Bachelor's Thesis

Bachelor of Engineering

2022

Beatrice Suominen

Biocatalytic Hydrolysis of Lignocellulosic Biomass

– Process Optimization

TURKU AMK
TURKU UNIVERSITY OF APPLIED SCIENCES

Bachelor´s Thesis | Abstract

Turku University of Applied Sciences

Chemical and Materials Engineering

2022 | 40 pages

Beatrice Suominen

Biocatalytic Hydrolysis of Lignocellulosic Biomass

- Process Optimization

The main goal of this thesis was to improve the function and stability of a cellulase enzyme cocktail with the help of metal ion/ions. The enzyme cocktail used for the study was MetZyme® SUNO™ hydrolysis solution which is used to release glucose from lignocellulose. The glucose released by the hydrolysis of the pretreated hardwood biomass could be used to produce biofuels. The client was MetGen Oy, which offers its tailored enzymatic solutions for optimizing biochemical processes.

During the practical part, metal ions were tested to find the ones that offer the highest boost to endoglucanase activity, measured by a robust endoglucanase enzymatic activity test using a model substrate. When the best metal ions were found, the thermostability of the enzyme in combination with the selected metal ions was tested with three different temperatures. Subsequently, hydrolysis of a biomass substrate was performed in the same conditions (most promising metal ions and temperatures) and with the same incubation times. The performance was compared to the endoglucanase activity with the model substrate. It was shown that hydrolysis performed at 60 ºC had higher yield than hydrolysis at the standard temperature of 50 ºC. These results were obtained after 24 hours of incubation. Zinc chloride was also found to improve the stability of the enzyme at 70 ºC.

Keywords:

enzyme, hydrolysis, metal ion, lignocellulose, sugar release

Opinnäytetyö (AMK) | tiivistelmä

Turun ammattikorkeakoulu

Prosessi- ja materiaalitekniikka

2022 | 40 sivua

Beatrice Suominen

Lignoselluloosabiomassan biokatalyyttinen hydrolyysi

- Prosessin optimointi

Tämän opinnäytetyön päätavoitteena oli parantaa selluaasientsyymin toimintaa ja kestävyyttä metalli-ionien avulla. Tutkimuksessa käytetty entsyymi oli MetZyme® SUNO™, jota käytetään glukoosin vapauttamiseen lignoselluloosasta. Esikäsitellyn lehtipuubiomassan hydrolyysissä vapautuvaa glukoosia voitaisiin käyttää biopolttoaineiden valmistukseen. Työn toimeksiantajana toimi MetGen Oy, joka tarjoaa asiakkailleen ratkaisuja biokemiallisten prosessien optimointiin.

Käytännön osuuden aikana testattiin metalli-ioneja yksinkertaisella endoglukanaasin entsyymiaktiivisuustestillä, jotta löydettäisiin parhaiten endoglukanaasin aktiivisuutta tehostavat ionit. Parhaiden metalli-ionien löydyttyä entsyymin lämpöstabiilisuus testattiin yhdessä valittujen metalli-ionien kanssa kolmessa eri lämpötilassa. Todellisen substraatin hydrolyysi tehtiin samoissa olosuhteissa (lupaavimmat metalli-ionit ja lämpötilat) ja samoja inkubaatioaikoja käyttäen, jotta suorituskykyä voitaisiin verrata todellisen substraatin ja keinotekoisella substraatilla suoritettujen endoglukanaasin aktiivisuustestien välillä. Työ osoitti, että hydrolyysi, joka suoritettiin lämpötilassa 60 °C sai aikaan suuremman saannon verrattaessa standardiin lämpötilaan 50 ºC 24 tunnin inkuboinnin jälkeen. Sinkkikloridin havaittiin parantavan entsyymin stabiilisuutta 70 °C lämpötilassa.

Asiasanat:

entsyymit, hydrolyysi, metalli-ionit, sokerin vapautuminen, lignoselluloosa

Table of content

Figures

Glossary

EDTA	Ethylenediaminetetraaceticacid
Zn	Zinc chloride
Mg	Magnesium chloride
Fe(II)	Iron chloride
Fe(III)	Iron oxide
Mn	Manganese chloride
Ca	Calcium chloride
Cu	Copper chloride
Ctrl	Control sample, a sample without metal ions
HPLC	High Performance Liquid Chromatography
rpm	revolutions per minute
kDa	kilodalton

1 Introduction

Our world is facing major environmental challenges such as human population growth, rapid industrialization, excessive utilization of fossil fuel and anthropogenic activities which have caused serious threats to the environment in terms of greenhouse gas emissions, global warming and air pollution. The loss of sustainability can be avoided by shifting from fossil fuels to biofuels. Lignocellulosic biomass is an attractive substrate for production of several high-value products such as bio-based chemicals and polymers due to its renewability, availability, and abundance. (Sharmili, et al., 2021.)

The main goal of this thesis was to improve the effectiveness and stability of the cellulase enzyme with the help of metal ion/ions. The biocatalyst used for the study was MetZyme® SUNO™, an enzymatic cocktail which is used to release glucose from lignocellulose. The glucose released from the pretreated hardwood biomass could be used to produce biofuels or as a building block for higher value products. During the practical part, metal ions were tested to find the ones that offer the highest boost to endoglucanase activity, measured by a robust endoglucanase enzymatic activity test using a model substrate. When the best metal ions were found, the thermostability of the enzyme in combination with the selected metal ions was tested with three different temperatures. Subsequently hydrolysis of a biomass substrate was performed in the same conditions (most promising metal ions and temperatures) and incubation time. The performance was compared to the endoglucanase activity with the model substrate. The hydrolysis was performed with biomass in 250 ml flasks from which samples were collected and the amount of glucose released was analyzed by HPLC.

MetGen Oy utilizes biotechnology to unleash the full power of pulp and paper enzymes for improving production efficiency and lowering energy consumption in an affordable way. The company was founded in 2008 in Kaarina, Finland, and their goal is to design and market innovative enzymatic solutions to meet the needs of customers. (MetGen Oy, 2022.)

2 Biomass valorization

Biomass is defined as renewable organic material which is provided by plants and animals. The biomass used for this study is a lignocellulose, derived from hardwood. Biomass is derived via photosynthesis, from the reaction with CO_2 in the air, water and sunlight. It produces carbohydrates and lignin that form the building blocks of biomass. Photosynthesis typically converts less than 1% of the available sunlight into stored, chemical energy. (McKendry, 2002.) Biomass can be burned directly to heat or converted to renewable liquid and gaseous fuels through various processes (U.S. Energy Information Administration, 2021). Valorization is the conversion of waste and biomass to energy, fuels and other useful materials, with particular focus on environmental indicators and sustainability goals (Nzihou, 2010).

The use of waste biomasses reduces the environmental hazards associated with their disposal. Impedance of lignin (opposition to alternating current) and the crystalline nature of cellulose pose major challenges in biomass-based energy. Several physio-chemical processes are recommended as mitigation routes, but none of them appear to be suitable for large-scale use. It appears that a combination of biological treatment and nanotechnology for efficient pretreatment and subsequent hydrolysis of biomass by ubiquitous enzymes is a promising alternative. (Nzihou, 2010.)

2.1 From biomass to biofuels

As a suitable feedstock for the synthesis of second-generation biofuels, lignocellulosic biomass plays an important role (Ragauskas, 2014). The research on biofuels aims at producing energy products such as ethanol, diesel, hydrogen, and biogas from biological sources, such as different waste biomasses (Elshahed, 2010). Biofuels are viewed as more environmentally friendly sources of energy since the plants that are the sources of feedstock for making biofuels, absorb carbon dioxide as they grow (U.S. Energy Information Administration, 2020). Fossil fuels are one of the limited primary energy resources stored in the Earth's crust, which have been used extensively to mediate nearly all modern technology. The availability of this limited primary energy resource is expected to diminish to the point that global dependence on fossil fuels will no longer be sustainable. To reduce the heavy reliance on fossil fuels and overcome serious challenges in energy resources, diversification in energy sources is essential for ensuring a stable supply of domestic energy and for ensuring environmental sustainability. (Elshahed, 2010.)

Enzymatic hydrolysis is the first challenging step in converting lignocellulose into fermentable sugar monomers (Ragauskas, 2014). In the beginning of the bioconversion process, lignocellulosic biomass is pretreated by reducing its crystalline integrity, leaving the biomass prone to enzyme attack. Enzymatic hydrolysis breaks down holocellulose polymers into sugar-based monomers. Bacteria and fungi can then convert these sugar fractions into ethanol via aerobic or anaerobic metabolic pathways in a process known as fermentation. (Demirbas, 2009.)

2.2 Lignocellulosic biomass

Lignocellulosic biomass, including forestry, agricultural, and agro-industrial waste, is an abundant, renewable, and inexpensive source of energy. Lignocellulose wastes are accumulated every year in large quantities, causing environmental problems. However, due to their chemical composition based on sugars and other compounds of interest, they could be utilized for the production of a number of value-added products, such as ethanol, food additives, organic acids, enzymes, and others. Therefore, besides the environmental problems caused by their accumulation in the nature, the non-use of these materials constitutes a loss of potentially valuable sources. (Nzihou, 2010.)

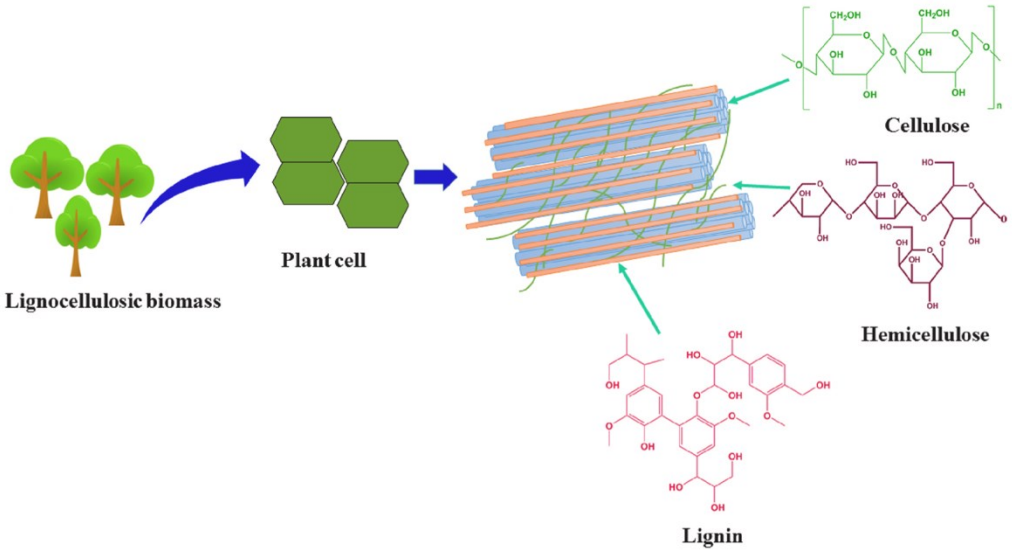

Figure 1. Structural overview of the lignocellulosic biomass (Nzihou, 2010).

A lignocellulosic material consists primarily of three polymers: cellulose, hemicellulose, and lignin (Figure 1). These polymers are associated in a hetero matrix with the varying degrees of association and relative composition depending on the biomass type, species, and even source. (Bajpai, 2016.)

2.3 Cellulose

Cellulose is the main component in the plant cell walls and it is generally synthesized by plants, but it is also produced by some bacteria. It is one of the most produced biomaterials on earth (Bringmann, et al., 2012). Cellulose is made of parallel unbranched D-glucopyranose units linked by β-1,4-glycosidic bonds. As a result of hydrogen bonds and Van der Waals forces, crystalline cellulose forms highly ordered microfibrils. Amorphous cellulose is formed when hydrogen bonds are broken, and the ordered arrangement is lost. Based on scattering data and data about cellulose synthase, microfibrils are reported to consist of 24 to 36 chains of cellulose. (Fernandes, et al., 2011). As a tough, fibrous, water-insoluble polysaccharide, cellulose is essential to the structure of cell walls in plants (Bringmann, et al., 2012).

2.4 Hemicellulose

The term hemicellulose is used to represent a family of polysaccharides that are found on the plant cell wall. They have different composition and structure depending on their source and the extraction method, and they are composed of the combination with pentose and arabinose and/or hexoses, galactose and glucose. The chemical nature of hemicellulose varies between species. In general, the main hemicelluloses of softwood are galactoglucomannans and arabinoglucuronoxylan, while the hemicellulose in hardwood is glucuronoxylan. (Pu, et al., 2008.)

Due to its highly branched structure and acetyl groups on the polymer chain, hemicellulose lacks crystalline structure and composition. Hemicellulose found from plants possesses a high degree of polydispersity, polydiversity and polymolecularity (a broad range of size, shape and mass characteristics) (Barakat, et al., 2007).

2.5 Lignin

Of the three major biopolymers that constitute wood, lignin is clearly different because it is an amorphous, cross-linked (Pla, 1992), and three-dimensional polyphenolic polymer (Davin & Lewis, 2005). Lignin is synthesized by polymerizing three types of phenylpropane units called monolignols: coniferyl, sinapyl, and p-coumaryl alcohol (Boerjan, et al., 2003).

Lignin plays an important role in the cell's endurance and development, as it affects the transport of water, nutrients and metabolites in the plant cell. By acting as a binder between cells, it creates a composite material that has remarkable resistance to impact, compression, and bending. It is also less hydrophilic than either cellulose or hemicelluloses and it has a general effect of inhibiting water adsorption and fiber swelling. (Pandey & Kim, 2010).

3 Enzymes

Enzymes are biological catalysts that is they accelerate chemical reactions. Enzymes are typically proteins, but RNA molecules can also be enzymes, referring to ribozymes (Powar & Chatwal, 2007). Without the action of enzymes, reactions would proceed too slowly, and life, as we know it, would not be feasible (Stenesh, 1998). The word ‘’enzyme’’ from Greek (meaning in yeast) was introduced by Kuhne in 1878 to refer to the occurrence in yeast of something responsible for its fermentative activity (Powar & Chatwal, 2007).

Enzymes accelerate reactions without consuming themselves in the process since they only need relatively low concentrations to act as catalysts (Robinson, 2015). In many protein enzymes, the active site consists of non-amino acids, often one or more metal ions coordinated to amino acids. These groups that help the enzyme are called cofactors, and the most common of these are copper, iron, and zinc ions. If the cofactor is an organic molecule, we are talking about a coenzyme. Cofactors and coenzymes attached to an enzyme are called prosthetic groups. The molecule upon which the enzyme acts is called a substrate. (Heino & Vuento, 2010.) Generally, enzymes catalyze the conversion of substrate molecules into product molecules. They can also be extracted from cells and then used to catalyze a wide range of commercially important processes. As an example, they can be used to make sweetening agents, modify antibiotics, manufacture washing powders and cleaning products, and they are crucial in the production of analytical devices and assays used in clinical, forensic, and environmental tests. (Robinson, 2015.)

3.1 Mechanism of Enzyme Actions

Probably the most frequently quoted mechanism of enzyme action is the lock-and-key model (Figure 2). This model assumes that the enzyme is a rigid three-dimensional body. (Powar & Chatwal, 2007.) Enzyme specificity is the result of a unique interaction between the enzyme and substrate, where the substrate binds to the enzyme at a particular part of the enzyme called the active site, but only when a structural complementarity between the substrate and the active site exists. To form an enzyme-substrate complex as an intermediate one in the reaction sequence, the substrate must fit stereochemically into the active site. This complex is generally, but not always, a noncovalent complex. Compounds that possess the stereochemical configuration of the substrate, or a configuration that is reasonably close to it, can bind at the active site and undergo reaction. (Stenesh, 1998.)

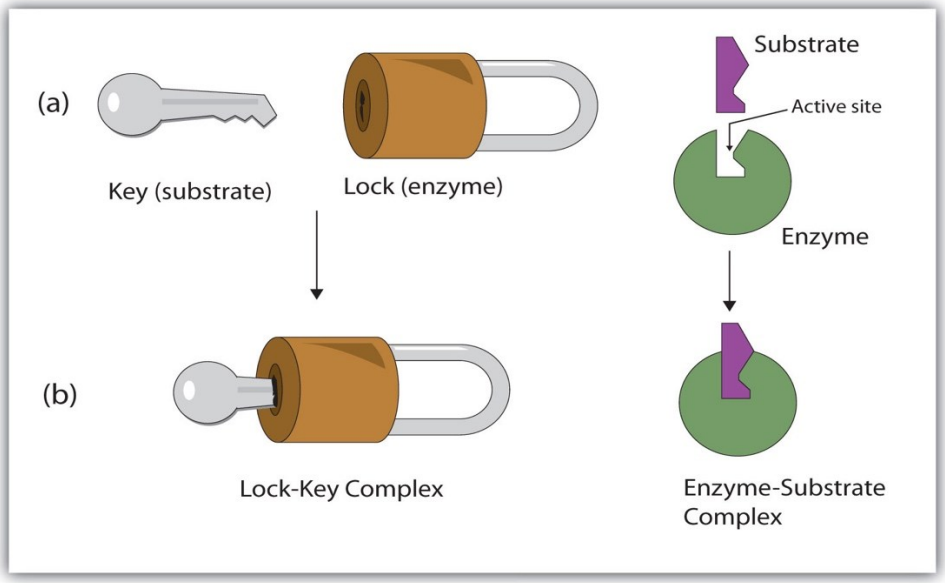

Figure 2. Representation of substrate binding to the active site of an enzyme (Saylor Academy, 2012).

According to the lock-and-key mechanism, an enzyme molecule has its own specific shape because that shape is necessary to maintain the active site in precisely the geometric alignment needed for that reaction.

The enzyme molecule is very large (usually 100 to 200 amino acid residues), but the active site is composed of just two or a few amino acid residues, located at different locations along the chain. Based on this model of enzyme action, substrates either fit or do not fit into a preformed active site. (Powar & Chatwal, 2007.) Based on this, the fundamental reaction in enzymology is:

$$E + S \leftrightarrows ES \leftrightarrows E + P$$

where E is an enzyme, S is a substrate, ES is the enzyme-substrate complex, and P is the product (Stenesh, 1998).

3.2 MetZyme® SUNO™

The biocatalyst used in this study is a mixture of different cellulase enzymes, including endoglucanase, exoglucanase and ß-glucosidase. The cellulase cocktails produced by MetGen Oy are developed to be thermophilic and thermotolerant, with a wide temperature operating window that goes from 20-70°C. However, the optimal window of temperature ranges from 40-50°C, giving the higher performance. Higher temperatures can improve the enzyme performance but at the same time it can heavily affect the enzyme stability and thus the activity. The enzymatic product used in this study is the hydrolysis solution MetZyme® SUNO™ produced in-house by MetGen Oy.

3.3 Endoglucanases

The endoglucanases cleave internal linkages in amorphous cellulose filaments, generating different sized oligosaccharides, which are attacked by exoglucanases (Aro, et al., 2005). The cellulolytic process is initiated by endoglucanases that randomly cleave internal linkages at the amorphous regions of the cellulose fiber and create new reducing and non-reducing ends that are susceptible to the action of cellobiohydrolases. Endoglucanases are monomeric enzymes with a molecular weight of 22 to 45 kDa and their optimum pH is at 4 to 5 and the temperature ranges from 50 °C to 70 °C. (Ding, et al., 2001.)

According to Zhang YHO (Zhang & Lynd, 2004), primary hydrolysis occurs on the surface of solid substrates and releases soluble sugars with a degree of polymerization (DP) up to 6 into the liquid phase upon hydrolysis by endoglucanases and exoglucanases. For the whole cellulose hydrolysis process, this depolymerisation step by endoglucanases and exoglucanases is the rate-limiting step. The second hydrolysis involves mostly the hydrolysis of cellobiose to glucose by β-glucosidases, although some β-glucosidases also hydrolyse longer cellodextrins. The combined actions of endoglucanases and exoglucanases modify the cellulose surface characteristics over time, resulting in rapid changes in hydrolysis rates. To assay endoglucanase activity, there are substrates that are used, such as carboxymethylcellulose (CMC), a soluble amorphous cellulose form that is an excellent substrate for endocellulases and its hydrolysis does not require cellolose-binding domains.

3.4 Exoglucanases

These enzymes, also known as cellobiohydrolases, catalyze the successive hydrolysis of residues derived from the reducing and non-reducing ends of cellulose, producing cellobiose molecules, which are hydrolyzed by β-glucosidases (Teeri, et al., 1998).

These enzymes are monomeric proteins with a molecular weight ranging from 50 to 65 kDa and their optimum pH is 4 to 5, with an optimum temperature from 37 to 60 °C, depending on the specific enzyme-substrate combination (Sadana, et al., 1984).

3.5 ß-glucosidases

β-D-glucosidases hydrolyze soluble cellobiose and other cellodextrins to create glucose in the aqueous phase in order to eliminate cellobiose inhibition. These enzymes have molecular weights ranging from 35 to 640 kDa and their optimum pH ranges from 3.5 to 5.5, and their optimum temperature ranges from 45 to 75 °C. β-D-glucosidase activities can be measured using cellobiose which is not hydrolysed by endoglucanases and exoglucanases. (Zhang & Lynd, 2004.)

4 Materials and methods

The materials and methods used in this thesis came from the laboratory of MetGen Oy. The study with enzymatic hydrolysis of lignocellulosic biomass (Hemminki, 2022) was performed previously, and the used methods and materials are similar with this thesis. The goal for both was to find a metal ion that would boost the enzyme performance, but in this study the conditions were changed to be able to have a deeper investigation of the metal ions' effect on the stability of the enzyme during the hydrolysis. Endoglucanase activity assays were performed to test the activity and the thermostability of MetZyme® SUNO™ with different metal ions. In enzymatic hydrolysis, cellulases are used to break down lignocellulosic biomass into fermentable sugars. HPLC was used to analyze the sample and detect the amount of glucose in the samples.

4.1. Endoglucanase Activity Assay

Thanks to Leonor Michaelis and Maud Menten (Michaelis & Menten, 1913) it is known that enzyme activity depends on defined conditions with respect to the temperature, pH, nature and strength of ions, and enzyme assays can reliably only be compared if such conditions are carefully considered. Because of the great diversity of enzymes and their optimum conditions, it is hard to define general rules for all enzyme assays. With the optimal conditions, enzymes can display their highest activity but deviations from the optimum cause a reduction of the activity, depending on the degree of the deviation.

There are many detailed descriptions of enzyme assays in the relevant literature (Methods in Enzymology; Advances in Enzymology and Related Areas of Molecular Biology (Nord, 2007)), but it is important that the procedure can be modified to suit the special characteristics of a particular enzyme or the instrumentation used. Whether it is the case of a new enzyme, but also when performing standard procedures, it is important to consider the general rules valid for all enzyme assays. (Bisswanger, 2012.)

Endoglucanase activity assays were used in this study in which an enzymatic reaction can be detected as the appearance of a colored compound so that it can be detected even with the eye. In the assay, the polysaccharides are stained with a dye and form noncovalent complexes with cellulose. Degradation of cellulose by endoglucanase results in a halo or zone of clearance is caused by the simultaneous degradation of the dye. (Shafiqur, et al., 2018.) In this procedure, the yield is not accurate and there is no reproducible data and therefore an appropriate instrument, a spectrophotometer, is applied to determine the color intensity (Cantor & Schimmel, 1980).

The assay is performed in MetGen Oy using as a substrate beta-glucazyme tablets from Megazyme and the enzyme is diluted 1:100 with the reaction buffer (50mM Citric Acid at pH 4.8) prior activity measurement (Megazyme, 2022). Incubation of the enzyme solution at the selected temperate for 10 minutes prior the addition of the substrate is necessary to activate the enzyme. Sodium Carbonate solution is used as a stopping solution (0.5M Na2CO3) and the assay is performed either in a 24-well plate or 15 ml falcon tubes. The samples obtained are pipetted to a 96 well spectra-plate with 160 µl of 0,5M Na2CO3 (stopping solution) and 40 µl of the supernatant and read with a plate-reader with the specific wavelength of 622nm. The dilution of the enzyme upon assay and of the samples produced before the spectrophotometer analysis is necessary to get results with absorbance <1 for more reliable results. (MetGen Oy, 2020.)

For the thermostability test, the same assay was performed and different temperatures and incubation times were tested. For incubation, time points at 30 minutes, 4 hours, 6 hours and 24 hours were selected. The temperature was either 50 ºC, 60 ºC or 70 ºC. The temperature of 50 ºC was chosen because it is optimal for the enzyme MetZyme® SUNO™ performance in standard conditions. Temperatures of 60 ºC and 70 ºC were chosen to study enzyme activity and thermostability at a higher temperature than the optimal.

4.2. Metal Ions

Metal ions play important roles in the biological function of many enzymes and can be bound by to proteins and form complexes with other molecules bound to enzymes. They can serve as electron donors or acceptors, Lewis acids, structural regulators, and some participate directly in the catalytic mechanism. (Riordan, 1977.) Metal ions can either activate or inhibit the enzymatic activity by interacting with the amine or carboxylic acid group of the amino acids (Ishida, et al., 1980). It has been found that besides ionic charge, ion radius size has a great influence on the activity and stability of the enzyme. Larger radius of the ion has less influence on catalytic amino acids, while the smaller radius can more intensely attract charged amino acids changing the enzyme's overall conformation and can damage the catalytic site. (Coolbear, et al., 1992.) (Zeng, et al., 2014.) In this study, seven different metal salt dilutions were used: zinc chloride, copper chloride, magnesium chloride, iron chloride, iron oxide, calcium chloride, and manganese chloride.

As all the selected metal ions were divalent, a chelating agent for divalent ions was selected and used as additive to sequestrate the divalent ions present in the enzyme solution. Fermentation broth of the enzyme production (both in fungal and bacterial fermentations) contains several trace elements which are also present in the final enzyme products. For this purpose, EDTA was chosen as the chelating agent because it is highly soluble, optically inactive, and does not interfere with most standard buffer chemicals. EDTA is commonly used as an efficient chelator of metal ion enzyme cofactors. These features make EDTA a common choice when attempting to generate metal-free conditions for biochemical and biophysical studies. (Lopata, et al., 2019.)

4.3 Enzymatic hydrolysis

Enzymatic hydrolysis is a heterogenous reaction with multiple steps. The first step is that lignocellulosic substrate is broken down at the solid-liquid interface. That step is followed by further liquid interface hydrolysis of soluble intermediates with specific catalytic proteins. In this study, cellulose substrate is initially decomposed by the synergistic action of endoglucanases and exoglucanases which is accompanied by further hydrolysis of soluble short β-glucosidases and cellobiose to produce glucose. (Shell & Duff, 1996.) Enzymatic hydrolysis consists of multiphase reactions involving the soluble enzymes working on insoluble substrates in the beginning and further liquid-phase hydrolysis of soluble intermediates (Qining, 2014).

The advantages with enzymatic hydrolysis are mild parameters (pH 4.5 -5.0 and temperature 40– 50°C), low corrosion problems, low utility consumption, low toxicity, less formation of inhibitory by-products, and high sugar yield (Shell & Duff, 1996). The enzymatic hydrolysis performed in this study was carried out in an incubator shaker. The agitation was 250 rpm and temperature for hydrolysis was 50°C. A buffer solution with pH of 4.8 (0.2M succinic acid) was used to control the pH during the reaction. After the samples were collected, they were centrifuged, and the supernatant was separated. The supernatant was then diluted with the factor of 10 to have a glucose concentration that could fit in the calibration curve of the HPLC for sugar analysis. The samples were analyzed with HPLC to find out how much glucose had been released from the biomass during the hydrolysis.

4.4 HPLC

HPLC is derived from the term ‘High Performance Liquid Chromatography’, though the term ‘High Pressure Liquid Chromatography’ is often preferred since high performance can also be achieved at low pressure. Chromatography is defined as a technique for the separation of mixtures by distribution between two or more immiscible phases. The compounds that are going to be separated

must be dissolved in a solvent, which is in these studies was purified water. (Wellings, 2006.) A schematic of a typical HPLC instrument is shown in Figure 3.

HPLC systems commonly have one to four solvent reservoirs which hold the mobile phase components. The mobile phase solvents must be filtered before they reach the column together with the samples. Filtering is necessary because small particles can lodge at the head of the packing and restrict the flow of solvent through the column. This might cause poor peak shapes, irreproducible flow control and exceptionally high pump pressures. (Vitha, 2016.)

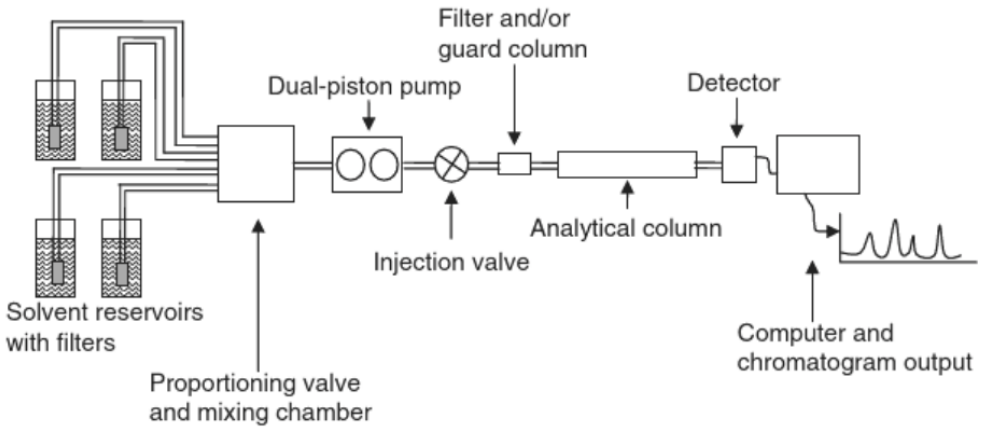

Figure 3. Schematic of a liquid chromatography system (Vitha, 2016).

A liquid mobile phase passes through a column made of porous particles as its mobile phase transports the molecules of analyte through the column. Analyte molecules are retained in the column due to interactions occurring between the particles' surfaces (primarily the surface inside the pores) or molecules chemically bonded to their surfaces (the stationary phase). Therefore, the total retention time of any solute depends on its affinity for the mobile phase compared with its affinity for the stationary phase.

Mobile phase molecules that are highly attracted to the stationary phase are only slightly retained, while molecules that have strong interactions with the

stationary phase but little affinity for the mobile phase are highly retained. Different compounds have different affinities in the mobile phase and stationary phase, allowing the complex mixtures of molecules to be separated. (Vitha, 2016.)

In this study, HPLC was utilized for the analysis of the hydrolysate samples produced by hydrolysis of the biomass. The HPLC used for this purpose is an Agilent 1260 Infinity II equipped with isocratic pump unit, autosampler column oven unit and refractive index detector (RID) and Aminex HPX-87P Column with water HPLC grade as mobile phase. Refractive index detector is commonly used for sugar detection in HPLC analysis due to the optical properties of the sugars in solution to affect its refractive index. In this set up, the functionalization of the stationary phase with Bio-Rad proprietary technology allows the separation of monomeric and dimeric sugars with water as eluent but others parameter like flowrate, column temperature and RID temperature might influence the shape and resolutions of the peaks. The analytical method used for the analysis of the samples produced in this work required a flowrate of 0.6 mL/min with a column temperature of 80 °C (in order to avoid any bacterial contamination) and the RID temperature set at 45 °C.

5 Results and discussion

The main purpose of the thesis was to identify a metal ion or ions that would influence and improve the enzymatic hydrolysis yields. Thermostability of the enzymes was tested to find out if different conditions would have an effect in the enzymatic hydrolysis. The range of reliability for the results was specified as ±5 % because of the analytical limitations and fluctuation of the data given by the colorimetric method. The used enzyme is MetZyme® SUNO™ produced by MetGen Oy.

The activity test and hydrolysis had been performed earlier in MetGen Oy by Lotta Hemminki. Results in her thesis showed that magnesium chloride improved the ability of the enzyme to break down the substrate into sugars with industrial pulp. EDTA showed a positive impact in dissolving pulp and zinc chloride activity assays but not in the hydrolysis. The conclusion was that there is not just one metal ion since the effect of different metal ions on the hydrolysis performance was dependent on the type of the substrate. The used substrates were dissolving pulp, industrial pulp and industrial wheat straw. (Hemminki, 2022.) With these results in mind, the studies were continued to confirm the hypothesis that was found in the previous study with pretreated hardwood. The main focus was to investigate the thermostability of the enzyme in the presence of metal ions, more specifically to find the metal ions that could allow to run hydrolysis in higher temperature while keeping the enzyme active.

5.1 Effect of Metal ions in endoglucanase activity assay and hydrolysis

MegaZyme provides beta-glucazyme tablets for enzymatic digestion to measure endoglucanase activity. It is possible to measure the dye released by digestion of dyed substrate by measuring the absorbance at 622 nm with a spectrophotometer. All the samples were prepared in triplicate, with a concentration of 10 mM metal ions and EDTA. The pH was 4.8 and it was

controlled with 50mM citric acid buffer. Iron oxide was discarded from the activity assay to be able to fit it on a 24-well plate.

The first assay was performed in a 24-well plate, the incubation time was 10 minutes and the temperature was 50 °C. The results are shown in Figure 4.

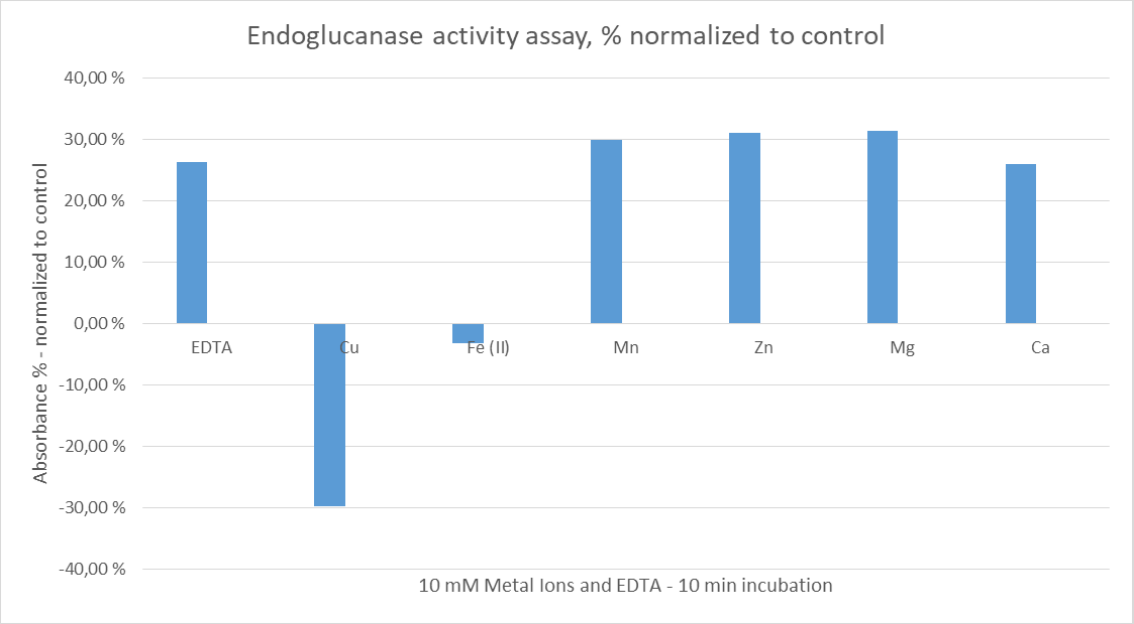

Figure 4. Endoglucanase activity assay result with metal Ions and EDTA, all data normalized to control.

Figure 4 shows that EDTA, manganese chloride, magnesium chloride, zinc chloride and calcium chloride are showing a positive impact. This suggests that these metal ions increase the activity of endoglucanase. The result obtained with copper chloride is not reliable due to a blue-shift of the absorbance peak making it impossible to measure the absorbance at 622 nm (sample stained purple). These results are similar to the endoglucanase activity assay performed in the same conditions earlier in MetGen Oy by Lotta Hemminki. (Hemminki, 2022.)

Because it had been discovered that the results from an enzyme activity test is not correlating when performing hydrolysis with different biomasses (Hemminki, 2022), first hydrolysis was performed with all metal ions, since the biomass was

different that used in the previous study. The first hydrolysis with pretreated hardwood was performed at 50 °C. The bottles were incubated for 48 h and the results are the average of the comparison against control in 24 and 48 h. All the samples were done in duplicate with the concentration of 10 mM metal ions and EDTA. Results from the hydrolysis performed with all metal ions are shown in Figure 5.

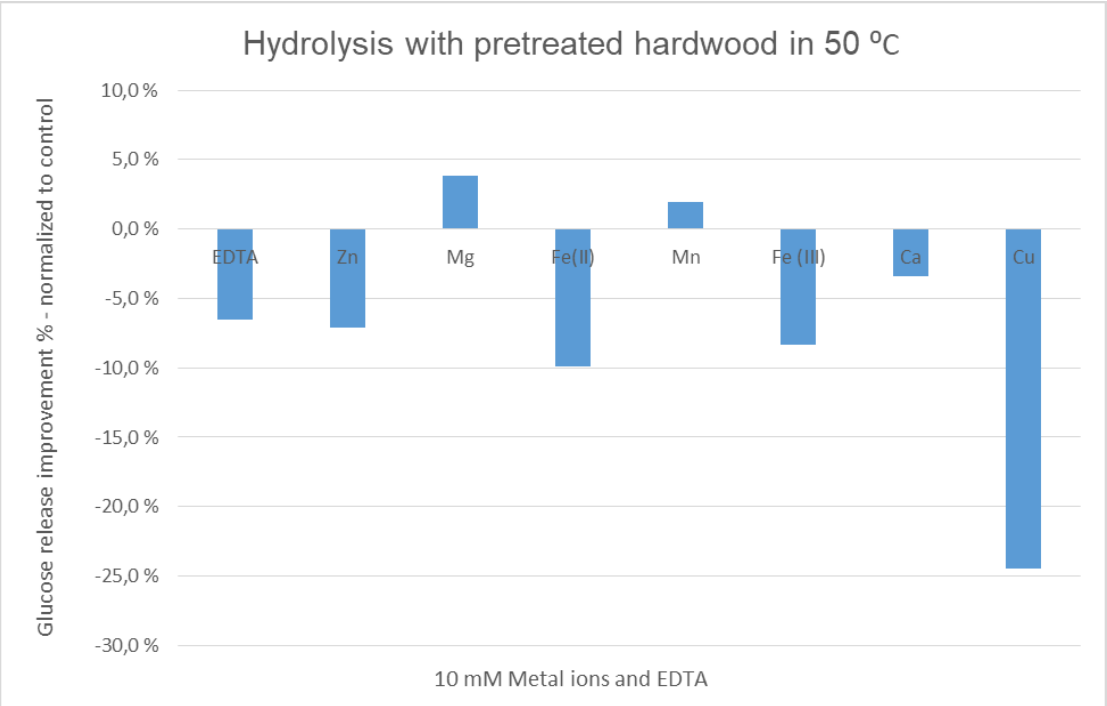

Figure 5. Hydrolysis with pretreated hardwood in 50 °C, all results normalized to control.

It is observed that magnesium chloride and manganese chloride are showing a positive impact also in the hydrolysis similarly to the results from the endoglucanase activity test. All the other samples are showing a negative impact.

The hypothesis of the earlier study was that magnesium chloride could improve the release of glucose. These results show that the hypothesis came to fruition, which is why the study was shifted to see if the metal ions would improve the thermostability of the enzyme, and the study was continued with selected ions. Since the magnesium chloride and zinc chloride were showing the highest positive impact on the endoglucanase activity test, the two metal ions were

chosen to continue the study for the enzyme thermostability test. Studies were continued including EDTA, to follow the performance of the enzyme when divalent ions are sequestered from the enzyme cocktail.

5.2 Endoglucanase activity assay with selected ions

The thermostability test was performed with magnesium chloride, zinc chloride, EDTA and a control sample. The conditions were pH 5 and the samples were taken after 30 min, 4 h, 6 h and last one after 24 h upon incubation at the selected temperatures of 50 ºC, 60 ºC and 70 ºC. Figure 6 shows the results with the first thermostability test which was performed in 50 ºC.

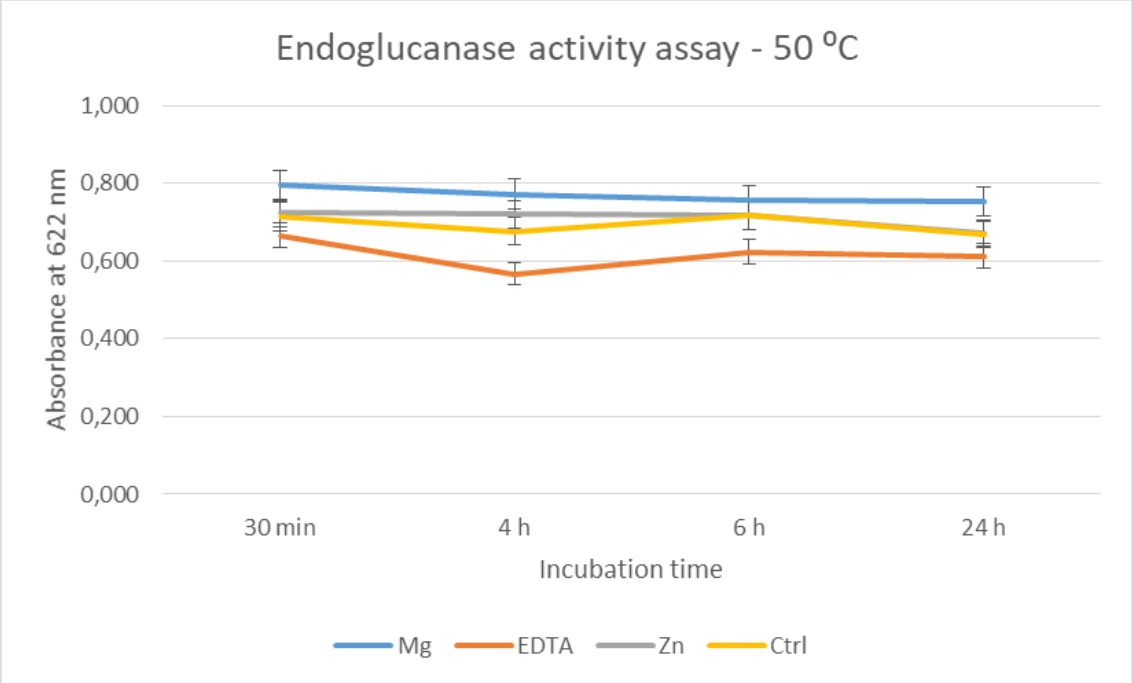

Figure 6. Endoglucanase activity assay performed at 50 ºC.

Magnesium chloride shows the highest absorbance in the activity assay performed at 50 ºC. The sample with EDTA is showing the lowest activity during the whole incubation time. The absorbance with zinc chloride is similar to the control one. All in all, the samples have a stable activity with the incubation and the activity does not drop after 24 h, which means the enzyme is not denatured after 24 h at 50 ºC. However, when taking into account that the range of

reliability for the results is ±5%, the results show no significant difference when compared to each other. The next activity assay was performed at 60 ºC and the results are shown in Figure 7.

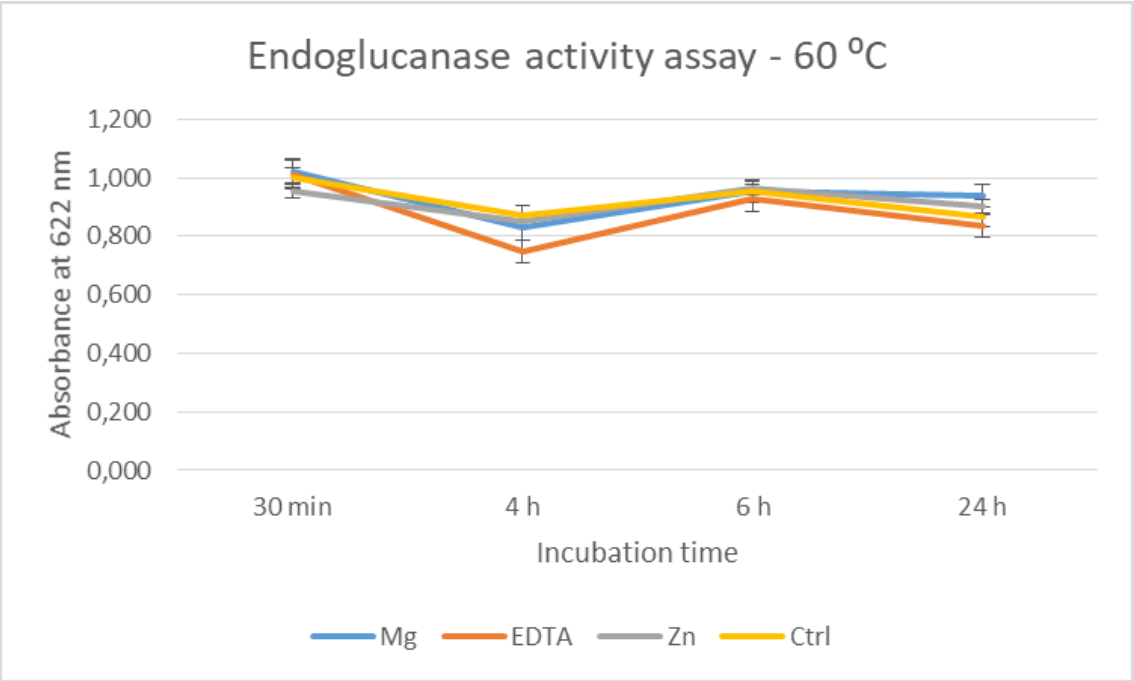

Figure 7. Endoglucanase activity assay performed at 60 ºC.

No significant differences were observed with the endoglucanase activity assays conducted at 60 ºC, as all the samples show similar absorbance in each timepoint. EDTA is showing the least activity, but magnesium chloride, zinc chloride and the control sample indicate very similar absorbance. Compared to the test conducted at 50 ºC, the starting activity is higher with all the samples, the biggest difference is with EDTA. The activity remains higher with all the samples even after 24 h than with 50 ºC and the activity is does not drop after 24 h, which means that the enzyme is not denatured after 24 h at 60 ºC. Similarly to the results obtained at 50 ºC, when taking into account the range of reliability of ±5%, the results are not showing any significant difference.

The last endoglucanase activity assay was performed at 70 ºC and the results are shown in Figure 8.

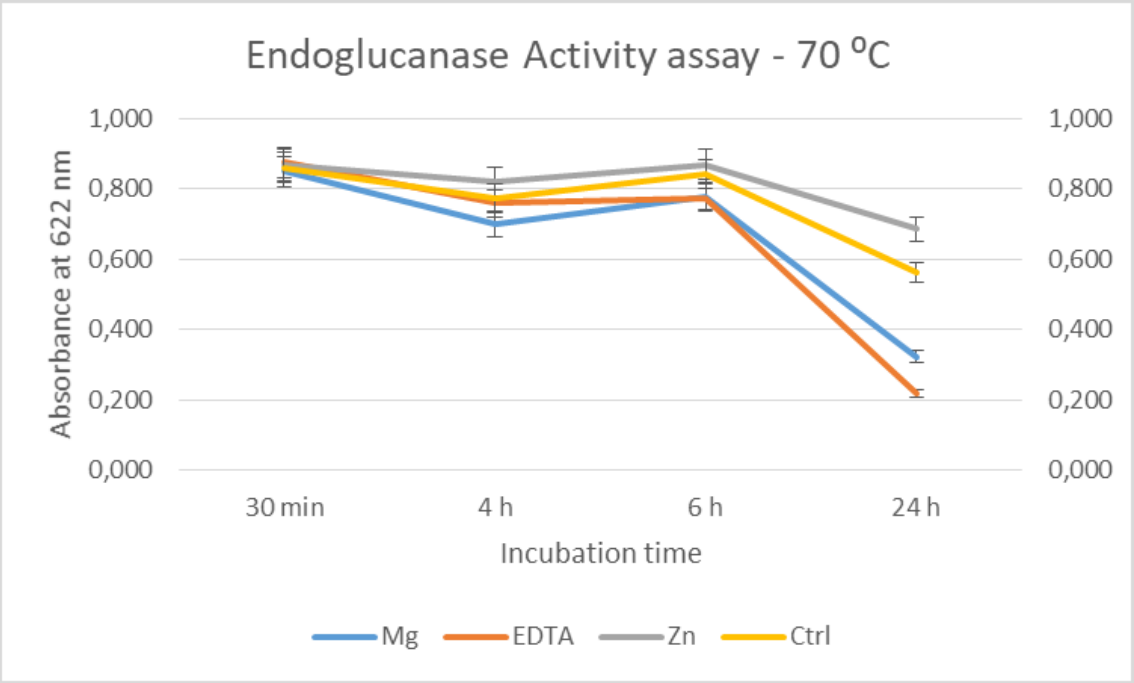

Figure 8. Endoglucanase activity assay performed at 70 ºC.

As can be seen comparing Figure 6, 7 and 8, the activity in the assay performed at 70 ºC is lower than in the assay performed at 60 ºC, but higher than in the one performed at 50ºC. Zinc chloride shows a positive impact comparing with the control and the activity remains higher during the whole incubation. The absorbance results are very close to each other but between 6 h and 24 h there is a significant drop in activity, and the enzyme is most probably getting denatured. This occurs with all the samples, EDTA and magnesium chloride are showing the largest drop in activity in 24 h, while zinc chloride appears to have a positive impact improving the stability comparing with the control. When considering that the range of reliability for the results is ±5%, the results are not showing a significant difference at earlier time points than 24 h.

To summarise, the temperature affects the endoglucanase enzyme activity, more specifically, it increases the activity. The enzyme appears to be thermostable at 50 ºC and 60 ºC, up to 24 h. Meanwhile, at 70 ºC, the enzyme loses its stability between 6 h and 24 h, within these conditions a significant

improvement of thermostability with zinc chloride was observed. Other than that, none of the metal ions or EDTA were showing a significant difference.

5.3 Enzymatic hydrolysis with selected ions

The enzymatic hydrolysis was done with pretreated hardwood biomass in a laboratory scale. The selected temperatures and time points were the same as in the endoglucanase activity assay so that the results are comparable. No samples were taken at 30 minutes timepoint since hydrolysis requires longer incubation time. The first hydrolysis was performed at 50 ºC and the results can be seen in Figure 9.

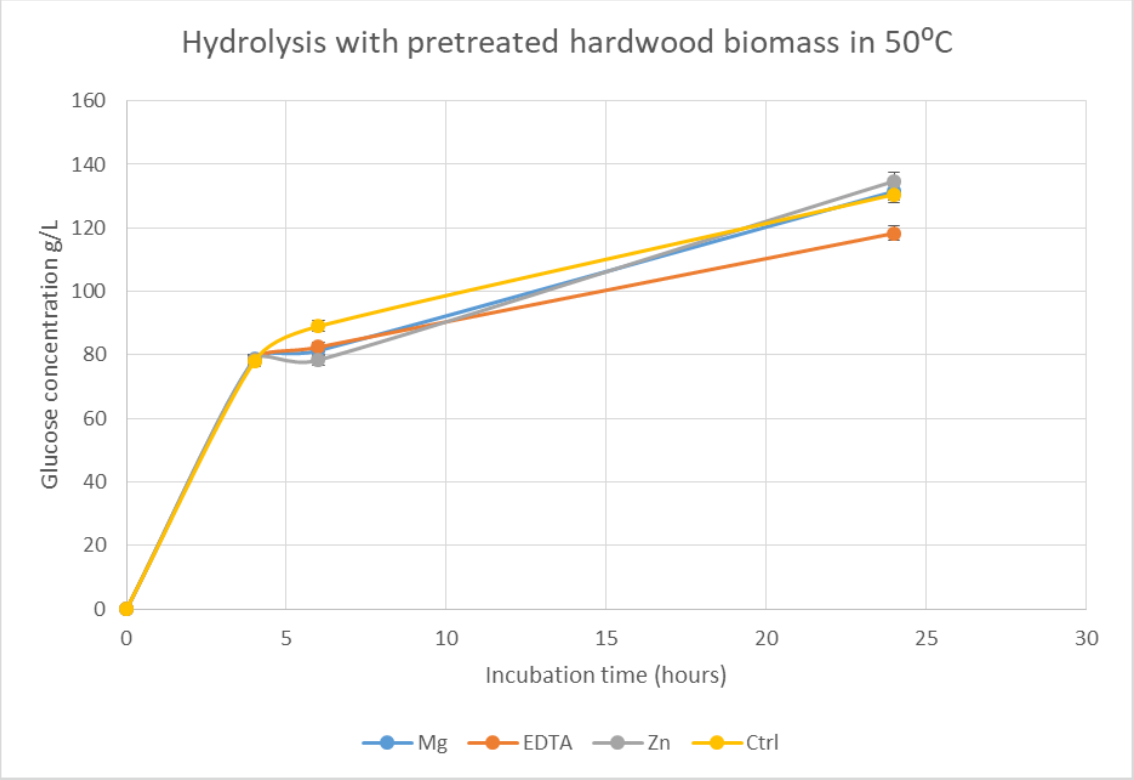

Figure 9. Hydrolysis with pretreated hardwood in 50 °C - selected ions.

Figure 9 shows the kinetics of the reaction which is allowing us to follow the glucose release during the hydrolysis reaction. It is noticed that the reaction is relative fast from 0 h to 4 h and then it goes into a slower phase between 4 h and 24 h. A reason for slowing down can be that the substrate in the hydrolysis

is being used up and the reaction becomes limiting. The samples were not showing significant difference with each other and when taking into the account that the range of reliability for the results is ±5%, the difference in the yields with magnesium, zinc and the control are not significant. However, hydrolysis performed in the presence of EDTA is showing the lowest glucose yield, suggesting a possible inhibition effect. The next hydrolysis was performed at 60 °C and the results are shown in Figure 10.

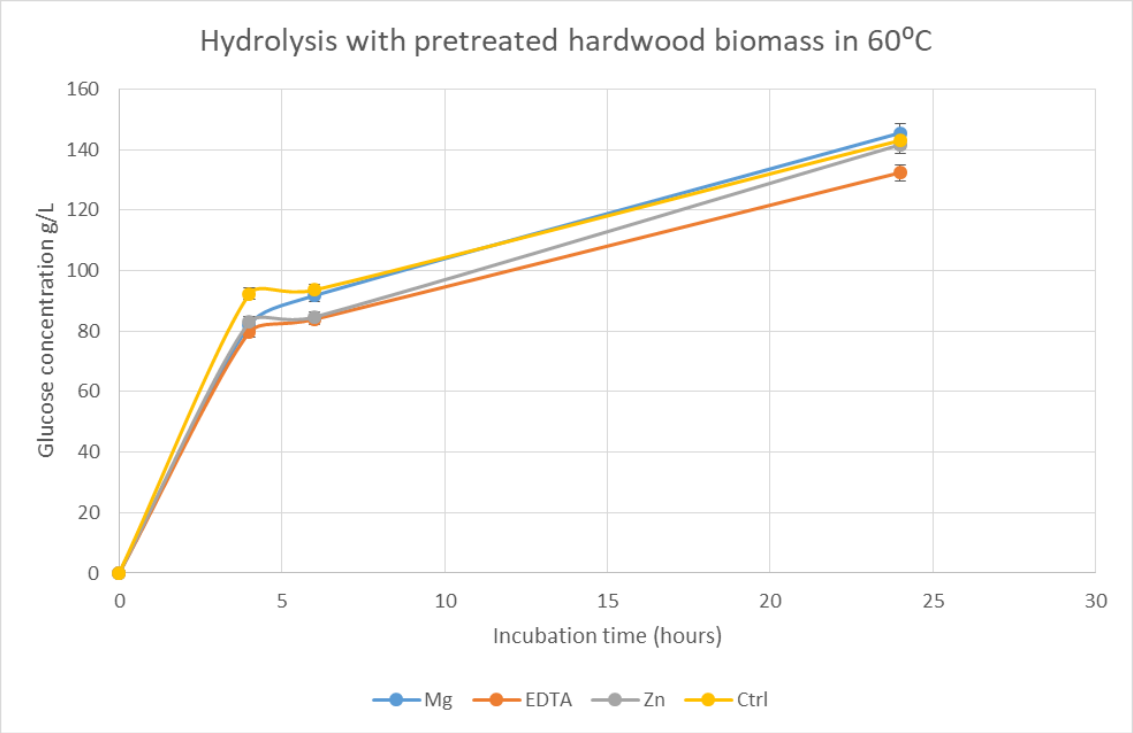

Figure 10. Hydrolysis with pretreated hardwood in 60 °C - selected ions.

When comparing figures 9 and 10 together, it can be seen that the slope is higher when the hydrolysis is performed at 60 ºC. This suggests that the first phase of the reaction is faster in the beginning when compared to the hydrolysis performed at 50 ºC. Also, the second phase of the reaction is giving a higher yield at 60 ºC than at 50 ºC. The samples are not showing a significant difference in relation to each other and when considering that the range of reliability for the results is ±5%, there is almost no difference at all. Samples made with EDTA are showing the lowest concentration.

The last hydrolysis was performed at 70 ºC and the results are given in Figure 11.

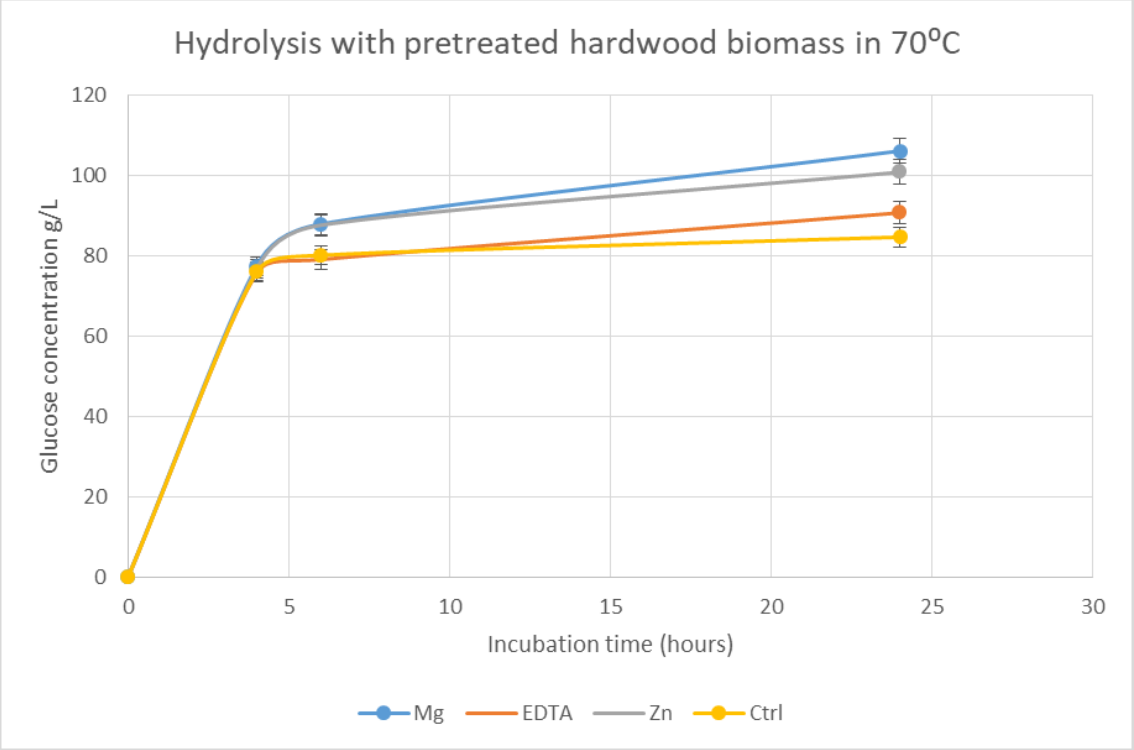

Figure 11. Hydrolysis with pretreated hardwood in 70 °C - selected ions.

From Figure 11 the slope is similar with the hydrolysis results shown earlier (Figure 9 and 10). After the slope, the second phase of the reaction is slower which is giving a lower yield as a result. Magnesium chloride and zinc chloride are showing higher concentration after 24 h, which indicates that these metal ions are helping with the thermostability of the enzyme. The control sample is showing the lowest concentration.

The progression with all the results is congruent and the slope is changing according to the operating temperatures. When all the results from the hydrolysis are collected, it is noticed that the hydrolysis performed at 60 ºC shows the highest concentration and gives the best yield after 24 h. This means that this temperature is improving the kinetics. EDTA is showing the lowest concentration, which could be caused by the chelating effect of the metal ions. However, this result is in contrast with the results obtained in the endoglucanase activity test, where EDTA was bringing a positive impact on the

activity. This might be explained by the higher complexity of the real biomass case, where the metal ions are already presented.

Comparing Figures from the results, it can be noticed that there is a fast reaction phase from 0 h to 4 h. The second phase is slower and it occurs between 4 h and 24 h. This is common for hydrolysis reactions due to the fast processing of the cellulose prone to be hydrolyzed in the first phase and the hydrolysis of the most recalcitrant cellulose in the second. However, as it can be observed, the slope of the reaction is affected by the temperature, showing a higher slope with a temperature at 60 ºC and a lower slope at 70 ºC.

6 Conclusions

For this thesis, a great deal of information was gathered about the effect of metal ions on the hydrolysis of lignocellulosic materials. The released glucose could be used to produce biofuels and be part of biomass valorization. MetZyme® SUNO™ was used throughout the study, a mixture of endoglucanase, exoglucanase and ß-glucosidase activities. The activity and thermostability of the enzyme were measured by endoglucanase activity assays, which were repeated to find out if a metal ion would improve the stability of the enzyme. The behavior of MetZyme® SUNO™ in the presence of metal ions was studied with the hydrolysis of pretreated hardwood biomass. The glucose released as a result of the enzymatic hydrolysis process of the biomass was determined by HPLC.

With earlier results in mind, the studies were continued to confirm the hypothesis of the previous study about magnesium chloride as a potential additive for improved performance. The main aim was to investigate the thermostability of the enzyme in the presence of metal ions, more specifically to find the metal ions that could allow to run hydrolysis at a higher temperature than the optimal (50 ºC) while keeping the enzyme more stable and so active. It was found that at 60 ºC the kinetics are improving and after 24 hours of incubation, the yield is higher than with hydrolysis conducted at 50 ºC. The yield was 10 % higher after 24 h with magnesium chloride, EDTA and control sample (without ions). With zinc chloride the yield was only 5 % higher but it was found to improve the thermostability at 70 ºC in both the endoglucanase activity assay and hydrolysis.

The study could be continued by testing the activity of exoglucanases and ß-glucosidases to see if certain metal ions improve the activities or the thermostability of these singular activities. This could be done similarly to how it was done with the endoglucanase activity assay with specific enzyme activity substrate digestion in the presence of metal ions and at different temperatures. Thereby, the best and most reliable combination could be found for improving

glucose production. For the thermostability test, longer incubation times should be investigated both at 50 ºC and 60 ºC to see how the activity changes after 24 h. If the enzyme thermostability is heavily affected at 60 ºC after 24 h, it could be tested as a process in which a first incubation at 60 ºC is performed for 24 h with the subsequent incubation at 50 ºC until the end of the hydrolysis run.

When comparing the results at 70 ºC, we can see that there seems to be a correlation between the lowest yield and the most probable inactivation of the enzyme. These results show that there seems to be a correlation between the endoglucanase activity assay results and the performance of the enzyme in real substrate hydrolysis.

In addition, if a certain metal ion or EDTA was found to improve glucose production from the pretreated hardwood biomass, its addition in the system would reduce the operating costs of glucose production with higher efficiency. It would also find a purpose for the pretreated hardwood biomass which currently ends up as waste. Even if this study did not find the metal ions or EDTA to have a significant difference in hydrolysis with pretreated hardwood biomass, the study could be repeated with other substrates to find out if the effect is dependent on the biomass more than on the enzyme cocktail.

7 References

Aro, N., Pakula, T. & Penttilä, M., 2005. *Transcriptional regulation of plant cell wall degradation by filamentous fungi,* Helsinki: FEMS Microbiology Reviews.

Bajpai, P., 2016. *Structure of Lignocellulosic Biomass. In: Pretreatment of Lignocellulosic Biomass for Biofuel Production..* Singapore: Springer.

Barakat, A. ym., 2007. Studies of xylan interactions and cross-linking to synthetic lignins formed by bulk and end-wise polymerization: A model study of lignin carbohydrate complex formation. pp. 267-81.

Bisswanger, H., 2012. *Enzyme assays,* 72076, Germany: Elsevier.

Boerjan, W., Ralph, J. & Baucher, M., 2003. *Lignin Biosynthesis,* s.l.: Annual Review of Plant Biology.

Bringmann, M. ym., 2012. Cracking the elusice aligment hypothesis: the microtube-cellulose synthase nexus unraveled. pp. 666-74.

Cantor, C. R. & Schimmel, P. R., 1980. *Biophysical Chemistry.* San Francisco: W. H. Freeman.

Coolbear, T., Whittaker, J. M. & Daniel, R. M., 1992. *The effect of metal ions on the activity and thermostability of the extracellular proteinase from a thermophilic Bacillus, strain EA.1.,* Portland: Biochemical Journal.

Davin, L. B. & Lewis, N. G., 2005. *Lignin primary structures and dirigent sites,* Washington, 99164-6340, USA: Current opinion in Biotechnology.

Demirbas, A., 2009. *Biorefineries: Current activities and future developments,* Sila Science, Trabzon, Turkey: Energy Conversion and Management.

Ding, S., Ge, W. & Buswell, J., 2001. *Endoglucanase I from the edible straw mushroom, Volvariella volvacea,* 268(22): European Journal of Biochemistry.

Elshahed, M. S., 2010. *Microbiological aspects of biofuel production: Current status and future directions,* s.l.: Journal of Advanced Research.

Fernandes, A. N. et al., 2011. *Nanostructure of cellulose microfibrils in spruce wood,* s.l.: Proceeidings of the national academy of sciences.

Heino, J. & Vuento, M., 2010. *Biokemian ja solubiologian perusteet.* Helsinki: WSOY.

Hemminki, L., 2022. *Enzymatic hydrolysis of lignocellulosic biomass.* [Online] Available at: https://www.theseus.fi/bitstream/handle/10024/704124/Thesis_Lotta_Hemminki_Ready%21.pdf?sequence=2&isAllowed=y

Ishida, N. ym., 1980. *Interaction of Amino Acids with Transition Metal Ions in Solution (I) Solution Structure of l-Lysine with Co(II) and Cu(II) Ions as Studied by Nuclear Magnetic Resonance Spectroscopy,* s.l.: Agricultural and Biological Chemistry.

Lopata, A. ym., 2019. *Beyond Chelation: EDTA Tightly Binds Taq DNA Polymerase, MutT and dUTPase and Directly Inhibits dNTPase Activity,* Hungary: Biomolecules.

McKendry, P., 2002. *Energy production from biomass (part 1): Overwiev of biomass,* Colchester CO5 9ES, UK: Bioresource technology.

Megazyme, 2022. *β-Glucazyme Tablets.* [Online] Available at: https://www.megazyme.com/beta-glucazyme-tablets?sSearch=beta-glucazyme [Haettu 21 04 2022].

MetGen Oy, 2020. *SOP for activity determination of MetZyme Products.* [Online] [Haettu 05 04 2022].

MetGen Oy, 2022. *MetGen - The friendly enzyme company.* [Online] Available at: https://www.metgen.com/our-story/ [Haettu 03 03 2022].

Michaelis, L. & Menten, M. L., 1913. *Die Kinetik der Invertinwirkung,* s.l.: Biochem. Z. 49..

Nord, F. F., 2007. *Advances in Enzymology and Related Areas of Molecular Biology.* North Carolina: Wiley-Interscience.

Nzihou, A., 2010. Toward the Valorization of Waste and Biomass. *Waste Biomass Valor*, 26 02, pp. 3-7.

Pandey, M. P. & Kim, C. S., 2010. *Lignin Depolymerization and Conversion: A Review of Thermochemical Methods,* s.l.: Chemical Engineering & Technology.

Pla, F., 1992. Vapor Pressure Osmometry. In: *Methods in Lignin Chemistry.* Berlin: Springer Series of Wood Science, pp. 509-517.

Powar, C. B. & Chatwal, G. R., 2007. *Biochemistry.* Global Media: Prouest Ebook Central.

Pu, Y., Zhang, D., Singh, P. M. & Ragauskas, A. J., 2008. The new forestry biofuels sector. *Biofuels, Bioproducts and Biorefining*, pp. 58-73.

Qining, S., 2014. Enzymatic Deconstruction of Lignocellulose to Fermentable sugars. Teoksessa: *Materials for Biofuels.* Atlanta, GA 30332, USA: Institute of Paper Science and Technology, School of chemistry and biochemistry, Georgia Institute of Technology, pp. 127-146.

Quiroz-Castaneda, R. E. & Folc-Mallol, J. L., 2013. *Hydrolysis of Biomass Mediated by Cellulases for the Production of Sugars,* s.l.: IntechOpen.

Ragauskas, A. J., 2014. *Materials for Biofuels,* s.l.: World Scientific Publishing Company.

Riordan, J. F., 1977. *The Role of Metals in Enzyme Activity,* Boston, MA 02115: Annals of clinical and laboratory science.

Robinson, P. K., 2015. *Enzymes: principles and biotehnological applications,* s.l.: Essays in Biochemistry.

Sadana, J., Lacke, A. & Patil, R., 1984. *Endo-(1-4)-beta-D-glucanases from Sclerotium rolfsii – purification, substrate specificity, and mode of action.,* s.l.: Carbohydrate Research.

Saylor Academy, 2012. *The Basics of General, Organic and Biological Chemistry.* [Online]
Available at: https://saylordotorg.github.io/text_the-basics-of-general-organic-and-biological-chemistry/s21-06-enzyme-action.html
[Haettu 26 04 2022].

Shafiqur, R. M. ym., 2018. *Engoglucanase (EG) Activity Assay,* s.l.: Natonal Library of Medicine.

Sharmili, R. et al., 2021. Recent nanobiotechnological advancements in lignocellulosic biomass valorization: A review. *Journal of Environmental Management*, 30 06, p. 14.

Shell, D. G. & Duff, B., 1996. *Handbook on Bioethanol: Production and Utilization.* Washington DC: Taylor & Francis.

Stenesh, J., 1998. *Biochemistrt.* Springer: ProQuest Ebook Central.

Teeri, T. ym., 1998. *Trichoderma reesei cellobiohydrolases: why so efficient on crystalline cellulose?,* s.l.: Biochem Soc Trans..

U.S. Energy Information Administration, 2020. *Biofuels explained.* [Online]
Available at: https://www.eia.gov/energyexplained/biofuels/biodiesel-and-the-environment.php
[Haettu 26 04 2022].

U.S. Energy Information Administration, 2021. *Biomass explained.* [Online]
Available at: https://www.eia.gov/energyexplained/biomass/
[Haettu 03 03 2022].

Wellings, D. A., 2006. *A Practical Handbook of Preparative HPLC.* s.l.:Elsevier Science & Technology.

Vitha, M. F., 2016. *Chromatography : Principles and Instrumentation.* s.l.:John Wiley & Sons, Incorporated.

Zeng, J. ym., 2014. *Effects of Metal Ions on Stability and Activity of Hyperthermophilic Pyrolysin and Further Stabilization of This Enzyme by Modification of a Ca2+-Binding Site,* s.l.: Applied and Environmental Microbiology.

Zhang, Y. & Lynd, L., 2004. *Toward an affregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems,* 88(7): Biotechnology and Bioengineering.