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MASTER THESIS

Characterization of the recombinantly expressed hydrolases *Pc*Cel5A and *Pc*Cel6A

to apply for the academic adgree Diplomingenior[in] (Master of Science)

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Wien, 27. Mai 2019

Abstract

Cellulose is the most abundant polysaccharide on earth. For a long time, industrial deconstruction of this natural resource was not possible or just not very efficient. The use of natural occurring organisms and enzymes that have the ability to deconstruct lignocellulosic biomass and the creation of enzymatic cocktails opened the door for cellulose as raw material for many industrial applications. Still, the efficient processing of lignocellulolytic biomass remains a challenge, because the currently used techniques for industrial deconstruction aim at the reduction of lignin and hemicellulose in biomass only. The main problems are the production of pure cellulose, hemicellulose and lignin for further processing and the efficient deconstruction of lignin.

Many organisms are able to deconstruct lignocellulosic biomass, at least to a certain extent. White-rot fungi are a prominent example for organisms that can deconstruct lignocellulose completely. In order to do that, they produce specialized enzymes which often work in a synergistic way. Understanding the interaction of enzymes and organisms with each other, is the first step for a successful introduction of enzymatic processes into industrial applications and the development of new, sustainable processes that follow natural models.

In this work, two hydrolases of the white-rot fungus *Phanerochaete chrysosporium* have been produced and characterized by using the production organism *Pichia pastoris*. Production of *Pc*Cel6A was successful and resulted in enough enzyme for enzymatic characterization. The optimum pH for *Pc*Cel6A with 3% CMC (w/w) as a substrate, was pH 5.0. The T_{50} value was determined to be 53.1°C and the pH stability was measured over the whole tested range from pH 2 – 9 for 48 hours. *Pc*Cel6A showed hydrolytic activity on different cellulosic substrates including PASC, lichenan, CMC and different oligosaccharides. The artificial substrate *p*NPC could not be hydrolyzed. High cellobiose concentrations above 10 mM inhibited the hydrolytic activity of *Pc*Cel6A. Synergies of *Pc*Cel6A with lytic polysaccharide monooxygenase from *Neurospora crassa* (*Nc*LPMO9C) have not been found.

Kurzzusammenfassung

Das Polysaccharid Zellulose ist weltweit verbreitet und es war lange Zeit nicht möglich diesen natürlichen Rohstoff effizient industriell abzubauen, von anderen Polymeren zu trennen und zu nutzen. Der Einsatz natürlich vorkommender Organismen und Enzyme, die lignozellulosische Biomasse abbauen können und die Erstellung von Enzymcocktails, führten zu einer deutlichen Verbesserung dieser Lage und der Nutzung von Zellulose als industriellem Rohstoff. Trotzdem stellt die Verarbeitung von Lignozellulose noch immer eine Herausforderung dar, weil die bisher verwendeten Techniken hauptsächlich eine Reduktion des Gehalts an Lignin und Hemizellulose bewirken. Die Hauptprobleme sind die Gewinnung von Reinstoffen (Zellulose, Hemizellulose und Lignin), für eine effiziente Weiterverarbeitung, sowie die energieeffiziente Verarbeitung von Lignin.

Viele Mikroorganismen sind in der Lage Lignozellulose auf natürliche Art, zumindest zum Teil, abzubauen. Weißfäulepilze sind ein markantes Beispiel für Organismen, die Lignozellulose komplett verstoffwechseln können. Zu diesem Zweck produzieren sie spezialisierte Enzyme, die häufig miteinander agieren. Das Verständnis über die Interaktion von Organismen und Enzymen miteinander, soll dazu beitragen, diese enzymatischen Prozesse in bestehende industrielle Anwendungen zu integrieren und neue Prozesse, basierend auf natürlichen Vorbildern, zu entwickeln.

Im Zuge dieser Arbeit, sollten zwei Hydrolasen von *Phanerochaete chrysosporium*, im Produktionsorganismus *Pichia pastoris*, produziert und charakterisiert werden. Die Produktion von *Pc*Cel6A war erfolgreich und erbrachte ausreichend Enzym für eine enzymatische Charakterisierung. Der optimale pH-Wert für *Pc*Cel6A mit 3% CMC (w/w) als Substrat lag bei pH 5.0. Der T_{50} Wert wurde bei 53.1°C berechnet und die pH Stabilität war über den ganzen getesteten Bereich, von pH 2 – 9, für 48 Stunden, gegeben. *Pc*Cel6A zeigte hydrolytische Aktivität mit verschiedenen zellulosischen Substraten, einschließlich PASC, lichenan, CMC und verschiedenen Oligosacchariden. Das künstliche Substrat *p*NPC konnte nicht hydrolysiert werden. Hohe Zellobiosekonzentrationen über 10 mM inhibierten die hydrolytische Aktivität von *Pc*Cel6A. Eine Synergie von *Pc*Cel6A mit lytischer Polysaccharidmonooxygenase von *Neurospora crassa* (*Nc*LPMO9C) konnte nicht festgestellt werden.

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Content

1. Introduction

1.1 Lignocellulose

Lignocellulose is a structurally complex arrangement of polymers that is responsible for the robust structural functionality of plants. The main components of lignocellulose are cellulose (40 – 50%), hemicellulose (20 – 40%) and lignin (20 – 30%). (BALDRIAN and VALÁSKOVÁ, 2008; BOMBLE et. al., 2017) The amounts of the different polymers in specific plants depend on the species of the plant or wood (softwood or hardwood) and the part of the plant investigated. Figure 1 gives a schematic view of the structure of the building blocks that form lignocellulose.

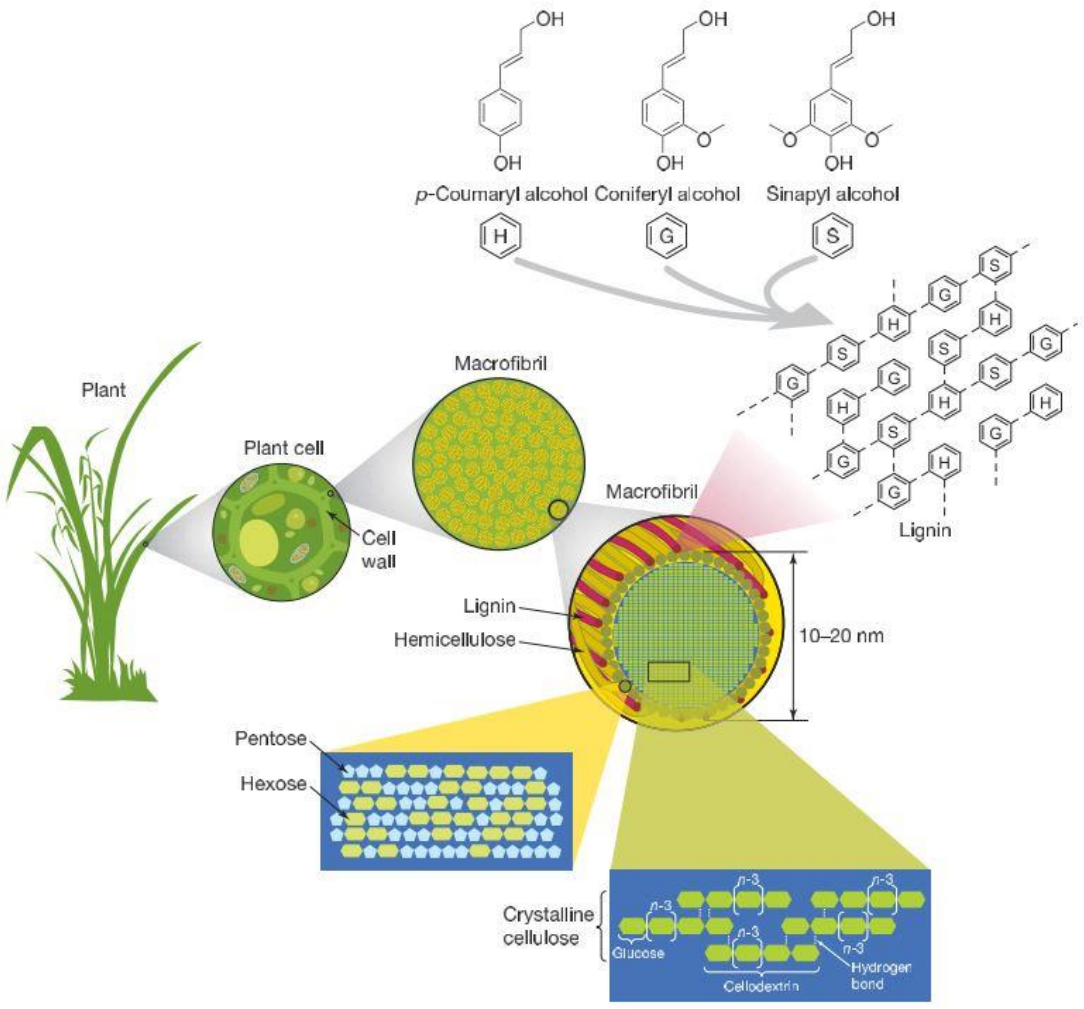

Figure 1: Structure of Lignocellulose (RUBIN, 2008).

1.1.1 Cellulose

Cellulose is the most abundant polysaccharide on earth, although the chemical composition of naturally occurring cellulose is quite simple. It consists of D-glucose residues which are linked by β-1,4-glycosidic bonds that form linear polymeric chains with hundreds or up to 10,000 glucose residues. (BALDRIAN and VALÁSKOVÁ, 2008) The single glucose residues can be linked to each other via hydrogen bonds, to form more dense, crystalline regions, or they assemble in less-ordered amorphous structures. Aggregates of cellulose chains form strong and highly ordered bundles of cellulose, micro- and macro-fibrils, that are interconnected with hemicellulose. Amorphous cellulose can be hydrolyzed easier by specialized enzymes than regions with high crystallinity. This indicates that the initial enzymatic hydrolysis rate is closely related to the crystallinity index of cellulose. The complete depolymerization of cellulose yields only one product, which is D-glucose. (BOMBLE et. al., 2017; HORN et. al., 2012; SUN et. al., 2016)

1.1.2 Hemicellulose

Hemicellulose consists of different non-cellulose polysaccharides with large variation. Examples for those polysaccharides are xylan, mannan and xyloglucan. The variation and the amount of the different polysaccharides is strongly interconnected with the type of plant or wood. Generally spoken, hemicellulose can be degraded easier than cellulose, still some structures are recalcitrant and hard to break because of complex branching and acetylation patterns. The degradation of hemicellulose results in a mixture of different sugars. (HORN et. al., 2012)

1.1.3 Lignin

Lignin fills the void volume of the plant cell wall. It aims to protect the plant integrity by offering a defense mechanism through its hard structure to prevent deconstruction by hydrolytic enzymes, secreted by pathogens and other invading organisms. The structure of lignin consists of different phenylpropanoid units (guaiacyl, syringyl and *p*-hydroxyphenyl) which are crosslinked with carbohydrates by ether or ester linkages. (BOMBLE et. al., 2017; HORN et. al., 2012)

1.2 Use of lignocellulosic biomass

Lignocellulose has been used as energy source, for textile applications and as a building material, for a very long time. It is available directly from natural resources or various waste streams like agricultural waste, municipal solid waste or industrial waste. (TAKKELLAPATI et. al., 2018) Additionally, to the traditional use lignocellulosic biomass serves as potential source for the production of value-able chemical products, like:

- Biofuel (Biogas, Biohydrogen, Bio-oil, Bioethanol)
- Furan molecules (furfuran, 5-hydroxymethylfurfural)
- Sugar alcohols (sorbitol, xylitol)
- Organic acids (succinic acid, lactic acid)

Another field in which lignocellulose can be used is environmental bioremediation and the development of advanced materials like adsorbents or nanocomposites. (PUTRO et. al., 2016)

1.3 Industrial hydrolysis of lignocellulose

To overcome the obstacle of the recalcitrant structure of lignocellulose and effectively degrade cellulose, many different industrial techniques have been developed. A pre-treatment process is necessary to remove high amounts of hemicellulose and lignin to make the biomass more accessible for hydrolytic enzymes and increase the digestibility of the residual cellulose. The pre-treatment process can consist of chemical, biological or physical methods or combinations hereof. The subsequent step is enzymatic hydrolysis which is conducted by cellulase enzyme cocktails or cellulolytic microorganisms. (LIAO et. al., 2016)

The currently used methods often require special equipment, intensive use of harsh chemicals or they consume a lot of energy and time. But the real drawback on the already used pre-treatment processes is the high costs which can make up to 40% of the total processing costs. (SINDHU et. al., 2016) All these points support the search for new methods that can be used industrially. At the moment research focuses on naturally occurring microorganisms and enzymes, which can be modified and improved to the needs of industrial processes in the future.

1.4 Deconstruction of lignocellulose in nature

The cellulose-hemicellulose-lignin matrix is highly recalcitrant. To overcome this obstacle, many fungi and bacteria have developed a diverse set of enzymes and strategies suited for the ecosystem in which they occur. (BOMBLE et. al., 2017)

Enzymes involved in polysaccharide deconstruction are grouped using the CAZy system (Carbohydrate Active EnzYmes). This system classifies enzymes acting on carbohydrates according their sequence, structure and similarity in their catalytic mechanisms. Cellulases are represented by currently more than 150 different glycoside hydrolase (GH) families. Additionally the CAZy database lists enzymes with auxillary activity (AA) and non-catalytic modules (carbohydrate binding modules, CBMs), which are highly interlinked with deconstruction of lignocellulosic biomass. (LOMBARD et. al., 2014; GUPTA et. al., 2016)

The classical scheme of cellulose deconstruction includes the following three main enzyme classes:

- Endoglucanases: non-processive enzymes that randomly cleave internal bonds anywhere along the cellulose chain
- Exoglucanases: processive enzymes that cleave a cellulose polymer from either the reducing or the non-reducing end of the polysaccharide chain
- β-Glucosidases: hydrolyze cellobiose dimers into glucose monomers

What all three enzyme classes have in common is that they are hydrolases. They cleave glycosidic bonds by addition of a water molecule. Their synergistic work leads to the complete deconstruction of cellulose to glucose monomers.

The kinetics of enzymatic deconstruction of lignocellulose are quite complex. Many different models have been proposed and withdrawn or improved due to new findings. The currently used model describes lignocellulose deconstruction as followed: endoglucanases and lytic polysaccharide monooxygenases cleave the cellulose chain and create new ends hereby, exoglucanases digest the created ends down to cellobiose units which are hydrolyzed by β-glucosidases. (BOMBLE et. al., 2017; BALDRIAN and VALÁSKOVÁ, 2008)

Knowing these mechanisms, nature provides still many interesting, not fully characterized and understood microorganisms, enzymes and many possible synergies which are not

investigated, until now. Industry is also very interested in naturally occurring enzymes for technical applications because they can be used in environmentally friendly processes under mild conditions. Genetic engineering of naturally occurring lignocellulolytic enzymes has great potential for many future applications.

1.5 Endoglucanases (EC 3.2.1.4)

Multiple endoglucanases are produced by bacteria, fungi, plants and animals, belonging to 14 different GH families. Until now endoglucanases could be isolated and characterized from different species of bacteria as well as of brown-rot and white-rot fungi. Due to different documentations, it seems as if this kind of enzymes were common among basidiomycetes. Usually this enzyme exists in a monomeric form and shows molecular masses ranging from 22 to 45 kDa. They may contain a catalytic domain and a cellulose-binding domain. Often endoglucanases are glycosylated to a low amount between 1 to 12%. Many already characterized endoglucanases exhibit endo-exo synergism with cellobiohydrolases (exoglucanases), which can be explained through the creation of new free end-groups that can be used as starting points for cellulose degradation by exoglucanases. (BALDRIAN and VALÁSKOVÁ, 2008)

1.6 Exoglucanases (EC 3.2.1.176 and EC 3.2.1.91)

Cellobiohydrolases are major components of the cellulase systems of many fungal species and are involved in the biodegradation of cellulose in nature. Until now, they could be isolated from many white-rot basidiomycetes, but they seem to be absent from most brown-rot fungi. (GUSAKOV et. al., 2011; BALDRIAN and VALÁSKOVÁ, 2008) These enzymes usually act on the ends of crystalline cellulose, sequentially cleaving off cellobiose units. They exist in monomeric form and consist of a bimodular structure with a C-terminal catalytic domain (CD) connected to a cellulose binding module (CBM) via a flexible linker region. (SUZUKI et. al., 2010; TEMPELAARS et. al., 1994) Cellobiohydrolases of type I (CBH I, EC 3.2.1.176, Cel7A) belong to GH family 7 and usually start their attack of the cellulose chain from the reducing ends. Cellobiohydrolases of type II (CBH II, EC 3.2.1.91, Cel6A) belong to GH family 6 and act primarily on cellulose chains from the non-reducing ends on.

CBHs are often glycosylated but only to a low amount. It was reported by (TAYLOR et. al., 2012) that small changes in the number of glycans attached, significantly influence the

enzymatic performance. Many scientific papers about CBHs are available and some CBHs are already well known and characterized. Cel7A from *Trichoderma reesei* is one of the most intensively studied cellulases to date. *Pc*Cel6A was already produced in *P. pastoris* by IGARASHI et. al., (2012) but the characterization aimed only at the comparison of velocities of degradation of crystalline cellulose with *Tr*Cel7A, but not at kinetic characteristics, enzyme stability or substrate specificity.

Cel6A enzymes are much less investigated than Cel7A enzymes to date. Nevertheless, kinetic information about Cel6A enzymes is important as well, because CBHs are one of the main "ingredients" of cellulase enzyme mixtures. Many papers also propose a possible exo-exo synergism of Cel6A enzymes with Cel7A enzymes, which would enhance their possible applications. This is the reason why the search continues for CBHs that work efficiently at high temperatures, low pH values and that are not inhibited by high cellobiose concentrations. (BADINO et. al., 2017; BALDRIAN and VALÁSKOVÁ, 2008)

1.7 Assays for the detection of hydrolases

During the course of time many different approaches for the detection of cellulolytic enzymes have been used. The DNS assay uses a substrate (3,5-dinitrosalicylic acid (DNS)), which is reduced to 3-Amino-5-nitrosalicylic acid and the carbonyl group of the sugar is oxidized to a carbonyl group. This reaction, which is only performed when reducing sugars are present, creates a color change of the DNS-solution from bright yellow to red-brown. (SHAO and LIN, 2018)

The NS assay also detects the amount of reducing sugars. It measures the reduction of copper in alkali solution by reducing sugars. The cuprous ions reduce the arsenomolybdate complex which is formed by the reaction of ammonium molybdate with sodium arsenate which results in the colorimetric reaction to molybdenum blue. (SHAO and LIN, 2018; SOMOGYI, 1945)

The *p*NPC assay works in a different way. If cellulases, including cellobiohydrolases, are present in the sample, the aglycone bond of *p*-nitrophenyl cellobioside is hydrolyzed. This reaction results in cellobiose-units and *p*-nitrophenol. The *p*-nitrophenol is an indicator substance which colors the enzyme solution yellow, if it is not bound anymore and can be detected photometrically. (DINGEE and ANTON, 2010)

1.8 Model organism – *Phanerochaete chrysosporium*

Phanerochaete chrysosporium belongs to the group of white-rot fungi and the phylum Basidiomycota. It is used as a model organism for wood decay, and has been studied intensively. Researchers are especially interested in its ability to produce lignocellulolytic enzymes, which enables the fungus to degrade all components of lignocellulose completely. Additionally, it produces other enzymes which degrade a variety of persistent environmental pollutants. Since 1974, when the teleomorph (sexual reproductive stage) of this fungus was identified, it is called *Phanerochaete chrysosporium*. Its distribution area spreads throughout Europe and North America, where it shows a rapid growth rate at a high optimum temperature for growth (40°C). In the year 2004, the whole genomic sequence of *Phanerochaete chrysosporium* (strain RP-78) was published by the group of MARTINEZ et. al., (2004). It was the first complete genome - of a white-rot fungus - that was sequenced.

The production of many hydrolytic enzymes, as well as extracellular enzymes, like cellobiose dehydrogenase (CDH) and the synergistic action with enzymes produced by other organisms, like lytic polysaccharide monooxygenases (LPMOs) produced by *Neurospora crassa*, lead to continuing interest in this organism. All mentioned characteristics and properties of *P. chrysosporium* make it an attractive candidate for different applications including the production of biofuels from lignocellulosic biomass. Nevertheless, much more scientific research is needed to completely understand the exact mechanism it uses and all possible synergistic effects that might be useful for the application of its enzymes. (MACDONALD et. al., 2012; BURDSALL and ESLYN, 1974; MARTINEZ et. al., 2004; PHILLIPS et. al., 2011)

1.9 Protein expression in *Pichia pastoris*

1.9.1 *Pichia pastoris* – Production organism

The expression levels of lignocellulolytic enzymes in the natural host *P. chrysosporium* are too low for characterization or even industrial applications. Therefore, it was decided to overexpress the enzymes in the well-known production organism *Pichia pastoris.* Many enzymes originating from different species have been produced successfully in *P. pastoris* to date. (MACAULEY-PATRICK et. al., 2005) One of the many advantages for choosing *P. pastoris* as production organism is that it only requires minimal media for fast growth and efficient production of foreign proteins. It is a well-known organism that offers the whole set of the eukaryotic expression machinery such as protein processing, protein folding and post translational modifications like glycosylation and disulfide bridge formation. Additionally, it is less expensive than other eukaryotic expression systems. (CEREGHINO et. al., 2002) One further major advantage using *P. pastoris* as a production host is its ability to secrete large amounts of heterologous proteins into the medium, whereas only little amounts of native proteins are secreted. (INVITROGEN, 2010)

1.9.2 Characteristics of the vector system pPICZ A

It was decided to use the vector system pPICZ A for foreign protein production in *P.* pastoris. This vector offers the feature of the resistance gene *She ble* from *Streptoalloteichus hindustanus* that causes a resistance to the antibiotic zeocin. It can be used as a selection marker in *E. coli* as well as in *P. pastoris* cells. Furthermore, the vector contains the AOX-1 promotor (of alcohol oxidase) native to *P. pastoris*, which allows high-level, methanol inducible expression of the gene of interest. The AOX-1 promotor needs the presence of a trigger substance, an inducer, to be activated. In this case the inducer is methanol, which is added to the liquid cell culture only after a certain growing period of the cells is over and a specific cell density is reached. Just then the artificial enzyme production is started by addition of methanol to the medium. This tight regulation leads to very high expression levels even when the produced protein harms the growth of the cells in suspension.

The choice of the right promotor for protein expression is essential for high level production. The use of the pPICZ A vector requires a change of carbon source from the pre-culture medium (YPD or BYPD) to the expression medium (BMMY), where carbon is added via the methanol

feed. Depending on the application of the produced enzyme, it might be questionable to use toxic substances like methanol in the production process. Nevertheless, the pPICZ vector system is more frequently used, than the pGAPZ system for foreign protein expression in *P. pastoris* cells. The scientific proposal is that the cells grow better, when the transcription of the gene of interest is tightly regulated and well controlled, which is the case for the pPICZ system. (MACAULEY-PATRICK et. al., 2005)

1.10 Previous work to this thesis

This master thesis was conducted in association to the ERC project OXIDISE. The aim of project OXIDISE is to resolve authentic conversion rates of fungal lignocellulose degrading enzymes and to investigate their distribution and interaction. These investigations are necessary because currently used processes for lignocellulose deconstruction are unspecific and produce only low amounts of industrial useable products. It aims for the use of specific biocatalysts for optimal segregation to damage cellulose and lignin structures only minimal and to provide high-quality feedstocks for industrial applications. Those specific biocatalysts do not need to be invented, they are naturally occurring fungal oxidoreductases which need to be characterized and optimized for specific industrial requirements. The first major scientific objective of OXIDISE is the heterologous expression and characterization of cellulose and lignin degrading enzymes naturally produced by *P. chrysosporium*.

As I joined the working group, literature research had already been done previously to identify the major oxidoreductases and hydrolases involved in the deconstruction of lignin and cellulose. This led to the selection of enzymes which should be objects of study for project OXIDISE. The genes encoding for the chosen enzymes were obtained from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank) or from the genomic database FungiDB (http://fungidb.org/fungidb). The genes were ordered from and synthesized by the company BioCat GmbH and cloned into the commercially available plasmid pPICZ A (Invitrogen, California, USA) for expression in the yeast *P. pastoris*. Some enzymes had already been produced successfully in *Trichoderma reesei* or *Pichia pastoris*. Because of the need to produce two further hydrolases (*Pc*Cel5A and *Pc*Cel6A) in *P. pastoris*, it was decided to continue with expressing and characterizing these selected enzymes.

2. Aim of the thesis

This master thesis aims at increasing the knowledge about different enzymes produced by the organism *Phanerochaete chrysosporium*. In this work two enzymes *Pc*Cel5A and *Pc*Cel6A should be produced in the host organism *Pichia pastoris*, a purification protocol and activity assays should be established. Furthermore, the produced enzymes should be characterized or compared to already published characterizations to gain new insights in the functionality of the enzymes, their interactions with already characterized proteins (possible synergies) and their possible applications for enzymatic cocktails. This shall contribute to enhanced utility of enzymes for industrial applications and eliminate the lack of reliable information on promising enzymes.

3. Material and Methods

3.1 Chemicals

All chemicals used in this work were purchased from Fluka/Sigma-Aldrich (Vienna, Austria), Honeywell Riedel-de Haën (Seelze, Germany), Invitrogen (California, USA), Megazyme (Wicklow, Ireland), Merck (Darmstadt, Germany), Primark (Dublin, Ireland), Roth (Karlsruhe, Germany) and VWR (Radnor, USA) and were of analytical grade or the highest purity available. All aqueous solutions were prepared using water deionized by reverse osmosis (RO-H_2O) or deionized and purified water (HQ-H_2O, 0.055 mS/cm) using a Siemens Ultra clear Basic UV SG system.

3.2 Equipment

- Shakers Unitron/Multitron Infors HT (Bartelt, Graz, Austria)
- Eppendorf Centrifuge 5804R and 5424R (Eppendorf, Vienna, Austria)
- Eppendorf Thermomixer F 1.5 (Eppendorf, Vienna, Austria)
- Laminar Flow Thermo Scientific Safe 2020 (Thermo Scientific, Waltham, Massachusetts, USA)
- Autoclave Thermo Scientific Varioklav (Thermo Scientific, Waltham, Massachusetts, USA)
- pH-Meter Metrohm (Metrohm Inula, Vienna, Austria)
- Bio-Rad Micropulser (Bio-Rad Laboratories, Vienna, Austria)
- Bio-Rad SDS Protean Tetra System (Bio-Rad Laboratories, Vienna, Austria)
- Bio-Rad Molecular Imager ChemiDoc XRS+ (Bio-Rad Laboratories, Vienna, Austria)
- Beckman Coulter DU800 Spectrophotometer (Beckman Coulter, Vienna, Austria)
- ÄKTA prime & ÄKTA pure Amersham Biosciences (GE Healthcare, Vienna, Austria)
- Biosan Thermo-Shaker TS-100 (Biosan, Riga, Latvia)
- Eppendorf Vapo.protect 6325 (Eppendorf, Vienna, Austria)
- Grant water bath VAB 12 EU (Grant Instruments, Cambridgeshire, United Kingdom)
- Shimadzu UV Spectrophotometer UV-1800 (Shimadzu, Duisburg, Germany)
- Agilent 5975C TAD Series GC/MSD System (Agilent Technologies, California, USA)

3.3 Buffer, media and solutions

3.3.1 Buffer

Potassium Phosphate Buffer (1 M, pH 6.0)

- 11.50 g Dipotassium-hydrogen-phosphate
- 59.06 g Potassium-dihydrogen-phosphate

Filled up to 900 mL with distilled water, pH adjusted to pH 6.0 with KOH, and addition of distilled water up to 1 L.

Hydrophobic interaction chromatography (HIC) buffers

HIC 1:

Buffer A (25% $(NH_4)_2SO_3$, 20 mM NaAc, pH 5.0)

- 250 mL saturated $(NH_4)_2SO_3$
- 1.15 mL 100% acetic acid

Filled up to 800 mL with distilled water, pH adjusted to pH 5.0 with NaOH, and addition of distilled water up to 1 L.

Buffer B (20 mM NaAc, pH 5.0)

- 1.15 mL 100% acetic acid

Filled up to 800 mL with distilled water, pH adjusted to pH 5.0 with NaOH, and addition of distilled water up to 1 L.

HIC 2:

Buffer A (10% $(NH_4)_2SO_3$, 20 mM NaAc, pH 5.0)

- 55.25 g $(NH_4)_2SO_3$
- 1.15 mL 100% acetic acid

Filled up to 800 mL with distilled water, pH adjusted to pH 5.0 with NaOH, and addition of distilled water up to 1 L.

Buffer B (20 mM NaAc, pH 5.0)

- 1.15 mL 100% acetic acid

Filled up to 800 mL with distilled water, pH adjusted to pH 5.0 with NaOH, and addition of distilled water up to 1 L.

Britton-Robinson buffer (0.5 M)

- 28.6 mL 100% acetic acid
- 30.915 g boric acid
- 33.71 mL phosphoric acid

Filled up to 800 mL with distilled water, pH adjusted with NaOH and addition of distilled water up to 1 L.

Running Buffer for SDS-PAGE gels (10x)

- 36.3 g Tris-Base
- 144 g Glycine
- 10 g SDS

Filled up to 1 L with distilled water.

Agarose Gel Electrophorese buffer (TAE 50x)

- 242 g Tris-Base

Solved in 750 mL with distilled water.

- 57.1 mL acetic acid
- 100 mL EDTA (0.5 M, pH 8.0)

Addition of acetic acid and EDTA and filled up with distilled water up to 1 L.

3.3.2 Media

All media used in this work, were prepared according to the "Easy select *Pichia* Expression Kit manual" (INVITROGEN, 2010) and the "*Pichia* Fermentation Process Guidelines" (INVITROGEN, 2002) both provided by Invitrogen. If required, zeocin was used as selective antibiotic and added to the medium after autoclaving in the following concentrations:

- *Escherichia coli*: 25 mg/L
- *Pichia pastoris*: 100 mg/L

Liquid media and agar plates were stored at 4°C in the dark, until usage.

Lysogeny broth (LB) low salt medium (*E. coli*)

- 10 g Peptone from casein
- 5 g Yeast extract
- 5 g Sodium chloride (NaCl)

For agar plates

- 15 g Agar-Agar

Filled up to 1 L with distilled water and autoclaved at 121°C for 15 minutes.

Yeast extract peptone dextrose (YPD) medium

- 20 g Peptone from casein
- 10 g Yeast extract
- 4 g D-glucose

For agar plates

- 15 g Agar-Agar

Filled up to 1 L with distilled water and autoclaved at 121°C for 15 minutes.

Buffered yeast extract peptone dextrose (BYPD) medium

- 20 g Peptone from casein
- 10 g Yeast extract
- 4 g D-glucose
- 100 mL Potassium Phosphate Buffer (1 M, pH 6.0)

Filled up to 1 L with distilled water and autoclaved at 121°C for 15 minutes.

Buffered methanol complex (BMMY) medium

- 20 g Peptone from casein
- 10 g Yeast extract
- 100 mL Potassium Phosphate Buffer (1 M, pH 6.0)

Filled up to 900 mL with distilled water and autoclaved at 121°C for 15 minutes.

Following solutions were sterile filtrated and added aseptically:

- 2 mL Biotin (500x)
- 100 mL Yeast Nitrogen Base (YNB) (10x)

Yeast Nitrogen Base (YNB) (10x)

- 100 g ammonium sulfate
- 34 g YNB without amino acids and $(NH_4)_2SO_4$

Filled up to 1L with distilled water and sterile filtrated though a 0.22-µm pore filter.

Biotin (500x)

- 200 mg Biotin

Filled up to 1 L with distilled water and sterile filtrated though a pore filter with a cut-off of 0.22µm.

3.3.3 Solutions

Production of phosphoric acid swollen cellulose (PASC)

Ten milliliters of ddH_2O were added to 2 g Avicel and mixed as well as possible. One hundred milliliters of 85% phosphoric acid were added and mixed well again to avoid the formation of lumps. This solution was kept at 4°C overnight. Four hundred milliliters of cold ddH_2O were added. The mixture was centrifuged at 10,000 rpm for 15 minutes. The supernatant was discarded. Approximately 150 mL ddH_2O were added to wash the pellet. The mixture was centrifuged again at 10,000 rpm for 15 minutes. The previously described washing procedure was repeated twice using fresh ddH_2O after each centrifugation step. About 40 mL sodium carbonate 2 M were added to each centrifuge tube to obtain a neutral solution, which was checked using pH-paper. The pellet was washed again with ddH_2O, two times like described before. The cellulose was solved in sodium acetate buffer with pH 5.0. Depending on the desired concentration of the PASC solution, the amount and concentration of the used buffer was calculated. (In this case 250 mL 110 mM sodium acetate buffer pH 5.0 were used to produce a 0.8% (w/w) PASC solution.)

Nelson-Somogyi assay solutions

Somogyi solution: The Somogyi reagent was prepared by mixing four parts of Solution I and one part of Solution II.

Solution I

Solution I was prepared by mixing 15 g potassium sodium tartrate tetrahydrate with 30 g sodium carbonate and solving the mixture in 300 mL ddH_2O. Twenty grams of sodium bicarbonate were added to the previously made solution and dissolved completely. An aliquot of 180 g of sodium sulfate anhydrous was dissolved in 500 mL ddH_2O separately and heated up to release bubbles, cooled and mixed with the first solution and the volume was completed to 1 liter with ddH_2O.

Solution II

The second solution was prepared by dissolving 5 g copper (II) sulfate pentahydrate and 45 g sodium sulfate anhydrous in 250 mL ddH_2O.

Nelson solution: The nelson solution was the mixture of one part of Solution III and two parts of 0.75 M sulfuric acid.

Solution III

Solution III was prepared by solving 25 g ammonium molybdate in 450 mL ddH_2O while stirring. Twenty-one mL concentrated sulfuric acid were added to the previous solution. Three g of sodium arsenate ($Na_2HAsO_4 \cdot 7\ H_2O$) were dissolved in 25 mL ddH_2O. Both solutions were mixed with stirring. The solution was incubated at 37°C for 24 to 48 hours in a dark brown bottle. After 48 hours the solution was finalized by mixing Solution III with 1 L 0.75 M sulfuric acid.

3,5-Dinitrosalicylic acid assay solutions

DNS solution (cuvette sized assay)

For preparation of the DNS solution two solutions were mixed: 5 g 3,5-dinitrosylicylic acid in 100 mL ddH_2O and 5 g NaOH in 100 mL ddH_2O. An aliquot of 250 mg of sodium sulfite (Na_2SO_3) was added to the solution and filled up to 500 mL with ddH_2O. The solution was filled in a dark brown bottle and stirred at 4°C for 48 hours.

DNS solution (96 well plate sized assay)

For preparation of the DNS solution the following components were dissolved 10 g DNS, 0.5 g Na_2SO_3, 10 g NaOH and 2 mL Phenol in 998 mL ddH_2O.

Rochelle salt solution

Rochelle salt solution was prepared by dissolving 40 g potassium sodium tartrate (Rochelle salt) in 100 mL ddH_2O.

3.4 Genetic techniques and protein production

For gene cloning and expression of enzymes, the following organisms and strains were used:

- *Escherichia coli* NEB 5-alpha New England Biolabs (Frankfurt/Main, Germany)
- *Pichia pastoris* X-33 Invitrogen (California, USA)

3.4.1 Primers

The primers listed below in Table 1 were ordered from Microsynth (Vienna, Austria), diluted with ddH_2O to a concentration of 100 µM and stored at -20°C. Working stock concentration of the primers was 10 µL.

Table 1: Used primers and their nucleotide sequences.

Primer Name	**5'-Nucleotide sequence-3'**
5' AOX-1 fwd	5'-GACTGGTTCCAATTGACAAGC-3'
3' AOX-1 rev	5'-GCAAATGGCATTCTGACATCC-3'
3' *Pc*CBH2	5'-GCTGGTGATAACGGAGGTG-3'
3' *Pc*EG38	5'-CCAAGACGAGGAAGAGCAC-3'

3.4.2 Enzymes

All enzymes, as well as DNA polymerases, were acquired from New England Biolabs (Frankfurt/Main, Germany). If not stated otherwise all reactions (Digestions and Ligations) were carried out according to the standard manuals provided by the enzyme suppliers.

3.4.3 Expression constructs

All genes used in this work were ordered and synthesized by the company BioCat (Heidelberg, Germany) and provided in a pPICZ A vector. The genes sequenced were obtained from database entries from GenBank or UniProt. The used entries are listed in the following Table 2. The expression vector pPICZ A is visualized in Figure 2.

Table 2: GenBank entry ID numbers of used genes.

Name of the gene	**GenBank entry ID number**
*Pc*Cel6A (CBH2)	S76141.1
*Pc*Cel5A (EG38)	AAU12275.2

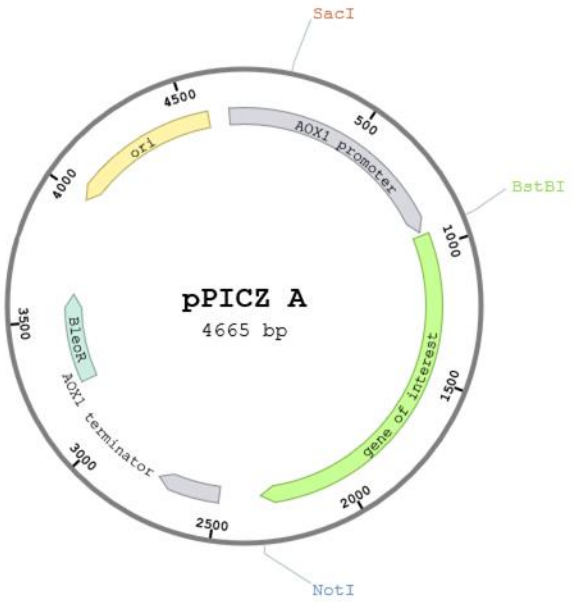

Figure 2: Expression vector pPICZ A.

3.4.4 Preparation of electrocompetent *P. pastoris* X-33 cells

Three pre-cultures of each 50 mL YPD medium in 250 mL baffled flasks were inoculated with a toothpick with *P. pastoris* X-33 (Invitrogen), from a frozen (-80°C) culture collection. The pre-culture flasks were incubated at 30°C and 140 rpm overnight.

The main culture in 500 mL YPD medium was inoculated with the total volume of 150 mL of the overnight cultures. After further incubation of the main culture of six hours an OD_{600} of 1 was reached. The *P. pastoris* cells were harvested by centrifugation in two centrifuge bottles at 4000 rpm, at 4°C for 10 minutes. The cell pellets were resuspended in 50 mL YPD medium and 1 mL 1 M HEPES buffer (pH 8.0) each. During gentle shaking of the centrifuge tubes, 1.25 mL 1 M dithiothreitol (DTT) was added. The resuspended cells were incubated at 30°C and 100 rpm for 15 minutes. After this incubation step it was essential to keep the cell suspension on ice all the time. The suspension was filled up to 200 mL total volume with cold, sterile ddH_2O and pelleted by centrifugation again at 3000 rpm and 4°C for 5 minutes. The previously described washing step was repeated another two times under the same conditions, each time using fresh cold ddH_2O and resuspending the cells properly after each step. After the washing, the remaining cell pellet was resuspended in 10 mL 1 M sorbitol and centrifuged again at 4000 rpm at 4°C for 10 minutes. As a final step the cell pellet was resuspended in 1 mL 1 M sorbitol. Aliquots of 50 µL cell suspension were made and frozen at -80°C until needed.

3.4.5 Restriction enzyme digest of *Pc*Cel6A in pPICZ A

The digests were performed according to the Double Digest Protocol from New England Biolabs and the pipetting scheme in Table 3.

Table 3: Pipetting scheme for SacI-HF digestion.

Amount	Reagent
2.5 µL	10x Cut Smart Buffer
1 µL	SacI-HF restriction enzyme
4 µL	pPICZ A plasmid with *Pc*Cel6A insert (286 ng/µL)
17.5 µL	ddH_2O

The digestion was performed at 37°C for two hours. Afterwards the digest was stopped by an incubation of the reaction at 65°C for 20 minutes. The digested vector was frozen until electroporation of *P. pastoris* cells was performed.

3.4.6 Preparation of agarose gels

For preparation of a 0.8% (w/w) agarose gel, 3.2 g agarose were weighed in 400 mL 1x TAE buffer. The agarose was melted in the buffer using a microwave, until the mixture was boiling and completely transparent without any turbid flakes.

For a small sized gel 50 mL of 0.8% (w/w) agarose in 1x TAE buffer with 3 µL of 20,000 peq-GREEN (peqlab, Erlangen, Germany) were used.

The gel was poured into a tray and a comb was introduced to create wells for sample application. After 30 minutes the gel was placed in the electrophoresis chamber which was filled with 1x TAE buffer until the gel surface was slightly covered. The DNA samples were prepared by mixing them with 6x Loading Dye (Thermo Fisher Scientific) and applied into the pockets of the gel. A 2-Log DNA Ladder or 1 kb Ladder (both New England Biolabs) was used as DNA standard on every gel. Electrophoresis was performed on a PowerPac 300 machine (Bio-Rad) at 90 V for 30 minutes.

The results could be observed under UV-light using a GelDoc 2000 machine (Bio-Rad). Analytical gels were used for verification purpose only, whereas preparative gels were used

for DNA purification and therefore the DNA bands of interest were cut out with a scalpel and transferred into a fresh, sterile and weighed Eppendorf tube.

3.4.7 Electroporation of electrocompetent *Pichia pastoris* X-33 cells

Previously to electroporation, the plasmid was digested to enhance the transformation efficiency by using a linearized vector instead of a circular one for this procedure. The SacI digested plasmid (3 µL; 40 ng/µL) was added to 50 µL electrocompetent *P. pastoris* cell suspension. This mixture was applied to a pre-cooled sterile 0.1 cm electroporation cuvette. Electroporation was carried out with 1.5 kV and 3 ms at manual settings. Immediately after electroporation the cells were resuspended in 500 µL cold 1 M sorbitol and 500 µL YPD medium in a fresh 2 mL Eppendorf tube. The cell mixture was incubated at 30°C and 100 rpm horizontal shaking for four hours. After incubation the cells were plated on prewarmed YPD selection plates containing zeocin using sterile glass beads and incubated at 30°C for two to three days until colonies appeared.

3.4.8 Colony PCR

Colony PCR is a quick and easy method for screening transformed colonies for the presence of the gene of interest. Depending on the gene, 5'AOX-1 and 3'AOX-1 primers or 5'AOX-1 and gene specific 3'-end primers were used. Colonies of transformed yeast cells were picked from the YPD transformation plates and streaked onto fresh YPD selection plates. After two days of incubation at 30°C big lumps of cells were resuspended in 30 µL sterile ddH_2O. The cell suspension was heated at 99°C for 10 minutes to lyse the cells (vortexed one time in between) and centrifuged at maximum speed (14,640 rpm) of the centrifuge for 5 minutes.

Ten µL of the supernatant were used as a template for the following PCR reaction which followed the pipetting scheme in Table 4 and the thermocycler protocol in Table 5.

For control reactions the template DNA was substituted with:

- Positive control: 1 µL 1:100 diluted plasmid + 9 µL ddH_2O
- Negative controls: 10 µL empty X-33 strain plus each reverse primer

The PCR product was applied to agarose gel electrophoresis. The amplified DNA fragments were detected using the GelDoc 2000 machine (Bio-Rad).

Table 4: Pipetting scheme for PCR mix.

Amount	Reagent
5 µL	5x Q5 Reaction buffer
0.25 µL	Q5-HF DNA Polymerase
0.5 µL	10 mM mixed dNTPs
1.25 µL	10 µM forward primer (5'AOX-1)
6.75 µL	ddH_2O
1.25 µL	10 µM reverse primer (3'AOX-1 or specific primer)
10 µL	template DNA from supernatant

Table 5: Protocol for colony PCR reaction.

Temperature	Time	Step	Cycles
95°C	∞		
95°C	2 min	Initialization step	1x
95°C	10 sec	Denaturation step	32x
60°C	20 sec	Annealing step	
72°C	2 min	Elongation step	
72°C	5 min	Final elongation step	1x
4°C	∞	Final hold	1x

3.4.9 Preparation of a cryo stock culture

To preserve *E. coli* and *P. pastoris* clones that carry the gene of interest, cryo stock cultures were created. An isolated clone was picked from a fresh inoculated agar plate and was used to inoculate 3 mL LB low salt medium (for *E. coli*) or 3 mL YPD medium (for *P. pastoris*) for an overnight liquid culture. The cultures were incubated at 37°C (for *E. coli*) or at 30°C (for *P. pastoris*) at 125 rpm. An aliquot of 500 µL of the overnight culture were mixed with 500 µL sterile 30% glycerol in sterile cryo-tubes. These tubes were frozen and stored at -80°C.

3.4.10 Protein expression in big shaking flasks with automatized methanol feeding

Single colonies from the transformation plates (YPD with zeocin) and non-transformed X-33 cells (YPD) as negative control, were used to inoculate overnight pre-cultures in 20 mL YPD

medium in 100 mL non-baffled flasks. The cultures were incubated at 30°C and 180 rpm for approximately 18 hours.

Protein expression was performed in baffled shaking flasks (1000 mL) in BMMY medium (200 mL each) that were inoculated with the whole volume of the overnight cultures. Each flask was equipped with one rubber tube and covered with aluminum foil before autoclaving. After inoculation of the big flasks the tubes were connected to a bottle containing 50% methanol solution for feeding the cells and hereby inducing protein expression. The methanol feed was controlled by a peristaltic pump system. Settings were chosen accordingly to maintain a methanol concentration of 1% in the flasks. This was accomplished by a feed of 1 mL 50% methanol per flask every 3 hours. Expression was carried out for 4 days and monitored by measurements of wet biomass, protein concentration (Bradford protein measurement) and SDS-PAGE electrophoresis.

3.4.11 96 deep well plate cultivation

For easy screening of high numbers of clones 96 deep well plate cultivation was used. The *P. pastoris* clones which should be screened were transferred to new YPD selection plates and incubated at 30°C for 2 days.

For the pre-culture 150 µL of BYPD medium with zeocin was transferred to each well of a sterile disposable 96 well plate. Every well was inoculated with one colony of the transformation plates and the used toothpick was also used to transfer the colony to a fresh YPD selection plate to maintain each colony also on solid medium. The 96 well plate was sealed with a Breathe-Easy® membrane and incubated at 30°C and 250 rpm at 80% humidity for 24 hours.

For an over-weekend culture a sterile 96 deep well plate was filled with 250 µL BYPD medium and inoculated with 10 µL of each well of the pre-culture plate. The new plate was sealed again with a Breathe-Easy® membrane and incubated 30°C and 350 rpm at 80% humidity. After 67 hours of incubation, protein expression was induced by addition of 250 µL BMMY medium with methanol. Fifty µL of 5% methanol were added to each well after further 8, 24 and 48 hours of incubation. The cultivation was stopped after 123 hours, and the cells were pelleted by centrifugation at 3500 rpm for 15 minutes.

The supernatant in the wells was divided to two fresh 96 well plates that were stored at 4°C and -20°C to keep them for screening procedures afterwards. The wells on the edges of the plates were not used for colony screening. This was due the fact that even though the Breathe-Easy® membranes were used, it could not be assured that evaporation of medium was prevented to 100%.

3.5 Protein purification

3.5.1 Harvesting of *P. pastoris* cells

The cultures were harvested by centrifugation at 6500 g and 4°C for 20 minutes. The supernatants of the flasks were pooled and filtrated over two cellulose filters.

3.5.2 Hydrophobic interaction chromatography (HIC)

Hydrophobic interaction chromatography was used as first and second step in protein purification. The descriptions and settings of the columns used for purification are listed in Tables 6 and 7.

Previously to the first HIC, solid ammonium sulfate was added to reach 25% saturation, the addition was performed slowly to prevent extensive precipitation, while the solution of the pooled supernatants was stirred. The solution was cleared from particles by centrifugation at 6000 rpm and 4°C for 10 minutes. Before the second HIC was performed the fractions of the protein peak were pooled again, concentrated and adjusted with saturated ammonium sulfate solution to 10% ammonium sulfate saturation.

Table 6: Column description and settings for 1st HIC - Phenyl Sepharose.

Column	**Phenyl Sepharose Fast Flow (GE Healthcare)**
Column Volume	250 mL
Buffer A	25% $(NH_4)_2SO_4$, 20 mM NaAc pH 5.0
Buffer B	20 mM NaAc pH 5.0
Flowrate Sample Application	20 mL/min
Gradient	0-100% buffer B in 1000 mL (4 column volumes)
Machine	ÄKTA prime (GE Healthcare)

Table 7: Column description and settings for 2nd HIC - Phenyl Source.

Column	Phenyl Source (GE Healthcare)
Column Volume	60 mL
Buffer A	10% $(NH_4)_2SO_4$, 20 mM NaAc pH 5.0
Buffer B	20 mM NaAc pH 5.0
Flowrate Sample Application	2.5 mL/min
Gradient	0-100% buffer B in 180 mL (3 column volumes)
Machine	ÄKTA pure (GE Healthcare)

3.5.3 Anion exchange chromatography (AEX)

Before the anion exchange chromatography was run, the fractions of the larger peak of the second HIC were dialyzed against 20 mM Tris-HCl buffer pH 8.0 using a Vivaflow 50 crossflow module (Fisher Scientific). AEX was used as third step in protein purification. The column description and settings for anion exchange chromatography can be found in Table 8.

Table 8: Column description and settings for AEX.

Column	Source Q15 (GE Healthcare)
Column Volume	15 mL
Buffer A	20 mM Tris-HCl pH 8.0
Buffer B	20 mM Tris-HCl pH 8.0, 500 mM NaCl
Flowrate Sample Application	5 mL/min
Gradient	0-100% buffer B in 240 mL (16 column volumes)
Machine	ÄKTA pure (GE Healthcare)

3.6 Protein characterization

3.6.1 Wet biomass measurement

Three mL samples of the shaking flasks were taken aseptically with 5 mL sterile pipettes into 5 mL Eppendorf tubes. The samples were divided into two weighted 1.5 mL tubes (each holding 1 mL sample). The tubes were centrifuged at 14,000 rpm for 3 minutes. After centrifugation the supernatant was separated from the cell pellet. The supernatant was frozen for additional measurements like protein concentration. The tube with the cell pellet was weighed again for determination of the wet biomass production in the shaking flasks.

3.6.2 Bradford protein determination

Total protein concentration of samples was determined using the Bradford assay. The Bradford stock reagent and the BSA standards were purchased from BioRad and prepared according to the instructions of the manufacturer. Six hundred microliters of the assay solution were mixed with 15 µL of diluted sample in a disposable cuvette. The sample solution was mixed well with a plastic spatula. After 15 minutes incubation and mixing again, the absorption of the sample was determined spectrophotometrically at 595 nm with a Beckman Coulter DU 800 UV/VIS spectrophotometer. The protein concentration was calculated using a calibration curve of BSA in the range of 0.1 – 1.0 mg/mL.

3.6.3 SDS-PAGE

Proper diluted protein samples (1 mg/mL) were mixed with Laemmli buffer (1:1) (Thermo Fisher Scientific) and denatured at 99°C for 5 minutes. After spinning down of the samples, 10 – 20 µL of each sample were loaded on a Mini-PROTEAN TGX Stain-Free (4 – 20%) precast gel (Bio-Rad). The gel was run at 120 V for approximately 90 minutes. After the run it was developed and could be observed using a GelDoc 2000 machine (Bio-Rad).

3.6.4 Thin layer chromatography (TLC)

Already pre-coated polyester sheets (POLYGRAM SIL G/UV_{254} 0.2 mm silica gel 60 with fluorescent indicator, Merck) were used for this method. One sheet was taken out of the storage box carefully to keep the silica coating intact. Approximately 1 cm from the lower border of the sheet a pencil line was drawn, and sample spots were marked. One microliter of

each sample or control was applied on the corresponding spot on the sheet. After 5 minutes of drying, another 1 µL of each sample was applied to the same spot again. For highly concentrated samples or positive controls application of 1 µL in total was enough. The bottom of a jam jar was covered with the running agent. For *Pc*Cel6A the running agent consisted of 3-parts pure butanol, 1-part concentrated acetic acid, and 1-part H_2O. Tweezers were used to grab the paper on the upper part and put it into the jam jar. It needed to be assured that the paper was standing upright and straight. The lid of the jam jar was closed tight. The chromatographic separation run was stopped by removing the TLC plate from the running solvent. The running agent was run until about 1 cm below the upper border of the paper.

After opening of the jar, the sheet was removed with tweezers by grabbing it at the dry upper part. It was put into a glass dish and the line of the running agent was marked with a pencil. For development of the paper it needed to be dry and was put into a developing solution which consists of 95% MeOH and 5% concentrated H_2SO_4. After the sheet was dry again, it was finally put it into a drying chamber at 100°C for 9 minutes. After heating the sheet, the result should be visible. Because the color on the sheet fades quickly, the visible dots were marked with a pencil.

3.6.5 Spectro photometrical assays

The enzyme stock concentration of *Pc*Cel6A was 11 mg/mL, before dilution, for all performed assays and kinetic measurements.

3.6.5.1 Nelson-Somogyi assay – NS assay

- 150 µL substrate (3% (w/w) CMC in 55 mM NaAc buffer pH 5)
- 100 µL enzyme dilution

The samples were incubated at 40 – 60°C and 1200 rpm, in a heating block for 10 – 60 minutes. The reaction was stopped by incubating the samples at 99°C in a heating block for 5 minutes.

The samples were centrifuged at 14,000 rpm for 3 minutes.

- 200 µL Somogyi reagent
- 200 µL supernatant of the previously incubated substrate & enzyme mix

The Somogyi reagent and the supernatant were pipetted into a 10 mL glass tube. The glass tubes were put in a metal rack and covered with aluminum foil. The whole rack was put into a boiling water bath at 99°C for 20 minutes.

The rack was cooled down in a cold-water bath which took about 5 minutes until the vials were cold enough to carry on

- 200 µL Nelson reagent were added to each glass vial
- 2.4 mL distilled water were added with a dispenser flask

The glass vials were vortexed, and aliquots of 1 mL were pipetted into 1.5 mL Eppendorf tubes. The samples were centrifugated at 14,000 rpm for 3 minutes and then measured at 540 nm in a spectrophotometer.

Due to the complexity of the assay, all steps are summarized in Figure 3.

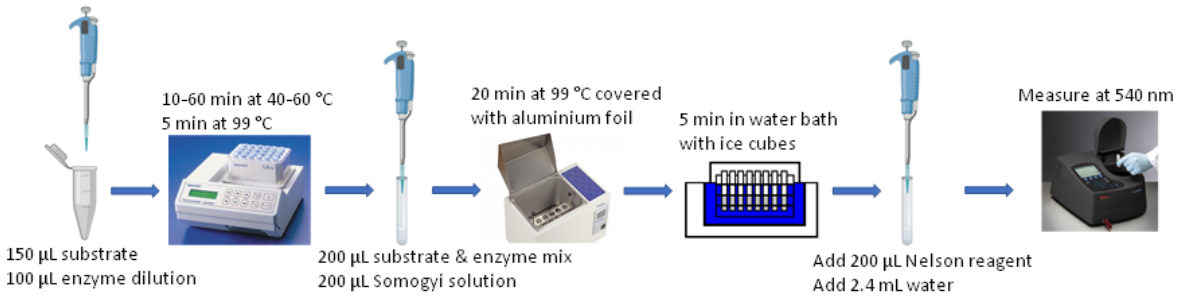

Figure 3: Pipetting scheme NS-assay.

3.6.5.2 DNS assay (cuvette size)

Five milligrams of CMC were weighed into a reaction tube and 1 mL sample solution (diluted with citrate buffer) was added. The samples were incubated in a heating block at 40°C and 900 rpm for 30 minutes. The samples were centrifuged at 10,000 rpm for 5 minutes and 600 µL of the incubated solution were added to 600 µL of DNS reagent in a new reaction tube. The mixture was heated up to 95°C for 15 minutes and after incubation 200 µL Rochelle salt were added. After cooling down on ice for 5 minutes, the absorption of the samples was measured at 575 nm.

3.6.5.3 DNS assay (96 well plate reader size)

Five milliliter DNS solution were mixed with 834 µL Rochelle salt solution to obtain the DNS assay solution. Thirty microliters of diluted enzyme solution (in citrate buffer) were mixed with

30 µL 3% (w/w) CMC solution in 50 mM sodium citrate buffer (pH 5.0) in a well of a 12 well PCR stripe. The samples were incubated at 50°C for 30 minutes in a thermal PCR cycler (BioRad). Sixty microliter DNS assay solution was added and the samples were heated up to 95°C for 5 minutes. An aliquot of 100 µL of each well was transferred to a 96 well plate and measured at 540 nm on the plate reader.

3.6.5.4 *p*-Nitrophenyl cellobioside assay

- 10 µL BSA 2% (w/w)
- 5 µL enzyme (undiluted)
- 35 µL ddH_2O
- 50 µL acetate buffer 200mM, pH 5.0
- 100 µL *p*NP cellobioside 0.2% (w/w) solved in water

The samples were incubated in a heating block at 50°C for 10 minutes. The reaction was stopped by addition of 500 µL Na_2CO_3 2% (w/w). The activity was measured at 410 nm in a spectrophotometer.

3.6.6 pH profile

For determination of pH profiles, a series of 9 Britton-Robinson buffer (0.2 M) solutions with pH values ranging from pH 2 to 10 was tested in triplicate measurements. Solutions of 3% (w/w) CMC were prepared with the different buffers. One microliter enzyme was diluted with 99 µL of the buffer solution and incubated with 150 µL of the corresponding 3% (w/w) CMC solutions at 50°C for 1 hour. The enzymatic reaction was stopped by incubation of the samples at 99°C for 5 minutes. Afterwards the NS assay was performed as described previously in this work.

3.6.7 Temperature optimum

The temperature optimum was determined by incubation of enzyme samples at different temperatures (30 – 50°C) during the NS assay. The measurement was performed in triplicates.

3.6.8 T_{50} value – thermostability

To evaluate the temperature at which the enzymes loses 50% of its initial activity after 2-hour incubation, samples were pre-incubated at different temperatures ranging from 39.9 to

60.1°C (blank at 4°C in the fridge). One microliter enzyme was diluted with 49 µL ddH_2O and incubated for 2 hours. The samples were then further diluted with 50 µL ddH_2O and analyzed using the NS assay.

3.6.9 pH stability

The stability of enzymes pre-incubated at different pH values was tested by using 1:50 enzyme dilutions in 20 mM Britton-Robinson buffer ranging from pH 2 to 10. The samples were tested in duplicates after 1-, 24- or 48-hour incubation at 4°C. After the pre-incubation 50 µL ddH_2O were added and the NS assay was started by addition of 3% (w/w) CMC solution (50 mM NaAc buffer pH 5.0).

3.6.10 Substrate comparison 24h

In order to test the enzymatic activity on different substrates a long-time incubation of 24 hours was used. Enzyme dilutions (in duplicates) were incubated with the substrate for 24 hours instead of the 30 minutes incubation of the NS assay. Further steps were conducted identical to the NS assay description previously mentioned.

3.6.11 Oligosaccharides as enzymatic substrates (TLC & GC)

The possible use of oligosaccharides as enzymatic substrates was tested using TLC or gas chromatography. An aliquot of 150 µL of 1:100 diluted enzyme was mixed with 150 µL 3% (w/w) CMC (55 mM in NaAc buffer pH 5.0) and incubated at 50°C and 1200 rpm for 24 hours. The enzymatic reaction was stopped by incubation of the samples at 99°C for 5 minutes. Thin layer chromatography was performed as previously mentioned. Gas chromatography samples were prepared by using 50 µL of the sample and 100 µL inositol and drying them with a Rotavapor R300 (BÜCHI Labortechnik, Flawil, Switzerland) at 70°C for 45 minutes. The samples were analyzed by gas-liquid chromatography as previously reported in BERNABÉ et. al., (2011), in an Agilent 5975C instrument equipped with an HP5-MS column and a flame ionization detector. Identification of the carbohydrates was performed comparing the retention time to previously measured standards. The samples were analyzed by an in-house service and the results were available online after 2 days.

3.6.12 Cellobiose inhibition

Possible product inhibition of the enzymes was evaluated by addition of cellobiose to the enzyme dilutions. The samples were measured using duplicate measurements. One hundred microliter of 1:100 diluted enzyme was mixed with 150 µL cellotriose (10 mM) and 50 µL of different cellobiose stocks. The samples were incubated at 50°C and 1200 rpm for 24 hours. The reaction was stopped by incubation of the samples at 99°C for 5 minutes. Afterwards the amount of formed glucose was measured using the Glucose-TR Trinder GOD-POD kit (Spinreact, Barcelona, Spain). The samples were measured at 505 nm using a spectrophotometer.

3.6.13 Interaction of PcCel6A with NcLPMO9C

Lytic polysaccharide monooxygenases are enzymes that act similar to endoglucanases. They cleave within the cellulose chain and create new chain ends, that could be used by *Pc*Cel6A. *Nc*LPMO9C has already been described by ISAKSEN et. al., (2014). This LPMO cuts the cellulose chain by oxidation of the C4-end, this results in new oxidized non-reducing chain ends. The possible use of pre-incubation of substrates with *Nc*LPMO9C before incubation with *Pc*Cel6A was tested using the NS assay. An aliquot of 150 µL of 1.4% (w/w) Avicel was mixed with 30 µL ascorbic acid (6.67 M) and 100 µL of 1:100 diluted *Nc*LPMO (69 mg/mL). This mixture was incubated at 50°C and 1200 rpm for 15 minutes or 60 minutes. After a 5-minute inactivation of the LPMO at 99°C, an aliquot of 100 µL of 1:100 diluted *Pc*Cel6A (11 mg/mL) was added. After this addition, a normal, already described, NS assay was performed.

3.6.14 Binding kinetics

The binding kinetics were determined for the substrates CMC (triplicates), lichenan (duplicates) and PASC (duplicates) at different initial substrate concentrations. The enzymatic activity was measured using the NS assay. The turnover number was calculated using SigmaPlot 14.0 (Systat Software, Chicago, USA) with a nonlinear least squares regression model based on the Michaelis-Menten equation.

4. Results and Discussion

4.1 Establishing the activity assays

The DNS assay was adapted and developed during production and purification of *Pc*Cel6A. Attempts were made to use the assay for detection of enzymatic activity in disposable cuvettes. Unfortunately, it was not easy to reproduce the results of the experiments in this size of the assay because of handling issues. We changed the composition of the DNS solution by addition of phenol and the sample size according to the use in 96 well plates. With these actions we were able to improve the reproducibility of the assay and to use the assay for activity screening of many clones.

For the DNS assay in 96 well plate format, a standard curve (Figure 4 A) for glucose with standards from 0.2 to 1.0 mg/mL was generated from each individual plate and used for determination of the amount of released sugars of the enzymatic reactions. Nevertheless, for enzyme characterization and routine measurements the NS assay was used instead of the DNS assay. The reason for changing the assay was that the DNS assay usually overestimates the amount of reducing sugars (GUSAKOV et. al., 2011). Additionally, the NS assay was already used as a routine method in the lab of María Jesús Martínez Hernández in Madrid, where *Pc*Cel6A was characterized.

For the NS assay, a standard curve (Figure 4 B) for glucose with standards from 0.01 to 0.1 mg/mL was generated and used to calculate the enzymatic activity of unknown samples.

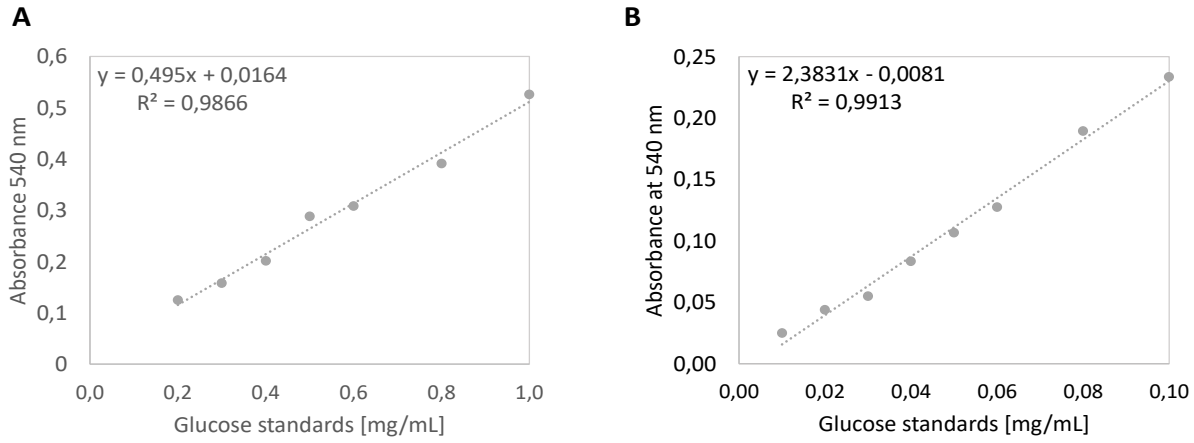

Figure 4: Glucose standard curve: (A): DNS assay (B): NS assay.

The enzyme activity [U/mL] is calculated using the following Equations 1 and 2.

$$\text{Liberated sugar } \left[\frac{\text{mg}}{\text{mL}}\right] = \frac{(\text{Blank corrected A540} - \text{d})}{\text{k}}$$

Equation 1: Amount of liberated sugar in a sample in mg/mL.

$$\text{Enzyme activity } \left[\frac{\text{U}}{\text{mL}}\right] = \frac{\text{Liberated sugar } \left[\frac{\text{mg}}{\text{mL}}\right] * \left(\frac{\text{Volume total } [\mu\text{L}]}{\text{Volume of sample dilution } [\mu\text{L}]}\right) * \text{dilution factor}}{\text{incubation time [min]}}$$

Equation 2: Enzyme activity in U/mL.

4.2 *Pc*Cel5A and *Pc*Cel6A production

The enzymes *Pc*Cel5A, an endoglucanase and *Pc*Cel6A, an exoglucanase should be produced. The genes encoding for both enzymes were ordered and synthesized by the company BioCat and provided in pPICZ A vectors for expression in *P. pastoris*. Linearization with SacI was performed for both enzymes.

4.2.1 Agarose gel electrophoresis

Agarose gel electrophoresis was used to verify the correct digest of both plasmids. The results of the electrophoresis are displayed in Figure 5 A and B. As expected, DNA bands are visible at 4443 bp for *Pc*Cel5A and at 4665 bp for *Pc*Cel6A.

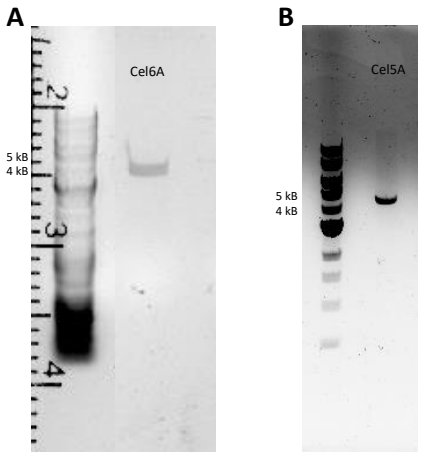

Figure 5: Result agarose gel electrophoresis: (A): *Pc*Cel6A (B): *Pc*Cel5A.

4.2.2 Electroporation of electrocompetent *P. pastoris* X-33

Electroporation of both expression vectors into *P. pastoris* cells yielded a sufficiently large number of single colonies on the YPD selection plates. Ten colonies were selected for screening in shaking flasks and transferred to new YPD plates containing zeocin.

4.2.3 Protein expression in big shaking flasks

Figure 6: Protein expression in big (1-L) shaking flasks.

The pre-culture flasks for the big shaking flask expression were directly inoculated from the new YPD selection plates. Due to problems with growing of the pre-culture flasks, only 7 clones for *Pc*Cel6A and 10 clones for *Pc*Cel5A were used for screening. The adjustment of the flasks in the shaker and the connection to the methanol feed flask via peristaltic pump is visible in Figure 6.

The success of foreign protein expression in the big shaking flask culture was observed by measurement of wet biomass, Bradford protein quantification and SDS-PAGE. A reliable activity assay was not available for verification of protein expression, at this step of production. The assay was developed, and its reproducibility was assured during protein purification. The results of determination of wet biomass and Bradford protein measurements are displayed in Figure 7 A and B. The amount of produced protein compared to the wildtype X-33 is not very prominent.

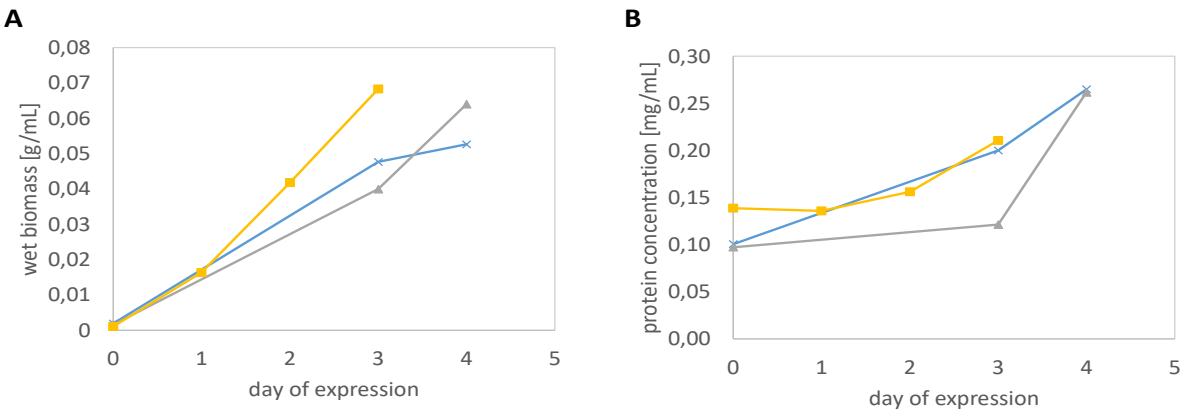

Figure 7: Shaking flask expression (A): Wet biomass determination *Pc*Cel5A and *Pc*Cel6A
(B): Bradford total protein concentration measurement *Pc*Cel5A and *Pc*Cel6A;
yellow line with squares (*Pc*Cel5A), blue line with crosses (*Pc*Cel6A), grey line with triangles (X-33).

However, the SDS-PAGE gels show that protein expression of *Pc*Cel6A was successful. The protein band visible for overexpressed protein at approximately 75 kDa in Figure 8 A, corresponds to *Pc*Cel6A. The theoretical molecular weight was calculated using the ExPASy tool (http://web.expasy.org/compute_pi) and gave 46 kDa. The difference between the calculated molecular mass and the visible expression band is most likely due to high glycosylation of the protein. Unfortunately, protein expression of *Pc*Cel5A was not successful, as can be seen on the SDS-PAGE gel in Figure 8 B, because no band for overexpressed protein is visible, which would have been expected around 38 kDa.

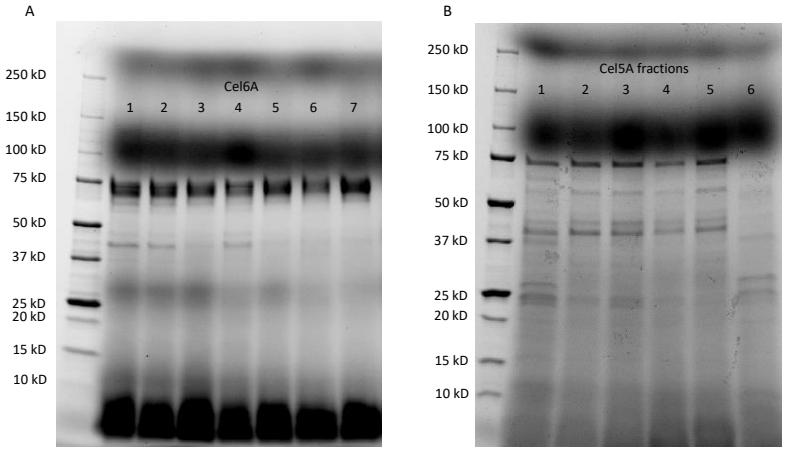

Figure 8: (A): SDS gel *Pc*Cel6A protein expression at day 4, (B): SDS gel *Pc*Cel5A protein expression at day 6.

Due to the fact, that protein expression of *Pc*Cel5A was not successful, the screening procedure was continued. The vector pPICZ A containing the gene for *Pc*Cel5A expression was digested again using SacI-HF. Electroporation was repeated using the previously mentioned settings and *P. pastoris* cells were grown on YPD selection plates again. The cells grew well and to verify the correct insertion of plasmids into the *P. pastoris* cells, colony PCR was performed. The colony PCR was done using gene specific primers resulting in a 300-base-pairs long fragment of the gene. As positive control, the plasmid containing the gene was used, and as negative control non-transformed X-33 cells were included.

The agarose gel, visible in Figure 9, shows that the gene was inserted into the host cells, compared to no insert in the wildtype X-33 cells.

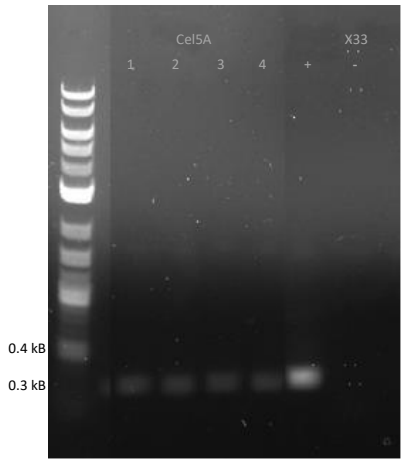

Figure 9: Agarose gel *Pc*Cel5A colony PCR.

After verification that the gene of interest was inserted into the host cells successfully, screening was continued in 96 well plates. This format of colony screening was chosen to be able to screen a larger number of colonies at once. The visual result of the assay can be seen in Figure 10 whereas the achieved absorption data from the plate reader is displayed in Table 9.

Glucose standards from 0.1 to 1.0 mg/mL and a blank containing just the buffer, as well as dilutions of a control (cellulase from *Trichoderma reesei*, Sigma-Aldrich) and negative controls, where the sample was added after the DNS solution was incubated, were included in the plate. The yellow marked values are blanks (A1, A7), the blue marked values are glucose standards (B1, C1, D1, E1, F1, G1, H1, H2, H3), the green marked values are positive controls (A2, A3, A4, A5, A6), the orange marked values are negative controls (H4, H5) and the samples of the expression clones with the highest absorption values are marked in red (B3, B7).

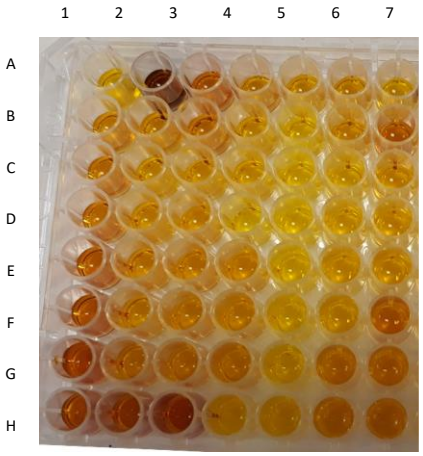

Figure 10: Result 96 well DNS assay *Pc*Cel5A, with highest absorption (dark orange) in sample wells B3 and B7.

Table 9: Result 96 well DNS assay *Pc*Cel5A: yellow = blank, blue = standard, green = positive control, orange = negative control, red = samples with highest absorption.

	1	2	3	4	5	6	7
A	0.000	1.722	0.285	0.097	0.049	0.080	0.014
B	0.117	0.160	0.252	0.063	-0.047	0.157	0.269
C	0.161	0.063	0.088	0.047	-0.049	0.026	0.043
D	0.174	0.094	0.121	-0.114	-0.050	0.056	0.033
E	0.272	0.128	0.166	0.139	-0.042	0.077	0.033
F	0.313	0.096	0.141	0.102	-0.046	0.044	0.187
G	0.466	0.173	0.164	0.136	-0.033	0.186	0.118
H	0.390	0.321	0.493	-0.007	0.006		

Due to the successful result of the DNS assay, the two transformants with the highest absorption values in wells B3 and B7 were chosen, to be used for 1-L shaking flask expression. Unfortunately, foreign protein expression of the desired protein *Pc*Cel5A of the promising clones B3 and B7 in big shaking flasks was not successful.

4.3 *Pc*Cel6A purification

4.3.1 Purification scheme of *Pc*Cel6A

Table 10: Purification scheme of *Pc*Cel6A.

Detection method		Volume [mL]	Protein concentration [mg/mL]	Total protein [mg]
Bradford	supernatant	1600	0.4	640
Bradford	HIC PheSepharose	500	n.d.	n.d.
A280	HIC PheSource	110	0.75	82.5
A280 1st pool	AEX, Source Q15	300	0.09	27.1
A280 2nd pool	AEX, Source Q15	48	0.79	37.9

Table 10 summarizes the purification steps that were conducted to obtain concentrated and purified *Pc*Cel6A protein. The results of the consecutive steps will be explained in the following sections.

4.3.2 Hydrophobic interaction chromatography (HIC)

The supernatants of all expression flasks were pooled and filtrated over cellulose filters. After salting in with ammonium sulfate to 25% saturation a hydrophobic interaction chromatography was performed using a Phenyl Sepharose fast flow column. Two protein peaks eluted at about 1% $(NH_4)_2SO_3$ as visible on the chromatogram in Figure 11.

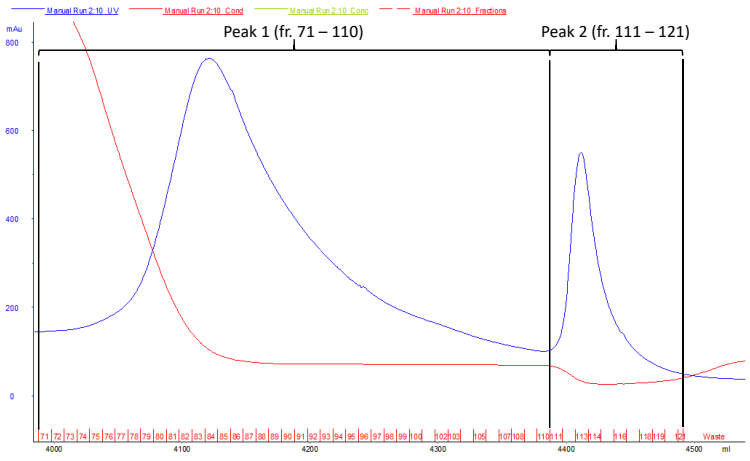

Figure 11: Chromatogram *Pc*Cel6A 1st HIC purification, peak 1 (fractions 71 – 110), peak 2 (fractions 111 – 121).

An SDS-PAGE was made after the HIC as well, to verify in which peak the protein was eluted. As clearly visible in Figure 12 A, the protein of interest was eluted in both peaks and the fractions of the second peak (113, 115) contain less impurities than the fractions of the first peak (80 – 91). Compared to the samples taken directly from the flasks 2 and 8 before ammonium sulfate was added and the HIC was run, it can be said that the HIC was successful because the protein received in the fractions was in a state of higher purity.

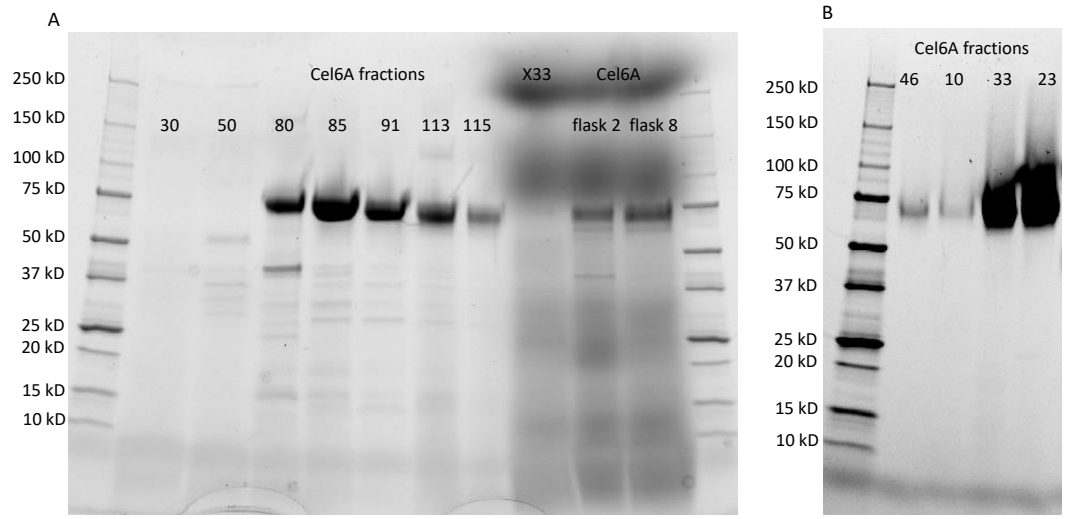

Figure 12: SDS gel *Pc*Cel6A (A): after 1st HIC (B): after 2nd HIC purification.

Nevertheless, the fractions of both peaks were pooled, concentrated and adjusted to 10% ammonium sulfate saturation. As second purification step another HIC was performed using a Phenyl-Source column. Again, the protein eluted in two peaks, as the chromatogram in Figure 13 shows.

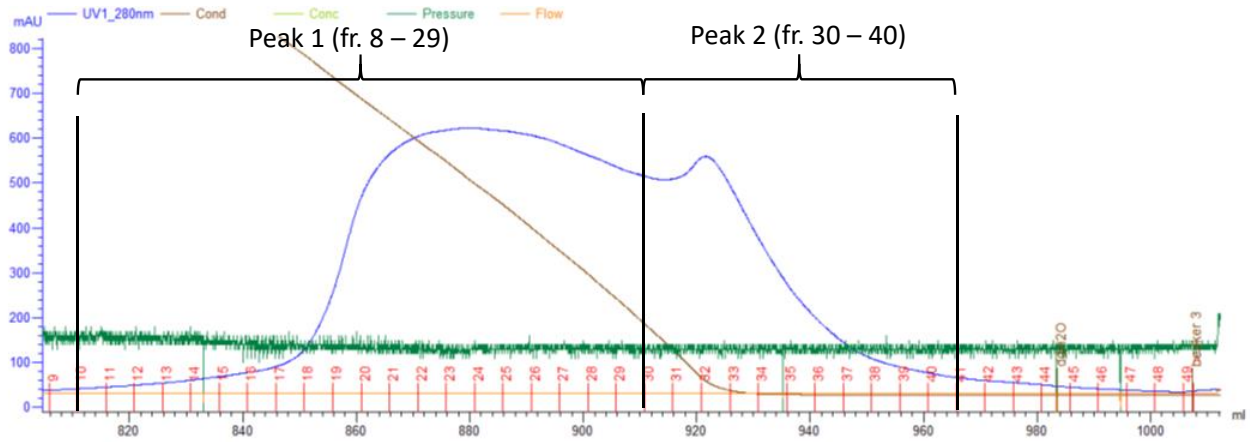

Figure 13: Chromatogram *Pc*Cel6A 2nd HIC purification, peak 1 (fractions 8 – 29), peak 2 (30 – 40).

The SDS-PAGE gel after the second HIC (Figure 12 B) revealed that the protein size of the proteins of both peaks is very similar.

4.3.3 MS analysis *Pc*Cel6A

The fractions of the two peaks were pooled separately and samples were evaluated using MS analysis (data not shown). The target protein *Pc*Cel6A was identified in different glycoforms. The first, larger peak contained more glycosylated proteins (64% not glycosylated), while the smaller peak contained protein with less glycosylation (83% not glycosylated). The MS analysis was commissioned by Lena Wohlschlager, who provided the results for this thesis.

According to measurements of the total protein concentration on a Nanodrop photometer, the highest level is reached in fraction 23. The fractions 8 – 29 (1st peak) and fractions 30 – 40 (2nd peak) were pooled and the fractions of the first peak were used for further purification steps whereas the fractions of the second peak were concentrated and stored at -80°C.

4.3.4 Anion Exchange chromatography (AEX)

The next purification step was dialysis of the pooled samples against 20 mM Tris-HCl buffer (pH 8.0) using a Vivaflow 50 crossflow module with a cut-off of 30 kDa. After dialysis an anion exchange chromatography was performed on a MonoQ Source 15 column. Unfortunately, the protein *Pc*Cel6A did not bind to the column properly. One fraction of the protein already eluted with the loading buffer, which can be seen on the chromatogram of Figure 14. To prevent further smearing and dilution of the sample, the gradient was started to elute the enzyme from the column. Analysis with an SDS gel (Figure 15) showed that both peaks contained the target protein, the first one in higher purity.

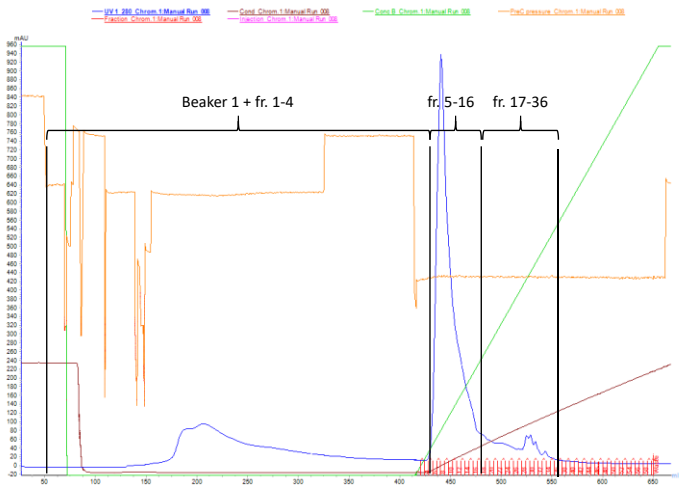

Figure 14: Chromatogram of *Pc*Cel6A on MonoQ Source 15.

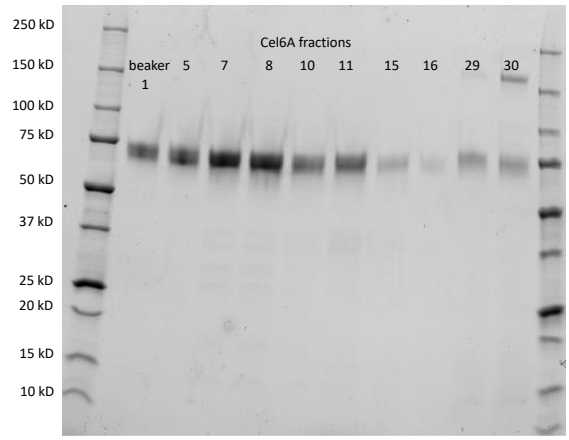

Figure 15: SDS-PAGE gel *Pc*Cel6A after MonoQ Source 15.

The fractions of both peaks were pooled separately, dialyzed against 50 mM sodium acetate buffer (pH 5.5) and concentrated using a Vivaflow 50 crossflow module. The total amount of protein obtained in both fractions is 65 mg.

4.4 *Pc*Cel6A characterization

The enzyme stock concentration of *Pc*Cel6A was 11 mg/mL, before dilution, for all performed assays and kinetic measurements.

4.4.1 Effects of pH and temperature on the activity of *Pc*Cel6A

The enzyme activity of purified *Pc*Cel6A was assayed by the standard NS assay with 3% (w/w) CMC in 55 mM sodium acetate buffer but with incubation at different pH values (pH 2 – 10) by addition of Britton-Robinson buffer or at various temperatures (30 – 70°C). Effects of the different assay conditions can be seen in Figure 16 A and B. The measurements were conducted using triplicate measurements.

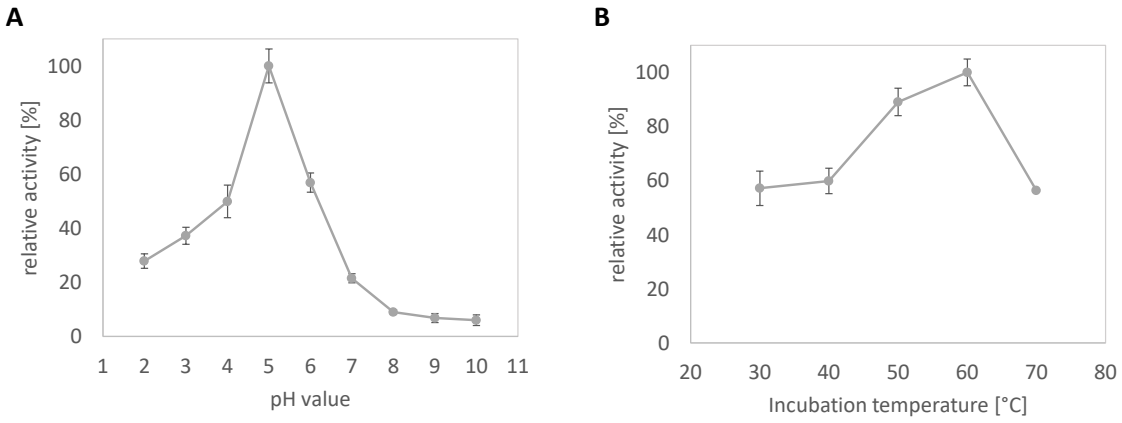

Figure 16: Effects of pH and temperature on the activity of *Pc*Cel6A (A): Activity profile over a range of pH values (B): Activity profile over a range of temperatures.

The optimum pH for *Pc*Cel6A with 3% (w/w) CMC as a substrate, was pH 5.0, with only 49.9% and 56.9% of the maximum activity appearing at pH 4.0 and pH 6.0, respectively. At pH values higher than pH 6.0, the enzymatic activity sharply decreased to less than 20% of maximum activity. This result corresponds well with measurements of BAUER et. al., (2006) and SONG et. al., (2010). Both investigations show pH optima around pH 5.0 to 5.5 for cellobiohydrolases belonging to GH class 6. Also, MANAVALAN et. al., (2015) states the optimum catalytic efficiency of most cellobiohydrolases from basidiomycetes in a narrow pH range between pH 4.0 and 5.0.

The optimum temperature for *Pc*Cel6A with 3% (w/w) CMC as substrate and sodium acetate buffer (55 mM, pH 5.0) was 60°C. *Pc*Cel6A also retained almost 90% of its activity when incubated at 50°C for 30 minutes. A similar behavior can be observed in the investigations of BAUER et. al., (2006). Whereas the measurements of SONG et. al., (2010) show 70°C as temperature optimum for *Trichoderma viride* CBH2 produced in *Saccharomyces cerevisiae*.

4.4.2 Thermostability of *Pc*Cel6A – T_{50} value

The T_{50} value is defined in this work as, the temperature, at which the enzyme lost 50% of its initial activity after two-hour pre-incubation without substrate.

This value gives information about the temperature stability and conformational stability of an enzyme. The initial activity of the enzyme was measured of a control sample, which was not pre-incubated but stored in 1:50 dilution at 4°C in the fridge. The enzyme samples were pre-incubated in 1:50 dilution with ddH_2O at different temperatures ranging from 39.9 to 60.1°C for 2 hours in a PCR machine. After this pre-incubation step, the samples were further diluted to a 1:100 dilution and a regular NS assay was performed. Obtained data from this experiment is presented in Figure 17. The corresponding T_{50} value for *Pc*Cel6A was calculated using the sigmoidal curve fitting of SigmaPlot 14.0 (Systat Software, Chicago, USA). *Pc*Cel6A shows 50% of its initial activity after 2-hour pre-incubation at 53.1°C.

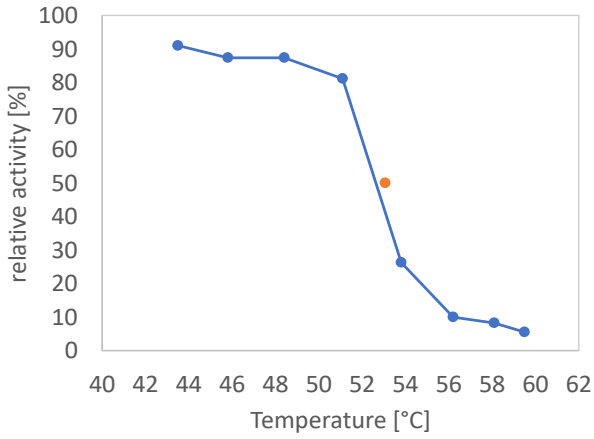

Figure 17: T_{50} value determination of *Pc*Cel6A.

The difference of the T_{50} value at 53.1°C to the optimal assay temperature at 60°C can be explained through the shorter incubation time of only 30 minutes during the assay and with the fact that enzymes are usually more stable and heat tolerant when they are incubated with their substrates rather than in dilutions with water, according to KLYOSOV and GERASIMAS (1979).

The obtained data are hard to compare with the findings of other groups, because all other published results for the T_{50} of GH class 6 enzymes were obtained in a different setting of reaction conditions (e.g. 10 minutes pre-incubation). Still if the results are compared to the findings of HEINZELMAN et. al., (2009), who evaluated T_{50} values between 51.1 and 75°C for CBH2 chimeras of *Hypocrea jecorina* and *Hypocrea insolens*, they are in a comparable range to our findings.

4.4.3 pH stability of *Pc*Cel6A

Purified *Pc*Cel6A was pre-incubated at different pH values (pH 2 – 9) in 1:50 dilution using Britton-Robinson buffers with corresponding pH values for the dilutions. This mixture was incubated without substrate for 1 hour, 24 or 48 hours in the fridge at 4°C. After pre-incubation the samples were diluted to a 1:100 dilution with ddH_2O without further adjustment of the pH value. For activity determination a regular NS assay with 3% (w/w) CMC in sodium acetate buffer (55 mM, pH 5.0) was performed. Figure 18 shows the obtained values for the activity of *Pc*Cel6A after pre-treatment. It can be seen that the enzyme remains activities higher than 70% over the whole tested pH range from pH 2 – 9. The large deviations in the activities are due to the difficult reproducibility of the NS assay under these conditions. The samples were measured in duplicates.

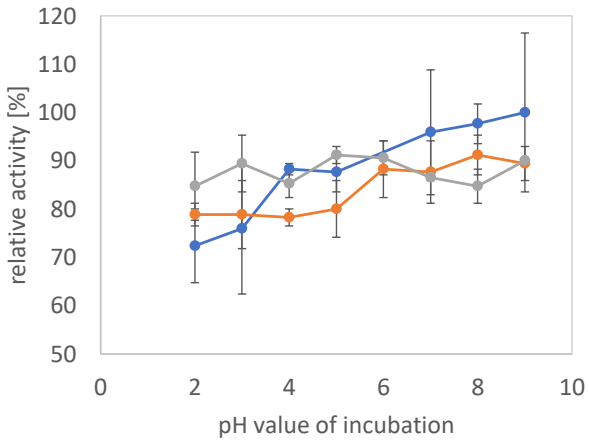

Figure 18: Results of the pH stability determination of *Pc*Cel6A, the blue line represents 1 hour, the red line 24 hours and the gray line 48 hours pre-incubation time at the pH value indicated.

4.5 Effects of different substrates on the activity of *Pc*Cel6A

The hydrolytic activity of *Pc*Cel6A was assayed on different polysaccharides, cello-oligosaccharides and the synthetic substrate *p*NPC. For polysaccharides, the amount of produced reducing sugars was measured using the NS assay, after incubation with the corresponding substrate for 30 minutes or 24 hours. For cello-oligosaccharides and CMC the reaction products were developed by thin layer chromatography (TLC) on silica gel plates (POLYGRAM SIL G/UV_{254} 0.2 mm silica gel 60 with fluorescent indicator, Merck). To analyze the reaction products produced by cellopentaose (C5) after 24-hour incubation, gas chromatographic analysis was performed. For the activity determination over *p*NPC as substrate, the previously described *p*NP assay was used.

The following substrates were tested for *Pc*Cel6A:

- α-cellulose
- Powdered cellulose
- Avicel
- Cotton pads
- PASC (phosphoric-acid swollen cellulose)
- CMC (carboxymethylcellulose)
- Laminarin
- Lichenan
- Cello-oligosaccharides (C3, C4, C5)
- *p*NPC (*p*-nitrophenyl cellobioside)

4.5.1 Effects of polysaccharides on the hydrolytic activity of *Pc*Cel6A

All polysaccharides were solved in 55 mM sodium acetate buffer pH 5.0.

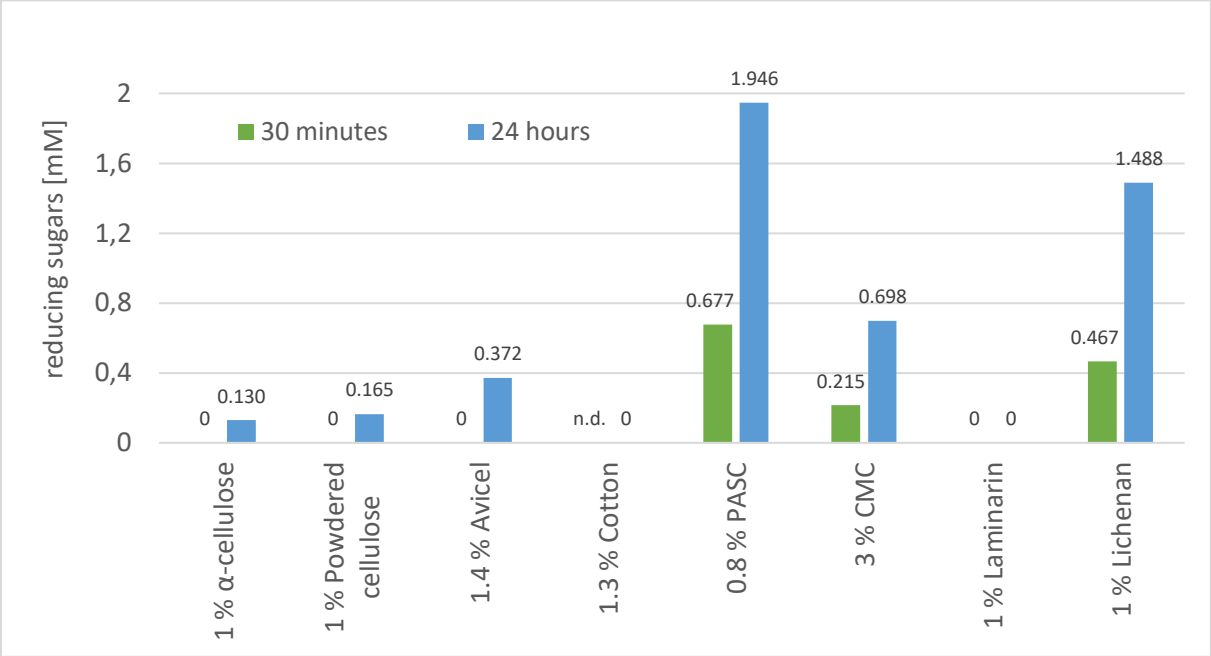

Figure 19: Amount of produced reducing sugars in mM after 30 minutes (green bars) and 24 hours of incubation (blue bars).

Figure 19 shows that after 30-minute incubation and activity measurement with the NS assay, *Pc*Cel6A only showed activity with PASC, lichenan and CMC. The other substrates tested, were not hydrolyzed after 30 minutes incubation. (Activity on cotton was not determined after 30 minutes of incubation).

After 24-hour incubation and activity measurement with the NS assay, *Pc*Cel6A showed highest activities with PASC (1.946 mM), lichenan (1.488 mM) and CMC (0.698 mM). Low activity was measured with α-cellulose (0.130 mM), powdered cellulose (0.165 mM) and 0.372 mM with Avicel. The other substrates laminarin and cotton were not hydrolyzed by *Pc*Cel6A.

Due to the different substrate concentrations used, only the amount of released sugars is comparable. This result suggests that *Pc*Cel6A is able to hydrolyze various cellulosic model substrates including CMC, PASC and Avicel, which represent different cellulose structures and degrees of crystallinity. A similar behavior relating the substrates, that can be hydrolyzed by cellobiohydrolases can be observed in the results of BAUER et. al., (2006) where the CBH of *Aspergillus nidulans* was tested and exhibited activity on CMC, lichenan and Avicel (PASC was not tested in this investigation). The results obtained in this work show that *Pc*Cel6A works better on amorphous cellulose (like Lichenan and PASC) than on more crystalline structures

(like Avicel and α-cellulose). These findings correspond well with the results of SUN et. al., (2016), that amorphous cellulose can be hydrolyzed more easily by enzymes than cellulose with high crystallinity.

4.5.2 Substrate specificity of cello-oligosaccharides as substrate for *Pc*Cel6A

The reaction products of cello-oligosaccharides (C3, C4 and C5) and CMC after 24-hour incubation with *Pc*Cel6A were developed by thin layer chromatography (TLC) on silica plates. The results of the developed plates are visible in Figure 20 A-C.

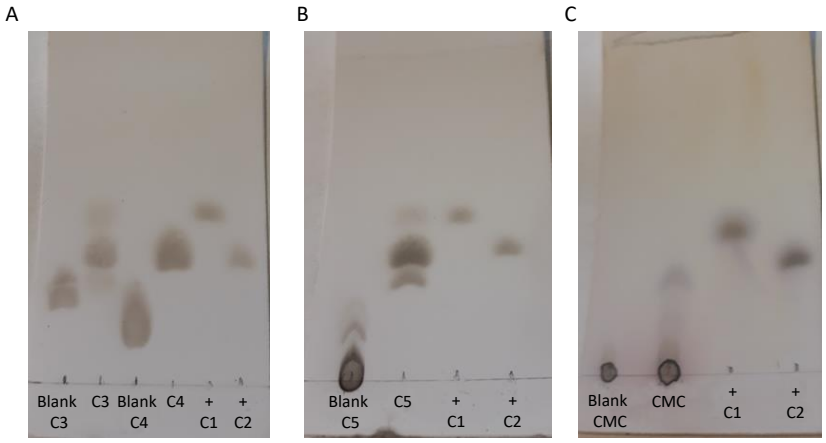

Figure 20: PcCel6A TLC analysis of (A): C3 and C4, (B): C5 (C): CMC.

The results are as expected, cellotriose (C3) is cut into cellobiose (C2) and glucose (C1) units; cellotetraose (C4) is cut into cellobiose (C2) units only and cellopentaose (C5) is cut into cellotetraose (C3), cellobiose (C2) and glucose (C1) units. The main hydrolysis product of CMC is cellobiose (C2) but also some larger bands are visible on the TLC plate, which shows that *Pc*Cel6A cuts not only cellobiose units but longer units as well. The reaction products of cellopentaose hydrolyzation were also investigated by GC analysis (Data not shown). The obtained results verify the results of the TLC plates. Interestingly more C3 units than C2 units could be measured, which is quite surprising because one would suppose that 1 mol C5 is split into 1 mol C3 and 1 mol C2 and the produced 1 mol C3 is split into 1 mol C2 and 1 mol C1.

Unfortunately, it was not possible to detect the larger bands than C3 with gas chromatography. Nevertheless, these results show that *Pc*Cel6A is not only active on different polysaccharides but as well on cello-oligosaccharides which corresponds well to the findings of BAUER et. al., (2006).

4.5.3 Hydrolysis of the synthetic substrate *p*NPC

Usually the *p*NPC method is used for selective determination of Cel7A activity. Nevertheless, we tested this substrate for *Pc*Cel6A as well. No activity was detected on this substrate, even after 24-hour incubation. This result corresponds well with the findings of BALDRIAN and VALÁSKOVÁ, (2008), that only enzymes which are acting from the reducing ends are able to liberate cellobiose from *p*NPC. It can be confirmed hereby that *Pc*Cel6A is a cellobiohydrolase that acts only from the non-reducing ends of cellulose.

4.6 Effect of cellobiose concentration on enzyme activity

Cellobiohydrolases mainly release cellobiose from polysaccharides and cello-oligosaccharides. As competitive inhibition by end products is a significant issue for many hydrolytic enzymes, it was to be tested if high cellobiose concentrations decrease the hydrolytic activity of *Pc*Cel6A. Because of the fact, that added cellobiose would also be detected by the NS assay as reducing sugar, it was not possible to use this detection method. A ready-to-use glucose detection kit was used instead. 15 mM cellotriose was used as substrate and cellobiose concentrations from 0 mM to 133 mM were tested. Higher cellobiose concentrations could not be tested because of solubility limitations.

The presented results in Figure 21 show that *Pc*Cel6A was inhibited by the presence of high concentrations of cellobiose. Amounts up to 10 mM cellobiose increased the hydrolytic activity of *Pc*Cel6A slightly. TAKAHASHI et. al., (2010), supposes that it might be a common feature of cellobiohydrolases of GH family 6 that high concentrations of cellobiose increase the hydrolytic activity. This assumption is not true for *Pc*Cel6A. Concentrations higher than 10 mM cellobiose reduce the specific hydrolytic activity of *Pc*Cel6A. At cellobiose concentrations higher than 112 mM, the specific activity is already lower than 70% of the initial enzyme activity.

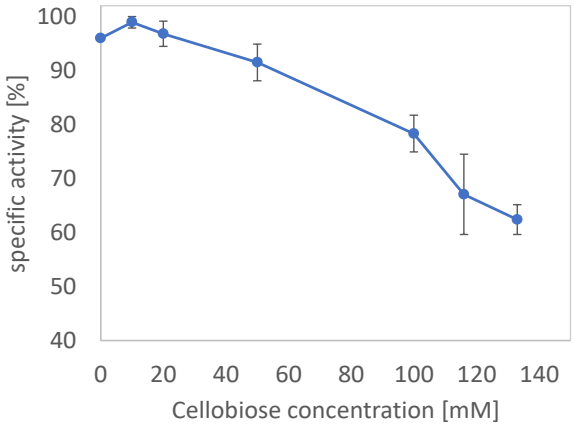

Figure 21: Test of cellobiose inhibition on hydrolytic activity of *Pc*Cel6A on 3% (w/w) CMC.

4.7 Interaction of *Pc*Cel6A with *Nc*LPMO9C

As *Nc*LPMO9C creates new chain ends, we suspected that activity of *Pc*Cel6A should be increased after the substrate was pre-incubated with the LPMO. The results are shown in Figure 22. Unfortunately, the results do not show an increase in the activity after a pre-incubation with LPMO. The samples show very similar absorption values after 15 or 60 minutes pre-incubation with *Nc*LPMO9C. This diversion from the expected results could be caused by the use of selectively this LPMO. *Nc*LPMO9C oxidizes the C4 ends and therefore produces oxidized non-reducing cellulose chain ends, due to the fact, that *Pc*Cel6A starts its work on the non-reducing ends, this might be the reason for not finding the suspected synergies between the tested enzymes. It could be possible that *Pc*Cel6A is not able to work from oxidized non-reducing chain ends of cellulose chains. This assumption shows that the interaction between Cel6A enzymes and LPMOs is still not very clear and needs more investigation.

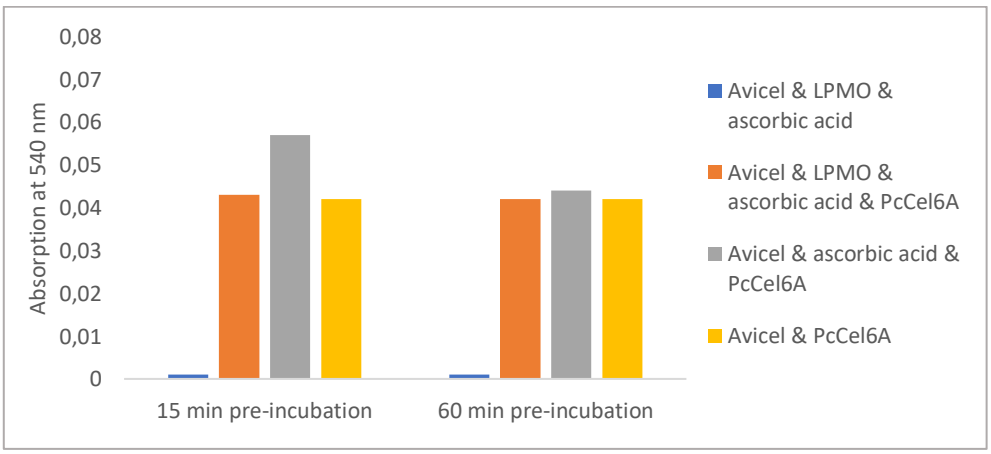

Figure 22: Absorption values at 540 nm after pre-incubation of 1.4% (w/w) Avicel with *Nc*LPMO9C for 15 (left) or 60 minutes (right) at 50°C and subsequent incubation with *Pc*Cel6A and NS assay.

4.8 Binding kinetics of *Pc*Cel6A

The turnover number of *Pc*Cel6A was determined for the substrates PASC, lichenan and CMC with different concentrations ranging from 0.025 to 8% (w/w) in sodium acetate buffer (55 mM, pH 5.0). The data obtained for the different substrates by NS assay measurements, are presented graphically in substrate vs. velocity plots, in Figure 23.

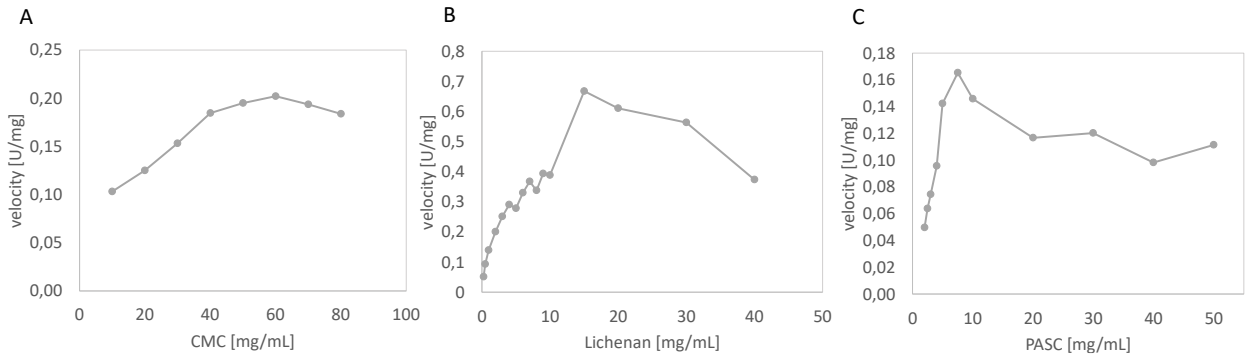

Figure 23: Substrate vs. velocity plots (A): CMC (B): Lichenan (C): PASC.

The catalytic constants were calculated with a nonlinear least squares regression model based on the Michaelis-Menten equation using SigmaPlot 14.0 (Systat Software, Chicago, USA).

The calculated turnover number numbers were as followed, for CMC 0.240 U/mg, for lichenan 0.483 U/mg and for PASC 0.605 U/mg. This shows the highest catalytic efficiency for PASC as substrate for *Pc*Cel6A. The blank values obtained with the NS assay for the different substrates (Table 11) are not linked to the calculated turnover numbers.

Table 11: Absorption values for the blank measurements of different substrates in the NS assay.

Substrate	Concentration	Absorption 540 nm
PASC	50 mg/mL	0.010
CMC	80 mg/mL	0.046
Lichenan	40 mg/mL	0.476

Inhibition of hydrolytic activity of *Pc*Cel6A was not found during the kinetic tests because the amount of formed cellobiose was too low. As already described, only cellobiose concentrations higher than 10 mM inhibit enzymatic activity of *Pc*Cel6A. The maximum amount of cellobiose that could be produced during the binding kinetic experiments, was for CMC 0.27 mM cellobiose, for lichenan 0.88 mM cellobiose and for PASC 0.21 mM cellobiose.

The amount of cellobiose that was formed, was calculated using the glucose calibration curve for the NS assay. As the NS assay measures the reducing ends in the sample and glucose has one reducing end and cellobiose has one reducing end and one non-reducing end, the absorption values for different cellobiose or glucose concentrations are comparable.

5. Summary, Conclusions and Outlook

During the course of this work, two enzymes, *Pc*Cel5A and *Pc*Cel6A should be produced. Only the production of *Pc*Cel6A was successful and yielded in enough product for enzymatic characterization. It might be possible to produce *Pc*Cel5A with the use of another vector like pGAPZ A instead of pPICZ A which incorporates a different promotor system, which could be one possible reason for the failure of production of this enzyme in this work. Another possible suggestion for future research on this enzyme would be the change of the signal peptide, from the native one to the alpha signal factor from *Saccharomyces cerevisiae*.

As the production of *Pc*Cel6A was successful the enzyme was purified and subsequently characterized. The optimum pH was determined at pH 5.0 and the optimum temperature at 60°C. The T_{50} value was identified at 53.1°C and the enzyme was stable over the whole tested pH rage (pH 2 – 9) for 48 hours. The effects of the use of different substrates on the hydrolytic activity of *Pc*Cel6A were evaluated. *Pc*Cel6A was able to hydrolyze various cellulosic model substrates including CMC, PASC and Avicel, which represent different cellulose structures and degrees of crystallinity. Additionally, cello-oligosaccharides (C3 – C5) could be hydrolyzed by *Pc*Cel6A. The artificial substrate *p*NPC could not be hydrolyzed by *Pc*Cel6A.

Due to the results on the TLC plate on which CMC was tested, it might be possible that *Pc*Cel6A produces not only cellobiose units but also longer reaction products. This possibility needs to be investigated further to evaluate the correct conclusion to this finding. Cellobiose concentrations of more than 10 mM are inhibiting the hydrolytic activity of *Pc*Cel6A.

A synergy between *Pc*Cel6A and *Nc*LPMO9C could not be confirmed. As already mentioned, it might be possible that *Pc*Cel6A can only start its work on a non-oxidized non-reducing end of a cellulose chain which would lead to the results we found. For future research in this field it will be interesting to investigate the possible synergy with *Nc*LPMO9F which oxidizes the C1 end, while cutting cellulose chains and should hereby produce new non-reducing chain ends that could be used by *Pc*Cel6A, because these ends would not be oxidized in this case.

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7. Appendix

7.1 Abbreviations

AOX ..... alcohol oxidase

BMMY ..... buffered methanol complex medium

BYPD ..... buffered yeast extract peptone dextrose medium

CAZy ..... Carbohydrate Active EnzYmes

CBH ..... cellobiohydrolase

CDH ..... cellobiose dehydrogenase

CMC ..... carboxymethylcellulose

DNS ..... 3,5-dinitrosalicylic acid

DTT ..... dithiothreitol

EG ..... endoglucanase

HIC ..... hydrophobic interaction chromatography

LB ..... lysogeny broth

LPMO ..... lytic polysaccharide monooxygenase

NaAc ..... sodium acetate

NaCl ..... sodium chloride

NS ..... Nelson Somogyi

PASC ..... phosphoric-acid swollen cellulose

*p*NPC ..... *p*-nitrophenyl cellobioside

SDS ..... sodium dodecyl sulfate

TLC ..... thin layer chromatography

YNB ..... yeast nitrogen base

YPD ..... yeast extract peptone dextrose medium

7.2 List of Figures

7.3 List of Tables

7.4 List of equations

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