THE EFFECT OF CATALASE ON ENZYMATIC SACCHARIFICATION OF WHEAT STRAW SLURRY

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“The effect of catalase on enzymatic saccharification of wheat straw slurry”

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Preface

This project was performed for the partial fulfilment of Master of Science in Biotechnology, at the Department of Biology and Biological Engineering, Chalmers University of Technology, Sweden. The project was initiated in January 2017 and finished in June 2017. The project was carried out under the supervision of Doctor Ausra Peciulyte, Chalmers University of Technology. Doctor Katja S. Johansen, a visiting researcher at Chalmers University of Technology was a co-supervisor and Professor Lisbeth Olsson, Chalmers University of Technology was an examiner.

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Abstract

Enzyme inactivation during enzymatic saccharification is a problem because it causes a slowdown in saccharification before the substrate is completely converted into sugars. Lytic polysaccharide monooxygenases can, under certain conditions, produce hydrogen peroxide which causes release of reactive oxygen species. These reactive oxygen species can interact either directly or indirectly with enzymes, causing their inactivation. Another reason for enzyme inactivation is precipitation. It has been shown that up to 30% of proteins can precipitate during standard saccharification conditions.

This project investigated the effect of catalase on both enzymatic saccharification and enzyme precipitation. During saccharification of 10% dry matter wheat straw slurry the addition of catalase decreased enzyme inactivation, and thereby the glucose release by 9.5% at a CTec3 loading of 15 mg/g DW. It was also found that acidifying reactions taking place during enzymatic saccharification were reduced by the addition of catalase meant that less KOH was needed to adjust the pH during the saccharification process. It was shown that at least part of the enzyme inactivation during saccharification can be caused by precipitation. In this project it was found that enzyme precipitation is higher at pH 4 than at pH 5 and 6. The results also showed that catalase can partially mitigate this precipitation. The enzyme cocktail used in this project is CTec3.

Keywords: LPMO, Catalase, Wheat straw, Saccharification, Precipitation, pH
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<table>
<thead>
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<th>Abbreviation</th>
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<tr>
<td>AA9</td>
<td>Auxiliary activity family 9</td>
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<td>BG</td>
<td>β-glucosidase</td>
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<td>CBH</td>
<td>Cellobiohydrolase</td>
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<td>CBM</td>
<td>Carbohydrate binding molecule</td>
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<td>CD</td>
<td>Catalytic domain</td>
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<td>DNS</td>
<td>Dinitrosalicylic acid</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>DW</td>
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<td>EG</td>
<td>Endoglucanase</td>
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<td>FPU</td>
<td>Filter paper unit</td>
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<td>GH</td>
<td>Glycoside hydrolase</td>
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<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<td>IUPAC</td>
<td>International union of pure and applied chemistry</td>
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<td>KOH</td>
<td>Potassium hydroxide</td>
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<tr>
<td>LPMO</td>
<td>Lytic polysaccharide monooxygenase</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate - polyacrylamide gel electrophoresis</td>
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<tr>
<td>SHF</td>
<td>Separate hydrolysis and fermentation</td>
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<td>SSF</td>
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1 INTRODUCTION

Society is very reliant on fossil fuels which also lead to the release of greenhouse gases that contribute to climate change. This reliance, in combination with the fact that peak oil might arrive in a not too distant future, has led to a search for alternative, preferably renewable, resources that can be used to produce fuels and chemicals. Lignocellulosic biomass has potential to be a major resource in the future for production of not just biofuels but also for energy, chemicals and building materials [1, 2].

The best way to utilize the biomass would be using a biorefinery. A biorefinery takes the biomass and breaks it down into its individual components which can be used to produce fuels, energy or other chemicals. The concept is analogous to an oil refinery except for the fact that the raw material is biomass instead of oil [3]. A biorefinery is built on the idea of a circular economy [4].

A linear economy is based on a linear flow consisting of “take-make-use-dispose”, which can be explained as (i) production of goods from virgin resources, (ii) use of them and, (iii) throw away goods as wastes no more usable [4, 5]. The linear economy is not feasible due to the fact that resources such as minerals and oil are finite which will cause shortages in the future. Unlike the linear economy, the circular economy aims to accomplish a closed loop system to maximize the recovery of raw materials derived from the waste at end-of-life. The concept was introduced during the 1980s by Walter Stahel who proposed that waste could become a resource [6]. Overall, the circular economy is based on the 3Rs – reduce, reuse and recycling of waste [4] and the fact that a biorefinery is built on this concept makes it a sustainable model.

One of the major products produced in a biorefinery are biofuels, such as bioethanol. The first generation of bioethanol is either starch- or sugar-based but since this is reliant on food sources, alternatives are needed [3]. A European Council decision in 2014 limited the use of first generation biofuels to 7% of the energy used in transport in 2020. The rest should come from second generation biofuels [7] which are derived from non-edible lignocellulosic biomass [1].

Lignocellulose is a complex matrix consisting of cellulose, hemicellulose (both are referred to as carbohydrate polymers) and lignin (aromatic polymer) and is one of the most abundant biopolymers on earth [2, 3, 8]. Cellulose is made up of glucose. Hemicellulose contains different types of sugars such as glucose, mannose, xylose etc. Lignin is bound to
both cellulose and hemicellulose and provides the plant cell wall with compressive strength and stiffness [9].

Despite all the possibilities in the use of lignocellulosic biomass it shows a remarkable resistance to being depolymerized [8]. This resistance makes it harder to achieve an economically beneficial production of bioethanol. Since the organisms used to produce bioethanol are incapable of using cellulose as a substrate, the lignocellulose first needs to be degraded into fermentable sugars.

The process of using enzyme cocktails to degrade polysaccharides is called enzymatic saccharification [3]. The main part of these cocktails is made up of glycoside hydrolases (GHs) known as cellulases, and hemicellulases. Cellulases and hemicellulases consist of several different types of enzymes that break down cellulose and hemicellulose respectively.

Lytic polysaccharide monoxygenases (LPMOs), which were only recently discovered, are oxygen dependent enzymes that use an electron donor and a copper cofactor to oxidatively induce chain breaks in several different types of polysaccharides [10]. These enzymes are capable of breaking glycosidic bonds using an oxidative mechanism unlike previously discovered enzymes that rely on hydrolytic cleaving [10]. The discovery of LPMOs have led to significant improvement in the efficiency of enzymatic saccharification, and thus in the production of biofuels [10]. The combination of LPMOs with traditional cellulases has a synergistic effect, thus increasing the saccharification rate.

LPMOs have been found to produce hydrogen peroxide ($H_2O_2$) in the absence of substrate and presence of oxygen and an electron donor, which leads to the release of reactive oxygen species (ROS) [11]. The ROS may interact either directly or indirectly with enzymes, causing enzyme inactivation [12]. Chylenski et al. have found that another reason for enzyme inactivation might be precipitation [13]. They have showed that the total protein precipitation during standard hydrolysis conditions can be as high as 30%, meaning that enzyme precipitation might be an important cause for enzyme inactivation. They have also showed that the enzyme precipitation during dilution was approximately 5-30%. Scott et al. have found that catalase can reduce enzyme inactivation by reducing $H_2O_2$ during saccharification [12]. Catalase (EC 1.11.1.6.) is an enzyme that turns $H_2O_2$ into oxygen and water, thereby preventing the formation of ROS.
2 AIMS AND OBJECTIVES

The work performed during this project investigates the effect of catalase in enzymatic saccharification of wheat straw slurry with and without warm-water washing. The work also investigates if enzyme precipitation is a probable cause for slowdown in the saccharification process over time. The work during this project also studies if catalase could affect precipitation. Finally, potential causes for slowdown in saccharification were evaluated. This project was divided into two major parts. In the first part of the project the effect of washing of the substrate to remove enzyme inhibitors and addition of catalase to the saccharification process was evaluated. The substrate was wheat straw slurry at 10% dry weight (DW). In the second part precipitation studies were performed both on Cellic CTec3, an industrial enzyme cocktail containing LPMOs, and monocomponent enzymes (catalase, LPMO and β-glucosidase (BG)). Also here the effect of catalase addition was evaluated with the hypothesis that catalase can prevent or at least reduce enzyme precipitation by neutralizing H₂O₂ and thereby preventing enzyme inactivation by ROS.

The objectives of the project are to:

- Investigate the possible effect of inhibitors on enzymatic activity by evaluating enzymatic saccharification of warm water washed and non-washed wheat straw slurry.
- Investigate if H₂O₂ has an effect on enzyme inactivation by evaluating the effect of catalase addition in enzymatic saccharification of wheat straw slurry.
- Follow pH development during enzymatic saccharification of wheat straw slurry in order to find out how pH develops during the different saccharification conditions.
- Evaluate potential causes for slowdown in saccharification.
- Investigate if enzyme precipitation is a possible cause for slowdown in saccharification of wheat straw and if catalase affects enzyme precipitation.
3 BACKGROUND

3.1 Biofuels

The term biofuel refers to solid (bio-char), liquid (bioethanol, bio-methanol, vegetable oils, biodiesel, bio-oil and Fischer-Tropsch liquids) and gaseous (biogas, bio-synthetic gas (bio-syngas) and bio-hydrogen) fuels predominantly produced from biomass [14]. Biomass refers to biological materials obtained from forest, agricultural residues, municipal solid wastes and other wastes from food and agro industries. Production of biofuels from renewable lignocellulosic resources may improve energy availability, decrease air pollution and diminish atmospheric CO$_2$ accumulation, as reviewed by Prasad et al. [15]. It is important that the amount of carbon released into the environment should be balanced, that the carbon cycle is closed as in Figure 3-1. The imbalance in the carbon cycle caused by the use of fossil fuels results in an increase of CO$_2$ released into the atmosphere [16].

![Figure 3-1. The closed carbon cycle, redrawn from [17].](image)

**Bioethanol**

Bioethanol is by far the bio-fuel most widely used for transportation worldwide [18]. It can be used either directly as a transportation fuel or it can be blended with gasoline. Bioethanol is most commonly blended with gasoline at concentrations of 90% gasoline and 10% bioethanol, forming 10% “gasohol”. Bioethanol contains 35% oxygen which reduces emissions of particles, carbon monoxide (CO) and nitrogen oxides (NO$_x$). By mixing gasoline with bioethanol the emissions of non-combusted hydrocarbons decrease while also reducing petroleum use and thereby greenhouse gas emissions [18]. There are three major groups of
feedstocks that can be used for bioethanol production [18]. The groups are sucrose containing feedstocks (e.g. sugar cane, sugar beet, sweet sorghum and fruits), starchy materials (e.g. corn, wheat, rice, potatoes, cassava, sweet potatoes and barley) and lignocellulosic biomass (e.g. wood, straw and grasses). The bioethanol is produced by microorganisms using the fermentable sugars available in the feedstock.

There are two major classes of bioethanol based on the raw material used for production. The classes are called first and second generation bioethanol.

**First generation bioethanol**

The first generation of bioethanol is derived from starchy materials or sucrose containing feedstocks [3]. Based on geographical location the availability of the different feedstocks varies. In Brazil, the major feedstock for bioethanol is sugar cane while in the United States it is corn [18]. In Europe the main feedstocks are beet molasses, wheat and barley. Production of first generation bioethanol involves separation of sugars from the raw material followed by the fermentation of sugars. Finally distillation and dehydration is performed to obtain the desired ethanol concentration.

The increase in use of food crops for bioethanol production has increased the food shortage in the world [19]. This limitation can be overcome by using non-edible biomass instead of food to produce the bioethanol. The established infrastructure and already implemented process used in the production of first generation bioethanol gave a necessary base for the development of the second generation bioethanol.

**Second generation bioethanol**

The second generation of bioethanol is derived from non-edible, renewable lignocellulosic biomass [3]. Lignocellulose is the major component of plant waste organic matter such as straw, cornhusk, wheat, sugarcane bagasse, corn stover, wood chips, saw dust, nut shells, leaves, the organic fraction of municipal waste etc. [15]. In order to produce bioethanol from the lignocellulosic biomass there are a few key steps needed. Pre-treatment of lignocelluloses is divided into two major steps [1]. The first step is to disrupt the fibers of the material using a thermochemical pre-treatment. The second step is an enzymatic saccharification process turning the polysaccharides into fermentable sugars. After the pre-treatment the sugars are fermented into ethanol. The saccharification and fermentation can be
performed either simultaneously (Simultaneous Saccharification and Fermentation, SSF) or separately (Separate Hydrolysis and Fermentation, SHF) [20]. The use of lignocellulosic waste for bioethanol production both limits the food competition and greenhouse gas emissions [21].

3.2 Lignocellulose composition

Lignocellulose is a complex matrix consisting mainly of cellulose (40-50%), hemicellulose (25-35%) and lignin (15-20%) [2, 22]. The compositions of different lignocellulosic materials vary between plant species [15]. Cellulose and hemicellulose are polysaccharides made from different types of sugar monomers while lignin is a highly complex aromatic [3]. An overview of the structure of lignocellulose can be seen in Figure 3-2.

![Figure 3-2. Structure of lignocellulose materials from [9].](image)

The lignin works as glue binding the cellulose and hemicellulose together. The three components form structures called microfibrils with crystalline cellulose in the center.
surrounded by a matrix consisting of hemicellulose and lignin. The microfibrils are then organized into larger macrofibrils that provide structural stability to the plant cell wall [9].

### 3.2.1 Cellulose

Cellulose is the main structural component of the plant cell wall and consists of long chains of \(\text{D-glucose}\) molecules linked together by \(\beta-1,4\)-glycosidic bonds (Figure 3-3). Cellulose polymers are linear, unlike the polymers in hemicellulose. In nature the cellulose chains can consist of more than 10 000 glucose units [23]. The polymer chains are linked together by hydrogen bonds forming highly crystalline cellulose which makes it resistant to degradation [9].

Each cellulose polymer chain is composed of anhydroglucopyranose units joined together by \(\beta-1,4\)-glucosidic linkages [24]. The repeating unit in the \(\beta-1,4\)-D-glucan polymer is cellobiose, which is composed of two anhydroglucose residues (Figure 3-3). Since a molecule of water is lost during the formation of a \(\beta-1,4\)-glycosidic bond, the glucose units in the polymer are referred to as anhydroglucose units.

![Cellulose structure](image)

*Figure 3-3. Molecular structure of cellulose (n= value of degree of polymerization) from [24]. The \(\beta-1,4\)-D-glucan polymer has a reducing and a non-reducing end. It is composed of repeating units of cellobiose, which consist of two anhydroglucose residues which are linked via \(\beta-1,4\)-D-glycosidic bonds.*

The C1-OH end of the polymer has reducing properties (reducing end), while the C4-OH end is an alcohol group which has non-reducing properties (non-reducing end). At the reducing end the ring can open to expose an aldehyde end-group. Different chemical and enzymatic reactions have a preference either for the reducing or the non-reducing end.

### 3.2.2 Hemicellulose
Hemicellulose is the second most abundant component in lignocellulosic biomass and consists of several different types of monosaccharides linked together. The monosaccharides are either hexoses (6-carbon sugars) such as glucose, mannose and galactose or they are pentoses (5-carbon sugars) such as arabinose and xylose [9]. The monosaccharides are linked together by β-1,4- or β-1,3-glycosidic bonds. Hemicellulose has a random, amorphous branched structure which makes it less resistant to hydrolysis than the highly crystalline cellulose [25].

3.2.3 Lignin

Lignin is an aromatic polymer composed of three major phenolic compounds: coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Figure 3-2). The ratio of the three components varies between different plants, wood tissues and cell wall layers. Lignin can be considered as cellular “glue” providing both the individual fibers but also the plant tissue with compressive strength. Lignin also provides the plant with resistance to insects and pathogens [9].

3.3 Process steps in the production of ethanol from lignocellulose

Lignocellulosic materials need to be processed in several different steps in order to produce bioethanol. An overview of the different process steps can be seen in Figure 3-4.
3.3.1 Pre-treatment of lignocellulose

For an industrial process that converts lignocellulosic materials into energy carriers such as ethanol to be economically feasible there are many requirements. One is that both the cellulose and the hemicellulose need to be hydrolyzed into sugars [26]. Thus, the ideal pre-treatment method needs to integrate several processes into one.

The recalcitrance (resistance to degradation) of lignocellulose is one of the major barriers to an economically sustainable production of bio-based fuels and chemicals. To reduce the recalcitrance the lignocellulosic material needs to be pre-treated to reduce the barriers formed by hemicellulose and lignin, making the cellulose more accessible to enzymes that convert the cellulose to glucose [27]. The drawback of the pre-treatment processes, besides the economic impact, is the generation of inhibitory compounds that can negatively affect the action of both enzymes and microorganisms. The inhibitors are produced as a result of extreme pH and high temperature treatment of the lignocellulose and therefore each pre-
treatment process should be carefully chosen and justified [28]. The pre-treatment can be classified as physicochemical, chemical and biological pre-treatment.

The wheat straw used in this thesis has been pre-treated using steam explosion which is a physicochemical pre-treatment. Physicochemical pre-treatments combine harsh physical conditions with the addition of chemicals such as acids or bases.

Steam explosion pre-treatment is one of the most commonly used pre-treatment methods. The method is based on subjecting the material to high pressure and high temperatures for a short period of time after which the pressure rapidly drops which disrupts the fiber structure of the material. As summarized by Brodeur, et al. [29] steam explosion is a cost effective method that transforms lignin and solubilizes hemicellulose. However, it also partially degrades the hemicellulose, generates toxic compounds and need an acid catalyst to make the process efficient at high lignin concentrations [29]. Specifically for the wheat straw substrate in this study, no acid catalyst is added since the hemicellulose in the wheat straw is acetylated meaning that acetic acid serves as catalyst.

Chemical pre-treatments, such as acid pre-treatment or alkaline pre-treatment use chemicals to break down the lignocellulosic material. Biological pre-treatment involves the use of microorganisms such as brown-, white-, and soft-rot fungi to degrade lignin and hemicellulose but leave the cellulose intact [29, 30].

3.3.2 Saccharification of lignocellulosic materials

After pre-treatment the solid part of the lignocellulosic materials consists mainly of cellulose and lignin as part of the hemicellulose has been solubilized during the pre-treatment process. In order to release sugars from the pre-treated material it needs to undergo enzymatic hydrolysis which is called saccharification. In this process enzymes are used to convert cellulose and residual hemicellulose to sugars. Enzymatic hydrolysis of lignocellulose is performed using enzyme cocktails [3]. The basis of the industrial enzyme cocktails is made up of carbohydrate degrading enzymes known as cellulases and hemicellulases. The enzymes are used to release fermentable sugars from the lignocellulosic material. Since part of the hemicellulose has been solubilized, cellulases are the most important enzymes needed in the hydrolysis [28].

The hydrolysis of cellulose differs from many other hydrolysis processes in the aspect that soluble enzymes act on an insoluble substrate. For the hydrolysis to take place the enzymes first need to adsorb to the surface of the insoluble substrate. Cellulases which have
Different structures and different binding sites work synergistically on the substrate to hydrolyze it into the final product glucose. The different actions of the different enzyme classes are illustrated in Figure 3-5.

Figure 3-5. Illustration showing how the different types of cellulases degrade cellulose, from [8]. β-glucosidase (BG) releases free glucose from cellobiose; cellobiohydrolase I (CBH I) releases cellobiose from the reducing (R) end of the cellulose chains; cellobiohydrolase II (CBH II) releases cellobiose from non-reducing (NR) end; and the endoglucanases EG I, II and III break the internal glycosidic bonds, creating free ends for CBH I and CBH II. The LPMO (AA9) is shown in red. Some cellulases contain cellulose-binding modules, depicted as blue triangles.

Several different types of microorganisms such as bacteria and fungi can produce cellulases, however only a few of these microorganisms produce cellulases in large quantities. Most of the cellulases produced commercially are produced in fungi. The most well-known fungi capable of utilizing cellulose as a primary carbon source are Sclerotium rolfsii, Phanerochaete chrysosporium, Trichoderma reesei and species of Aspergillus, Penicillum and Schizophyllum [28]. Especially T. reesei and derived mutants have been extensively studied [28]. Interest in producing enzymes using T. reesei increased during the oil crisis in the 1970s when bioethanol became economically attractive as an alternative.

3.3.3 Fermentation

After saccharification a fermentation process takes place. During the fermentation, sugars released by the saccharification process are consumed by a microorganism to produce ethanol [28]. There are several different microorganisms that can ferment the pentoses and hexoses released during saccharification into ethanol. Organisms commonly used for ethanol
production are *Escherichia coli*, *Zymomonas mobilis*, *Saccharomyces cerevisiae* and *Pichia stipitis* [28] but there are several other microorganisms that can be used for the same purpose.

Enzymatic saccharification and fermentation can be performed either separately or simultaneously, processes which are referred to as SHF and SSF. In the SHF process enzymatic saccharification and fermentation processes takes place in two different vessels at 45-50°C and 30°C respectively meaning that the different processes both can be performed at their temperature optimum. In this process it is also possible to recycle the microorganism. However, the sugars released during the saccharification process can have an inhibitory effect on the saccharification enzymes. The SHF process also involves two different process steps, which can increase the cost. In SSF both saccharification and fermentation are carried out as one process step. This method minimizes product inhibition of the enzymes since the sugars released are consumed by the microorganism to produce ethanol. However, when using this method the process conditions are a compromise between what is optimal for saccharification and what is optimal for fermentation. During these conditions it is also not possible to recycle the microorganism.

In the initial phase of fermentation, yeast will use available oxygen to grow but once the oxygen is depleted fermentation, which is anaerobic, starts. In the case of SSF yeast and enzymes are inoculated simultaneously, which causes competition for oxygen between the yeast and LPMOs. Theoretically this would mean that SSF conditions results in less oxidized carbohydrates, implying that the LPMOs are less active, being formed compared to SHF. This was confirmed by Cannella et al. [31]. In SSF the ethanol production was lower than for the SHF, where enzymes were introduced before the yeast. Cannella et al. found that the anoxic environment generated during SSF was inhibitory for LPMOs and that the conditions were more favorable during SHF where enzymes and yeast do not compete for dissolved oxygen [31]. Similar results have been found by Müller et al. [32] who compared SSF with SHF in production of lactic acid using lactic acid bacteria. The production of lactic acid was higher in the SHF setup, since in the SSF setup the microorganism competes with LPMO for oxygen. They conclude that activation of LPMOs by aeration is feasible, but may cause production of undesirable side-products such as acetic acid [32].

3.4 Cellulolytic enzymes

3.4.1 Cellulases
Most cellulases are modular enzymes that are composed of independently folded, structurally and functionally discrete units which are referred to as either domains or modules [33]. Most commonly, cellulases consist of one catalytic domain (CD) and one carbohydrate binding molecule (CBM) [33]. The two parts are usually linked together by a relatively long (30-44 amino acids) linker peptide. There are also cellulases with multi-catalytic domain enzymes which possess several binding molecules [34]. The primary role of the CBM is to accommodate physical contact of the enzyme to the cellulose, thus increasing the effective concentration of the enzyme as well as the time that the enzyme will spend in near proximity of the substrate. Enzymes without CBM, i.e. enzymes with only the CD, still have the ability to absorb to the cellulose but often with lower affinities compared to enzymes with both CD and CBM [35].

Cellulolytic enzymes, (GHs), hydrolyze the glycosidic bond through acid catalysis [36]. The acid catalysis requires a proton donor and a nucleophile/base. Hydrolysis leads to breaking of bonds by addition of water, as the name suggests and can occur in two different ways. In the retaining mechanism there are two major steps. First an intermediate is formed through nucleophilic attack. In the second step a water molecule frees the hydrolysis product from the enzyme and recharges the proton donor. In the inverting mechanism the glycosidic oxygen is protonated and the release of hydrolysis product is accompanied by a concomitant attack by a water molecule that has been activated by the base residue [36].

At least three major groups of cellulases are involved in the hydrolysis process. The first group is endoglucanases (EG or EC 3.2.1.4.) which attack regions of low crystallinity in the cellulose fiber, randomly introducing breaks in the chain thus creating free chain ends [37, 38]. The second group is exoglucanases or cellobiohydrolases (CBH or EC 3.2.1.91.) which degrade the molecule further by removing cellobiose units from the free chain-ends. The third group is β-glucosidases (BG or EC 3.2.1.21) which hydrolyse cellobiose to produce glucose, thereby preventing cellobiose inhibition of the other enzymes. The rate limiting step is the depolymerisation of the insoluble cellulose by the EGs and CBHs.

3.4.2 Lytic polysaccharide monooxygenases (LPMOs)

Reese, et al. [39] were the first to suggest that there could be undefined enzymes participating in the disruption of the recalcitrant cellulose structure, thus allowing the traditional cellulases (EGs, CBHs and BGs) to attack. In 2010 the activity of fungal LPMOs,
which are classified as auxiliary activity family 9 (AA9), previously GH61, was described [40]. There are also bacterial LPMOs which are classified as AA10, previously CBM33 [41].

LPMOs are oxygen dependent enzymes that use an external electron donor and a copper cofactor [2] to oxidatively introduce chain breaks in cellulose, chitin, xylan, xyloglycan, glucomannan, lichenan, β-glucan and starch as reviewed by Hemsworth et al. [3]. The chain breaks induced by the oxidation boosts the activity of classical cellulases by introducing new chain-ends upon which the cellulases can act while also disrupting the crystalline structure of the cellulose [42].

LPMOs have been found to produce superoxide that is spontaneously transformed into H₂O₂ in the absence of substrate which leads to the release of ROS [8, 11, 12, 42]. ROS are chemically reactive molecules containing oxygen that can react with for example enzymes, thus inactivating them.

3.5 Challenges with enzymatic saccharification

The enzyme cocktails used for enzymatic hydrolysis contain several different types of enzymes such as cellulases and LPMOs. In this project the Cellic® CTec3 cocktail from Novozymes A/S was used. For efficient enzymatic saccharification it is essential that enzymes work in cooperation, or synergistically. Synergy between cellulolytic enzymes means that the combined action of the different enzymes leads to higher action than the sum of the individual actions. The individual actions of the enzymes can be seen in Figure 3-5.

There is a number of challenges with enzymatic hydrolysis of lignocellulosic materials at a commercial scale [28]. The cellulases are expensive and therefore, to reduce cost, the enzyme loadings should be minimized. However lower enzyme loadings increase the time needed to completely hydrolyze the substrate. To reduce the cost of distillation, the ethanol concentration and thereby also the sugar concentrations should be maximized. The simplest way of retrieving higher final concentration is to increase the substrate concentration. However, using higher substrate concentration increases the product inhibition as well as the presence of inhibitory compounds which are produced during the pre-treatment which causes the enzymatic performance to decrease. Furthermore, the enzymatic performance is depending on a number of factors such as temperature, pH and lignin concentration.

Enzymatic saccharification of lignocellulosic material is normally preformed at substrate concentrations of around 10-15 % DW [28], since higher substrate concentrations cause trouble with mixing. Using the whole slurry as obtained after pre-treatment of the
material can significantly reduce the performance of the enzymes as a result of end product inhibition by sugars and other inhibitory compounds formed during the pre-treatment. Other degradation products, such as furfurals and acetic acid, present in the pretreated material can also have inhibitory effects on the enzyme activity [43]. One way to increase the efficiency can be washing of the solid material before hydrolysis at the expense of an increased operation cost [44]. Therefore warm water washing of wheat straw slurry was evaluated in this thesis.
4 MATERIALS AND METHODS

4.1 Enzymes and lignocellulosic substrate

Unless otherwise stated all reagents were laboratory grade. Steam pretreated wheat straw was retrieved from Novozymes A/S, purchased from Lund University. The enzymes used in the experiments were Cellic® CTec3, catalase, LPMO and BG. CTec3 is a commercial enzyme mixture. All additions of CTec3 were made using mg of mixture rather than mg of protein. All enzymes were kindly provided by Novozymes A/S and prepared as described by Scott et al. [12]. The catalase stock had a protein concentration of 16 g/l and the molecular weight of the enzyme is 75 kDa. The LPMO stock had a concentration of 73 g/l and the molecular weight of the enzyme is 24 391 Da. The more specific name for the LPMO used in this study is TaAA9A where Ta is an abbreviation of the name of the organism of origin (*Thermoascus aurantiacus*), AA9 is the collective name of LPMOs from fungal origin and A shows that it is the first LPMO reported from this organism. The BG had a stock concentration of 34 g/l and a molecular weight of 120 kDa (recombinant enzyme, SDS-PAGE [45]) or 94.75 kDa (theoretical, [46]).

All enzymes were stored in aliquots at -20°C and a new aliquot was used for each experiment. During the experiments the enzymes were thawed either at +4°C or on ice and all enzymes and enzyme dilutions were kept on ice until they were added to the experiments.

The wheat straw substrate was stored in aliquots at -20°C and new aliquots were used for each experiment. The substrate was thawed in a cold water bath.

Dilutions of enzymes and substrate in this project were made with tap water. This is because tap water is more in line with what is used in an industrial setting. The pH was adjusted by addition of 1 M KOH which allowed studies of changes in pH during the saccharification process. The fact that the pH is adjusted using base instead of buffers is also closer to an industrial setting.

4.2 Filter Paper Unit (FPU) measurements of Cellic CTec3 enzyme cocktail

The filter paper assay is an enzyme assay developed to evaluate the combined activity of cellulose degrading enzymes. The assay was performed in 50 mM triethylammonium acetate buffer at pH 5. The assay is based on the conversion of filter paper (cellulose) into
glucose by enzyme preparations and thereafter the detection of glucose using dinitrosalicylic acid (DNS) reagent. In the reducing sugar assay, DNS reagent reacts with the reducing ends of carbohydrates and changes color from yellow to red. Not only glucose is a reducing sugar but also glucose oligomers have a reducing end that can be detected by the DNS assay. Glucose oligomers are often present if the BG levels are insufficient which means that the glucose oligomers released by endoglucanases and cellobiohydrolases are not completely degraded to glucose monomers. The color of each sample is measured at 540 nm and is proportional to the amount of reducing sugars present in the sample.

The International Union of Pure and Applied Chemistry (IUPAC) accepted and recommended the filter paper assay in 1987 and it has been used ever since [47]. In 2004 Xiao et al. presented a scale down of the filter paper assay [48]. The new version of the assay is performed in a microtiter scale rather than in 25 ml test tubes which significantly increased the number of samples that can be run simultaneously, as well as reducing the reagent consumption.

Glucose solutions for the standard curve as well as the triethylammonium acetate buffer (henceforth referred to as buffer) were prepared according to Appendix 8.1. Enzyme dilutions were prepared according to the Table 4-1. First the enzyme stock solution was diluted 4x in buffer to make it easier to pipette since the stock solution is quite viscous. Table 4-1 also shows the final dilutions (in brackets) in the 20 µl of enzyme sample that is added to the reaction, considering the initial 4x dilution.

| Table 4-1. Preparation of enzyme dilutions using serial dilutions for the Filter Paper Unit assay, all volumes are in µl. |
|---|---|---|---|---|---|---|---|
| Enzyme dilutions | 25x (100x) | 50x (200x) | 125x (500x) | 167x (668x) | 334x (1336x) | 835x (3340x) | 1670x (6680x) |
| Enzyme | 12 | 150 | 140 | 250 | 100 | 80 | 100 |
| Buffer | 288 | 150 | 210 | 84 | 100 | 120 | 100 |
| Total volume | 300 | 300 | 350 | 334 | 200 | 200 | 200 |

From the 4x dilution, the dilutions were serial and the 25x dilution was used to make the 50x, the 50x to make the 125x, the 125x to make the 167x, the 167x to make the 334x, the 334x to make the 835x and the 835x to make the 1670x. Numbers in brackets show the final dilutions.

The dilution 668x is the final dilution in the enzymatic saccharification (as described in section 4.3) with the enzyme loading of 15 mg/g DW which is why this was chosen as a midpoint. All incubations were performed in an Eppendorf ThermoMixer® C.
**FPU assay:**

Circles of Whatman no.1 filter paper were produced using a paper puncher. One circle of filter paper was put into the wells of a flat bottomed 96-well plate (Sarstedt) except for the wells that contained the enzyme control (referred to in the Table 4-2 as B, blank). For the layout of the 96-well plate see Table 4-2. Then 40 µl of buffer was added to each well. 20 µl of buffer, glucose standard or enzyme solution was then added to the wells. The enzyme reaction was performed at 50°C for 60 min in an Eppendorf ThermoMixer® C. After incubation 120 µl of DNS reagent was added to each well and then color development was performed for 8 minutes at 95°C in an Eppendorf ThermoMixer® C. A second plate was prepared with 160 µl milliQ water in each well and after incubation, 36 µl of the reaction was transferred to the second plate. Absorbance was measured at 540 nm using a microplate reader (FLUOstar Omega).

*Table 4-2. The layout of the microplate. B stands for blank and means that no filter paper was added to that well. 0.5, 1, 2, 3, 4 and 5 represents the glucose concentrations in g/l in the added glucose standards (for glucose dilutions see Appendix 8.1.)*

<table>
<thead>
<tr>
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<th>1</th>
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<th>3</th>
<th>4</th>
<th>5</th>
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<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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<tbody>
<tr>
<td>A</td>
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<tr>
<td>B</td>
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<td></td>
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</tr>
<tr>
<td>C</td>
<td>Buffer</td>
<td>Buffer</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>5</td>
<td>6680x</td>
<td>6680x</td>
<td>6680x</td>
<td>3340x</td>
<td>3340x</td>
<td>3340x</td>
<td>3340x</td>
<td>1336x</td>
<td>1336x</td>
<td></td>
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<tr>
<td>E</td>
<td></td>
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<tr>
<td>G</td>
<td></td>
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</tbody>
</table>

Glucose was used as a standard for the assay and a control using only buffer and DNS reagent was also included. Filter paper was added both to the glucose standard and the control. Each sample is incubated in the assay with and without (blank) filter paper. The blank samples are used for measuring the glucose/reducing sugars present in the initial sample and this value is subtracted from the value of reducing sugars measured in the samples with filter paper.
paper. This gives the net production of reducing sugars from the saccharification of the filter paper.

The filter paper reaction is non-linear. As stated by Ghose [47]: “Twice as much enzyme will give equal sugar in half the time, but it will not give twice as much sugar in equal time.”. Therefore the filter paper unit is based on equal conversion of substrate in the reaction time, also known as the critical amount of glucose to be released. In the microscale filter paper assay the critical amount of glucose to be released is 0.08 mg per 20 µl of enzyme. Therefore, to be able to determine the filter paper activity correctly, each sample should be diluted to release the critical amount of glucose. This is very difficult practically and instead several different dilutions are prepared from each sample with the aim to find one dilution releasing a little more and one releasing a little less than the critical amount of glucose.

One FPU is defined as the amount of enzyme that releases 0.08 mg of glucose equivalents in one hour at 50°C, for more information on how to calculate FPU activity, more detailed methods as well as explanations of the data processing methods, see Appendix 8.1.

**Preparation of DNS reagent:**

First 472 ml of distilled water, 3.53 g of 3,5-dinitrosalicylic acid and 6.6 g of sodium hydroxide was mixed until the solids had dissolved. Once dissolved, 102 g of potassium sodium tartare tetrahydrate (also known as Rochelle salts), 2.53 ml of phenol (melted in water bath at 50°C) and 2.77 g sodium metabisulfite was added and the solution was mixed properly. The reagent was then put in a glass bottle and wrapped in aluminum foil to be stored in room temperature.

**4.3 Enzymatic saccharification of steam exploded wheat straw slurry**

**4.3.1 Preparation of the washed wheat straw slurry**

About 45 ml of slurry was added to a 50 ml conical centrifuge tube. The tube was centrifuged at 5311 x g at 4°C for 20 minutes. The supernatant (about 15 ml supernatant was retrieved per 45 ml slurry) was collected and stored at -20°C until it was used for the enzyme precipitation experiments (described in section 4.6).
The pellet (insoluble part of the slurry) was removed from the tube into a beaker and mixed with ~25 ml of 60-70°C tap water, put back into the conical centrifuge tube and centrifuged at 5311 x g at 4°C for 20 minutes. The supernatant was discarded and the procedure was repeated 3 more times.

4.3.1 Dry weight determination of the washed wheat straw slurry

The dry weight of the washed wheat straw slurry was determined by first weighing two small containers and noting the weights. Then a small amount of slurry was added to each container and the container + wet weight of the slurry was weighted, from which the wet weight can be calculated. Then the containers were put at 105°C so that the wheat straw slurry would dry. The container + dry weight was then measured after one and two days. As the weight didn’t change between the measurements, the wheat straw was dry and the dry weight was calculated. Using the wet weight and the dry weight, the DW of the wet slurry was calculated according to the following equation which gave a DW percentage that was then averaged between the two different samples:

\[
\frac{\text{Dry weight}}{\text{Wet weight}} \times 100
\]

4.3.2 Preparation of samples for enzymatic saccharification

Slurry of 10% DW was prepared by diluting 14.2% slurry 1.4 times (the 17.3% DW washed slurry was diluted 1.7 times) with tap water. The dilutions were made by weight. 120 µl of vitahop lactostab antimicrobial agent was added per one kg of 10% DW slurry. The pH was then adjusted using 1 M KOH. Finally, water was added to achieve 10% DW. The slurry was loaded into 50 ml conical centrifuge tubes with 22 g per tube, leaving 20 g per tube after time 0 sampling 20 g of 10% slurry per tube means that there was 2 g of dry weight in each tube initially.

Catalase was diluted 10x and CTec3 was diluted 4x in tap water. CTec3 was loaded at 15 mg/g DW (w/w) (called as “high”) or 5 mg/g DW (w/w) (called as “low”). Since the DW contents in each tube is 2 g, after dilution the enzyme loadings correspond to 120 µl (high) and 40 µl (low). Catalase was loaded at 79.2 µg of protein/g DW. Since the DW content in each tube is 2 g, after dilution the catalase loading corresponds to 100 µl/tube. Enzymes or corresponding amount of water was added to both the slurry and the washed slurry according to Table 4-3, with duplicates 1 and 2 for each enzyme loading.
Table 4-3. Enzyme loading for the different samples. L stands for low loading of Ctec3, H for high loading of CTeC3 and C for catalase loading. All volumes are in µl.

<table>
<thead>
<tr>
<th>Added solution</th>
<th>L1, L2</th>
<th>H1, H2</th>
<th>LC1, LC2</th>
<th>HC1, HC2</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x Diluted CTec3</td>
<td>40</td>
<td>120</td>
<td>40</td>
<td>120</td>
</tr>
<tr>
<td>10x Diluted Catalase</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Tap water</td>
<td>180</td>
<td>100</td>
<td>80</td>
<td>-</td>
</tr>
</tbody>
</table>

**Incubation:**

The samples were placed in a mini LabRoller™ rotator (Labnet) for free fall mixing and incubated at 50 °C with incubation start defined as time=0.

**Sampling:**

Time 0 samples were taken before the addition of enzymes. After time 0 samples there was 20 g of 10% slurry in each sample tube. During the sampling first the pH was measured and samples with pH below 5 were adjusted up to 5 using 1 M KOH. At each sampling point 2 g of sample was removed from the slurry and centrifuged at 10 000 x g for 10 min at 4°C. Supernatants were filtered through 0.2 µm nylon filters and stored in 2 ml microcentrifuge tubes at -20°C until analysis.

**Preparation of samples for enzymatic saccharification of slurry**

The slurry was prepared as described above. For the 4x dilution of CTec3, 228.8 mg of enzyme was diluted in 686 µl of water. pH was adjusted to 5.3 using 1 M KOH. Samples were taken after 25 h, 42.7 h, 71.8 h, 90.5 h and 168.1 h, as described above.

**Preparation of samples for enzymatic saccharification of washed slurry**

The slurry was prepared as described above. The final pH of the slurry was 5.6 rather than 5.3. For the 4x dilution of CTec3, 235.5 mg of enzyme was diluted in 707 µl of water. Samples were taken after 25.1 h, 43.7 h, 72.7 h, 91.3 h and 168.8 h, as described above.
4.4 Recycling of enzymes in enzymatic saccharification on wheat straw slurry

In this context, recycling of enzymes means addition of fresh substrate to the enzymatic saccharification process to investigate the remaining activity of the enzymes after 48 hours since the glucose release rate had decreased significantly at this point. Figure 4-1 gives an overview of the experimental process. There were two different types of recycling experiments, both performed in duplicates. In the Figure 4-1 the different types of recycling are referred to as “Without concentration” and “With concentration”. The procedure in this experiment is inspired by Andersen et al. [49] and Rodrigues et al. [50] papers.

![Flow chart](chart.png)

Figure 4-1. A flow chart describing the different steps in the enzyme recycling experiment. For the samples labeled “without concentration” fresh substrate was simply added to the 10 g of old slurry while for the samples labeled “with concentration” the enzymes in the supernatant were concentrated using a 10 kDa cutoff filter, which retained all enzymes with molecular weights above 10 kDa. The concentrated enzymes were added back to the slurry before addition of fresh slurry.
First 10% slurry was prepared as described in section 4.3.2 and divided into 50 ml conical centrifuge tubes, 20 g per tube.

For the 4x dilution of CTec3 164.8 mg of enzyme and 495 µl of water was used. CTec3 was loaded at 15 mg/g DW. Since the DW contents in each tube is 2 g, after dilution the enzyme loading corresponds to 120 µl.

Once the slurry was prepared and enzymes were loaded, 2 g of time 0 samples were taken from each tube. The incubation and sampling procedure was performed as in section 4.3.2 samples were incubated at 50°C and 2 g samples were taken at each sampling point, samples were taken after 24 and 47.7 h. After the sampling at 47.7 h, 10 g of the remaining slurry was removed. For the “with concentration” samples the slurry was centrifuged at 5525 x g at 4 °C for 20 min. The supernatant was removed and transferred to an Amicon® Ultra-4 10K centrifugal filter device. Using the 10 kDa cutoff filter the supernatant was concentrated 10x (from 7 ml to 0.7 ml). The concentrated enzyme solution was then washed with 3 ml of tap water by adding it to the filter and spinning it through. This was done to wash away residual sugars. The concentrated enzymes were then added back to the pellet and tap water was added to get back up to 10 g of slurry.

For both the samples “without concentration” and the samples “with concentration” 10 g of fresh slurry was added to each tube and the slurry was mixed properly. Then the pH was adjusted up to 5.3 for each sample and 2 g of new time 0 samples were taken. The samples were then incubated as before with samples taken at 23.2 and 47 h.

4.5 Enzyme precipitation in supernatant from wheat straw slurry

4.5.1 Preparation of samples for precipitation experiments

First the supernatant from the non-washed wheat straw slurry was filtered through a 0.2 µm filter and then it was diluted 1.5 times to resemble the saccharification experiments. The supernatant was then aliquoted into different 15 ml conical centrifuge tubes. There is one tube for each day (0, 1 and 3) and pH (4, 5 and 6). The experiment was performed in duplicates.

The volume of supernatant in each tube was 5 ml. The enzymes were then added on top of this volume as described below. There was also a slight increase in volume when the pH was adjusted, due to addition of KOH.
Enzymes were diluted and then added accordingly: CTec3 was added at 1.5 mg/ml (w/v), which is representative of the high enzyme loading in the saccharification experiments and catalase was added at 7.9 µg of protein/ml, also representative of the saccharification experiments.

After the enzymes had been added to the tubes, the pH was adjusted to 4, 5 or 6 using KOH. For the pH adjustment, the initial pH was recorded (3.9) and then the pH was adjusted. Once all samples were prepared, the samples that were going to be incubated were filtered through 0.45 µm nylon filters to remove precipitates caused by the initial sample preparation. This was done to make sure that the pellets in the incubated samples were formed due to incubation and nothing else. Both day 1 and day 3 samples were put in an Eppendorf ThermoMixer® C at 50°C and 400 rpm.

Time 0 samples are samples that instead of being filtered and incubated after preparation were directly divided into 3 of 2 ml microcentrifuge tubes/sample (2 ml of sample/tube) and centrifuged at 4200 x g for 15 min at 4°C. The supernatants were transferred into new tubes and both supernatants and pellets were put to -20°C for storage. The same was done for the day 1 and day 3 samples after 22.2 and 68.6 h respectively.

**Dilution of CTec3**

The CTec3 enzyme stock was diluted 10x with tap water, 773.2 mg enzyme and 6959 µl water and enzyme was added at 1.5 mg/ml (150 µl/ml → 750 µl/tube)

**Dilution of CTec3 and catalase**

The CTec3 stock was diluted 5x with tap water, 716.85 mg enzyme and 2867 µl tap water and the catalase stock was diluted 10x with tap water, 250 µl enzyme and 2250 µl water.

Enzymes were then added accordingly: CTec3 was added at 1.5 mg/ml (75 µl/ml of 5x dilution → 375 µl/tube) and catalase was added at 7.92µg/ml (50 µl/ml of 10x dilution→ 250 µl/tube).

**4.5.2 Precipitation of catalase, β-glucosidase and TaAA9A with and without catalase**
The following enzymes and enzyme combinations was studied for precipitation in supernatant:

- TaAA9A
- TaAA9A + catalase
- Catalase
- β-glucosidase

This precipitation experiment was only performed at pH 5. The catalase loading was the same as in the initial experiment at 7.92 µg/ml. Based on Scott et al. [12], the BG and the TaAA9A was loaded at 0.03024 mg protein/ml.

The TaAA9A stock solution was 73 g/l. To achieve the same protein loading as Scott et al. [12], 0.03024 mg of enzyme/ml was required, leading to the following calculation to determine the corresponding volume of stock solution:

\[ V = \frac{m}{c} = \frac{0.03024 \cdot 10^{-3}}{73} = 4.1425 \cdot 10^{-7} \approx 0.414 \, \mu l/ml \]

The TaAA9A stock was diluted 20 times. Meaning that for 5 ml of supernatant, 41.4 µl of TaAA9A was added.

BG stock solution was 34 g/l. To achieve the same protein loading as Scott et al. [12], 0.03024 mg of enzyme/ml was required, leading to the following calculation to determine the corresponding volume of stock solution:

\[ V = \frac{m}{c} = \frac{0.03024 \cdot 10^{-3}}{34} = 8.894 \cdot 10^{-7} \approx 0.889 \, \mu l/ml \]

The BG stock was diluted 20x. This means that for 5 ml of supernatant, 88.9 µl of BG was added.

**Preparation of samples**

First the supernatant from the non-washed wheat straw slurry was filtered through a 0.2 µm filter and then it was diluted 1.5 times. The supernatant was then aliquoted into different 15 ml conical centrifuge tubes according to Table 4-4.
The enzymes were diluted according to Table 4-5 and then added according to Table 4-4. This gave a total volume of 5 ml.

<table>
<thead>
<tr>
<th>Solution added</th>
<th>Catalase</th>
<th>TaAA9A</th>
<th>Catalase + TaAA9A</th>
<th>BG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase 10x</td>
<td>250 µl</td>
<td>-</td>
<td>250 µl</td>
<td>-</td>
</tr>
<tr>
<td>TaAA9A 20x</td>
<td>-</td>
<td>41.4 µl</td>
<td>41.4 µl</td>
<td>-</td>
</tr>
<tr>
<td>BG 20x</td>
<td>-</td>
<td>88.9 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant 1.5x</td>
<td>4750 µl</td>
<td>4958.6 µl</td>
<td>4708.6 µl</td>
<td>4911.1 µl</td>
</tr>
</tbody>
</table>

The pH was adjusted to 5 using 1 M KOH. Once all samples were prepared, the samples that were going to be incubated were filtered through 0.45 µm nylon filters to remove precipitates caused by the initial sample preparation. This was done to make sure that the pellets in the incubated samples were formed due to incubation and not sample preparation. Both day 1 and day 3 samples were put in an Eppendorf ThermoMixer® C at 50°C and 400 rpm.

The Time 0 samples were aliquoted into 2, 2 ml microcentrifuge tubes/sample (2 ml/tube) and centrifuged at 4200g for 15 min at 4°C. The supernatants were transferred into new tubes and both supernatants and pellets were put to -20°C for storage. The same was done for the day 1 and day 3 samples after 22.2 and 68.6 h respectively. For day 0 and 1 there were no visible pellets.

### 4.6 Analytical measurements

#### 4.6.1 HPLC analysis

High performance liquid chromatography (HPLC) was used to measure glucose, xylose and acetate in the saccharification samples.
HPLC is a chromatographic technique used to separate analytes in order to facilitate analysis. The setup consists of a column containing a stationary phase that will weakly bind the analytes, causing them to slow down, and a mobile phase that carries the analytes through the column. The amount of retardation and thereby the retention time differs between analytes depending on its physical or intramolecular properties [51]. In HPLC a high pressure is applied to push the analyte through the column, thus saving time.

The saccharification samples were analyzed in a Dionex, UltiMate™ 3000 HPLC using a Resex ROA organic Acid H+ (300mm x 7.8mm) column. Samples were run using a flow rate of 0.8 ml/min at a temperature of 80°C and a program time of 18 min in the isocratic elution mode. The eluent used was a 5 mM solution of sulfuric acid and the wash buffer was a 5% methanol solution. All compounds were detected with an RI detector. An example of a chromatogram can be seen in Appendix 8.2. All samples from the saccharification experiments were analyzed with HPLC. Samples were diluted in milliQ water between 2 and 10 times depending on how far into the hydrolysis experiments they were taken. Data analysis was performed using Chromeleon software. For information about standard preparations see Appendix 8.2.

4.6.2 SDS-PAGE analysis

Samples from the precipitation experiments were analyzed by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to evaluate how the protein amounts varied during the time of the experiment. The principle of the SDS-PAGE is that first you add sample buffer to your sample and boil it in order to denature the proteins. The sample buffer contains sodium dodecyl sulfate (SDS), a negatively charged molecule that interacts by hydrophobic interaction with the protein and covers it, thereby making the proteins negatively charged. The charge/weight is constant for all proteins. This solution would then be run through a highly crosslinked polyacrylamide gel by applying a voltage causing the proteins to wander towards the positive pole. Larger proteins will wander slower than the smaller proteins through the gel causing a size separation [52].

Sample preparation

The supernatants from the precipitation experiment with CTec3 and CTec3 with catalase were diluted 10 times, with a final dilution after addition of sample buffer of 13.3x
while the pellets (from 2 ml aliquot) were first washed by resuspending them in 400 µl of milliQ-water and centrifuging at 4200 x g, 4°C and 15 min. The water was then removed and the pellets were suspended again in 200 µl of milliQ-water. Pellet suspensions were non diluted and had a final dilution of 1.3x after addition of sample buffer. Supernatants from experiments with TaAA9A, catalase and BG were diluted in total 1.3x after addition of sample buffer.

The samples were analyzed using SDS-PAGE with the Bio-Rad ChemiDoc MP imaging system. The gels used were Mini-PROTEAN® TGX Stain-Free™ precast Protein Gel, 10 well, 30 µl from Bio-Rad and electrophoresis was run at 100 V for about 1 h and 20 min in 25 mM Tris/192 mM glycine/0.1 % (w/v) SDS, pH 8.3 running buffer. The Bio-Rad Precision Plus Protein™ Unstained Standard was used as a protein standard. 10 µl of standard was used and 15 µl of sample was mixed with 5 µl of 4X sample loading buffer and boiled at 95°C for 10 min leading to a loading volume of 20 µl. Before analysis the gels were stained with PageBlue Protein Staining solution, from Fermentas, and de-stained overnight in deionized water. Due to poor resolution on the small bands on the stain free gels they were stained, giving a better resolution.

Gels with supernatants from precipitation experiments with CTec3 and CTec3 with added catalase were exposed for 0.046 s using the BioRad ChemiDoc MP imaging system, while pellets from the same experiments were exposed for 0.045 s. Gels with supernatants from precipitation experiments with TaAA9A, catalase and BG were exposed for 0.050 s. For the complete SDS-PAGE procedure, see Appendix 8.3.
5 RESULTS AND DISCUSSION

5.1 Cellic CTec3 Filter Paper Unit activity

In order to measure enzyme activity in the CTec3 enzyme mixture a FPU assay was performed. Since the protein concentration of the CTec3 is not revealed by Novozymes A/S the FPU result could be used to calculate the amount of FPUs that are loaded per g of DW which makes it possible to compare saccharification results between different enzyme mixtures and other papers where the enzyme loading is described in FPUs. Based on the duplicate experiments the filter paper unit activity of CTec3 was calculated to be 262±1 FPU/ml.

5.2 Enzymatic saccharification experiments

5.2.1 Enzymatic saccharification of wheat straw slurry and warm water-washed slurry

In order to investigate the possible effect of inhibitors produced during pre-treatment of the substrate on the enzymatic activity two sets of saccharification experiments were set up, one experiment with wheat straw slurry and one where the wheat straw slurry had been washed with warm water. Scott et al. [12] has shown that catalase interrupts oxygen dependent radical reactions mediated by LPMOs that are damaging to one or more components of the enzyme mixture. Therefore the saccharification experiments were also performed with and without addition of catalase, which converts \( \text{H}_2\text{O}_2 \) into water and oxygen. This was done in order to investigate if \( \text{H}_2\text{O}_2 \) has an effect on enzyme inactivation.

The overall experimental procedure is based on the work of Scott et al. [12]. The experiments on the slurry are repeats of the Scott experiments while the experiments on the washed substrate are new in this thesis.

Figure 5-1 shows the glucose released over time during 168 h of incubation for the wheat straw slurry (A) and washed slurry (B). Even though the high enzyme loading was three times higher than the low loading it did not result in three times more glucose (Figure 5-1 A and B). This could be due to several different reasons such as enzyme inactivation, substrate depletion and end point inhibition. In Figure 5-1 A, the addition of catalase gave a faster glucose release from the slurry. Table 5-1 shows the average glucose release rate for the
different samples between the sampling points for the slurry. For the low enzyme loading the release rate for samples with catalase was either higher or the same as for samples without catalase (Table 5-1). For the samples with high enzyme loading the initial rate was higher without catalase, however after the initial sampling point the rate was higher with catalase added (Table 5-1). Even though the initial rate was higher for the high CTec3 without catalase the total glucose released was higher with the addition of catalase (Figure 5-1 A). The difference between the glucose released, due to catalase addition, seemed to increase over time (Figure 5-1 A).

Catalase is preventing H$_2$O$_2$ from forming ROS that can inactivate the enzymes [11]. Therefore in the sample without catalase the amount of inactivated enzymes would increase more over time than in the samples without added catalase. This would cause an increased glucose release rate for samples with added catalase, which in turn led to a higher final glucose concentration. This explains why the difference in glucose amount would increase over time. These results match the results produced by Scott et al. [12]. In Figure 5-1 B, which shows the results for the washed slurry, the glucose release seems to decrease with the addition of catalase which was surprising.

More glucose was released from the washed substrate (Figure 5-1 A vs. B). This indicated that washing of the substrate can remove at least part of the soluble inhibitors that are produced during the pre-treatment process [28].

<table>
<thead>
<tr>
<th>Time</th>
<th>Low CTec3</th>
<th>Low CTec3 + Catalase</th>
<th>High CTec3</th>
<th>High CTec3 + Catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-25</td>
<td>0.29</td>
<td>0.31</td>
<td>0.79</td>
<td>0.73</td>
</tr>
<tr>
<td>25-43</td>
<td>0.15</td>
<td>0.19</td>
<td>0.40</td>
<td>0.44</td>
</tr>
<tr>
<td>43-72</td>
<td>0.13</td>
<td>0.14</td>
<td>0.09</td>
<td>0.17</td>
</tr>
<tr>
<td>72-91</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>91-168</td>
<td>0.04</td>
<td>0.04</td>
<td>0.03</td>
<td>0.03</td>
</tr>
</tbody>
</table>

The final glucose concentrations (g/l) after 168 h were 19.0±0.1 for low CTec3, 20.8±0.1 for low CTec3 with catalase, 33.7±0.1 for high CTec3 and 35.8±1.1 for high CTec3 with catalase for the experiment on slurry. For the washed slurry the final glucose concentrations were 31.2±0.4 for low CTec3, 31.5±0.1 for low CTec3 with catalase, 45.0±2.0 for high CTec3 and 44.6±0.2 for high CTec3 with catalase. This means an increase in final glucose with 9.5% and 6.2% by adding catalase to the low and high CTec3 respectively for
the slurry, while for the washed substrate the addition of catalase gives no clear difference in final glucose concentration. However it seems like the addition of catalase slows down the saccharification process for the washed slurry (Figure 5-1 B), which would need to be confirmed by further experiments.

Figure 5-1. Glucose release plotted against the incubation time. Low CTec3 means that CTec3 was loaded at 5 mg/g DW (w/w) and High CTec3 that CTec3 was loaded at 15 mg/g DW (w/w). The 5 mg/g DW of CTec3 corresponds to an FPU of 5.2 FPU/g DW while the 15 mg/g DW corresponds to 15.7 FPU/g DW. Catalase was loaded at 79 µg/g DW. (A) shows the results for the slurry and (B) shows the results for the washed slurry. The Low CTec3 sample is represented by black diamonds, the Low CTec3+catalase by black squares, High CTec3 by black triangles, High CTec3+catalase by black circles and the blank, without enzyme, by white squares. Each point represents the average values of two duplicates and the error bars show the standard deviation.

Figure 5-2 shows the glucose release from a repeat experiment on the slurry. The results for the lower CTec3 loading confirm the results in Figure 5-1 A, where addition of catalase causes more glucose to release. However for the high CTec3 loading (Figure 5-2) the addition of catalase causes lower release of glucose, which was surprising.
Figure 5-2. Glucose release plotted against the incubation time, repeated experiment on the slurry. Low CTec3 means that CTec3 was loaded at 5 mg/g DW (w/w) and High CTec3 that CTec3 was loaded at 15 mg/g DW (w/w). The 5 mg/g DW of CTec3 corresponds to an FPU of 5.2 FPU/g DW while the 15 mg/g DW corresponds to 15.7 FPU/g DW. Catalase was loaded at 79 µg/g DW. (A) shows the results for the slurry and (B) shows the results for the washed slurry. The Low CTec3 sample is represented by black diamonds, the Low CTec3+catalase by black squares, High CTec3 by black triangles, High CTec3+catalase by black circles and the blank, without enzyme, by white squares. Each point represents the average values of two duplicates and the error bars show the standard deviation.

Figure 5-3 shows the glucose release plotted against the enzyme dose multiplied by time, where A shows the results for the slurry and B for the washed slurry. If there was no time dependent enzyme inactivation all lines in each figure would superimpose [12]. The fact that the lines deviate is consistent with time dependent enzyme inactivation [53].

Figure 5-3 A shows that the enzyme inactivation is lower for the samples with catalase addition indicating that catalase can lower the rate of enzyme inactivation in the slurry caused by H₂O₂. The enzyme inactivation was lower for the washed slurry (Figure 5-3 B) than for the slurry (Figure 5-3 A) which can be seen by the fact that, for the washed slurry the lines representing the high and the low CTec3 loading overlap longer than for the slurry. However in the case of the washed substrate (Figure 5-3 B) the addition of catalase seems to lead to more enzyme inactivation, especially in the case of the higher enzyme loading.
The addition of catalase seems to have little to no effect on the xylose release as can be seen in Figure 5-4 A and B. It is also clear that the total xylose release was decreased for the washed slurry (B) as compared to the slurry (A). It can be observed from Figure 5-4 that for the slurry there was about 1.8 g/l of xylose initially while for the washed slurry the value is close to zero. Another cause for the decrease in xylose release is probably that soluble xylo-oligomers were removed during the washing process and therefore there was less remaining potential xylose in the washed substrate. The soluble xylo-oligomers are produced during the pre-treatment process where most of the hemicellulose is solubilized [29]. The solubilized hemicellulose was washed away during the washing process so the xylose that was released during the saccharification of the washed slurry came from hemicellulose in the solid fraction.

The concentrations of xylose (g/l) after subtraction of blank (slurry without enzymes), after 168 h were 14.1±0.2 for low CTec3, 14.0±0.1 for low CTe3 with catalase, 16.2±0.0 for high CTec3 and 16.2±0.1 for high CTec3 with catalase for the experiment on slurry. On the washed slurry the final xylose concentrations (g/l) were 4.6±0.0 for low CTec3, 4.5±0.0 for low CTec3 with catalase, 5.0±0.2 for high CTec3 and 5.0±0.1 for high CTec3 with catalase.
The release of xylose plotted against the incubation time. Low CTec3 means that CTec3 was loaded at 5 mg/g DW (w/w) and High CTec3 that CTec3 was loaded at 15 mg/g DW (w/w). Catalase was loaded at 79.2 µg/g DW. (A) shows the results for the slurry and (B) shows the results for the washed slurry. The Low CTec3 sample is represented by black diamonds, the Low CTec3+catalase by black squares, High CTec3 by black triangles, High CTec3+catalase by black circles and the blank, without enzyme, by white squares. Each point represents two duplicates and the error bars show the standard deviation.

The setup of the saccharification experiment was based on the setup used by Scott et al. [12]. The saccharification of the wheat straw slurry confirms their results that the addition of catalase to the saccharification slurry decreases the time dependent enzyme inactivation in ambient oxygen conditions. The current project, additionally to investigating the effect of catalase under ambient oxygen as performed by Scott et al. [12], also compared saccharification of slurry and warm water washed slurry as well as investigated the effect of catalase on washed slurry in ambient oxygen conditions.

The saccharification experiments show that more glucose is released and that the enzyme inactivation is lower in the washed slurry as compared to the slurry. This indicates that there are water-soluble compounds, such as organic acids and furfurals [43], in the slurry that contribute to enzyme inactivation, which are at least partially washed away during the washing process.

As mentioned before, Scott et al. [12] has shown that the addition of catalase to saccharification experiments at ambient oxygen conditions can reduce the inactivation of a cellulase mixture containing LPMOs. They have also shown that catalase has no apparent effect on the catalytic rate, which implies that catalase interrupts oxygen dependent radical reactions that are mediated by the LPMOs and that are damaging to the enzyme mixture. The fact that the catalase has positive effect, with regard to glucose release, on the slurry confirms...
these results. However the additions of catalase had no or even reverse effect on the washed slurry. This indicates either that the H$_2$O$_2$ is not produced by LPMOs in the washed slurry and therefore catalase has no effect or that H$_2$O$_2$ is not only produced by LPMOs in the slurry but also by another route, such as aerobic degradation of organic matter [54]. Since this positive effect by addition of catalase only was seen in the slurry and not in the washed slurry this could indicate that the H$_2$O$_2$ is produced by aerobic degradation of a soluble compound that is washed away during the washing process.

As mentioned before, saccharification of the washed substrate resulted in less enzyme inactivation and higher glucose release. However, there is potential xylose lost in the washing procedure and the fact that the washing adds a process step to consider when choosing a substrate for industrial use.

### 5.2.2 Acetate release and pH adjustment

In order for the enzymes to work as efficiently as possible the pH needs to be kept close to the optimal pH for the enzyme cocktail. The optimal pH for CTec3 is between 4.75 and 5.25 [55]. The pH was initially adjusted to 5.3 since the pH drops during enzymatic saccharification and then it was adjusted up to 5 if necessary at each sampling point, to keep the saccharification process as close to the optimal pH as possible. The pH was adjusted by adding 1 M KOH to the samples, the progress curves of KOH addition over time can be seen in Appendix 0. In this thesis, no buffer was used for the saccharification experiments since the use of buffer is less relevant in an industrial setting. This setup also made it possible to study the changes in pH during incubation which otherwise would be masked by the addition of buffer.

During the saccharification process acetic acid is released from the hemicellulose [56]. Since the release of acetic acid causes the pH to drop, the acetate levels during the saccharification process were evaluated.

As can be seen in Figure 5-5 A, the addition of catalase showed slightly less acetate released in the slurry compared to slurry without catalase. By comparing the slurry (Figure 5-5 A) with the washed slurry (Figure 5-5 B) it can be concluded that the washing of the slurry removed acetate that was released during the pre-treatment process. It can also be observed in Figure 5-5 A (blank) that some acetate is released from the substrate even without the addition of enzymes, which was surprising.
The reason that the results for the washed experiment (Figure 5-5 B) only show the results up to 72.7 h is that at this point some of the samples started to show signs of contamination (first the pH rose up to 5.8 and later the contamination could be confirmed visually as a white mold). The HPLC results from samples after the sudden rise in pH show no acetate present and therefore the data is only shown up to the point when contamination occurred.

The final acetate concentrations (g/l) due to enzymatic release (Figure 5-5 A) were 1.2±0.0 for low CTec3, 1.2±0.0 for low CTec3 with catalase, 1.3±0.0 for high CTec3 and 1.3±0.0 for high CTec3 with catalase for the experiment on slurry. For the washed slurry (Figure 5-5 B) the acetate concentrations (g/l) due to enzymatic release after 72.7 h were 0.4±0.0 for low CTec3, 0.4±0.0 for low CTec3 with catalase, 0.5 for high CTec3 and 0.4±0.1 for high CTec3 with catalase.

![Figure 5-5. Acetate release during the saccharification experiments. Low CTec3 means that CTec3 was loaded at 5 mg/g DW (w/w) and High CTec3 that CTec3 was loaded at 15 mg/g DW (w/w). Catalase was loaded at 79.2 µg/g DW. (A) shows the results for the slurry (up to 168 h) and (B) shows the results of the washed slurry (up to 73 h). The Low CTec3 sample is represented by black diamonds, the Low CTec3+catalase by black squares, High CTec3 by black triangles, High CTec3+catalase by black circles and the blank, without enzyme, by white squares. Each point represents the average value of the two duplicates and the error bars show the standard deviation.](image)

Figure 5-6 A shows the sum of the 1 M KOH that was added at each sampling point for the different samples after 25, 73 and 168 h of incubation. It is clear that by adding catalase, less KOH was needed in total to adjust the pH. One partial explanation for this is the fact that slightly less acetate is released when catalase is added (Figure 5-5). A lower acetic acid release means a lower drop in pH and therefore less base is needed to raise the pH to 5 at
each sampling point. Figure 5-6 A also shows that most of the KOH is added at the first sampling point. In Figure 5-5 A it is clear that the most acetate is released up to the first sampling point and then the slope declines. This could partially explain why the initial pH drop is larger and why the most KOH therefore is needed in the beginning.

Figure 5-6 B shows the sum of the KOH that was added at each sampling point for the different samples after 25 and 73 h. By adding catalase, less KOH was needed in total to adjust pH to 5 at each sampling point. As mentioned before, after 73 h some samples were contaminated and acetate levels dropped to 0, causing the pH to rise for the contaminated samples. Since the contaminated samples therefore needed no addition of KOH, the remaining sample points were not included in the summation of KOH. The most of the KOH was added at the first sampling point for all samples but the blanks (Figure 5-6 A and B).

It is also noteworthy that also for blanks, without enzymes the pH drops and needs to be adjusted (Figure 5-6 A and B). In Figure 5-5 A and B, some acetate release can be seen for the blanks which might to some extent explain why the pH drops even without enzymes.

![Figure 5-6. The total amount (mg) of 1 M KOH added during 25, 73 and 168 h of saccharification experiment to adjust the pH up to 5 for the slurry (A). The total amount (mg) of 1 M KOH added during 25 and 73 h of saccharification experiment to adjust the pH up to 5 for the washed slurry (B). The blank (without enzyme) is represented by striped bars, the Low CTec3 is represented by black bars, the Low CTec3 + Catalase is represented by grey bars, the High CTec3 by white bars and the High CTec3 + Catalase by dotted bars. Each point represents two duplicates and the error bars show the standard deviation.](image)

Acetic acid release is believed to be a major cause of the decrease in pH during enzymatic saccharification [1]. For both the slurry and the washed slurry the addition of catalase resulted in less KOH needed to be added in order to adjust the pH up to 5 at each sampling point. Figure 5-5 (A vs. B) shows that the washed substrate contains less acetate and
therefore less base needed to be added to the washed substrate in order to adjust the pH (Figure 5-6 A vs B). For both the slurry and the washed slurry the largest increase in acetate was seen between the time 0 and 25 h (Figure 5-5 A and B). This can, at least partially, explain why the major part of the KOH is added at the first sampling point since more acetate means a larger drop in pH.

5.2.3 Enzyme recycling

In this experiment the residual enzyme activity was investigated after 48 h of initial saccharification, since at this point the glucose release rate had dropped significantly as seen from previous saccharification experiments. After 48 h either fresh slurry (10% DW) was added at 1:1 or first enzymes were concentrated (sugars and other soluble compound with molecular weights lower than 10 kDa were removed) and then fresh substrate was added. The two different conditions, with and without enzyme concentration were evaluated in order to investigate if the slow-down in saccharification was due to substrate depletion, end-point inhibition and/or enzyme inactivation.

The result of the glucose release is shown in Figure 5-7. In Figure 5-7 the release of glucose slowed down in the first half of the experiment. The removal of sugars (concentrating of enzymes) led to a higher glucose release rate (Table 5-2) compared to not removing sugars (without concentration of enzymes).

![Figure 5-7. The total glucose released plotted against the incubation time. The recycling is marked by the vertical line at 47.7 h. CTec3 was loaded at 15 mg/g DW (w/w). Catalase was loaded at 79.2 µg/g DW. Diamonds represent samples without enzyme concentration and squares represent samples with enzyme concentration.](image)
Table 5-2 shows that the removal of sugars, which occurs during the concentration of enzyme, increased the glucose release rate (With vs without enzyme concentration) and that after the addition of fresh substrate the rate of glucose release increased (before and after 48 h). This indicates that by the addition of fresh substrate, more accessible cellulose is added.

Table 5-2. Glucose release per hour between the different sampling points. The table shows the data corrected with the dilution factor since the samples, and therefore the enzymes, were diluted 2x with the addition of fresh substrate after 48 h.

<table>
<thead>
<tr>
<th>Time</th>
<th>Without enzyme concentration</th>
<th>With enzyme concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-24 h</td>
<td>0.96</td>
<td>0.96</td>
</tr>
<tr>
<td>24-48 h</td>
<td>0.21</td>
<td>0.16</td>
</tr>
<tr>
<td>48-71 h</td>
<td>0.33</td>
<td>0.45</td>
</tr>
<tr>
<td>71-95 h</td>
<td>0.11</td>
<td>0.15</td>
</tr>
</tbody>
</table>

From the recycling experiment it can be observed that after 48 h the glucose release rate is considerably lower than the initial release rate. The addition of fresh substrate without concentration of enzymes caused the release rate to increase (after correction for enzyme dilution factor). This indicates that part of the reason for the slowdown in glucose release rate can be explained by substrate depletion. Rodrigues et al. [50] have shown that denatured proteins bind to the substrate and thereby block other enzymes from degrading it. This indicates that the slowdown in saccharification is not only due to substrate depletion but also due to limited access to the substrate. They have also shown that enzymes adsorb onto lignin without being inactivated and that the enzymes can desorb from one substrate to another which can be induced by adding fresh substrate, causing the enzyme activity to increase.

The fact that addition of fresh substrate after concentration of the enzymes (and thereby also removal of sugars) resulted in a higher glucose release rate than without enzyme concentration indicates that during the conditions evaluated in this experiment the enzyme activity is also affected by endpoint inhibition which has also been shown by Andersen et al. [49].

The glucose inhibition can be studied further by for example adding glucose at different concentrations to fresh substrate before adding enzymes and see how much the glucose release rates are affected. In order to investigate further how the substrate depletion compares to enzyme inactivation experiments can be performed where old enzymes are added to fresh substrate and fresh enzymes are added to old substrate.
5.3 Enzyme precipitation

5.3.1 Precipitation of Cellic CTec3 with and without addition of catalase

In order to investigate if enzyme precipitation is a possible cause for slowdown in saccharification of wheat straw slurry and if catalase would affect the precipitation, precipitation experiments were designed. The hypothesis for these experiments is that at least some of the precipitation can be explained by enzyme inactivation caused by H$_2$O$_2$ and that this could be counteracted by adding catalase to the experiment. As mentioned before, LPMOs produce H$_2$O$_2$ when they have access to oxygen and electron donors, but no access to substrate [11].

The precipitation was performed in supernatant from the wheat straw slurry to resemble the conditions of the saccharification. CTec3 was added at 1.5 mg/ml, which is representative of the high enzyme loading in the saccharification experiments and catalase was added at 7.92 µg/ml, also representative of the saccharification experiments. The supernatant is a simplified model that doesn’t contain solids but contains the same solubles as in the saccharification experiments of wheat straw slurry.

During the experiments each sample was centrifuged in order to separate the precipitate (pellet) from the supernatant.

In acknowledgement of the material transfer agreement with Novozymes, the actual gel-pictures with CTec3 samples are not shown.

**Supernatant:**

Cellic Ctec3 is a complicated cocktail of many different enzymes. This was also evident from inspection of the SDS-PAGE pictures (not shown throughout the report) with the samples from the precipitation experiment. It could be seen that the enzymes in the supernatant decrease over time for all the different pH values. It can also be seen that the decrease is most apparent for the samples at pH 4 and less for pH 5 and 6, indicating that more precipitation occurs at pH 4, due to the low pH. Some enzymes, like the 3 bands around 25 kDa and the band at 19 kDa, seem to precipitate completely during the time of the experiment, while most of the enzyme amounts decrease over time.
The same pattern is seen for samples where catalase has been added. Especially smaller enzymes decrease or disappear over time. At pH 4 the loss of enzyme is the most clear, both for the smaller enzymes but also two protein bands around 100 kDa.

However, when comparing the two gels, the most obvious difference is for the band at around 30 kDa. The band clearly fades over time at all pH values when no catalase was added to the CTec3 cocktail. The relative amount of this band was quantified using the GelAnalzyer densitometry tool. The result is shown in table 5-3. When catalase has been added to the experiment, this enzyme is more stable over time, and the band doesn’t change. This indicates that catalase might be able to reduce enzyme precipitation. The 30 kD band very likely represents the heterologous AA9 LPMO in Ctec3.

<table>
<thead>
<tr>
<th>A</th>
<th>pH 4</th>
<th>pH 5</th>
<th>pH 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation [days]</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Band intensity [%]</td>
<td>17</td>
<td>13</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>pH 4</th>
<th>pH 5</th>
<th>pH 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation [days]</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Band intensity [%]</td>
<td>16</td>
<td>17</td>
<td>13</td>
</tr>
</tbody>
</table>

**Precipitates:**

As enzyme amounts decreases in the supernatant, the amount increases in the precipitate instead, indicating that most of the enzyme loss can be accounted for by precipitation. It is important to note that since the samples were filtered before incubation, the pellets after one and three days were formed completely during incubation, while the day 0 pellet was formed during preparation of the samples. It can be observed that the precipitation is most prominent at pH 4 and decreases as the pH rises up to 5 and 6. The enzymes which are most sensitive to precipitation are the enzymes at 90 and 120 kDa.

The SDS-PAGE of the pellets from the precipitation experiments on CTec3 with added catalase was also prepared. The precipitation at day 0 for pH 4 and 5 is significantly larger than for the other samples. However the day 0 pellets, when resuspended in water,
formed lumps which was not the case for pellets from day 1 and 3. This means that it was hard to get a representative result for these samples (day 0, pH 4, 5 and 6). In a repeat gel, the results are similar for pH 4 and 6 but the pH 5 differs.

It could be observed that catalase, at least at pH 4, can reduce the overall precipitation during incubation. For pH 5 and 6, the catalase is less clear. It also seems like a wider spectrum of enzymes precipitate during sample preparation (day 0) while during incubation (day 1 and 3) it is mostly the enzymes at 90 and 120 kDa that precipitate. However, more of the 30k Da band (likely to be the LPMO) is seen in the pellets from samples without catalase (see table 5-4).

*Table 5-4. Densitometry analysis of the 30 kDa band in pellet samples of A) Cellic Ctec3 and B) Cellic Ctec3 + catalase after SDS-PAGE. The default setting for identifying lanes and bands were used, as was the rolling-ball diameter for determining the baseline for the densitometry peaks. The band intensity in percentage is relative to the intensity of the 25 kDa molecular weight marker. The lane for sample pH 4, day 3 has too dark background for quantification of the band intensity.*

<table>
<thead>
<tr>
<th></th>
<th>pH 4</th>
<th>pH 5</th>
<th>pH 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation [days]</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Band intensity [%]</td>
<td>0</td>
<td>12</td>
<td>nd</td>
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</tbody>
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<table>
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<tr>
<th></th>
<th>pH 4</th>
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<tbody>
<tr>
<td>Incubation [days]</td>
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<td>1</td>
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</tr>
<tr>
<td>Band intensity [%]</td>
<td>0</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

The reason that a wider spectrum of enzymes precipitate initially could be due to dilution of the enzyme mixture or possibly due to addition of KOH to adjust the pH. Chylenski et al. [13] has noted that cellulase preparations from *T. reesei* form precipitates during dilution. If the initial precipitation was due to the addition of base, there should be more precipitate formed at pH 6 since more base is added to these samples, implying that this might not be the best explanation.

Chylenski et al. [13] studied precipitation of commercial cellulase preparations in 50 mM sodium citrate buffer, pH 4.8 at 50°C and found that up to 15% of proteins precipitated after 24 h. This indicates that overall hydrolysis rates slow down because large fractions of active enzyme fall out of solution.

The precipitation experiments show that precipitation is higher at pH 4 than at pH 5 or 6. Since the pH drops during the saccharification process this indicates that it is important to
monitor and adjust the pH during the saccharification as it could help prevent enzyme inactivation. The results indicate that the addition of catalase can affect enzyme precipitation at least at pH 4, it is harder to tell if it has any effect at pH 5 and 6. It might also be interesting to note that the enzymes that precipitate mostly are the enzymes at 90 and 120 kDa during all circumstances and therefore it might be beneficial to add these enzymes in higher quantities to enzyme mixtures.

Some enzymes are more sensitive to precipitation than others. One explanation for this could be that the more sensitive enzymes have more hydrophobic parts and therefore are more likely to cluster together and precipitate. It might also be that some enzymes are inherently more unstable and therefore more sensitive to ROS inactivation, which could explain why the addition of catalase can decrease the precipitation.

5.3.2 Precipitation of catalase, β-glucosidase and TaAA9A with and without catalase

LPMOs have been shown to produce H$_2$O$_2$ in the absence of substrate with access to electron donors and molecular oxygen. To investigate if the production of H$_2$O$_2$ has an effect on enzyme precipitation, samples containing TaAA9A, which is a fungal LPMO, was investigated. No additional electron donors were added to the supernatant. There was also a control with added catalase to investigate if the precipitation is due to formation of H$_2$O$_2$ or not.

As can be seen in Figure 5-8 the amount of TaAA9A decreases when incubated. For the samples with added catalase however the TaAA9A seems to be more stable during incubation since the band after 3 days of incubation is almost identical to the band at day 0 (Table 5-5).
Figure 5-8. SDS-PAGE gel with supernatant samples from the precipitation of catalase, LPMO and catalase + LPMO from day 0, 1 and 3. A, B and C are catalase from day 0, 1 and 3 respectively. D, E and F are TaAA9A from day 0, 1 and 3 respectively and G, H and I are catalase+TaAA9A from day 0, 1 and 3 respectively.

Table 5-5. Densitometry analysis after SDS-PAGE of the TaAA9 band in supernatant samples of this monocomponent sample with or without addition of catalase. The default setting for identifying lanes and bands were used, as was the rolling-ball diameter for determining the baseline for the densitometry peaks. The band intensity in percentage is relative to the intensity at day 1.

<table>
<thead>
<tr>
<th>Condition</th>
<th>no catalase</th>
<th></th>
<th>+ catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation [days]</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Band intensity [%]</td>
<td>100</td>
<td>82</td>
<td>60</td>
</tr>
</tbody>
</table>

Figure 5-9 shows precipitation result for BG. The gel shows the BG as a band at around 130 kDa and faint traces of protein that can be either fragments of the BG as a result of protease activity or simply proteins that were not removed during the purification of the protein.

Figure 5-9. SDS-PAGE gel with supernatant samples from the precipitation of BG from day 0, 1 and 3 which are represented by A, B and C respectively.
From the precipitation experiments with catalase, TaAA9A, catalase + TaAA9A and BG it seems that catalase can limit LPMO precipitation. It would be interesting to repeat this experiment at higher enzyme concentrations since this concentration was too low for the precipitates to be visible. It would also be interesting to repeat all precipitation experiments in a buffer to see if the precipitation is effected by the composition of the slurry supernatant in which the experiments were performed.
6 CONCLUSION

The saccharification was performed on two different wheat straw slurrys. One slurry and one warm-water washed slurry. The glucose release was lower from the steam pretreated wheat straw slurry (33.7 g/l at 15 mg/g DW of CTec3) than in the washed slurry (45.0 g/l at 15 mg/g DW of CTec3). This suggests that there are inhibitors present in the slurry that were washed away during the washing of the substrate.

The glucose release from the slurry can be increased by the addition of catalase, about 9.5% increase at 5 mg/g DW of CTec3 and 6.2% increase at 15 mg/g DW of CTec3. The addition of catalase can also positively affect the time dependent enzyme inactivation in the slurry. In the washed slurry the catalase did not affect the time dependent inactivation and the overall time dependent enzyme inactivation was lower in the washed slurry (Figure 5-3 A vs B).

The xylose release was higher from the slurry (16.2 g/l at 15 mg/g DW of CTec3) than the washed slurry (5.0 g/l at 15 mg/g DW of CTec3). This is most likely due to the fact that soluble xylo-oligomers were washed away in the washing process and therefore the available potential xylose is lower.

The addition of catalase to the saccharification process decreases the amount of KOH that needs to be added in order to adjust the pH. After 168 h of saccharification an average of 400 mg of 1 M KOH had been added to the samples with a CTec3 loading of 15 mg/g DW and to the samples where catalase had been added 293 mg of KOH was added. Between 53% and 100% of the KOH was added at the first sampling point.

The results from the recycling experiments show that the slowdown in saccharification is caused at least partially by endpoint inhibition and substrate depletion. Some of the enzyme inactivation during saccharification can also be explained by enzyme precipitation. However in this thesis the precipitation model has been simplified so that it only contains the supernatant from the slurry and no solid substrate. Precipitation was studied on CTec3 with and without addition of catalase. The precipitation experiment performed during this work indicates that precipitation is a cause for enzyme inactivation during incubation at 50°C, especially at pH 4. The experiments also show that addition of catalase can mitigate this effect. However it is important to note that precipitation takes place already at time 0.

Future perspectives
For future experiments the saccharification experiment on the washed wheat straw slurry would need to be repeated, both to confirm the results and to investigate if the contamination had an effect on other things than the acetic acid. It would also be interesting to repeat the precipitation experiments with TaAA9A, catalase and BG at higher enzyme concentrations to make sure that precipitates that form would be enough for analysis.

Causes for slowdown in saccharification could also be evaluated further. The glucose inhibition can be studied by for example adding glucose at different concentrations to fresh substrate before adding enzymes and see how much the glucose release rates are affected. In order to investigate how the substrate depletion compares to enzyme inactivation, experiments can be performed where old enzymes are added to fresh substrate and fresh enzymes are added to old substrate.
7 REFERENCES


[33] B. Henriessat, T. T. Teeri and R. A. J. Warren, ”A scheme for designating enzymes that hydrolyse the polysaccharides in the cell walls of plants.,” *Fes Letters*, vol. 425, nr 2,
50


[38] B. Seiboth, C. Ivanova and V. Seidl-Seiboth, ”Trichoderma reesei: a fungal enzyme producer for cellulosic biofuels,” *Rijeka: INTECH open access publisher, 2011.*


8 APPENDIX

8.1 Microtiter scale Filter Paper Unit (FPU) assay

8.1.1 Standard preparation

A glucose stock of 10 g/l was prepared by dissolving 440 mg of glucose monohydrate in 40 ml of Triethylammonium acetate buffer, 50 mM, pH 5. The stock solution was aliquotted into 1 ml aliquots and stored at -20°C.

For each run of the FPU assay the glucose standard was diluted in a serial dilution, meaning that the 5 g/l was used to make the 4 g/l and so on, according to Table 8-1.

<table>
<thead>
<tr>
<th>Glucose concentration</th>
<th>Solution added</th>
<th>5 g/l</th>
<th>4 g/l</th>
<th>3 g/l</th>
<th>2 g/l</th>
<th>1 g/l</th>
<th>0.5 g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose stock</td>
<td>150</td>
<td>160</td>
<td>105</td>
<td>66</td>
<td>50</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>150</td>
<td>40</td>
<td>35</td>
<td>33</td>
<td>50</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Total volume</td>
<td>300</td>
<td>200</td>
<td>140</td>
<td>99</td>
<td>100</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

8.1.2 FPU assay

The filter paper assay is an enzyme assay developed to evaluate the combined activity of cellulose degrading enzymes. The assay is based on the conversion of filter paper (cellulose) into glucose by enzyme preparations e.g. industrial enzyme cocktail, and thereafter the detection of glucose using the reducing sugar assay, also referred to as DNS assay.

50 mM Triethylammonium acetate buffer was prepared by diluting a 1M stock (25 ml) with milliQ water to a total of 250 ml and pH was adjusted to 5.0 using 100% acetic acid. The solution was stored at 4°C.

The filter paper reaction is non-linear, which can be seen in Figure 8-1 where the glucose released is plotted vs enzyme concentration where the enzyme concentration is retrieved by 1/dilution factor. The filter paper unit calculation is based on equal conversion of substrate in the reaction time, also known as the critical amount of glucose to be released. In the microscale filter paper assay the critical amount of glucose to be released in 0.08 mg per 20 μl of enzyme. Therefore, to be able to determine the filter paper activity correctly, each
sample should be diluted to release the critical amount of glucose. This is very difficult practically and instead several different dilutions are prepared from each sample with the aim to find one dilution releasing a little more and one releasing a little less than the critical amount of glucose as represented by the red dots in Figure 8-1. The calculation procedure is described below together with figures showing an example procedure.

Figure 8-1. Example showing the glucose released during the filter paper assay against enzyme dilutions. Samples were measured in duplicates and the error bars show the standard deviation. Samples in red are the samples surrounding the critical amount of glucose. The enzyme dilution that gives 0.08 mg glucose/20 µl is retrieved from the equation \( y = 0.0138x + 0.0003 \).

The calculation procedure to determine the filter paper activity of the enzyme mixture:

1. Construct a linear glucose standard curve using the absolute amounts of glucose (mg/20µl) plotted against absorbance at 540 nm. The data for the standard curve should fit closely to a calculated straight line with an \( R^2 \) very close to 1, for example see Figure 8-2.
2. Using the standard curve, determine the amount of glucose released for each sample after subtraction of enzyme blank.

3. Estimate the concentration of enzyme which would have released exactly 0.08 mg of glucose using a plot of glucose liberated (mg/20 µl) against enzyme concentration. To find the required enzyme concentration, take 2 data points that are very close to 0.08 mg and draw a straight line between them. Then use this line to interpolate between the two points to find the enzyme dilution that would produce exactly 0.08 mg glucose equivalents of reducing sugars. See example below in Figure 8-1.

Note: here as well as in the equation for calculating FPU, the term “enzyme concentration” refers to the enzyme dilution, which is calculated as 1/dilution factor. This enzyme dilution refers to the concentration, i.e. dilution, in the 20 µl of enzyme solution added to the filter paper assay.

4. Calculate FPU according to the equation (1):

\[
FPU = \frac{0.37}{[\text{enzyme}] \text{ releasing } 0.08 \, \text{mg glucose}} \text{ units/ml} \tag{1}
\]
Where \([\text{enzyme}]\) is the enzyme concentration as described above and the 0.37 comes from the following the equation (2):

\[
\frac{[\text{Glucose}_{\text{crit}}]}{V_{\text{enz}}M_{\text{glucose}}h} = \frac{0.08 \text{ mg glucose}}{0.02 \text{ ml} \cdot 0.18 \text{ mg glucose/µmole} \cdot 60 \text{ min}} = 0.37
\]  

(2)

In the equation (2) \([\text{Glucose}_{\text{crit}}]\) is the critical amount of glucose to be released (0.08 mg), \(V_{\text{enz}}\) is the volume of enzyme added to the reaction mixture (0.02 ml), \(M_{\text{glucose}}\) is the molecular weight of glucose (0.18 mg/µmole) and \(h\) is the reaction time (60 min). If the enzyme solution that releases 0.08 mg of glucose is non-diluted giving a \([\text{enzyme}]\) of 1, the enzyme solution has the activity of 1 FPU/ml.

In the example shown in Figure 8-1 the FPU is calculated by \(0.37/0.001404=263.532\approx264\) units/ml.

### 8.2 High performance liquid chromatography (HPLC)

#### 8.2.1 Example of chromatogram

Figure 8-3 shows an example of a chromatogram. The chromatogram is from sample L1, day 2 from the experiment on washed wheat straw where L means low CTec3 loading 5 mg/g DW (w/w) and 1 means that it was duplicate 1.

*Figure 8-3. Example of chromatogram, sample L1 day 2. 1st peak shows glucose (retention time 7.383 s), second peak shows xylose (retention time 7.875 s) and the third peak shows acetate (retention time 11.392 s).*
8.2.2 Preparation of HPLC standards for analysis of Glucose, Xylose and Acetate

Two different stock solutions were prepared, one with glucose and xylose and one with acetic acid. The glucose and xylose stock solution was prepared by adding 11.0433 g of glucose, 5.0000 g of xylose and milliQ water to a total volume of 50 ml in a 50 ml volumetric flask. The solution was heated to 50 °C and stirred until all sugars had dissolved. The stock solution was then aliquoted into 1 ml aliquots and stored at -20°C. The final concentrations were 220.9 g/l of glucose and 100 g/l of Xylose.

The acetic acid stock was prepared by adding 500 mg of 100 % acetic acid and milliQ water to a total volume of 25 ml in a 25 ml volumetric flask. The stock solution was then aliquoted into 1 ml aliquots and stored at -20°C. The final concentration of acetic acid was 20 g/l.

Preparation of standard solutions for HPLC analysis was performed mostly as a serial dilution. The 1x dilution was prepared using 150 µl of the glucose+xylose stock and 150 µl of the acetic acid stock diluted in 1200 µl of milliQ water. The 1x dilution was then used to make the 2x and 5x dilutions. The 10x dilution was made from the 5x and the 20x was made from the 10x. For volumes added see the standard preparation table (Table 8-2).

Table 8-2. Standard preparation table, all volumes are in µl. The stocks are one stock with glucose and xylose and one stock with acetic acid.

<table>
<thead>
<tr>
<th>Solution added</th>
<th>1x</th>
<th>2x</th>
<th>5x</th>
<th>10x</th>
<th>20x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock</td>
<td>150+150</td>
<td>500</td>
<td>240</td>
<td>600</td>
<td>500</td>
</tr>
<tr>
<td>MilliQ</td>
<td>1200</td>
<td>500</td>
<td>960</td>
<td>600</td>
<td>500</td>
</tr>
<tr>
<td>Final volume</td>
<td>760</td>
<td>1000</td>
<td>600</td>
<td>700</td>
<td>1000</td>
</tr>
</tbody>
</table>

The final concentrations of the diluted standards are represented in Table 8-3.

Table 8-3. Representation of final concentrations (g/l) of compounds in standards.

<table>
<thead>
<tr>
<th>Compound</th>
<th>1x</th>
<th>2x</th>
<th>5x</th>
<th>10x</th>
<th>20x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>22.0866</td>
<td>11.0433</td>
<td>4.41732</td>
<td>2.20866</td>
<td>1.10433</td>
</tr>
<tr>
<td>Xylose</td>
<td>10</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>2</td>
<td>1</td>
<td>0.4</td>
<td>0.2</td>
<td>0.1</td>
</tr>
</tbody>
</table>
8.3 SDS-PAGE

For analysis of proteins by gel-electrophoresis samples were mixed with sample buffer and denatured. 15 µl of sample was mixed with 5 µl of 4X sample loading buffer and incubated at 95°C for 10 min. The loading buffer was prepared by mixing 900 µl of 4X Laemmli Sample Buffer (Bio-Rad) with 100 µl of 2 M dithiothreitol (DTT). After denaturation the samples were put on ice and briefly centrifuged to collect the mixture at the bottom of the tube. The samples were then stored at room temperature until loading them on the SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gel.

The SDS-PAGE gel (Mini-PROTEAN® TGX Stain-Free™ precast Protein Gel, 10 well, 30 µl from Bio-Rad) was removed from its package and rinsed with deionized water to remove the buffer. The tape at the bottom of the gel as well as the comb at the top was removed and gels were assembled in a gel holder (Mini-PROTEAN® Tetra cell system) and put into a gel chamber (Mini-PROTEAN® Tetra cell system). Running buffer (1X Tris/Glycine/SDS running buffer, 25mM Tris, 192 mM glycine, 0.1 % (w/v) SDS, pH 8.3, diluted from Bio-Rad stock of 10X) was poured into the inner chamber of the Mini-PROTEAN® Tetra cell until it was completely filled and approximately 10 cm of used running buffer was poured into the outer chamber. 20 µl of each sample was then loaded into the wells of the gel. The first well was loaded with 10 µl of protein ladder (Precision Plus Protein™ Unstained Standards, Bio-Rad). The Mini-PROTEAN® Tetra cell was then connected to a power supply and the gel was run at 100 V for 1 h and 20 min.

After ended run, the gel was rinsed with deionized water and lifted from the plastic cassette. The gels were washed by putting the gel into a tank, adding 100 ml of deionized water and rinsing for 10 min before discarding the wash, this was done 3 times. Then 20 ml of PageBlue™ Protein Staining Solution was added and the gel was stained during gentle agitation for 1 hour. After 1 h the staining solution was removed and 100 ml of deionized water was added and then removed to rinse away residual staining solution before the gel was destained overnight in 100 ml of deionized water.

When imaging the gel it was placed on a white light conversion screen, making sure to remove air bubbles underneath the gel and then the conversion screen was put into the BioRad ChemiDoc™ MP imaging system. In the ImageLab software the application was set to Comassie Blue and the gel type was set to Bio-Rad Mini-PROTEAN Gel.
8.4 SDS-PAGE gels from duplicates

All precipitation experiments were run in duplicates.

Figure 8-4. SDS-PAGE gel with supernatant samples from the precipitation of catalase, LPMO and catalase + LPMO from day 0, 1 and 3. A, B and C are catalase from day 0, 1 and 3 respectively. D, E and F are TaAA9A from day 0, 1 and 3 respectively and G, H and I are catalase+TaAA9A from day 0, 1 and 3 respectively.

Figure 8-5. SDS-PAGE gel with supernatant samples from the precipitation of BG from day 0, 1 and 3 which are represented by A, B and C respectively.
### 8.5 KOH addition over time

The addition of KOH at each sampling point was monitored and is represented below.

#### Wheat straw slurry

![Graph showing KOH addition over time for wheat straw slurry.](image)

*Figure 8-6. Addition of KOH at each sampling point. Low CTec3 means that CTec3 was loaded at 5 mg/g DW (w/w) and High CTec3 that CTec3 was loaded at 15 mg/g DW (w/w). The 5 mg/g DW of CTec3 corresponds to an FPU of 5.2 FPU/g DW while the 15 mg/g DW corresponds to 15.7 FPU/g DW. Catalase was loaded at 79 µg/g DW. The Low CTec3 sample is represented by black diamonds, the Low CTec3+catalase by black squares, High CTec3 by black triangles, High CTec3+catalase by black circles and the blank, without enzyme, by white squares. Every point represents an average of two duplicates and the error bars show the standard deviation."

#### Warm water washed wheat straw slurry

![Graph showing KOH addition over time for warm water washed wheat straw slurry.](image)

*Figure 8-7. Addition of KOH at each sampling point. Low CTec3 means that CTec3 was loaded at 5 mg/g DW (w/w) and High CTec3 that CTec3 was loaded at 15 mg/g DW (w/w). The 5 mg/g DW of CTec3 corresponds to an FPU of 5.2 FPU/g DW while the 15 mg/g DW corresponds to 15.7 FPU/g DW. Catalase was loaded at 79 µg/g DW. The Low CTec3 sample is represented by black diamonds, the Low CTec3+catalase by black squares, High CTec3 by black triangles, High CTec3+catalase by black circles and the blank, without enzyme, by white squares. Every point represents an average of two duplicates and the error bars show the standard deviation."