a 55°C oven. 1.5 g-DM substrate is added to 100 ml Erlenmeyer flasks. Distilled water is added to the substrate to reach 75% moisture content (except for the “temperature/moisture” experiment, where some of the flasks contain 65% and 80% moisture). All flasks are autoclaved at 121°C for 20 min. Sterile distilled water (to correct moisture loss) and $5x10^6$ spores/g-DM substrate are added to the flasks in a flow bench (except for the “time and light/dark” experiment, where the loss of moisture in the autoclavation process is not corrected, and for the “spore-substrate ratio” experiment, where $1x10^5$, $5x10^5$, $1x10^6$ and $1x10^7$ spores/g-DM is used in some of the flasks). The flasks are incubated for 9 days (except for the “time and light/dark” experiment, where an incubation time of 0, 2, 5, 7, 12, and 14 days is also used) at room temperature (except for the “temperature/moisture” experiment, where some of the flasks are incubated at 20°C and 30°C), and with the availability of light (except the “time and light/dark” experiment, where some flasks are covered in aluminum foil, and the “temperature/moisture” experiment, where all flasks are covered in aluminum foil). Supernatant used for the enzymatic hydrolysis (2.3.3.2) and enzymatic hydrolysis and grinding (2.3.4.3) is created from 5 g-DM substrate in 250 ml Erlenmeyer flasks.

2.3.2.5 Enzyme harvesting

After the incubation period, 25 ml of sterile distilled water is added to each flask in a flow bench and incubated at room temperature for 4 hours, 200 rpm. The content of the flasks is added to 50 ml Falcon tubes and centrifuged at 13,880 g, 4°C for 20 min. 3x1ml supernatant is extracted into Eppendorf tubes, which are stored at -18°C. For supernatant used for enzymatic hydrolysis (2.3.3.2), 85 ml sterile distilled water is added, two Falcon tubes are used for each Erlenmeyer flask, and the supernatant is centrifuged two times.

2.3.2.6 Activity analysis

2.3.2.6.1 Filter Paper Assay (FPA)

Standard curve: Different dilution of 10 g/L glucose stock is prepared using 0.05 M sodium citrate buffer, pH 4.5 (0-6.67 g/L glucose). 120 µl of each dilution is added to different 2 ml Eppendorf tubes. Tubes are incubated at 50°C for 60 min. The tubes are cooled on ice and 240 µl DNS reagent is added. The tubes are boiled for 10 min and afterward cooled on ice. 1300 µl distilled water is added. 100 µl sample is added to specified wells in a 96 well microtiter plate. The plate’s absorbance is read in a plate reader at wavelength 540 nm. The absorbance is read 2-4 times for each standard.

Assay procedure: All supernatants are diluted 3 times using 0.05 M sodium citrate buffer, pH 4.5 (except for the time and light/dark experiment, where a 2 times dilution is used). 2 Eppendorf tubes are prepared for each diluted supernatant. 120 µl of the dilution are added to Eppendorf tubes and incubated at 50°C for 5 min. In half

of the tubes, 2 filters with a diameter of 0.6 cm (approximately 4 mg) are added (VWR European Cat. No. 516-0847. Quantitative filter papers, 474). Tubes are incubated at 50°C for 60 min. The tubes are cooled on ice and 240 µl DNS reagent is added. The tubes are boiled for 10 min and afterward cooled on ice. 1300 µl distilled water is added. 100 µl sample is added to specified wells in a 96 well microtiter plate. The plate's absorbance is read in a plate reader at wavelength 540 nm. The absorbance is read 1-4 times for each sample.

2.3.2.6.2 Endoglucanase assay

Standard curve: Different concentrations of cellulase from Aspergillus niger (0.84 U/mg) is prepared using 100mM sodium acetate, pH 4.5 (0-190 mU/ml). 125 µl 4M CM-cellulose, pH 4.5 is added to all Eppendorf tubes and incubated at 40°C, 700 rpm. Every 30 sec., 125 µl of the diluted cellulase is added to a new tube and vortexed. After exactly 30 min, 625 precipitant solution is added to each tube and vortexed. The tubes are centrifuged at 14500 rpm for 10 min. 100 µl sample is added to specified wells in a 96 well microtiter plate. The plate's absorbance is read in a plate reader at wavelength 620 nm. The absorbance is read 2-4 times for each standard.

Assay procedure: All supernatants are diluted 5 times using 100mM sodium acetate, pH 4.5 (except for the "time and light/dark" experiment, where some are not diluted at all). 125 µl 4M CM-cellulose, pH 4.5 is added to all Eppendorf tubes and incubated at 40°C, 700 rpm. Every 30 sec., 125 µl of the diluted supernatant is added to a new tube and vortexed. After exactly 30 min, 625 µl precipitant solution is added to each tube and vortexed. The tubes are centrifuged at 14500 rpm for 10 min. 100 µl sample is added to specified well in a 96 well microtiter plate. The plate's absorbance is read in a plate reader at wavelength 620 nm. The absorbance is read 2-4 times for each sample.

2.3.2.6.3 Exoglucanase assay

Standard curve: Different dilution of 10 g/L glucose stock is prepared using 0.01 M sodium acetate buffer, pH 5 (0-5 g/L glucose). 100 µl of diluted stock and 200 µl DNS solution is mixed in Eppendorf tubes. The tubes are boiled for 10 min and cooled on ice. 100 µl sample is added to specified wells in a 96 well microtiter plate. The plate's absorbance is read in a plate reader at wavelength 540 nm. The absorbance is read 2-4 times for each standard.

Assay procedure: 2 Eppendorf tubes are prepared for each supernatant: One containing 140 µl 0.01 M sodium acetate buffer, pH 5 and one containing 140 µl 1% Avicel suspended in 0.01 M sodium acetate buffer, pH 5. 140 µl supernatant is added to each tube and they are mixed and spun down. The tubes are incubated at 40°C, 1400 rpm for 24 hours. 5 µl of 5 times diluted Novozyme 188 is added to each tube. The tubes are incubated at 40°C, 1400 rpm for 2 hours. The tubes are centrifuged for 10 min at 14500 rpm. 100 µl sample is transferred into new tubes containing 200 µl DNS solution. These tubes are incubated at 99°C for 10 min and cooled on ice. 100 µl sample is added to specified wells in a 96 well microtiter plate. The plate's absorbance is read in a plate reader at wavelength 540 nm. The absorbance is read 1-4 times for each sample.

2.3.2.6.4 B-glucosidase assay

Standard curve: Different dilution of 10 mM pNP stock is prepared using 0.05 M sodium acetate buffer, pH 5 (0-2.5 mM pNP). 50 µl 1M Na_2CO_3 is added to the used wells in a 96 well microtiter plate. 30 µl of the diluted stock is added to the specified wells in the plate. The plate's absorbance is read in a plate reader at wavelength 405 nm. The absorbance is read 1-4 times for each standard.

Assay procedure: 50 µl 1M Na_2CO_3 is added to the used wells in a 96 well microtiter plate. 50 µl 4mM pNPG is added to Eppendorf tubes and incubated at 40°C, 700 rpm. Every 30 sec., 5 µl supernatant is added to a new tube. After exactly 30 min of the supernatant addition, 30 µl of the liquid is added into the specified well in the plate. The plate's absorbance is read in a plate reader at wavelength 405 nm. The absorbance is read 1-4 times for each sample.

2.3.3 Optimization of enzymatic hydrolysis

2.3.3.1 FPA

Standard curve: Different dilution of 10 g/L glucose stock is prepared using 0.05 M sodium citrate buffer, pH 4.5 (0-6.67 g/L glucose). 40 µl of each dilution and 80 µl of 0.05 M sodium citrate buffer, pH 4.5 is added to 2 ml Eppendorf tubes. Tubes are incubated at 50°C for 60 min. The tubes are cooled on ice and 240 µl DNS reagent is added. The tubes are boiled for 10 min and afterward cooled on ice. 1300 µl distilled water is added. 100 µl sample is added to specified wells in a 96 well microtiter plate. The plate's absorbance is read in a plate reader at wavelength 540 nm. The absorbance is read 4 times for each standard.

Assay procedure: Different dilutions of the supernatant is prepared using 0.05 M sodium citrate buffer, pH 4.5 (0-13.3 times). 40 µl of each dilution and 80 µl of 0.05 M sodium citrate buffer, pH 4.5 is added to 2 ml Eppendorf tubes and incubated at 50°C for 5 min. In half of the tubes, 2 filters with a diameter of 0.6 cm (approximately 4 mg) are added (VWR European Cat. No. 516-0847. Quantitative filter papers, 474). Tubes are incubated at 50°C for 60 min. The tubes are cooled on ice and 240 µl DNS reagent is added. The tubes are boiled for 10 min and afterward cooled on ice. 1300 µl distilled water is added. 100 µl sample is added to specified wells in a 96 well microtiter plate. The plate's absorbance is read in a plate reader at wavelength 540 nm. The absorbance is read 4 times for each sample.

2.3.3.2 Enzymatic hydrolysis

Four different experiments analyzing the optimal condition of 1-2 parameters are performed. The experiment tested are "time/units" experiment (time for hydrolysis and the number of Units added per g-DM of substrate), "pH" experiment (effect of pH on the hydrolysis), "temperature" experiment (influence of the temperature), and the "brown juice" experiment (Test if diluted brown juice can be used for pH regulation).

1 g-DM substrate is added to 100 ml Erlenmeyer flasks. 5 FPU/g-DM supernatant is added (except for "time/units" experiment, where some flasks contain 2, 7.5, and 10 FPU/g-DM). Ampicillin is added to reach the concentration of 100 µg/ml. 0.05 M sodium citrate buffer, pH 5 is added to reach a total volume of 25 ml (except for "pH" experiment, where pH 4, 4.5, and 5.5 are also used and for "brown juice" experiment, where both 1/20 brown juice and 0.05M sodium citrate buffer at pH 5.5 are used). substrate control is made, where no supernatant is added. Enzyme control is made, where no substrate is added. Samples are taken. All flasks are incubated at 50°C for 24 hours (except "time/units" experiment, where samples are also taken after 1, 3, 48, 72, and 96 hours and for "temperature" experiment, where 40°C and 55°C are also used). All samples are boiled for 10 min. Each optimization experiment is performed in duplicates.

2.3.3.3 DNS

Standard curve: Different dilution of 10 g/L glucose stock is prepared using the same buffer as in enzymatic hydrolysis (0-6.67 g/L glucose). 100 µl of each dilution and 200 µl DNS are added to Eppendorf tubes. The tubes are boiled for 10 min and afterward cooled on ice. 1 ml distilled water is added. 100 µl sample is added to

specified wells in a 96 well microtiter plate. The plate's absorbance is read in a plate reader at wavelength 540 nm. The absorbance is read 2-4 times for each standard.

Samples: All samples are centrifuged at 14500 rpm for 10 min. 15-100 µl sample, 0-85 µl of buffer used in the enzymatic hydrolysis (total volume of sample and buffer is 100 µl), and 200 µl DNS is added to Eppendorf tubes and boiled for 10 min. Tubes are cooled on ice and 1 ml distilled water is added. 100 µl sample is added to specified wells in a 96 well microtiter plate. The plate's absorbance is read in a plate reader at wavelength 540 nm. The absorbance is read 4 times for each sample.

2.3.4 Effect of enzymatic hydrolysis

2.3.4.1 Test for best commercial enzyme

Standard curve: Different dilution of 30 g/L glucose stock is prepared using 0.12 M succinic acid buffer, pH 5 (0-30 g/L glucose). 25 µl of each dilution, 75 µl buffer, 3 µl 10% sulfuric acid, and 200 µl DNS are added to Eppendorf tubes. The tubes are boiled for 10 min and afterward cooled on ice. 1 ml distilled water is added. 100 µl sample is added to specified wells in a 96 well microtiter plate. The plate's absorbance is read in a plate reader at wavelength 540 nm. The absorbance is read 4 times for each standard.

Samples: 75 mg milled alfalfa press cake (2.3.1.2) is added to Eppendorf tubes. Enzyme cocktails (cellulases) and 0.12 M succinic acid buffer, pH 5 are added according to the table below (table 13). 100 µl sample of each tube is extracted and frozen down. Tubes are incubated at 50°C, 1400 rpm for 48 hours. All samples are boiled for 10 min and cooled on ice. 3 µl 10% sulfuric acid for every 100 µl volume is added to the tubes. Tubes are centrifuged at max speed for 10 min. For samples taken before incubation, 25 µl sample, 75 µl buffer, and 200 µl DNS is added to Eppendorf tubes. For samples taken after incubation, 10 µl sample, 90 µl buffer, and 200 µl DNS is added to Eppendorf tubes. All tubes are boiled for 10 min and afterward cooled on ice. 1 ml distilled water is added. 100 µl sample from each tube is added to specified wells in a 96 well microtiter plate. The plate's absorbance is read in a plate reader at wavelength 540 nm. The absorbance is read 4 times for each sample.

Table 13: Amount of buffer and undiluted enzyme volume added to each tube.

Content	enzyme	buffer
Control	NA	1500 µl
Own enzyme (0,431 FPA/ml)	50 µl	1450 µl
Novozyme 188	50 µl	1450 µl
CelliCtec	50 µl	1450 µl
CelliHtec	50 µl	1450 µl
CelliCtec II	50 µl	1450 µl
Celluclast	50 µl	1450 µl
Viscozymes	50 µl	1450 µl
Celluclast 4/5 Novozyme 188 1/5	40 µl 10 µl	1450 µl
Viscozymes 4/5 Novozyme 188 1/5	40 µl 10 µl	1450 µl
CelliCtec 1 + 2 and CelliHtec 1	16.6 µl of each	1450 µl

2.3.4.2 Enzyme harvesting

The supernatant used in this section derives from enzyme production 5 g-DM substrate flasks (2.3.2.4), which are harvested with 75 ml distilled sterile water (2.3.2.5). The supernatant contains an activity on 0.834 FPU/ml (2.3.3.1). The supernatant is called created enzyme cocktail.

2.3.4.3 Enzymatic hydrolysis and grinding

Two different enzyme cocktails (created enzyme cocktail and Viscozyme) and a control are tested in duplicates at 3 different incubation times.

3 g of fresh clover grass (Copenhagen university greenhouse, 14/4-2021), 15 ml 0.05 M sodium citrate buffer pH 5, and 5 ml distilled water is added to flat bottom falcon tubes. The Ultra-Turrax mill is used to grind the substrate using the following settings: 30 sec grinding at speed 11000 rpm, at least 1 min break where all solids are pushed to the bottom of the tube, 30 sec grinding at speed 11000 rpm, at least 1 min break where all solids are pushed to the bottom of the tube, 30 sec grinding, where the speed is slowly increased to 14000 rpm. 5 ml distilled water, 5 ml enzyme cocktail with 0.834 FPU/ml or 2 ml Viscozymes + 3 ml distilled water is added to each tube. Ampicillin is added to reach a concentration of 100 µg/ml. At incubation time 0, the mixture is directly filtered. At incubation time 4, tubes are incubated at room temperature for 4 hours, before filtration. At incubation time 24, the content of the falcon tubes is moved to 100 ml Erlenmeyer flasks and first incubated at room temperature for 4 hours and afterward incubated at 50°C, 150 rpm for 20 hours. After the incubations, the flask content is filtered.

2.3.4.4 Filtration and centrifugation

All the milled and hydrolyzed substrate is added to a 100 ml syringe with a 100 µm mesh filter at one end. The filter is scraped gently until all visible liquid has passed through. The liquid is squeezed through the filter using the plunger. Solids not passed through the filter are recovered and frozen down. The liquid that passed through the filter, is recovered into a 50 ml falcon tube through a funnel. Falcon tubes are centrifuged at 13880 g for 10 min and the supernatant is extracted into a syringe. The supernatant is filtered through a 0.45 µm filter into 15 ml falcon tube/1.5 ml Eppendorf tubes. Supernatant is frozen down. The centrifuged falcon tubes containing a pellet are frozen down to -80°C together with some freshly harvested clover grass. The frozen pellets and clover grass are freeze dried for 1 day with the settings: freezing chamber -50°C, tray temperature: 30 °C, pressure 0.5 mbar. The freeze dried material is stored in a desiccator. The clover grass is ground for 25 sec., and bead milled using the following settings: 600 rpm, 30 sec. milling, 30 sec. break, 15 rounds.

2.3.4.5 TS/VS

TS is performed on the pellets and the fresh clover grass. One sample is performed per pellet and TS is performed on fresh clover grass in triplicates. TS/VS is performed on bead milled clover grass in triplicates. TS on pellets is performed using small trays made of aluminum foil. The weight before and after the addition of the substrate is recorded with 0.001 mg accuracy. TS is performed on fresh clover grass using aluminum foil trails. The weight before and after adding the substrate is recorded with 0.0001 g accuracy. TS is performed on fresh clover grass using crucibles. The weight before and after adding the substrate is recorded with 0.0001 g accuracy. All samples are incubated at 105°C for 1 day and the weight is recorded. For VS, the crucibles are burned at 550°C for 3 hours and the weight is recorded.

2.3.4.6 BCA

BCA is performed according to the Pierce ™ BCA Protein Assay Kit protocol. Working reagent is prepared by mixing reagent A with reagent B in volume 50:1. Different dilution of BSA is made (0-2 mg/ml) using 0.9% saline solution. Controls consisting of diluted created enzyme cocktail and diluted Viscozymes are prepared. Control samples and samples containing the created enzyme cocktail are diluted 5 times using 0.9% saline solution. Samples containing Viscozymes are diluted 10 times using 0.9% saline solution. 3 x 25 µl sample or BSA standard are added to a 96 well plate. 200 µl working reagent is added to the sample or standard containing wells. The plate is covered with parafilm and first incubated at 37°C for 30 min and afterward incubated at room temperature for 5 min. The absorbance is measured at wavelength 562 nm with 3 measurements for each standard and sample.

2.3.4.7 Elemental analysis

Solids: Samples (one for each pellet, two for the milled clover grass) are prepared the same way as Elemental analysis (2.3.1.5). A urea sample is analyzed after every 10 samples.

Liquid: Controls consisting of diluted created enzyme cocktail and diluted Viscozymes are prepared. 20 µl supernatant or control is added to specific hard elemental analyze containers, with one sample for each. The hard containers are dried in a 40°C oven overnight and stored in a desiccator until they are analyzed. Analysis of the samples is performed the same way as the solid samples.

2.3.4.8 HPLC

Samples are added to HPLC vials (has already been filtered in the “Filtration and centrifugation”). Controls consisting of diluted created enzyme cocktail and diluted Viscozymes are prepared. The controls are centrifuged, and 1 ml is filtered through a 0.45 µm filter into HPLC vials. The HPLC vials are closed and analyzed using the method for HPLC sugar analysis pH 5-7 (2.3.1.6.2). One HPLC vial is prepared for each sample.

3 Results and discussion

In this section, results from each of the four sections of the experiments will be presented and discussed one at a time.

3.1 Substrate analysis

"Substrate analysis" is performed on four different substrates. TS/VS is used to determine the dry matter, organic matter, and ash content of the different substrates (2.3.1.4). HPLC analysis of the strong acid hydrolysis liquid fraction is performed to determine the sugar content of the different substrates (2.3.1.6.2). Elemental analysis is performed on the freeze dried and milled substrates to determine the protein content (2.3.1.5). TS/VS is performed on the strong acid hydrolysis solids to determine non-acid degrading lignin (2.3.1.4). All results are presented in the table below (table 14). The standard deviations are based on the triplicates for TS/VS and the biological duplicates for the rest.

Table 14: Content of the four different substrates used for the "optimization of enzyme production" section. Alfalfa press cake is also used for the "optimization of enzymatic hydrolysis". All numbers above the thick line are given in g/100 g of substrate. The number below the thick line is given in g/100 g-DM substrate.

	Alfalfa press cake	Clover grass press cake	Brewers spent grains	Wheat bran/ sphagnum peat
Moisture content (g/100g)	65.4 ± 0.7	79.6 ± 2.7	77.3 ± 0.6	47.4 ± 17.9
Dry matter (g/100g)	34.6 ± 0.7	20.4 ± 2.7	22.7 ± 0.6	52.6 ± 17.9
Organic matter (g/100g)	31.4 ± 0.7	18.6 ± 2.8	21.8 ± 0.6	49.1 ± 17.8
Ash (g/100g-DM)	9.3 ± 0.04	8.8 ± 0.09	3.7 ± 0.004	6.5 ± 0.14
Protein (g/100g-DM)	13.8 ± 0.08	13.6 ± 0.007	25.1 ± 0.01	11.9 ± 0.02
Cellulose (g/100g-DM)	31.7 ± 0.4	33.3 ± 0.3	35.9 ± 0.4	44.8 ± 0.8
Xylan (g/100g-DM)	28.8 ± 2.0	21.1 ± 0.05	31.5 ± 0.4	32.5 ± 0.7
Arabinan (g/100g-DM)	3.6 ± 0.006	2.1 ± 0.005	11.0 ± 0.8	11.8 ± 0.4
Klason lignin (g/100g-DM)	15.7 ± 0.4	15.8 ± 0.05	18.0 ± 0.07	17.1 ± 7.1

As mentioned, the dry matter/water content results originate from the TS/VS performed. For alfalfa press cake, DM is further determined three times in triplicates. If these determinations are included, the DM is determined to be 35.6 ± 0.03 instead of 34.6 ± 0.7. The reason multiple determination of DM is performed is due to the heterogeneity of the substrate used in the experiments. Therefore, a new DM determination is performed before each "optimization of enzyme production" experiment. The three other substrates are only used for one experiment and are thus only analyzed once. The heterogeneity of the clover grass is observed since all three measurements in TS/VS yielded different results (18.4-23.6 g/100g). For the wheat bran/sphagnum peat substrate, one of the measurements is much higher than the two other (42.2-73.3 g/100g) which is reflected in the high standard deviation. Since this substrate is created, it is expected to be more homogenous than the others, but this is not the case. Maybe the substrate is not mixed properly and thus is not homogenous. For

brewers spent grains, the weight for one of the crucibles is not recorded and thus is only performed in duplicates. The organic matter is based on the DM and thus has almost the same standard deviations.

The content of the DM for each substrate is presented in figure 8. Ash is calculated from the inorganic matter left after burning the crucibles. The standard deviations are much lower and thus much more reliable. The protein content is calculated from Elemental analysis results, using freeze dried and bead milled substrates. These substrates are thus in the form of powder and are assumed to be homogenous. This is also reflected in the low standard deviation. Due to the high precision of the analysis method, it is only performed in duplicates. To determine the amount of cellulose, xylan, arabinan, and lignin present in the substrates, strong acid hydrolysis, followed by HPLC and TS/VS is used together with the freeze dried and milled substrates. In the measurement of sugars, some of the tubes have an addition of a STAM solution, which allows the calculation of the recovery factor. To measure the klason lignin, the solids from strong acid hydrolysis are collected on a filter. Unfortunately, not all solids are collected, since part of it is on the glass container surrounding the filter. Therefore, the measurement is not recording 100% of the non-acid degrading lignin. When measuring the lignin, the crucibles used for brewers spent grains weight more after burning than before substrate addition. This is also observed in the filter control (empty filters), which has a weight difference from -0.0018 to -0.0033 g. Therefore, the lignin results are not reliable. The acid degrading lignin is also measured but the results are unreliable and thus not included in the report. In total, more than 100% of the substrate is recorded for three of the substrates. This shows that the methods or the calculations themselves are not 100 % reliable.

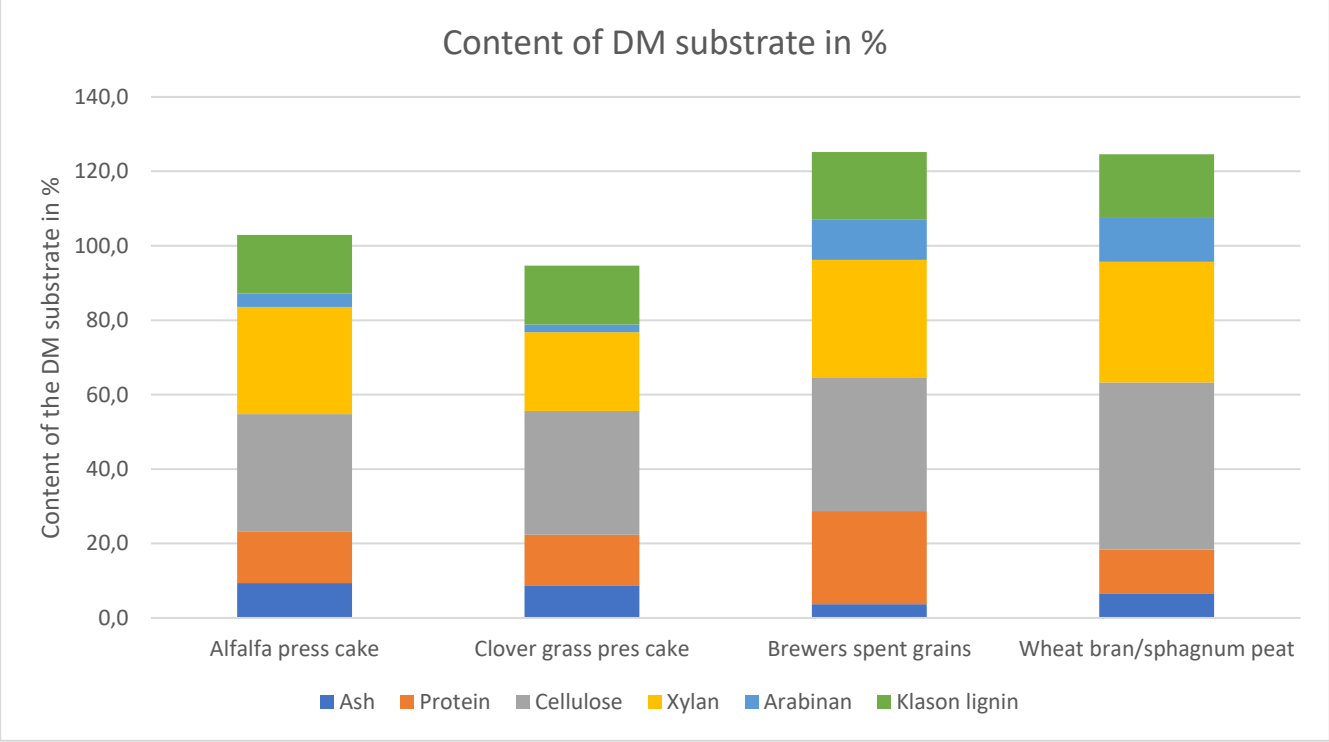


Figure 8: Graphical representation of the content of the different substrates in the percentage of the substrates dry matter.

Comparing the different substrates, the alfalfa press cake, and clover grass press cake are similar, with clover grass press cake containing less xylan. Brewers spent grains contain less ash, more protein, and much more arabinan compared with alfalfa press cake. Wheat bran/sphagnum peat contains less ash, more cellulose, and much more arabinan compared with alfalfa press cake. All substrates contain a similar amount of lignin.

Comparing the alfalfa press cake with literature, then the amount of ash measured correspond with the amount on fresh alfalfa grass. The amount of lignin is higher. The amount of xylan and arabinan measured is also higher than in fresh alfalfa grass. Hemicellulose is calculated by NDF-ADF. For cellulose, the measurement is again higher than fresh alfalfa grass. Cellulose is calculated by ADF-lignin (OECD 2015, Yilmazel et al., 2015). Compared with Santamaría-Fernández et al., (2018), this alfalfa substrate has a higher DM and organic matter content (table 2 (1.2.1)). It also has a higher total nitrogen content. In Santamaría-Fernández (unpublished), DM, protein content, and ash correspond more or less with the measured results (table 15). The amount of cellulose, xylan + arabinan, and the amount of lignin measured in this report on the other hand are all larger.

Table 15: Alfalfa press cake composition. NDF: neutral detergent fibers, ADF: acid detergent fibers, ADL: acid detergent lignin (Santamaría-Fernández, unpublished).

Component	Alfalfa press cake
Dry matter (%)	29.9 ± 2.9
Crude protein (%DM)	15.8 ± 1.8
Ash (%DM)	8.1 ± 1.6
NDF (%DM)	46.2 ± 4.9
ADF (%DM)	25.2 ± 1.6
ADL (%DM)	4.0 ± 0.77

Compared with a previous study (Rafique et al., 2019 (7^{th} semester project)) using the same substrate (substrate derives from the same plastic bag, measured a year previous (table 16)), the dry matter, organic matter, ash, and lignin is similar. The amount of cellulose, xylan, arabinan, and protein is higher in this project. Compared with this project, Rafique et al. (2019 (7^{th} semester project)) first pretreated the substrate with dilute acid pretreatment, measured hemicellulose with weak aid hydrolysis, and protein content is based on BCA measurements of the control supernatant after an enzyme extraction. A potential reason why fewer sugars are detected in Rafique et al. (2019 (7^{th} semester project)) is that multiple steps are performed on the substrate instead of performing SAH directly. The protein used in this report is measured using elemental analysis, which is a more reliable method and measures the nitrogen content directly on the substrate and not what is released doing enzyme production and harvesting (Rafique et al., 2019 (7^{th} semester project)).

Table 16: Substrate analysis (Rafique et al., 2019 (7^{th} semester project)).

Component	Alfalfa press cake
Dry matter (%)	33.7 ± 1.22
Organic matter (%)	30.6 ± 1.12
Cellulose (% DM)	24.9 ± 0.2
Hemicellulose (% DM)	18.9 ± 0.89
Insoluble Lignin (% DM)	18.3 ± 1.1
Ash (% DM)	9.04 ± 0.04
Protein (% DM)	9.7 ± 1.3

3.2 Optimization of enzyme production

First, in this section, the different spore suspension results are explained and discussed. Thereafter, the differences in the production for each experiment are explained and the potential influence of the results is discussed. At last, the results from the different assays are presented and discussed.

3.2.1 Spore suspension

The spore suspensions used are made from four (five) different PDA plates (figure 9). The green spores from *T. reesei* are present on plates 1, 3, and 4, while spores on plate 2 are only slightly visible. This may be due to the technique used to cultivate the plate. On plate 2, a lot of viable cells are added to the plate using the spread plate technique and thus the plate is more or less filled with spores/colonies. Therefore, the plate is overgrown before the production of spores can begin and thus fewer spores will be extracted. Compared with the other plates, then plate 1 (spore suspension consists of two plates, but only one is shown) is prepared with a 1 year old spore suspension. In this spore suspension, the number of viable spores is low and thus only a few colonies are produced on the plate (non-green circles). In plates 3 and 4, the streak plate technique is used, which creates a space where no spores are present and thus more room for the production of spores. From plate 3 it is also observed that in regions where different colonies meet, no spores are produced. Thus the best inoculation method is where spores are only added to the middle of the plate.

For all four spore suspensions made, the inoculation size is counted in a 0.2 mm Fuchs-Rosenthal counting chamber. The spore concentration in the different spore suspensions are 1: $2.68*10^8$ spores/ml (used for "time and light/dark" experiment, and "temperature/moisture" experiment), 2: $5.67*10^7$ spores/ml (used for "spore-substrate ratio" experiment, and "substrate" experiment), 3: $2.63*10^8$ spores/ml (used for "spore-substrate ratio" experiment), 4: $6.34*10^7$ spores/ml (used for enzyme production to produce supernatant for "optimization of enzymatic hydrolysis" and the created enzyme cocktail). The spore suspensions are only used for experiments until they are a few weeks old. The number of counted spores is high. It is expected to be around 1-30 million spores, not above 100 million. In literature, it is reported that *T. reesei* plates can yield a spore concentration on $2*10^6$ spores/ml (PDA media) to $1*10^8$ spores/ml (Malt extract agar) (Sathendra et al., 2019, Zhang et al., 2017). Thus, both spore suspension 2 and 4 is inside the expected range of spore concentration. The amount of liquid used in the spore suspension in these pieces of literature is not known, and thus a different amount of liquid may have been added. Both spore suspensions 1 and 3 contain more than twice the expected spore concentration and thus a mistake in the counting may have happened. When counting, non-viable spores, mycelium, and other components may be present in the sample, which can result in a higher spore counting than is present. Alternatively, counting of the spore forming units/colony forming units (SFU/CFU) can be used. In this method, the supernatant is diluted and plated, and the number of viable spores/cells can be counted at dilutions forming between 20-300 colonies. This method would be much more precise since only viable spores/cells are counted. Compared with using a counting chamber, this method takes much longer (counting chamber takes up to a couple of hours, SFU/CFU takes days) and more material (for dilution rows and plates). Therefore, only counting is performed, even though the results are much higher than expected.

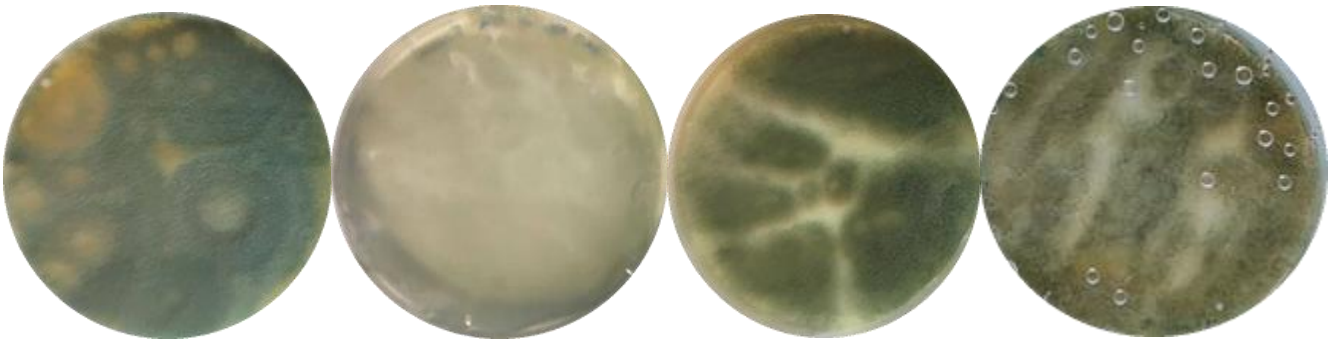


Figure 9: The plates used for spore suspension. From the left: Plate used for first, second, third, and fourth spore suspension.

3.2.2 Optimization parameters

In this section, the known mistakes/differences of each enzyme production are discussed together with the known mistakes in each of the four different assays used. In the end, results from each analysis method are analyzed and compared with different pieces of literature.

3.2.2.1 Time and light/dark

The initial moisture concentration in the different flasks is different since some of it is lost in the autoclavation and is not corrected afterward. All inoculation of the flasks is performed at the same time using the same inoculation volume from the same spore suspension. Incubation happens on a windowsill, where the temperature is not constant throughout the day. Also, of the incubated duplicates, number 1 is closest to the window, while number 2 is farthest away (figure 10). The flasks closer to the window receives more sunlight and thus the incubation in light does not have the exact same growth condition. Every 2-3 days, incubation ends for 2-4 flasks. At the same time, the flasks that are still incubated are shaken. This means that mixing of the flasks happens infrequently and the entire fermentation is very heterogeneous. Also, no conditions (pH, temperature, moisture content, oxygen level, and more) are checked under incubation and thus cannot be controlled. This exact method of incubation may be impossible to control and replicate again, but it is cheap and the less control, the less monitoring and power input of the incubations is needed. This specific setup does not need any sensors, artificial light, and a controlled temperature/environment. In general, solid state fermentation is hard/impossible to control but can be much easier to treat afterward due to the lower reaction volume compared with the same kind of setup for a submerged fermentation.

Figure 10: Cultivation flasks incubated on a windowsill. Biological duplicate 1 is closest to the window, while biological duplicate 2 is the closest to the camera. From left: control (no spore inoculation) with 14 days incubation (D14 C2), 14 days incubation, 12 days incubation, 12 days incubation in darkness, 9 days incubation, 7 days incubation, 7 days incubation in darkness, 5 days incubation, 2 days incubation (D2 1).

In the harvesting of enzyme part, the enzymes are harvested the same day the incubation ended. The exact time used for the incubation for harvesting changed slightly for each time and thus may have a small influence on the results but is always around 4 hours. After the harvesting incubation, all content of the flasks is added to 50 ml falcon tubes. The amount of substrate added to the falcon tubes changes a little for each time depending on how much is collected from the Erlenmeyer flasks (some substrate is stuck to the side of the flasks). The substrate is also more fermented and easier to get out of the flasks at longer fermentation times. Observing the substrate in the flasks, white mycelium is observed after 5 days of incubation. After centrifugation, the substrate is more compact in the samples incubated with spores for at least 5 days. The longer the incubation time with spores, the darker the supernatant becomes (figure 11).

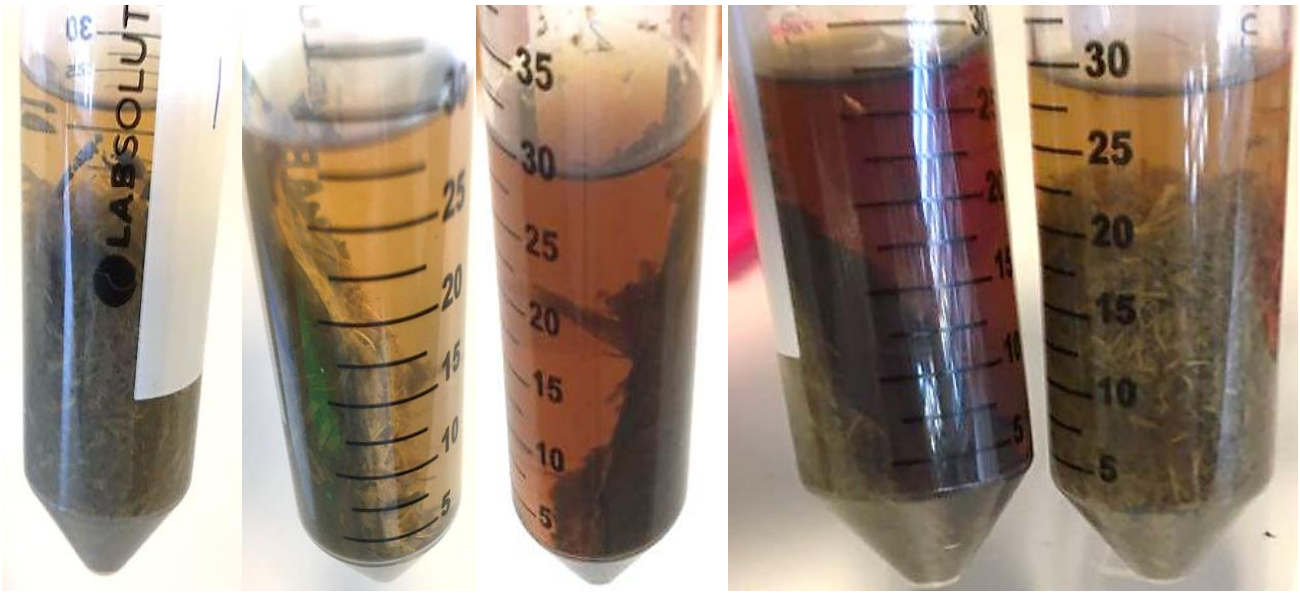


Figure 11: Content of flasks after centrifugation. From the left: after 0 days of incubation, after 5 days of incubation, after 9 days of incubation, after 14 days of incubation, where the tube to the right is the control.

3.2.2.2 Temperature and moisture

Substrate is dried in a 55°C oven before incubation, otherwise, moisture content of 65% is not possible to reach. By drying the substrate, it may have influenced the degradability and thus could influence the experiment. Moisture content is measured after autoclavation by loss of weight and additional sterile distilled water is added to each tube to correct moisture content. The flasks are placed at three different temperatures, one at around 20-22°C, one at room temperature (around 25°C), and the last in an incubator at 30°C. After five days of incubation, the temperature in the room on 20-22°C is increased to 25°C and thus fermented at room temperature for the next four days. Therefore, the results given at temperature 20°C in the different assays are not representative of growth at 20°C. Since all flasks are covered in aluminum foil, it is not possible to observe the growth. In the process of transferring the content of the flasks into falcon tubes, and after the centrifugation, it is observed that one of the biological duplicates of the flasks containing 65% moisture is similar to non-fermented substrate or at least substrate incubated for a couple of days. Therefore, it is expected that almost no enzymes are produced and that the standard deviation in the assays will be quite big for the 65% moisture results.

3.2.2.3 Spore-substrate ratio

Spore suspension 2 originates from the year old spore suspension. To check that no mutation with influences on the enzyme production has happened, spore suspension 3 is made from a cryostock and is added to flasks with the spore concentration of $5*10^6$ spores/g-DM substrate. The flasks are incubated on a windowsill using the same setup as "time and light/dark" experiment. After six days of incubation, growth is only observed in one of the duplicates containing $5*10^6$ spore/g-DM supernatant using spore suspension 2, $1*10^7$ spore/g-DM substrate, and $5*10^6$ spore/g-DM substrate using spore suspension 3. Therefore, it is expected that large standard deviations are present in the different assays for these three conditions. The rest of the flasks is not expected to produce lots of enzymes. Reasons behind the amount of growth observed at this point could be due to the spore suspensions used, the incubation place beside the window (experiment happened at the end of November and the windowsill is colder than the previous experiment), and that solid state fermentation is heterogeneous, which influence the growth. At the end of the incubation period, clear growth is observed in both duplicates of the previously mentioned flasks. When transferring the content of the flasks into flacon tubes, it is observed that the substrate with no visible growth is much harder to extract.

3.2.2.4 Different substrates

The different substrates have different structures, which can influence enzyme production. Alfalfa press cake and clover grass press cake have a similar structure with fibrous leaves. Clover grass press cake is darker than alfalfa press cake. Brewers spent grains are light brown and consist of small pieces that do not clump together. Wheat bran/sphagnum peat is black soil with some small white clumps in it (figure 12). After six days of incubation, clear growth is observed in all non-control flasks except for brewers spent grains. Due to the color and structure of brewers spent grains, there is a possibility that the growth is not visible. After eight days of growth, it is still not possible to observe any mycelium on brewers spent grains. Though, the incubated substrate does have a darker color than the control, which could indicate growth. After transferring all liquid into falcon tubes, they are frozen down, since it is not possible to centrifuge them the same day. The tubes are completely thawed before being centrifuged. By freezing the tubes, it may have changed how efficient the enzyme extraction is and thus may have influenced the assay results.

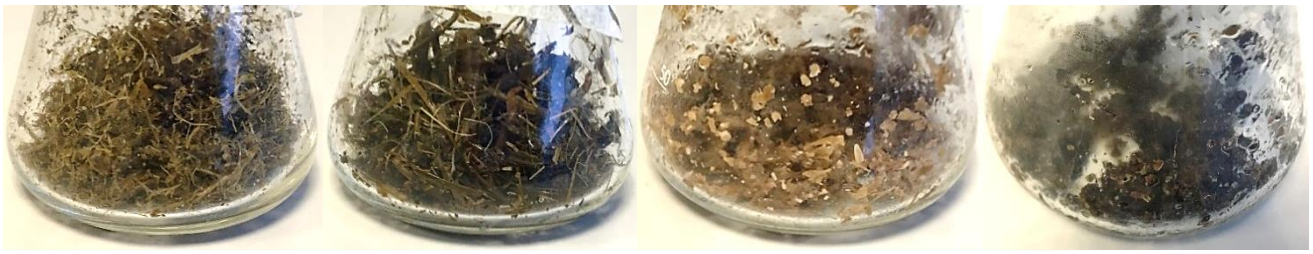


Figure 12: Erlenmeyer flasks with the four different substrate controls after 2 days incubation. From the left: alfalfa press cake, clover grass press cake, brewers spent grains, wheat bran/sphagnum peat.

3.2.2.5 FPA assay

FPA assay is used to determine the overall activity of an enzyme cocktail by degrading a cellulose filter. To find the specific activity, it is necessary to find the dilution of the enzyme cocktail that degrade 2 mg/ml of glucose in 1 hour. This FPA analysis is not following this principle. In this analysis, the specific activity is not found, but instead, the degradation of filters in 1 hour is compared. Therefore, the annotation for the FPA assay is in U/ml, not FPU/ml. This means that the activity may not be correct, but higher activity still yields a higher degradation of the filters and thus is used in the comparison. The FPA assay is used for each "enzyme production" experiment and the results are converted into U/ml (figure 13). U (units) is defined as µM/min of released sugar.

In the "time and light/dark" experiment, the best incubation time is determined to be 9 days. Due to the standard deviation for both day 9 and 12, the activity for these two days may be similar, but day 9 still yielded the highest overall activity. For the experiment fermented in darkness, a higher yield is observed. For the 7 day production, the yield increases by more than 25%. This means that fermentation in darkness enhances enzyme production. For the flasks fermented in darkness for 12 days, one of the flasks has very low activity compared with the other and thus the figure shows a large standard deviation (figure 13). When measuring the results in a spectrophotometer, each experiment is only read once. When reading the absorbance, an air bubble, or some kind of disturbance in the liquid or on the plate itself may have influenced the results, but it cannot be determined. This could have been prevented by pipetting each sample into more than one well each. Generally, between 7-12 days of incubation is needed to reach the optimal enzyme production.

In the "temperature/moisture" experiment, the best temperature is 30°C and the best moisture content is 80%. As mentioned in "temperature and moisture" (3.2.2.2), the incubation at 20°C is only incubated at 20°C for around 5 days and thus the result is not representative for lower temperatures. The standard deviation for both 20°C, 65%, and 80% are high which may indicate heterogeneous growth. Due to these high standard deviations, it is impossible to determine the exact best condition without performing this experiment again, but the condition used in the previous experiment showed promising results and are also the simplest to implement (no drying of substrate or use incubator). In this experiment, each absorbance is measured twice, and thus measure absorbance should be reliable.

In the "spore-substrate ratio" experiment, results show that at least $5*10^6$ spores/g-DM substrate needs to be added. As mentioned in the "spore-substrate ratio" (3.2.2.3), growth is only observed in three different flasks after 6 days of incubation. This is seen in the high standard deviation. The activity for the flasks with observed growth after 6 days is around 0.1 U/ml. The results for the supernatant created from spore suspension 3 is not present in the graph but is similar to the results for $1*10^7$ spores/g-DM substrate and thus it is not expected that

any mutations influencing the enzyme production have happened in the year old spore suspension. As with "temperature/moisture" experiment, each absorbance is measured twice.

In the "substrate" experiment, the substrates clover grass press cake and wheat bran/sphagnum peat produce the highest activity at around 0.25 U/ml. Alfalfa press cake and brewers spent grains have similar activity, which is less than half of the activity of the other two substrates. From these results, it can be concluded that alfalfa press cake is not the optimal substrate. Since wheat bran/sphagnum peat is a created medium, clover grass press cake is the most optimal natural substrate of these three to use for enzyme production.

In general, it may be needed to perform optimization for incubation temperature, moisture content, spore-substrate ratio, and fermentation in darkness again due to the high standard deviation.

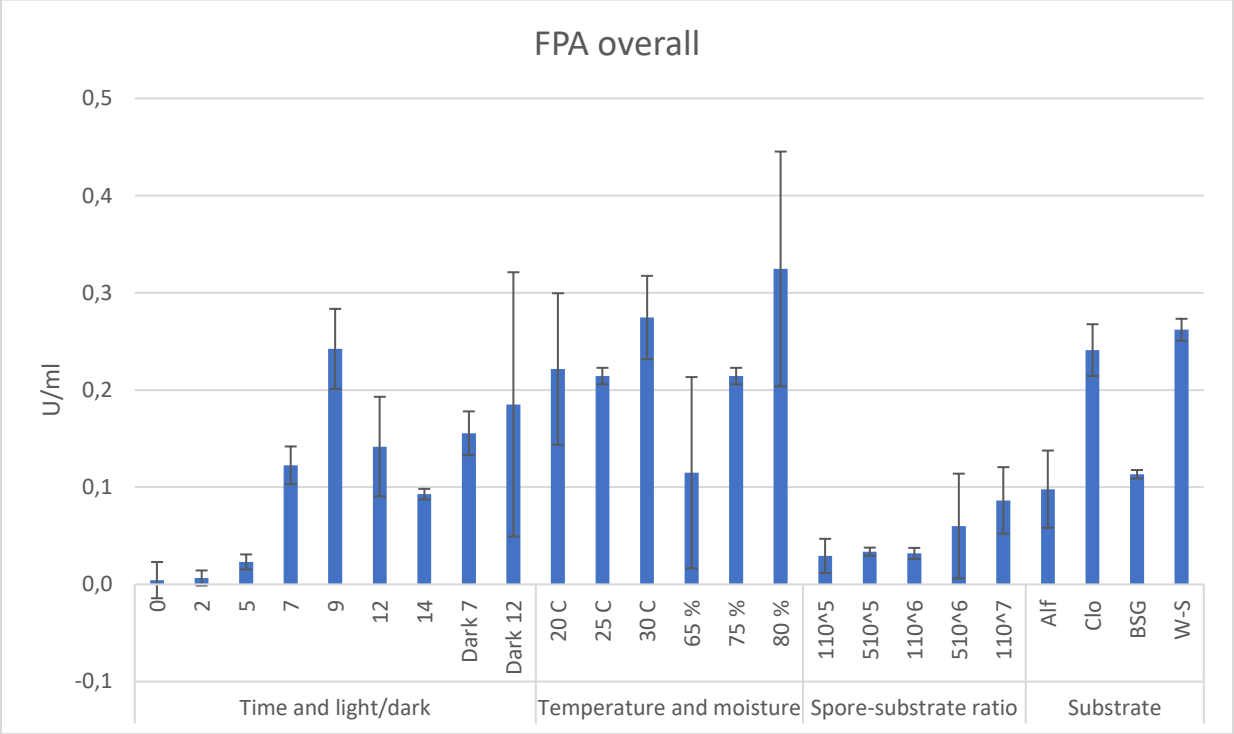


Figure 13: The overall enzyme activity for all enzyme production optimization parameters. "Time and light/dark" have the unit days, where Dark 7 + 12 is the flasks cultivated in darkness. "Temperature and moisture" either have the unit °C (temperature) or % moisture (moisture content). The result at 25°C and 75% is the same. "Spore-substrate ratio" has the unit spores/g-DM substrate (110^5 is $1*10^5$). "Substrate" indicates which substrate is used; Alf = alfalfa press cake, Clo = clover grass press cake, BSG = brewers spent grains, W-S = wheat bran/sphagnum peat.

Since the four different "optimization of enzyme production" experiments are performed at different times in the year, the flasks produced under the same conditions (9 days, room temperature, 75% moisture content, $5*10^6$ spores, and alfalfa press cake substrate) are compared (figure 14). The "temperature and moisture" experiment is performed in darkness and thus the activity may be higher than expected. It is observed that the closer to the new year the experiment is performed, the lower activity is achieved. This may be due to the temperature used under cultivation. The last experiment has a higher enzyme production than the one from the period 24/11 – 3/12. This may be due to where the incubation is performed. For the experiment at the period

24/11 – 3/12, the flasks are incubated on the windowsill, which is colder than the rest of the room. In the period 9/12 – 18/12, the incubation is performed on the table right beside the windowsill. Flasks still receive sunlight, but it is not as cold as the windowsill itself.

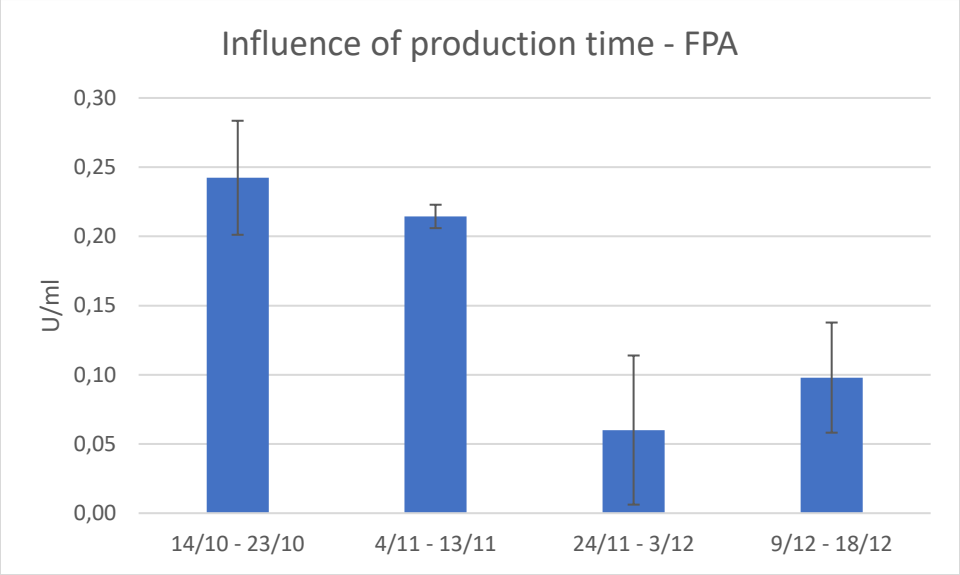


Figure 14: Comparison of the different production optimization experiments for FPA using the same growth conditions. "Time and light/dark" is 14/10-23/10 2020. "Temperature/moisture" is 4/11-13/11 2020. "Spore-substrate ratio" is 24/11-3/12 2020. "Substrates" is 9/12-18/12 2020.

In Rafique et al. (2019 (7^{th} semester project)), an overall enzyme activity on 0.18 U/ml is reached. This supernatant is created at conditions 10 days incubation, 0.51 g-DM alfalfa press cake, room temperature on a windowsill, $1.37*10^7$ spores/g-DM substrate, shake every 2-3 days. Comparing these results with the results gained in this report, the activities are similar. Kolasa et al. (2014) reached an overall enzyme activity for *T. reesei* on around 8 nmol glucose/ml/min. The conditions used to create the supernatant are: 10 days incubation, 16.4 g-DM wheat bran/sphagnum peat, 25°C, $3.05*10^5$ spores/g-DM, shake twice a day. The amount of glucose released for FPA in Kolasa et al. (2014) is very low, but the incubation time used is 26 hours, which may explain the low numbers. Novozyme 188 is added in the last 2 hours of incubation. Novozyme 188 will degrade all cellobiose, and thus the analysis is not 100% similar to the one used in this experiment. The activity reached in this report for wheat bran/sphagnum peat is 0.26 U/ml (mM/min/ml).

3.2.2.6 Endoglucanase assay

Endoglucanase assay is used to determine the endoglucanase activity by the degradation of azo-CM-cellulose. Azo-CM-cellulose contains a low weight blue dye, that is released from the substrate by endoglucanase. After centrifugation, the low weight dye will remain solubilized while the substrate will form a pellet. As a standard, endoglucanase extracted from *A. niger* with a known unit concentration is used to degrade the substrate and make a standard curve. The substrate and precipitant solution are both hard to pipette since they stuck to the pipette sides themselves. This is also a problem when pipetting into the wells and thus there may be a slight volume change, even though most is added. The standard curve has the unit U/ml. In each experiment, two wells are used per condition, and wells in "spore-substrate ratio" and "substrate" experiments are measured twice.

In the “Time and light/dark” experiment, the best incubation time is determined to be 9 days, which is the same as the FPA results. Compared with FPA, the standard deviation for the days 0-12 is low. In the Dark 7, 80% more endoglucanase activity is measured than after 7 days. There is no significant difference between day 12 light and dark. After the 9th day of incubation, the endoglucanase activity is the same or slowly decreasing. In the “temperature and moisture” experiment, the best temperature is 30°C and the best moisture content 80%, which is the same as the FPA results. As with FPA, both 20°C, and 65% have a high standard deviation. The standard deviation at moisture content 80% is much lower than for FPA. In the “spore-substrate ratio” experiment, at least 5*10^6 spores/g-substrate need to be added to have any endoglucanase activity, even if the standard deviations are high. The results for the supernatant created from spore suspension 3 are not present in this graph but the activity is around 65% lower than 5*10^6 spores/g-DM substrate and has a similar standard deviation. In the “substrate” experiment, wheat bran/sphagnum peat produce the highest activity at around 17 U/ml. The activity for clover grass press cake is around half of the activity of wheat bran/sphagnum peat, while the activity for alfalfa press cake and brewers spent grains is around half of clover grass press cake. Both Alfalfa press cake and clover grass press cake have a high standard deviation (figure 15).

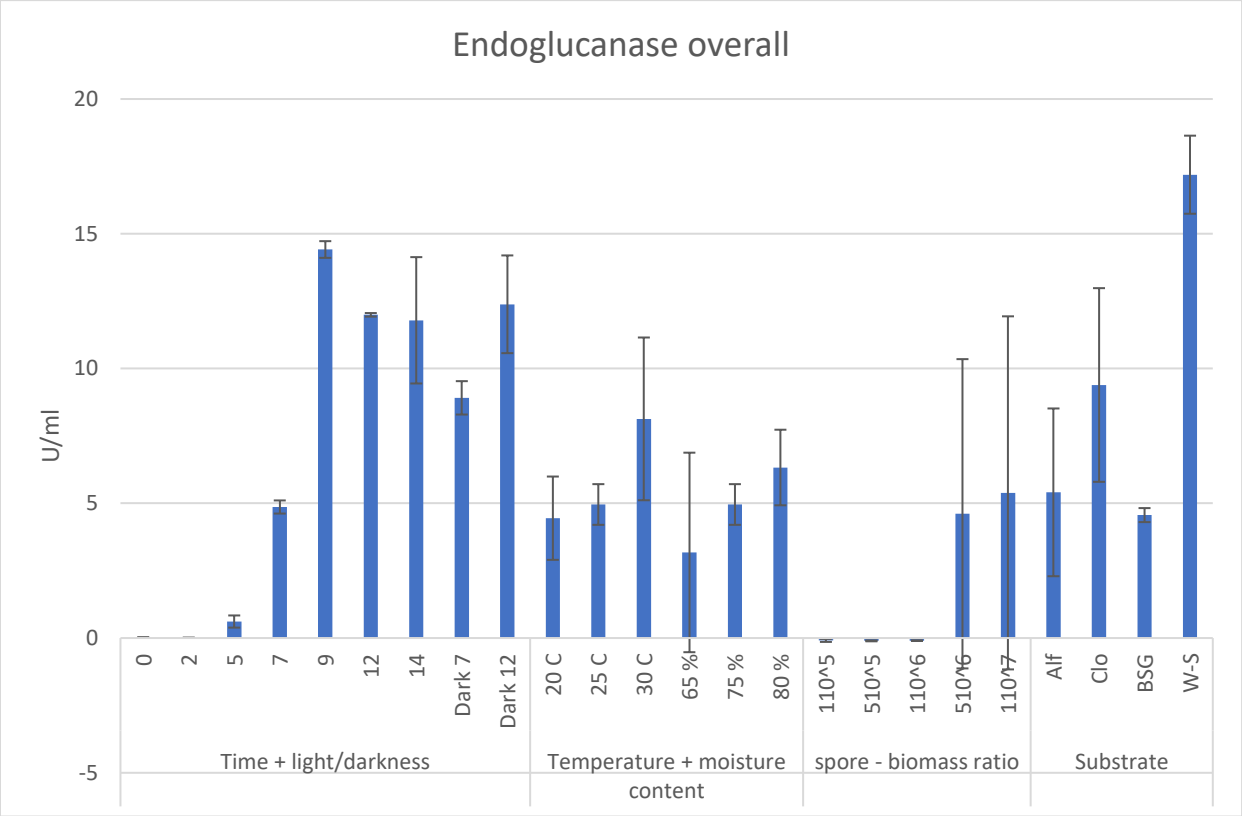


*Figure 15: The endoglucanase activity for all enzyme production optimization parameters. “Time and light/dark” have the unit days, where Dark 7 + 12 is the flasks cultivated in darkness. “Temperature and moisture” either have the unit °C (temperature) or % moisture (moisture content). The result at 25°C and 75% is the same. “Spore-substrate ratio” has the unit spores/g-DM substrate (110^5 is 1*10^5). “Substrate” indicates which substrate is used; Alf = alfalfa press cake, Clo = clover grass press cake, BSG = brewers spent grains, W-S = wheat bran/sphagnum peat.*

In Rafique et al. (2019 (7th semester project)), an endoglucanase activity on 2.5 U/ml is reached. This activity is much lower than what is measured at the optimal condition in this report, which is unexpected. The reason for the difference in activity is not known. Kolasa et al. (2014) reached an endoglucanase activity for *T. reesei* on 13 U/ml, using similar conditions as this report. The numbers gained in this report is around 3 times higher for wheat bran/sphagnum peat, but compared with some of the other experiment, the number is in the expected range.

3.2.2.7 Exoglucanase/CBH assay

Exoglucanase assay is used to determine the exoglucanase activity by the degradation of Avicel. With the addition of Novozyme 188 (β-glucosidase), the results can be analyzed through DNS quantification. Avicel is microcrystalline cellulose, that consists of short chains of cellulose. It is insoluble in water and dilute mineral acids. It is important to shake Avicel solution before each extraction of substrate to increase the homogeneity of the solution. The standard curve has the unit g/l glucose. In the "time and light/dark" experiment, each sample is only measured once. In samples for "temperature and moisture" experiment, two wells are used for each sample. For the last two experiments, two wells are used for each sample, which is measured twice. The reaction spanned a period of 26 hours, which may be a reason for a low activity (mU instead of U) compared with the FPA and endoglucanase assays.

In the "Time and light/dark" experiment, the best incubation time is determined to be 7 days, which is two days shorter than the FPA results, but due to high standard deviations in day 0-9 and for dark 7, it is hard to determine with certainty. Therefore, it is also impossible to determine if light/darkness affects exoglucanase production. The activity of exoglucanase is the same on day 0 as on day 12, and thus no activity is measured after 12 days of incubation. The measured sugars at day 0 may indicate that some Avicel is solubilized and thus measured in the DNS analysis. In the "Temperature and moisture" experiment, the best temperature is 30°C and the best moisture content 65%, which is the same temperature as the FPA results, but the opposite moisture content. Thereby, a higher exoglucanase concentration is reached at lower moisture content and 30°C. In the "Spore-substrate ratio" experiment, at least $5*10^6$ spores/g-DM substrate is needed before any exoglucanase activity is measured, which is the same as the FPA results. The standard deviations are still high and the flasks containing spores from spore suspension 3 have the same activity as the flasks containing $5*10^6$ spores/g-DM substrate. In the "substrate" experiment, wheat bran/sphagnum peat produce the highest activity on around 2 mU/ml, but due to the high standard deviation, it may be much lower. Clover grass press cake has no exoglucanase activity at all, which is surprising since it has the highest/second highest activity in FPA/endoglucanase assay. Alfalfa press cake and brewers spent grains has the same activity on around 1. In a control for clover grass press cake (no enzyme cocktail added), an exoglucanase activity on 4 mU/ml is reached, while no activity is measured for the other specific enzyme assays. An FPA activity on 0.1 U/ml is also reached. The reason behind this is unknown (figure 16).

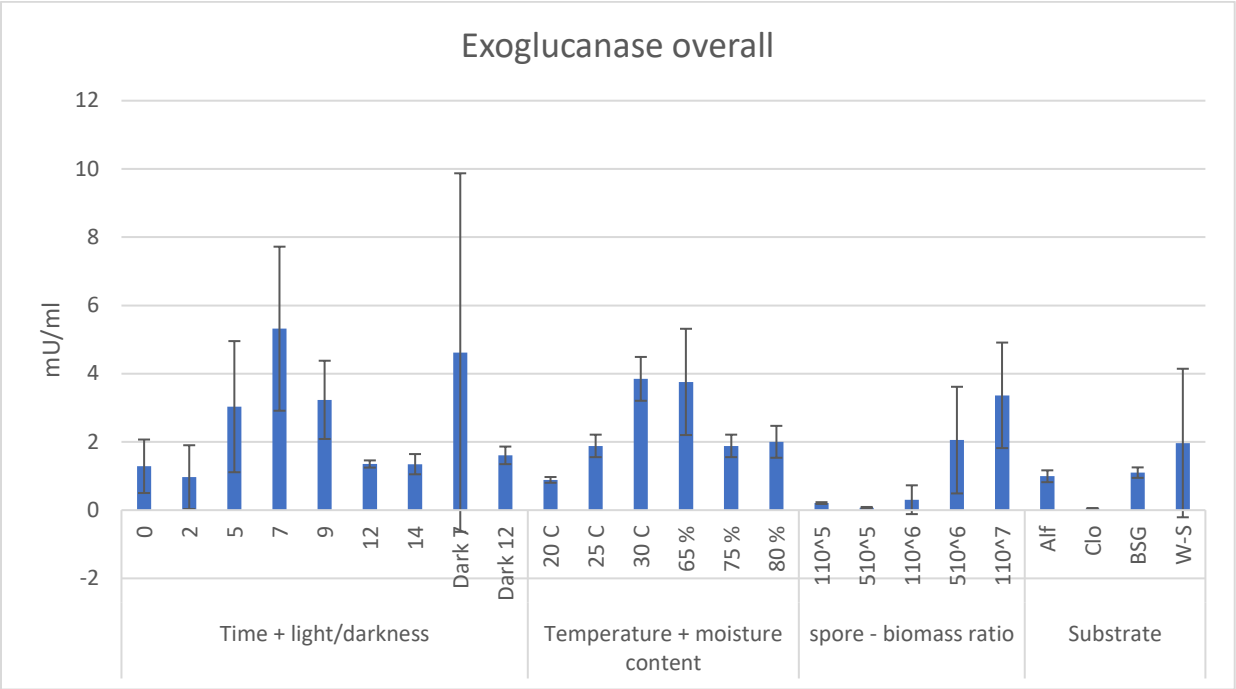


Figure 16: The exoglucanase activity for all enzyme production optimization parameters. "Time and light/dark" have the unit days, where Dark 7 + 12 is the flasks cultivated in darkness. "Temperature and moisture" either have the unit °C (temperature) or % moisture (moisture content). The result at 25°C and 75% is the same. "Spore-substrate ratio" has the unit spores/g-DM substrate (110^5 is $1*10^5$). "Substrate" indicates which substrate is used; Alf = alfalfa press cake, Clo = clover grass press cake, BSG = brewers spent grains, W-S = wheat bran/sphagnum peat.

In Rafique et al. (2019 (7th semester project)), an exoglucanase activity on 0.84 mU/ml is. This result is similar to the activity in the "substrate" experiment for alfalfa. Kolasa et al. (2014) reached an exoglucanase activity for *T. reesei* on 0.6 U/ml, using an incubation time of 2 hours with no extra addition of Novozyme 188. The results are much higher than measured in this report. A possible reason for this could be the much shorter incubation time. If the same absorbance is reached after 2 hours instead of 26 hours, then the results should be 13 times higher, and thus alfalfa press cake after 7 days may reach up to 0.07 U/ml, which is still 10 times lower than Kolasa et al. (2014).

3.2.2.8 β-glucosidase assay

β-glucosidase assay is used to determine the β-glucosidase activity by the degradation of pNPG, which dye the sample yellow. The standard curve has the unit mM pNP. Each sample from each experiment is added to one well. "Time and light/dark" and "temperature and moisture" experiments are measured once each, while the two other experiments are measured four times each. Therefore, the last two experiments should theoretically be more precise. The measurement of the samples is in the range of mU.

In the "time and light/dark" experiment, the best incubation time is in the range of 9 and 12 days, which is similar to the FPA results. All the standard deviations are also quite low except for day 5 and Dark 7. It seems that the enzyme activity is stable after reaching 9 days. Dark 7 has a lower enzyme activity than day 7, while day 12 and Dark 12 have similar activity. In the "temperature and moisture" experiment, the optimal temperature is 20-25°C and the best moisture content is 75%, which is different from the FPA results. Only 65% has a high standard

deviation. In general, the temperature does not have that much effect on the β-glucosidase production. In the "spore-substrate ratio" experiment, at least $5*10^6$ spores/g-DM substrate is needed before β-glucosidase activity is detected, which is the same as the FPA results. The standard deviations are still quite high. The results from the flasks containing spores from spore suspension 3 have the same activity as the flasks containing $5*10^6$ spores/g-DM substrate. Spore concentration between $1*10^5$ and $1*10^6$ has the same activity as fermentation day 0 and 2. In the "substrate" experiment, wheat bran/sphagnum peat produce the highest activity on around 33 mU/ml, which is almost the same activity as clover grass press cake and brewers spent grains, which is on 31 mU/ml. Alfalfa press cake has the lowest activity on 20 mU/ml, but with the high standard deviation, it may be similar to the other substrates. This means that *T. reesei* produces around the same amount of β-glucosidase on all four substrates (figure 17).

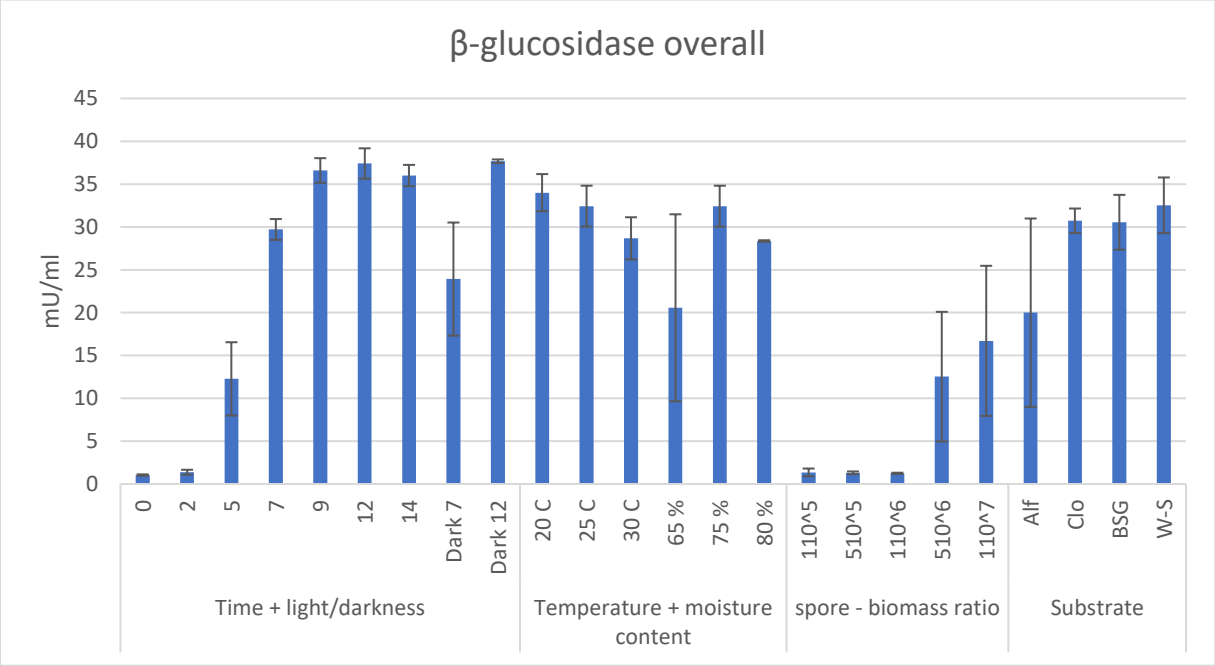


Figure 17: The β-glucosidase activity for all enzyme production optimization parameters. "Time and light/dark" have the unit days, where Dark 7 + 12 is the flasks cultivated in darkness. "Temperature and moisture" either have the unit °C (temperature) or % moisture (moisture content). The result at 25°C and 75% is the same. "Spore-substrate ratio" has the unit spores/g-DM substrate (110^5 is $1*10^5$). "Substrate" indicates which substrate is used; Alf = alfalfa press cake, Clo = clover grass press cake, BSG = brewers spent grains, W-S = wheat bran/sphagnum peat.

In Rafique et al. (2019 (7th semester project)), a β-glucosidase activity on 4.9 mU/ml is reached, which is almost 10 times lower than what is reached in this report. In Kolasa et al. (2014), the β-glucosidase activity for T. reesei is not possible to determine. The result is a little above 0 U/ml, which may be similar to the concentration gained in this experiment, but a number could not be determined and thus it is not known if the results gained in this report are similar. The experiment in Kolasa et al. (2014) is also only incubated for 15 min, not the 30 min used in this report.

3.2.2.9 Optimal parameters

In the three specific enzyme assays, the results show that different conditions affect the specific enzyme activity differently. Therefore, it is not possible to conclude the best conditions using these assays. Therefore, the optimal conditions are decided based on the FPA analysis and on which condition uses the least number of resources. The optimal time is 9 days. The incubation is performed in the light at room temperature since the flasks do not need to be covered and no incubator/oven is needed. If incubation in darkness is preferred, then it is possible to use a cupboard at lab scale. If the setup is used on a larger scale than laboratory, then incubation in darkness is preferable. For moisture content, 75% is used since no significant difference is present between 75% and 80%. For inoculum size, $5*10^6$ spores per g-DM substrate are used. Potentially more could have been used, but in an industrial process, the fewer resources needed, the more practical. Even though it is the substrate with the least U/ml, alfalfa press cake will continue to be used. A reason the different substrates are analyzed in the first place is that different pieces of literature claim *T. reesei* does not produce β-glucosidase. This experiment is thus included to check that this is true for multiple substrates. Compared with exoglucanase, the amount of β-glucosidase produced is more than 5 times higher, though compared with the FPA or endoglucanase produced, then the amount of β-glucosidase produced is between 5 to 100 times lower. Compared with the results in Kolasa et al. (2014), then the aspergillus species produces much more β-glucosidase and thus β-glucosidase production in *T. reesei* is inferior to the aspergillus species. In Rafique et al. (2019 (7^{th} semester project)), the β-glucosidase activity is the same for *T. reesei* and three different *Aspergillus* species, which is different from Kolasa et al. (2014).

In Idris et al. (2017), the optimal cellulose/substrate ratio is 1g/5g, optimal inoculum size is $3*10^6$ spores/g substrate, and optimal moisture content is 57.5%. Compared with this report, the substrate with the highest activity is the one with either the highest or lowest amount of cellulose/g-DM substrate. For the optimal inoculum size, the result is close to the optimal condition found in this report. The optimal moisture content is lower than this report, but since *T. reesei* can successfully be cultivated in moisture content ranging from 38-80%, then it is possible that the optimal condition is not between 65-80% (Idris et al. 2017). In Abdullah et al. (2016), the optimal temperature is 30°C, the moisture content is 60%, inoculum size $5*10^5$, and 7 days of incubation yielded greater enzyme production compared with 5 days. Optimal temperature and days are comparable with the results obtained in this report. For moisture content and inoculum size, they are lower than the results obtained. In Singhania et al. (2006), the optimal temperature is 30°C and the optimal moisture content is 37.5%. While the temperature corresponds with this report, the moisture content is much lower. This could be due to a local maximum, where *T. reesei* can produce great yields at multiple moisture content. It could also be the substrate in question. For fresh substrate, it is an advantage to use a substrate that is not dried to reach optimal moisture content, while it is an advantage to use dried or synthetic substrates without additional water. In Maurya et al. (2011), the optimal conditions are 6 days incubation, 70% moisture content, and an incubation temperature of 30°C. Compared with this report, the moisture content and temperature are similar, while the incubation time is lower. This may be due to the different substrate or possible other conditions, but it is not known.

While results are generated by changing one parameter at a time, the method used in this report does not include the interactions between each parameter. Potentially an incubation temperature of 30°C could lower the optimal incubation time. Testing all parameters at once would be impossible since it would include at least 2520 single experiments using the different parameters (7 times parameters, 2 light/dark parameters, 3 temperature

parameters, 3 moisture content parameters, 5 spore-substrate parameters, 4 different substrate parameters). Instead, a mathematical analysis method could be used such as the Plackett and Burman method (1.10). Using this method, lots of different parameters can be analyzed at once and the parameter with the largest effect can afterward be analyzed further. This is also a great technique when multiple of the parameters have little effect since they can be discarded as unimportant. If this method is used, it should identify the parameters with the most effect on the enzymatic activity and thus which parameters are the most important in the incubation. Some of the parameters not tested in this report are the size of the substrate, addition of minerals/supplementary nutriment, addition of lactose, and pH. The reason these parameters are not analyzed is due to using the simplest method and the difficulty of maintaining a specific pH.

3.3 Optimization of enzymatic hydrolysis

First in this section is the results from the FPA analysis. Thereafter, the difference in each enzymatic hydrolysis experiment is explained and the potential influence it can have on the results. Afterward are the results from the DNS analysis. In the end, the optimal parameters are explained and compared with pieces of literature.

3.3.1 FPA analysis

The supernatant used for the enzymatic hydrolysis is produced from Erlenmeyer flasks containing 5g-DM substrate. Two separate batches containing three Erlenmeyer flasks are prepared, using spore suspension 4 as inoculum.

First batch: After 6 days of incubation, growth is observed in all flasks. Due to the total volume, the content of each flask is transferred into two falcon tubes containing the same weight. After both centrifugations, the same volume of supernatant from each centrifuged falcon tube is mixed in new falcon tubes, which all should contain the same activity. Thereby, only one of the mixed supernatants is used for the FPA analysis. In the FPA analysis, the samples are diluted to find the dilutions closest to the activity reaching 2 mg/ml/h glucose. These dilutions are plotted in a semilogarithmic scale and the dilution for reaching a concentration of 2 mg/ml/h is calculated. This dilution is added to formula 2 to find the FPU (Adney and Baker, 1996). The activity calculated is 0.861 FPU/ml. The FPU/ml calculated is more than 3 times higher than the calculated FPA U/ml from the "optimization of enzyme production" (3.2.2.5). Part of this can be explained with the water added in the "enzyme harvesting", where only 85 ml sterile water is added instead of 125 ml (17 ml/g-DM substrate instead of 25 ml/g-DM substrate). The rest can be explained in the difference in the calculations and that the FPA method is only reliable at measuring activity around the concentration of 2 mg/ml glucose.

$$Formula\ 2\text{:}\ \frac{0.37\ \mu mol/minute - mL}{dilution\ needed\ to\ reach\ a\ degradation\ of\ 2\frac{mg}{ml}\ glucose} = FPU/ml$$

Second batch: After 4 days of incubation, growth is observed in all flasks. Falcon tubes are frozen down before centrifugation and are thawed the next day. Doing the thawing process, some supernatant is lost and thus reducing the volumes. All tubes are centrifuged at 1000 rpm for 1 second, where even more liquid is lost. The tubes are weighed again, and the centrifuge is dried. Afterward, all tubes are centrifuged two times. By freezing the tubes, some supernatant is lost but it is not expected to affect the FPU. The same mixing method of the supernatant as in batch 1 is used. The activity calculated for the second batch is 0.431 FPU/ml. This is around half of the activity of the first batch. While growth in this batch may have been observed after four days, it is not a lot and thus may not have produced the same amount of FPU.

It is found out that the 0.37 µmol/minute-ml used in formula 2 may not be the true value needed to calculate the FPU/ml. This value is calculated in formula 3. The new value is 4.63 µmol/minute-ml instead of 0.37 µmol/minute-ml, since the supernatant dilution volume is 0.04, not 0.5 ml as in this protocol. This increases the activity of the supernatants 12.5 times. The procedure used in this report is similar to the protocol, just using reduced volume, and is thus unknown if 4.63 µmol/minute-ml should be used for future calculations (Adney and Baker, 1996). When performing the experiments, the value 0.37 µmol/minute-ml is used and thus is continued to be used in this report even though that the activity is 12.5 times higher based on the calculation.

$$Formula\ 3\text{: } \frac{(2.0\ mg\ of\ glucose\ /\ 0.18016\ mg\ glucose/\mu mol)}{(supernatant\ dilution\ volume\ (ml) * 60\ \mathrm{min})} = \mu\mathrm{mol/minute} - \mathrm{ml}$$

3.3.2 Time/unit

The substrate used for the hydrolysis experiments is not autoclaved or otherwise pretreated except for the screw pressing process. To prevent contamination, a concentration of 100 µg/ml ampicillin is added. A total volume of 25 ml is used at the start of the incubation. The buffer pH is measured to be 5 using a pH indicator strip. Using a pH meter, the pH is measured to be 4.8. After around 0, 1, 3, 24, 48, 72, and 96 hours of incubation, samples are extracted and frozen down. The sample size is 250 µl to prevent more than 10% of the total volume from being removed. The samples consist of liquid and degraded substrate. Extracting samples is also problematic due to substrate blocking the pipette tips. Due to the shaking of the incubator used, it is assumed that the reaction is homogenous and thus extracting samples from one place should be representable. When sampling before incubation, the flasks are shaken to mix content and is thus also assumed homogenous. When sampling after 72 hours, the samples are not frozen down and thus are stored at room temperature. Therefore, the results for the samples after 72 hours are not used since they are not reliable. To terminate the enzymatic hydrolysis, samples are incubated at 99°C to inactivate enzymes. Before performing DNS analysis, the samples are centrifuged to remove the solids from the supernatant.

3.3.3 pH

Using a pH meter, the pH of the buffer is adjusted to the correct pH (4.5-5.5). Due to the volume of the pH adjusted buffer, the reaction volume is reduced to 15 ml and the substrate is reduced from 1 g-DM to 0.6 g-DM. Due to the same ratio between volume and substrate, it is not assumed to affect the results. Samples taken before incubation are frozen down. Under incubation, the content of enzyme blanks (contains no substrate) for pH 5 and 5.5 are lost and thus results may be affected. Based on the results from “time/unit” experiment, the substrate blanks (contains no enzyme) have the highest number of released sugars (above 1 g/L reduced sugars) of the two blanks, and thus affect the hydrolysis the most. The enzyme blank on the other hand contains less than 0.2 g/L sugars and is thus assumed to contribute the least to the reduced sugar content in the samples.

3.3.4 Temperature

Samples taken before incubation are frozen down. After 2 hours of incubation, it is discovered that the incubator at 40°C is not shaking and thus mixing for this incubation first starts two hours later. This may affect the results.

3.3.5 Brown juice

Brown juice is created in the green biorefinery concept after protein precipitation and centrifugation. This specific brown juice is created by heat precipitation. The brown juice has a pH of 5.5. The entire goal with this optimization is that valorizing a “waste stream” from another part of the concept reduces the number of

chemicals needed for the process and thus makes it more sustainable. To minimize the amount of brown juice used and to dilute it, the brown juice is diluted 20 times. It is possible that the brown juice can be diluted further but it has not been researched. To minimize the difference between the brown juice and the buffer, the buffer's pH is increased to 5.5. Samples taken before incubation are frozen down.

3.3.6 DNS analysis

To measure the effect of enzymatic hydrolysis, the samples are analyzed with DNS to measure the reduced sugars created under hydrolysis. The more reduced sugars are measured, the more substrate is degraded. While DNS is less precise than HPLC and it is not possible to distinguish which sugar molecules are present, it is still used. In enzymatic hydrolysis, more than 150 samples are analyzed, not including the standards. Assuming each DNS reaction costs 0.1 DKK (only for the reaction itself, not for the equipment used), then all analysis performed costs around 23 DKK. If an HPLC analysis is performed instead, then the results may have been much more precise, but using the same standards will cost around 2300 DKK. DNS is also much faster to analyze. Therefore, the less precise method is used since it is still possible to compare the results. It is also possible to measure the protein content in the supernatant/sample, but it is not done to reduce the amount of analysis performed. In the "temperature and units" experiment, each sample is only added to one well but measured two times. In the "pH" experiment, the standard curve sample is added to one well, while the normal sample is added to two wells. Absorbance is measured twice. In the "temperature" and "brown juice" experiments, each sample is added to two wells and absorbance is measured twice. The result from each experiment is expressed as the % of the released sugars present in the substrate. Since DNS analysis cannot distinguish between the reduced sugars, it is not known if only cellulose is hydrolyzed, or if some hemicellulose (mainly xylose) is also hydrolyzed. Therefore, the results in the "Time/unit" experiment are given in % of the total released sugars (both cellulose and hemicellulose (figure 18)). The other experiments are given in both total released sugars and if only cellulose is degraded (figure 19). There is around the same amount of cellulose and hemicellulose present in alfalfa press cake (3.1). For all experiments, the standard curve is based on glucose. Therefore, the measurement of mixed sugars may yield a different absorbance at the same concentration.

In the "Time/Unit" experiment, the optimal degradation of substrate happens when at least 7.5 FPU enzymes/g-DM substrate is added (figure 18). The fastest degradation of the substrate happens in the first 3 hours, whereafter the degradation is slowed. After two days, the degradation is slowed even further. Since only the reduced sugar concentration is measured, it is not known if the protein concentration changes over time, and thus it is hard to determine the optimal incubation time. If hydrolysis is implemented into the green biorefinery concept, then the length of the hydrolysis is important. The shorter the process, the easier it is to implement. Therefore, potentially 3 hours of hydrolysis can be used. In this report, a longer incubation time is chosen. Using hydrolysis for a day, around 8% of the total sugars are released using 7.5 FPU enzymes/g-DM substrate, which corresponds to around 16% of the glucose content. While 7.5 FPU enzymes/g-DM substrate is the optimal enzyme loading, the low activity in the FPA (first batch 0.861 FPU/ml, second batch 0.431 FPU/ml) indicates that large volumes of the supernatant are needed. Due to the necessary volume, the enzyme concentration used in the rest of the experiments is 5 FPU enzymes/g-DM substrate which releases 5% of the total sugars after 1 day.

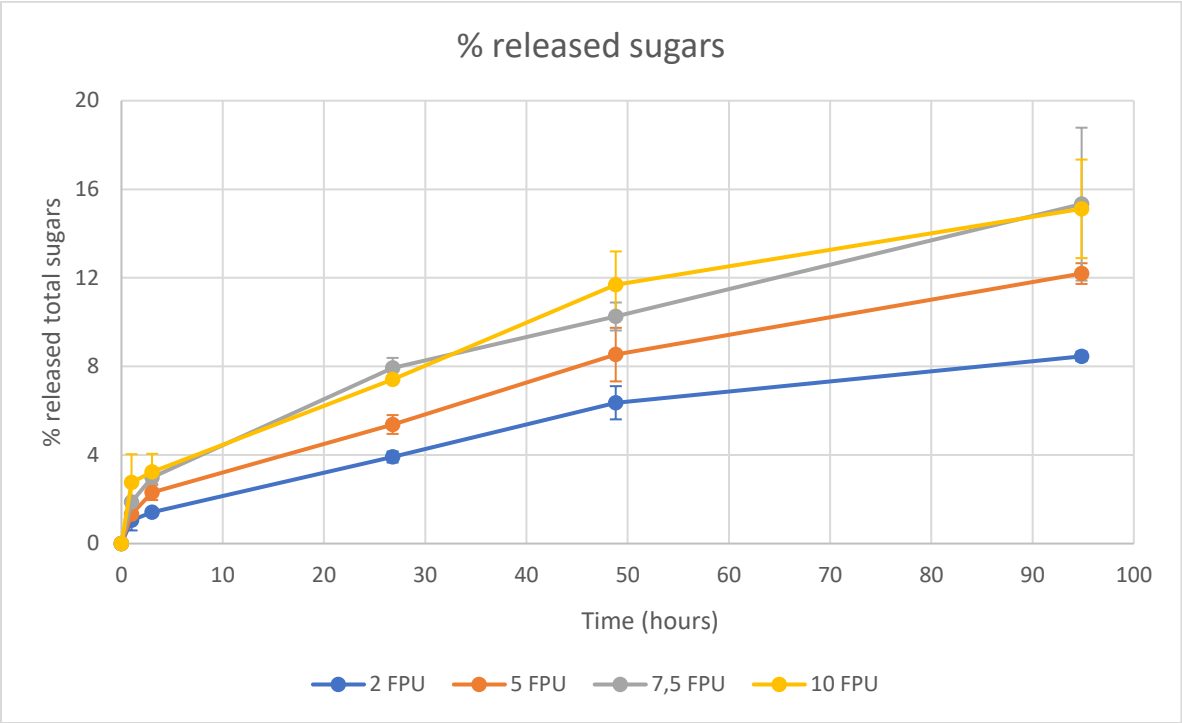


Figure 18: Optimization of the enzymatic hydrolysis in the experiment "time/units". Blue represents 2 FPU/g-DM substrate, Orange represents 5 FPU/g-DM substrate, Gray represents 7.5 FPU/g-DM substrate, Yellow represents 10 FPU/g-DM substrate.

In the "pH" experiment, the optimal pH on the graph is pH 5 (figure 19). When looking through the absorbance measured for pH 5, one of the wells measures a much higher absorbance than the others. If this high number is removed, then the % released sugars is similar to the other three pH measurements (13.4 ± 2.9 % released glucose) and the standard deviation lower. Therefore, it is expected that the difference in the graph is due to the absorbance measurements, not because of a difference in the hydrolysis. It can thus be concluded that pH does not influence hydrolysis in the pH range of 4-5.5. The standard curves used in this experiment span from a pH of 4-5.5. There is no difference in the absorbance and thus it can be concluded that pH in this range does not influence the DNS coloration and the absorbance. In the "temperature" experiment, 50°C is the optimal temperature for hydrolysis. The other temperatures may yield close to the same released sugars, but 50°C has the highest yield. In the "brown juice" experiment, the yield of released sugars is the same. pH is also measured before and after hydrolysis, which is also the same. Thus, it is successful to use the 20 times diluted brown juice at pH 5.5 as a buffer for hydrolysis. If the process is implemented into the green biorefinery concept, then it is more sustainable to use brown juice as a buffer, than using a chemical based buffer.

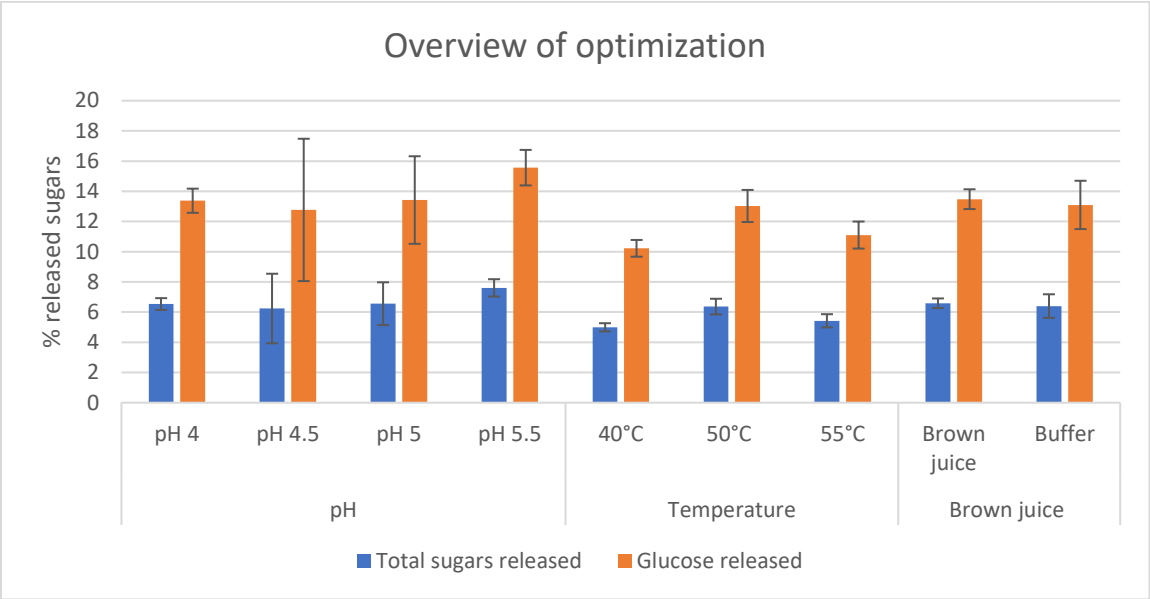


Figure 19: Optimization of the enzymatic hydrolysis in the experiments "pH", "temperature", and "brown juice". Blue blocks represent the % of released sugars if both cellulose and hemicellulose is degraded. Orange blocks represent the % of released sugars if only cellulose are degraded.

In Chen et al. (2008), an optimal enzyme loading on 20 FPU/g-substrate is found using pretreated maize straw as substrate. This is almost 3 times higher enzyme loading than this report found. This may show that the calculation of FPU in this report is done wrong and is thus much higher. It could also be due to the different substrate used. By pretreating the substrate before hydrolysis, the structure is easier to degrade. This is reflected in the hydrolysis yield, which is around 80% after 48 hours, compared with the 12% achieved in this report. Another difference is the possible cellobiose inhibition. While it is not analyzed in this report, in Chen et al. (2008), a 10 percentage point increase in the hydrolysis yield is observed when extra β-glucosidase is added. This decreases cellobiose inhibition. At 7 FPU/g-substrate, a 30% hydrolysis yield is achieved after 24 hours (Chen et al., 2008). Compared with this report, then only 8% is reached after 24 hours for 7.5 FPU/g-DM substrate. In Vásquez et al. (2007), the optimal temperature for conversion of cellulose to glucose is at 42°C, while the highest glucose concentration is achieved at 47°C. The temperature to reach the highest glucose concentration correspond with the optimal temperature found in this report, and thus a temperature around 50°C is the best. A solid loading of 10% yields the highest glucose concentration (Vásquez et al., 2007). This solid loading is higher than this report, but since Vásquez et al. (2007) did not measure at higher solid loading, then it is not known if it is the optimal solid loading. An enzyme loading around 25 FPU/ml yields the best results (Vásquez et al., 2007). This enzyme loading is much higher than this report and again supports that the calculation performed in this report is properly wrong. In Haldar et al. (2018), the same temperature and the same pH range as this report is reported as the optimal conditions. Again, a higher enzyme loading than 7.5 U/g-DM substrate is reported as the optimal one.

If a more statistical analysis method is used, then pH and the different buffers will not be further analyzed. This is due to their low influence on the hydrolysis results. This can change which parameters are analyzed. Other potential parameters that could have been researched are the substrate size, pretreatment of substrate,

agitation, addition of Novozyme 188/β-glucosidase to reaction, and substrate concentration. Samples could also be analyzed using other methods than measuring the number of reduced sugars present in the samples.

The optimal condition found is pH in the range of 4-5.5, usage of either 20 times diluted brown juice or using 0.05M sodium citrate buffer, incubation temperature on 50°C, and enzyme loading on 7.5 FPU/g-DM. Time is depending on the length of the experiment, but most of the hydrolysis happens in the first 3 hours.

3.4 Effect of enzymatic hydrolysis

First in this section is the "test for the best commercial enzyme" which analyzes which of the available commercial cellulase enzyme cocktails are the most efficient in degrading alfalfa press cake. Next is the FPA analysis. Then is the process of processing the fresh substrate. Thereafter is the different analysis method used and in the end a comparison of the results.

3.4.1 Test for best commercial enzyme

This test is performed to find which of the commercial enzyme cocktails degrades freeze dried and milled alfalfa press cake most efficiently. The most efficient enzyme cocktail is used in the later experiment as a comparison to the created enzyme cocktail. Fresh substrate is not used in this experiment. The fresh substrate is used right after it is harvest for the later experiments. It is therefore impossible to perform this experiment first. Potentially, fresh substrate harvested earlier could be used, but the substrate still needs to be freeze dried and milled first. Alfalfa press cake is the substrate used for the optimization experiments and therefore it is chosen to be used in this experiment.

This is a substrate degradation test, which tests the different enzyme cocktails' ability to degrade and subsequently release reduced sugars. While FPA can also be used to find an activity, only cellulases are tested, not other sugars that may be present in the enzyme cocktails. Some enzyme cocktails also have low FPA activity even though their true activity is much higher. When taking samples before incubation, some of the samples are stuck inside the pipette tip (one of CelliCtec and one of Celluclast 4/5 + Novozyme 188 1/5). To remove the sample, 100 µl buffer is added, thus diluting these samples. This should not influence the results. In one of the Novozyme 188 reactions, a drop of buffer is lost which makes the reaction slightly more concentrated, but it should not affect the results much. Two of the mixed reaction are created to find if the addition of β-glucosidase increases the degradation of the substrate. The last mixture is made to check if a mixture of CelliCtec and CelliHtec degrades more sugars. The number of enzymes added to each reaction is based on volume since the activity of the enzyme cocktails is not known.

The enzyme cocktail with the highest % released sugars is Viscozyme® (figure 20). Changing 1/5 of the volume with Novozyme 188 does not yield a greater % released sugar and does thus not enhance the degradation of the substrate. The next best enzyme cocktail to degrade the substrate is CelliCtec 2. Celluclast 4/5 and Novozyme 188 1/5 also degrades the substrate quite efficiently. In Gabiatti Jr. et al. (2020) FPA is used to measure the activity of Celluclast and Viscozyme®. Celluclast activity is measured to 75 FPU/ml, while Viscozyme® is measured to be 10 FPU/ml. In Solano (2011), degradation of oil palm open fruit bunch using 1 ml Celluclast or Viscozyme® is tested, where Celluclast yields 27% fermentable sugars, while Viscozyme® yields 9% fermentable sugars. Both these articles have the opposite results compared with this study. A reason behind the difference in FPA could be that the activity of Viscozyme® is not measured correctly. Another reason behind the difference in this study compared with the literature could be the age of the commercial enzymes. The age of Celluclast is not known,

but CelliCtec, CelliHtec and Novozyme 188 are all from 2009, CelliCtec 2 from 2015, and Viscozyme® is from May 2020. Comparing the results from Viscozyme® with the enzyme cocktail produced in this report (created enzyme cocktail, 0.431 FPU/ml), then Viscozyme® degrade 5.25 times more substrate than the produced enzyme cocktail. To equalize the degradation, more volume of the created enzyme cocktail is added corresponding to this difference.

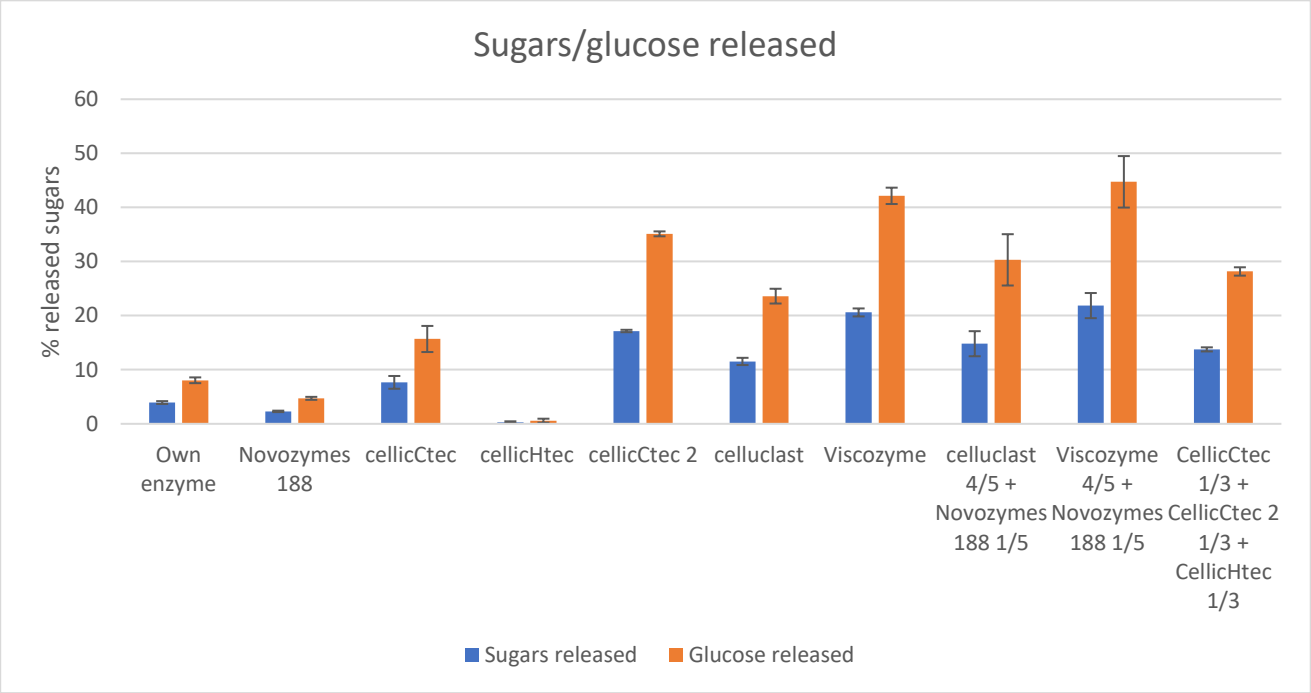


Figure 20: % of the released total sugars or the released glucose in the degradation.

3.4.2 FPA analysis

The supernatant used for the enzymatic hydrolysis is produced from Erlenmeyer flasks containing 5g-DM substrate. A batch containing four Erlenmeyer flasks is prepared, using spore suspension 4 as inoculum (third batch).

Third batch: Less sterile water is added to this batch compared with the first and second batch and is thus more concentrated. 1 hour and 30 min after the start of enzyme extraction it is discovered that the shaking is at 1 rpm, not 200 rpm. Thus, the flasks are only shaken at 200 rpm for 2 hours and 30 min instead of 4 hours. Extraction, centrifugation, and mixing of the different supernatant are all performed the same way as batch 1 (3.3.1). Using formula 2, it is calculated that the activity of the supernatant is 0.834 FPU/ml. This activity is around the same as in the first batch, even though the extraction volume is lower.

3.4.3 Processing of fresh substrate

The substrate used in this part is fresh clover grass, which is harvested few hours before this analysis. The clover grass consists of long grass leaves with a bit of clover in between. When working with the substrate, it is hard to homogenize it. To try, all grass leaves are broken into pieces that are up to 5 cm long. The grass and some clover are mixed in flat bottomed falcon tubes. The substrate consists mostly of the grass and only a small fraction of

clover. When milling the substrate, it is observed that it is hard to grind the grass leaves and thus some longer pieces of leaves are still present in the mixture. Grinding of one sample takes around 5-10 min. This makes measuring of time for incubation hard. Thus, the incubation is assumed to start when Viscozyme®/created enzyme cocktail/distilled water is added and end when the content is filtered through a mesh filter. The substrate is not pretreated or sterilized before the grinding and enzymatic hydrolysis. Enzymes are not added before grinding due to the incubation time, the state of the substrate and to make sure that the grinding does not affect the enzymes themselves. Of the three different incubation times, the substrates at incubation time 0 and 4 hours are green, the same color as the fresh clover grass (figure 21, A). For the substrate incubated at 24 hours, the substrate is a brown color (figure 21, B). From this observation, it seems that chlorophyll is degraded or inactivated in the incubation at 50°C. Since the control also has this color change, it seems that the temperature is the reason behind the color change.

Of the three fractions created in the filtration and centrifugation step, the largest substrate fraction consisting mainly of non-grinded and large parts of the substrate. The pellet consists of substrate with a size of 100 µm or less. The supernatant consists of the liquid fraction. The largest substrate fraction is not used in any of the analyses. The supernatant is used as it is. The pellet is freeze dried to remove water. Afterward, the pellet is used directly for the elemental analysis, where the rest of the pellet is used to determine the dry matter. To analyze the substrate itself, it is cut into pieces with a length of max. 5 cm. Mostly grass but also some clover. The substrate is freeze dried and bead milled to homogenize the sample. Since the substrate itself is very heterogeneous, it is impossible to know if this sample is representative. The color of the supernatant change depending on which enzyme cocktail is added. For the samples containing created enzyme cocktail, the supernatant is light yellow. For the samples containing Viscozyme®, the supernatant is yellow. For the control samples, the supernatant is transparent (figure 21, C).

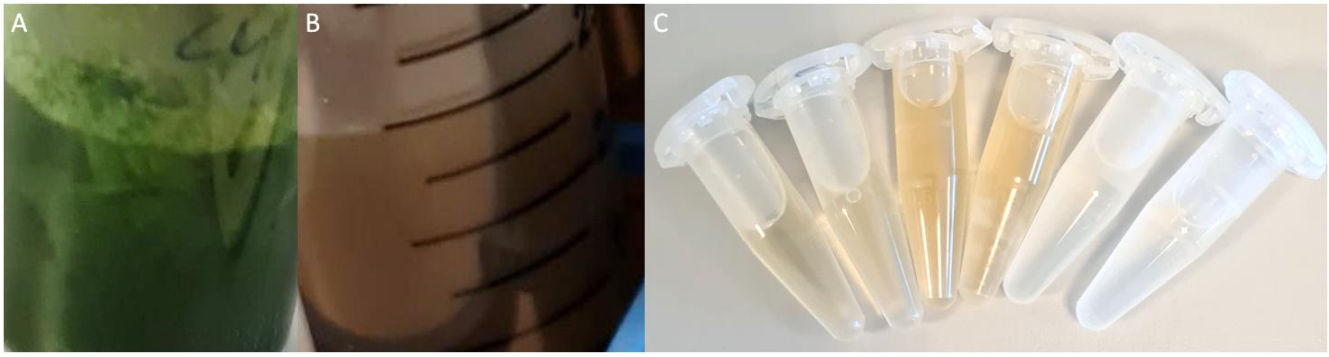


Figure 21: A: substrate incubated for 4 hours. B: substrate incubated for 24 hours. C: Eppendorf containing supernatant for substrate incubated for 3 hours. The two tubes to the left contain created enzyme cocktail. The two tubes in the middle contain Viscozyme®. The two tubes to the right contain no enzyme cocktail.

This process is based on an AAU protocol received from the "Laboratory engineer, Rocio del Rio de Diego". The reason behind the four hours of incubation is that the protocol is designed to extract proteins from fresh substrate using chemicals. After at least 3 hours of incubation, the pH of the buffer will change. In this experiment, the pH did not change after 4 hours, but the incubation is still performed. In the green biorefinery concept, a screw press is used to separate solids from liquids. It is possible to use a small screw press to simulate a similar process in lab scale, but then at least 200 g of fresh substrate is needed for each sample. Using the Ultra-

Turrax mill instead, 3 g of fresh substrate is used for each sample. Thus, while the screw pressing process would have been more similar to the concept, the Ultra-Turrax mill needs less substrate to function, which is great when experimenting at lab scale with a limited fresh substrate supply.

3.4.4 TS/VS

The dry matter of the pellet, the dry matter of fresh clover grass and the TS/VS of freeze dried and milled clover grass is analyzed. The different pellets have different dry matters, but all in the range of 94-99%. The fresh clover grass has a dry matter of 12.5% and the freeze dried and milled substrate has a dry matter of 95.2% and volatile solids of 80.7%. Converting the volatile solids for the dried and milled substrate into the fresh, the fresh clover grass volatile solids is on 10.6%. The substrate contains more moisture than clover grass press cake which is expected since it has not been screw pressed, which extracts liquid. The sugar content of fresh clover grass has not been analyzed and thus the composition is not known. The protein concentration is analyzed in the elemental.

3.4.5 BCA

BCA is used to determine the protein concentration in the supernatant. It is assumed that the incubation period degrades the substrate structure, which will then release structure bound proteins into the supernatant. Observing the results, this did not happen (figure 22). Compared with the control of diluted enzyme cocktails measured (same enzyme concentration as the samples), then the control contains more proteins than in the samples themselves. The proteins measured are expected to be the enzymes present. If this is true, then it seems that the concentration of enzyme decreases with time for the Viscozyme® containing samples. A reason behind the lessening in protein concentration is that some of the enzymes/proteins may not be extracted in the filtration and/or centrifugation step. Thus, some enzymes may still be present together with the solids. A potential reason for the decrease in the protein concentration over time is that a larger number of enzymes is with the solids or that the enzymes are degraded. BCA can only detect proteins, not their monomeric form of amino acids and thus potentially some of the proteins are degraded. To check this, a method called SDS-page can be used. SDS-page is a qualitative method that separates the proteins present based on size. If the proteins are degraded, then it can be observed by this method. This method is not used and thus the reason behind the decrease in protein concentration is unknown.

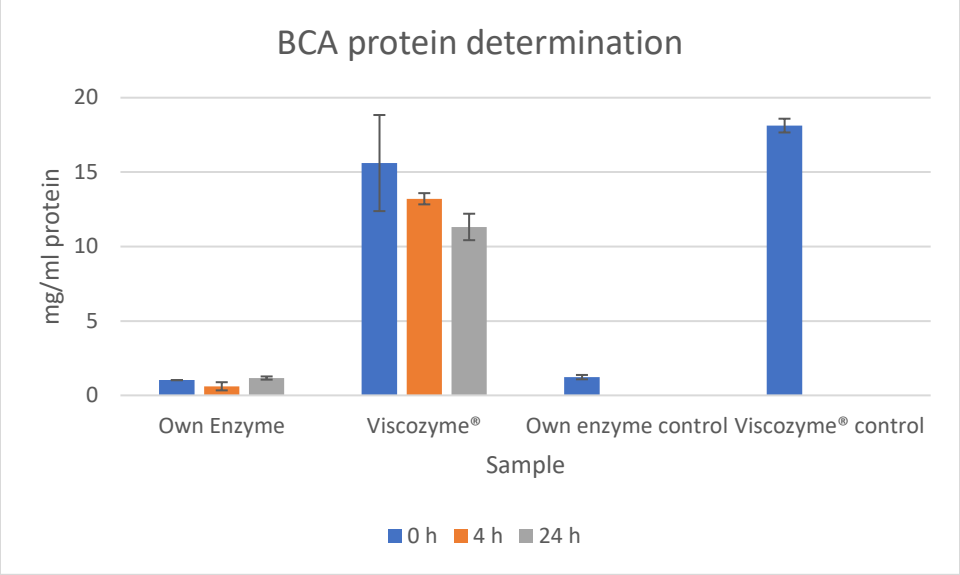


Figure 22: Results from the BCA protein determination kit.

3.4.6 HPLC

HPLC is used to determine sugar concentration in the supernatant (figure 23). In samples containing created enzyme cocktail, the glucose concentration almost doubles after 4 hours. Compared with the concentration after 4 hours, the concentration increases 5 times after 24 hours to around 15 g/100g-DM substrate. Thus, the degradation of cellulose and the structure of the substrate is successful. For xylose, galactose, and arabinose, all three sugars are in higher concentration after 24 hours, but the concentration never reaches above 2 g/100g-DM substrate. This means that some hemicellulase is present, but mainly glucose is degraded. No concentrations are negative, which means that they have a similar or higher concentration than the enzyme control. In the sample containing Viscozyme®, the glucose concentration also increases after 24 hours. The xylose concentration on the other hand decreases after 24 hours of incubation. The reason behind this is not known. The galactose concentration increases a little while the arabinose concentration is almost the same after an incubation of 24 hours. Thereby, it can be concluded that cellulases are present in Viscozyme®, while no specific hemicellulase activity is determined. Comparing the glucose concentrations for the samples containing created enzyme cocktail and Viscozyme®, it seems that the glucose concentration after 24 for created enzyme cocktail samples has degraded most glucose. If the concentration at incubation time 0 is changed to zero, then the samples containing Viscozyme® are the ones to degrade most glucose. The glucose concentration produced by the two enzyme cocktails is similar and thus proves that around the same activity of enzymes is added. For the control samples, then the glucose concentration increases from 1 g/100g-DM substrate to 3.3 g/100g-DM substrate in the span of 24 hours. The rest of the sugars are either the same or increase very little at the same incubation time. For each sample, only one HPLC measurement is performed. This means that the results are not 100% reliable. The reason for this is to reduce the number of samples analyzed using the HPLC machine.

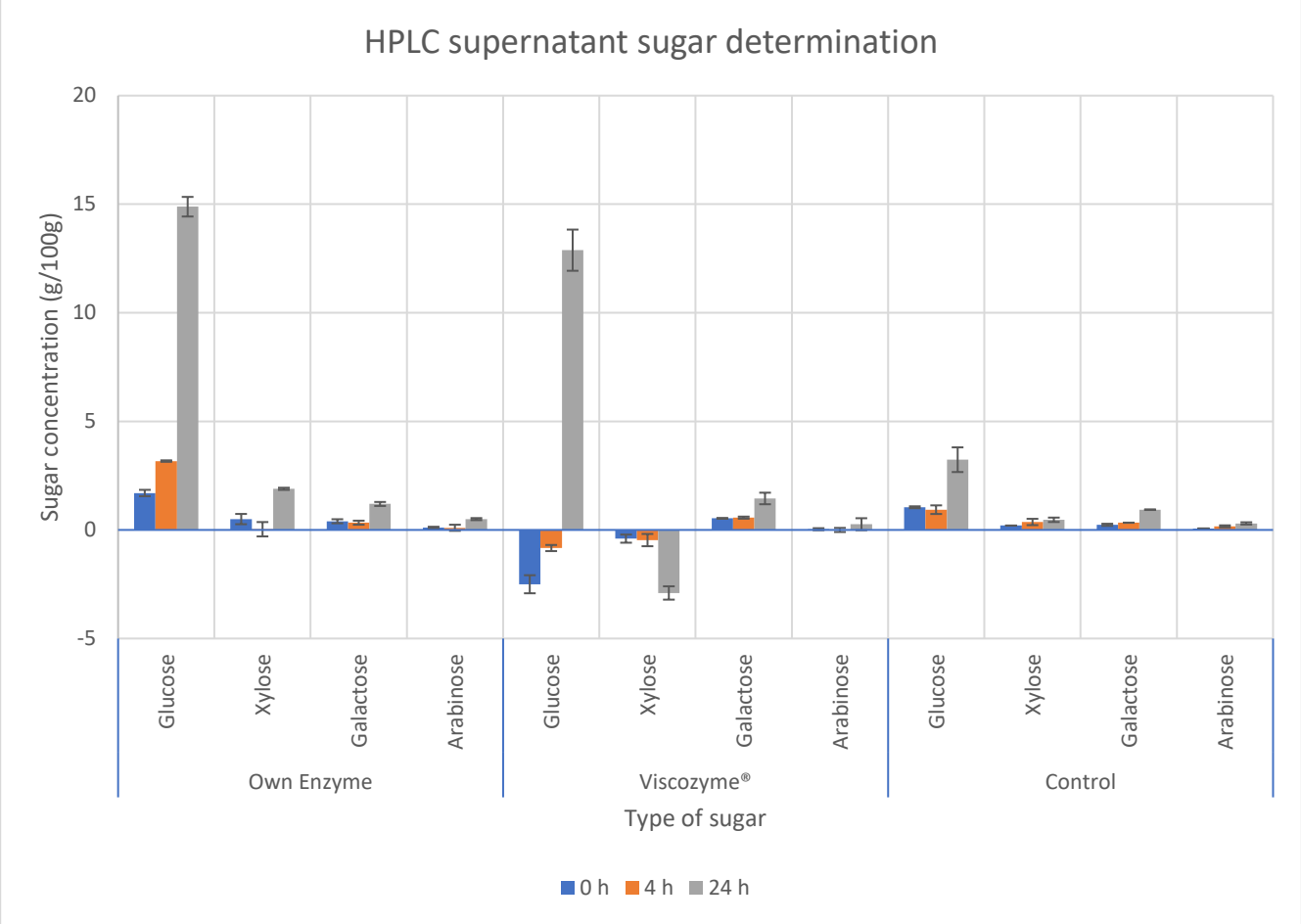


Figure 23: HPLC result of the sugar concentration in the supernatant after the second filtration.

3.4.7 Elemental analysis

Elemental analysis is performed on both the pellet and the supernatant to measure the nitrogen content (figure 24 and figure 25). In the measurement of the solids, the nitrogen content of freeze dried and milled substrate is also measured. From the results, it can be observed that the nitrogen concentration in the pellets decreases after incubation with an enzyme cocktail. Comparing incubation time 0 and 24 hours, the nitrogen concentration for the samples containing created enzyme cocktail decreases by 17%, while samples containing Viscozyme® decreases by 34%. This decrease in nitrogen concentration shows that some nitrogen in the substrate is released after enzymatic hydrolysis. In the control, no difference in the nitrogen concentration is observed. The nitrogen concentration in the freeze dried and bead milled substrate is much lower than the rest of the samples. The concentration in the control is more than twice the concentration in the substrate. The change in the nitrogen concentration may be due to the composition of the sample. When the samples are ground with the Ultra-Turrax mill, a lot of the grass leaves are still whole/in large pieces. Thus, when the content is filtrated the first time, only the smallest pieces of the substrate pass through. This could contain mostly clover, which should contain more protein than the grass itself. If the solids that did not pass through the filter are measured using elemental analysis, then the amount of nitrogen may be lower than the substrate measured. For each sample, only one

measurement is performed. This means that the results are not 100% reliable. The reason for this is to reduce the number of samples analyzed.

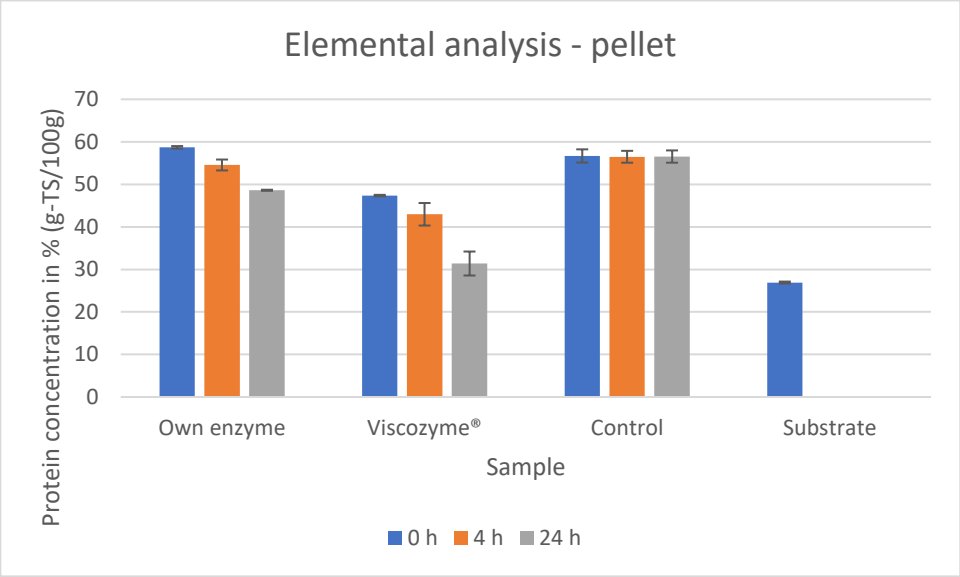


Figure 24: Elemental analysis of the freeze dried pellet and the freeze dried and milled clover grass.

For the elemental analysis of the supernatant, the protein concentration increases after 24 hours of incubation. Comparing incubation time 0 and 24 hours, for the samples containing created enzyme cocktail, the nitrogen concentration increased by 38%, the samples containing Viscozyme® increased by 76%, and the control samples increased by 75% (figure 25). Compared with the pellet control, then the nitrogen concentration in the pellet does not change, while it increases for the supernatants containing enzyme cocktails (figure 24). From this, it seems that the nitrogen in the samples is extracted by the buffer and/or water after milling without adding enzymes. Also, the enzymes extracted seem to derive from the solids not measured in this report. Around the same increase of nitrogen in the supernatant happens in the samples containing Viscozyme®. For the samples containing created enzyme cocktail, the nitrogen concentration only increased by around half as much as the other samples. The nitrogen content after 4 hours incubation is also lower than after 0 hours incubation. A reason behind this difference could be due to the milling or due to the heterogenicity of the substrate itself. For each sample, only one measurement is performed. This means that the results are not 100% reliable. The reason for this is to reduce the number of samples analyzed.

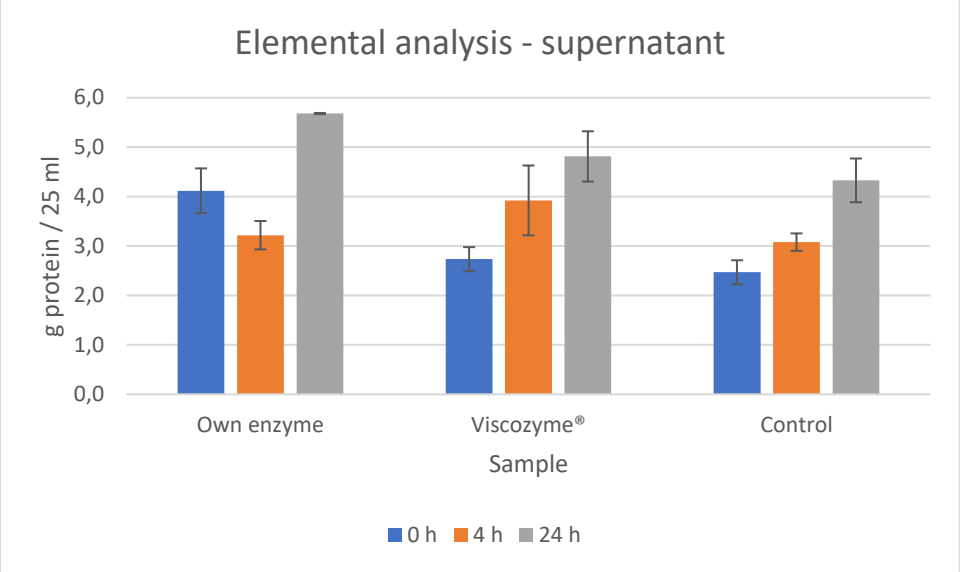


Figure 25: Elemental analysis of the supernatant.

3.4.8 Comparison of results

Based on the results generated in this section, it cannot be concluded with certainty that using enzymatic hydrolysis before separating the solids from the liquid increases the protein content in the supernatant. While almost no proteins in samples containing created enzyme cocktail are measured in the BCA protein kit, it has the highest concentration of nitrogen out of all of the samples in the elemental analysis of the supernatant. It is also the one that has the highest measured glucose concentration in the HPLC analysis. The decrease in the nitrogen concentration in the pellet shows that less nitrogen is present at longer incubation times, but there is no concrete data that proves that more protein is extracted, just that the cellulose is degraded. Besides measuring a higher protein concentration using the BCA kit, the samples containing Viscozyme® have similar results as the created enzyme cocktail.

Comparing the results gained in the BCA analysis and the results gained in the elemental analysis, it is clear that elemental analysis measures more proteins. This may be due to the state of the proteins. If the proteins are degraded into their monomeric form, then the BCA analysis cannot detect them. Also, BCA can only detect proteins in liquids, while the elemental analysis can detect nitrogen in both solids and liquids, as long as the sample is homogenous. A problem with the elemental analysis is that the state/form of the proteins is not known, and thus extra analysis methods have to be performed to find this information.

Comparing the HPLC analysis in this part with the DNS analysis in “optimization of enzymatic hydrolysis”, it is shown in the HPLC analysis that mostly cellulases are present in the different enzyme cocktails. Therefore, it should be sufficient to measure sugar content in samples while using a glucose standard curve, especially when comparing released sugars in the samples using these enzyme cocktails. It should be noted though that the substrate used for incubation before either the DNS or HPLC analysis is different and thus have different compositions. It would probably have been a great idea to measure the first “enzymatic hydrolysis” experiment

using HPLC, or at least some of the samples to check which sugars are present in the supernatant and thus which enzymes are present, based on the products.

The enzyme cocktail supernatant produced in the "enzyme production" is efficient. It has a degradation efficiency of 1.9-5.2 times lower than commercial enzymes at activity on 0.431 FPU/ml. Since some of the substrate is still left after the incubation, it could potentially be possible to sterilize the substrate again and add fresh spores to utilize more substrate. If submerged fermentation is used instead of solid state fermentation, then the supernatant will be more diluted. The process also needs much more resources, especially regarding aeration and agitation. On the other hand, if this process is implemented into the green biorefinery concept, then it is easier to scale up the submerged fermentation due to limited/missing equipment designs. In the third batch of enzyme cocktail supernatant, *T. reesei* spores and 20 g-DM substrate produced around 250 ml supernatant with activity on 0.834 FPU/ml. If each g-DM substrate hydrolyzed requires 5 FPU enzymes, then around 42 g-DM substrate can be hydrolyzed. This means that around half of the press cake produced in the green biorefinery process is needed to hydrolyze all of the substrate.

2.4.9 Future perspective

At the moment, it is not known which specific cellulases are present in the produced enzyme cocktail. If it is analyzed in the future, the proteomic analysis SDS-page and/or the transcriptomic analysis PCR can be performed. Since potential cellulases have been identified in the NCBI database, the sequences can be used. If the proteins themselves are analyzed, then the proteomic analysis SDS-page is used to determine the size of the protein. The specific gel used has to be able to separate molecules that are close in size since all potential enzymes length is in the range of 242-932 amino acids. While this result is not 100% accurate since the sizes are close to each other, the endoglucanase is, in general, the smallest, while the β-glucosidase is, in general, the longest. If the cells are disrupted and RNA extracted, then a PCR can be performed to analyze the produced enzymes. To do this, a primer pair can be designed for every potential enzyme and PCR can amplify the sequence if it is present. Thus, RNA strands that are present in the cells themselves can be identified and it can be known which cellulases are produced.

4 Conclusion

The different enzyme production activity test does not show the same results. This is because the different parameters affect the different cellulase activities differently. Instead, only the FPA results, and the simplicity of the experiments are taken into considerations. Therefore, the parameters used in the start for enzyme production are also the ones chosen to be the most optimal for the and/or the easiest to implement in the enzyme production process. The optimal conditions used to produce enzyme cocktails (the three batches) are 9 days of incubation at room temperature beside a windowsill with a moisture content of 75% and $5*10^6$ spores/g-DM substrate. For the different substrates, alfalfa press cake and brewers spent grains produce the lowest enzyme activity of the analyzed substrates. Incubating on clover grass press cake produces much higher enzyme activity than the other natural substrates, even though no exoglucanase activity is measured. The β-glucosidase activity for alfalfa press cake is much lower than the other substrates in this study, but due to the large standard deviation, then it may not be lower at all. For the rest of the substrates, the same b-glucosidase activity is produced.

The optimal conditions for enzymatically hydrolyzing alfalfa press cake are 7.5 FPU/g-DM substrate at an incubation temperature at 50°C and with a pH in the range of 4-5.5. While the exact optimal incubation time is not found, 8% of the total released sugars are measured as degraded after 24 hours. The buffer used and the 20 times diluted brown juice both have similar results and pH after enzymatic hydrolysis. Thus, if this is implemented into the green biorefinery concept, then it is possible to perform without using any chemicals. Even though the optimal parameters for each of the optimization experiments are analyzed, the interaction between each parameter is not, and thus it is not known how they interact with each other.

The commercial enzyme tested with the highest degradation efficiency is Viscozyme®. CelliCtec II has the second highest degradation efficiency. Therefore, Viscozyme® is used to compare with the created enzyme cocktail. In the BCA test, the concentration of proteins measured is either the same or decreases over time, with the enzyme controls containing higher concentrations of proteins than the samples. In the elemental analysis, the concentration of nitrogen increases in the supernatant over time for all samples and controls, and the nitrogen concentration decreases in the pellets for samples over time. The nitrogen concentration in the control pellets stays the same. From these results, it is not possible to determine whether the nitrogen concentration in the supernatant increases over time due to the enzymatic hydrolysis. For the HPLC results, it is shown that the glucose concentration increases after enzymatic hydrolysis, and thus the enzymatic hydrolysis works. If enzyme production and enzymatic hydrolysis are implemented into the green biorefinery concept, then half of the produced press cake is needed to generate the necessary enzymes.

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