University of Natural Resources and Life Sciences

Department for Agrobiotechnology, IFA-Tulln

Institute of Environmental Biotechnology

Master Thesis

Morphological and molecular characterization of the chitosan producing fungi *Absidia glauca*

Author: **Lisa Berndorfer, B.Sc.**

Master Program Biotechnology

Supervisor: **Univ.Prof. Dipl.-Ing. Dr.techn. Georg M. Gübitz**

Institute for Environmental Biotechnology

Co-Supervisor: Mag. Dr. Doris Ribitsch

Dipl.-Ing. Dr.nat.techn. Günther Bochmann

Institute for Environmental Biotechnology

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ABSTRACT (ENGLISH)

Enzymes are powerful biocatalysts acting and enabling processes, in the blue, green, red or white sector of biotechnology. Lower process time and costs, low energy input, and nontoxic or eco-friendly characteristics are features indispensable for implementing sustainable approaches. Identification of new enzymes concerning basic catalytic mechanism, structure-function relationships, or also substrate specificity is of considerable relevance these days. Novel enzymes have demonstrated paths regarding the omnipresent and widely debated climate crisis in concern to e.g. plastic and CO_2 degradation or biofuel formation in combination with recovery of the industrial waste. Consequently, characterization of yet unknown microorganisms in the immense microbial world for enriching acknowledge about their behavior and about the repertoire of known enzymes is of big relevance for the future.

In this work the filamentous soil fungus *Absidia glauca* was used for the development of a submerged fermentation technique with the purpose of identification and characterization of extracellular enzymes. Protein expression was induced with a medium containing ammonium sulfate, dipotassium phosphate, magnesium sulfate heptahydrate, calcium chloride dihydrate and sodium chloride. In combination with this medium, a chitosan yield of 1.99 ± 0.01 % with an acetylation degree of 58.94 ± 7.11 % was achieved. In order to obtain a closed process with the utilization of both, biomass and supernatant, the secreted enzymes were visualized with SDS chromatography, promising bands were cut out and a sequencing procedure was carried out. A protein belonging to the glycoside hydrolase family 16 (GH-16) was identified and small scale fermentations in baffled flasks were performed with the reason of identifying substrate specificity of the glycoside hydrolase. Latest experiments aiming the purification of the 35 kDa GH-16 protein were carried out using ion-exchange chromatography.

ABSTRACT (GERMAN)

Enzyme sind leistungsfähige Biokatalysatoren, unabhängig ob es sich um deren Einsatz in Prozessen der blauen, grünen, roten oder weißen Biotechnologie handelt. Geringere Prozesszeiten und -kosten, geringer Energieeinsatz sowie ungiftige oder umweltfreundliche Eigenschaften sind unverzichtbare Merkmale für die Umsetzung nachhaltiger Ansätze. Die Identifizierung neuer Enzyme hinsichtlich grundlegender katalytischer Mechanismen, Struktur-Funktionsbeziehungen oder auch Substratspezifität ist heute von großer Bedeutung. Neuartige Enzyme können Lösungswege in Bezug auf die allgegenwärtige und viel diskutierte Klimakrise zeigen, z.B. in Bezug auf den Abbau von Kunststoffen und CO_2 oder die Erzeugung von Biokraftstoffen in Kombination mit der Verwertung von Industrieabfällen. Folglich ist die Charakterisierung von noch unbekannten Mikroorganismen zur Erweiterung der Kenntnisse über ihr Verhalten und zur Erweiterung der Gesamtheit an bekannten Enzymen von großer Bedeutung für die Zukunft.

In dieser Arbeit wurde der filamentöse Bodenpilz *Absidia glauca,* für die Entwicklung einer Submersfermentation mit dem Ziel der Identifizierung und Charakterisierung von sekretierten Enzymen im Überstand, verwendet. Die Proteinexpression wurde mit einem Medium induziert, welches Ammoniumsulfat, Dikaliumphosphat, Magnesiumsulfat-Heptahydrat, Calciumchlorid-Dihydrat und Natriumchlorid enthält. In Kombination mit diesem Medium wurde eine Ausbeute von 1.99 ± 0.01 % Chitosan mit einem Acetylierungsgrad von 58.94 ± 7.11 % erreicht. Um einen geschlossenen und nachhaltigen Prozess mit der Nutzung von Biomasse und Überstand zu erhalten, wurden die extrazellulären Enzyme mithilfe von SDS-Chromatographie visualisiert, vielversprechende Banden ausgeschnitten und ein Sequenzierungsverfahren durchgeführt. Ein Protein der Glycosidase Familie 16 (GH-16) wurde identifiziert. Letzte Experimente zur Aufreinigung des 35 kDa GH-16 Proteins wurden mit Ionenaustauschchromatographie ausgeführt.

1 INTRODUCTION

1.1 ABSIDIA GLAUCA

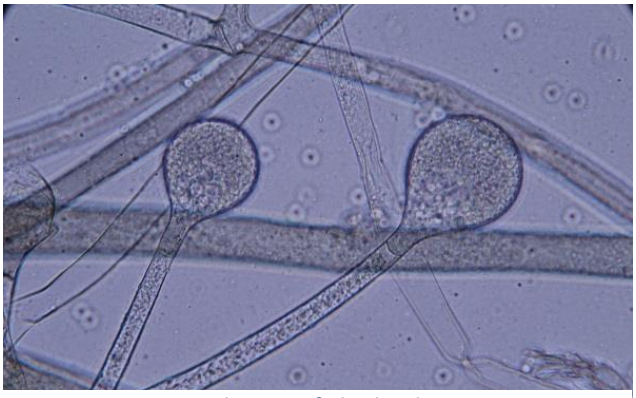

Figure 1: Microscopical image of Absidia glauca.

The filamentous fungus *Absidia glauca* belongs to the class Mucoromycetes (Zygomycetes) within the Cunninghamellaceae family. Figure 1 shows the microscopical image of the fungi and was taken with the microscope Olympus BX43 with 40 x enlargement.

Absidia spp. is a widely spread soil fungus with rapid growth which can be subdivided in three different groups: thermotolerant, mesophilic and mycoparasitic fungi, with A*bsidia glauca* belonging to the mesophilic one [1]. *Absidia spp.* exhibit clinical importance as opportunistic human pathogen. Those opportunistic infections, called "mucormycosis", can be initiated by the most important *Absidia* species *A. corymbifers* and concerns primary immunocompromised individuals [2].

Less is known about the species *A. glauca* concerning characteristics in general, metabolic pathways or the production of proteins within economically relevant research areas. One promising topic appears to be the feasible extraction of chitosan from industrial waste mycelia. Out of thirty three screened fungi, *A. glauca* seems to exhibit the best production capabilities [3].

1.2 CHITIN AND CHITOSAN

After cellulose, chitin appears to be the second most abundant polysaccharide arising in nature and point out various important utilizations in high quantities [4]. Chitin is found not only in exoskeletons of crustaceans like crabs and shrimp. It is additionally found in cell walls of fungi and insects and consists out of repeated N-acetyl-glucosamine (GlcNAc) units which are covalently β-1,4 linked. The presence of hot alkali can result in the N-acetylation of the biopolymer chitin, leading to the formation of the promising and well-known molecule chitosan (Figure 2). High

expectations are set on the chitosan production from waste fungal mycelia especially in various fields like medicine, agriculture, food and non-food industries [5]. Fungal mycelia is promising because it is a promising alternative to the production from crab and shrimp shells and would lead to environmental sustainability.

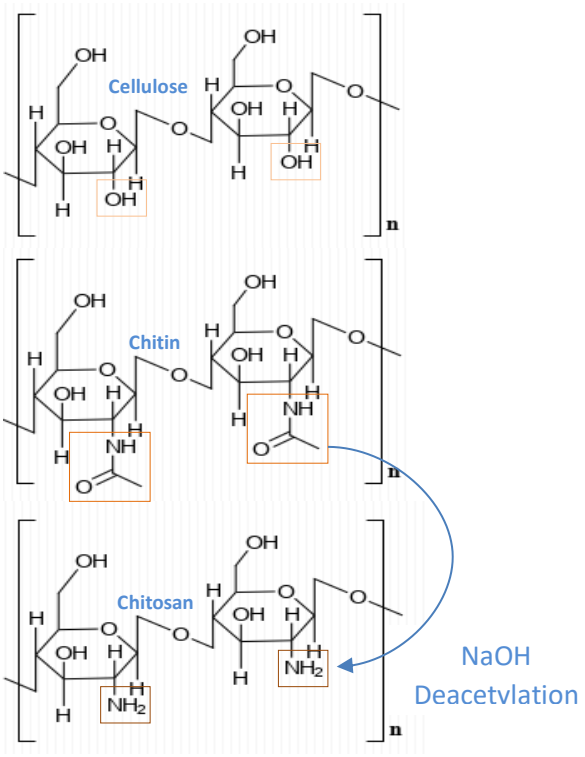

Figure 2: The structure of cellulose is displayed on the top of the figure. The generation of chitosan from the polysaccharide chitin due to the presence of hot alkali is presented below [5].

The applications in medicine and pharmacy places special interest on chitosan's property like biodegradability, biocompatibility and its low toxicity. Compared to chitin, chitosan seems to be more of concern in this field because of its solubility in water. Not only the solubility of chitosan but also the biodegradability is dependent on the acetylation grade , the pH of the environment and on its molecular weight [6]. Thankfully a shift away from the demand of chemical pesticides to natural derived compounds like chitosan is observed these days. Hence the antifungal and antibacterial property of chitosan is more and more examined and utilized to control progression of crop plant diseases and further to avoid huge production losses [7].

Concerning the examined fungus *A. glauca* and its chitosan production it must be noted that this polymer exhibits the best adsorption capacity for (Cu)II [3]. Adsorption capacities are of big interest in the field of wastewater treatment. Chitosan was already undergone several studies concerning application for treatment of polluted water in a way to adsorb heavy metal, to remove dyes (textile wastewater) or to be used as coagulation/flocculation agent [8]. These examples are just a small selection of how interesting and promising chitin and its derivative chitosan are and that big hope can be set on these molecules for the future.

1.3 SUBMERGED FERMENTATION

The research in fermentation aims to optimize the production of the highest possible amount of product within definite time and volume [9]. Maintaining optimal and homogeneous reaction conditions, minimizing microbial stress and increasing the metabolic accuracy yields in high product quality and concentration [10]. Biological conversion of substrates into complex compounds with fungi or bacteria can be obtained with different fermentation techniques. During the metabolic breakdown of the used substrates additional compounds called secondary metabolites or also “bioactive compounds” can be released from the microorganism. Secondary metabolites vary from antibiotics to growth factors, enzymes and peptides. Many microbial enzymes are expressed and secreted out of the cell into the supernatant of the fermentation broth like amylases, proteases, pectinases, lipases, xylanases, cellulases, and laccases [11]. Fermentation can be classified according to the used substrate, either liquid or solid, therefore a distinction between submerged (liquid) fermentation (SmF) and solid state fermentation (SSF) can be met. Submerged fermentation provides the fungi a liquid fermentation media with all the nutrients the organism needs for cell growth, metabolism and protein expression in contrast to solid state fermentation, where a solid support is used. Limiting factor concerning SmF can be the development of viscosity due to the fungal mycelium and therefore a hindrance in impeller action, which can lead to oxygen availability and mass transfer limitations [12].
In the scope of this work, *A. glauca* grew submerged in a complex liquid medium. The cultivation of the fungus in batch operation modus is followed by the generic well known growth curve. After inoculation with the fungal liquid preculture, the lag phase is initiated, characterized by an adaption of the microorganism to the new environment and almost no growth. After that the exponential phase begins equaling a rapid growth and development of biomass and also the decrease of pO2 (partial pressure of dissolved oxygen). This stadium merges then into the stationary phase where the energy source is depleted, growth stops and the organism enters the death or decline phase.

The process includes a heat exchanger, peristaltic pumps, air flow in and out, and the main process controller. As acidic pH is preferred for fungal growth the pH during this fermentations was set to 4 [13]. Counteraction against a deviation from the set point measured by the pH electrode is accomplished with peristaltic pumps. Also the production of foam is antagonized by the addition

of antifoam. The temperature is controlled and kept on a stable level with the return condenser. Complex control needs to be ensured to guarantee optimal and preset conditions for the organism.

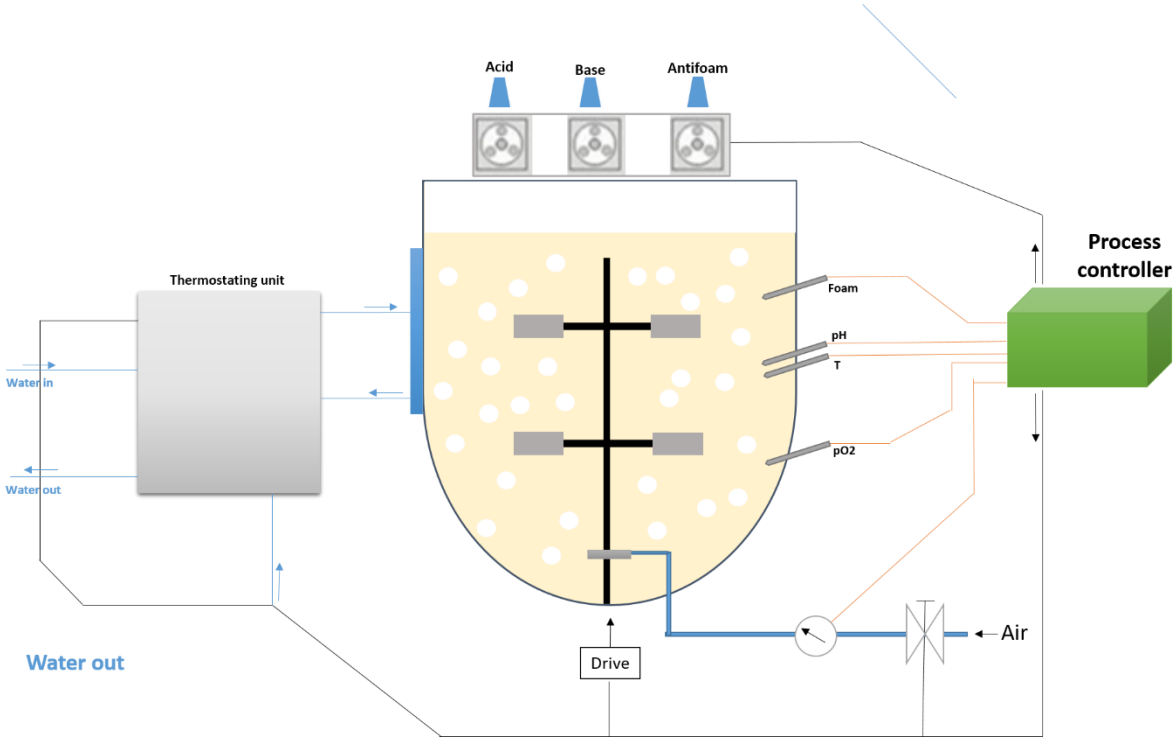

Figure 3: Batch Fermentation with process controller unit (green) and thermostating unit (grey). Acid, base and antifoam pumps are indicated on top of the main reactor. The Antifoam, pH, temperature and pO2 probes were installed before autoclaving procedure. Air flow into the reactor was filtered through 0,2 µM nylon a pump and controlled by a pump.

1.4 ENZYMES AND ITS APPLICATION IN INDUSTRY

The fast-paced world and the more and more demanding society and economy urges to come up with solutions to cope with the omnipresent depletion of natural resources, to reduce any production costs or to enhance environmental safety by the usage of enzymes. Developing strategies to recycle or reuse industry residues to circumvent those mentioned issues is already a hot contested object in research [14]. Environment and its protection are one of the main topics for future and ubiquitous in all discussions. To decrease the presence of harsh materials to make the environment friendlier or to use resources still present in waste products, enzymes are continuously more and more deployed [15].

The early use of microorganism goes back to 6000 BC, where yeasts were utilized for the commercial production of alcoholic beverages. From then on, hardly illustrating progress was accomplished, just mentioning recombinant DNA technology and interference and manipulation in the metabolism of microorganisms with protein engineering technologies [16]. Microbial enzyme-based processes are of great importance in various industrial fields like food, agriculture, chemicals and pharmaceuticals. High concentration of extracellular enzymes can be produced by various microorganism. Especially bacteria and fungi are suitable for these processes due to their high growth rate and easy handling. [17]

Enzymes, built up from amino acid chains connected via peptide bonds, are biocatalyst speeding up reactions with high specificity, activity and selectivity [18]. Ranging from microbes to humans, life without them would not be possible and they are doubtless necessary for sustainable life. Depending on the enzymatical reaction they can be classified in six different groups. Oxidoreductases transfer either hydrogen, oxygen or electron between different molecules. Transferases catalyze the transfer of atoms from molecules to another. The hydrolytic cleavage of bonds is carried out by hydrolases. In contrast to hydrolases, lyases do not use water and cleavage of bonds go along with elimination or addition reactions. Isomerases catalyze the isomerization of different molecules by intramolecular rearrangements. And the last group called ligases consume adenosine triphosphate (ATP) in order to be capable of joining molecules [19].

1.4.1 Laccases

In industrial fields like pulp and paper, textile and food one of the focus is on oxidation reactions using molecular oxygen as electron acceptor. Encouraging results are therefore obtained with the enzyme group laccases [20].

Laccases are multi-copper containing proteins and dependent on at least four copper atoms per active protein unit in order to exhibit catalytical activity. Like displayed in Figure 4 the type one copper contains two histidine, one cysteine ligands and one variable amino acid. In case of fungal laccases, mostly leucine or phenylalanine is present. The covalent copper-cysteine bond leads to the blue color of multi-copper proteins, which is due to the high electronic absorbance. Type 2 copper is coordinated close to type 3 copper and connected to two histidines. It has no specific absorption features in the visible region and is therefore named as “normal/non-blue” copper. Type 3 copper is coordinated with six histidines and the oxidized form absorbs at 330 nm [21].

Laccases can perform a radical-catalyzed reaction mechanism and have quite low substrate specificity making it very attractive to all kinds of sectors of Biotechnology due to the very broad range of substrates. The molecular weight of these enzymes can vary between different organisms from 50 to 130 kDa. In the course of this reaction molecular oxygen is reduced while substrates containing aromatic compounds like lignin are oxidized. The radicals, which are released undergo reactions which can lead to the crosslinking of monomers, degradation of polymers or to the ring cleavage of aromatics [22]. Laccases are predominately found in fungi and plants, whereas in fungi they can be responsible for lignin degrading processes, for pigment and spore formation and also, depending on the species, for plant pathogenesis [23].

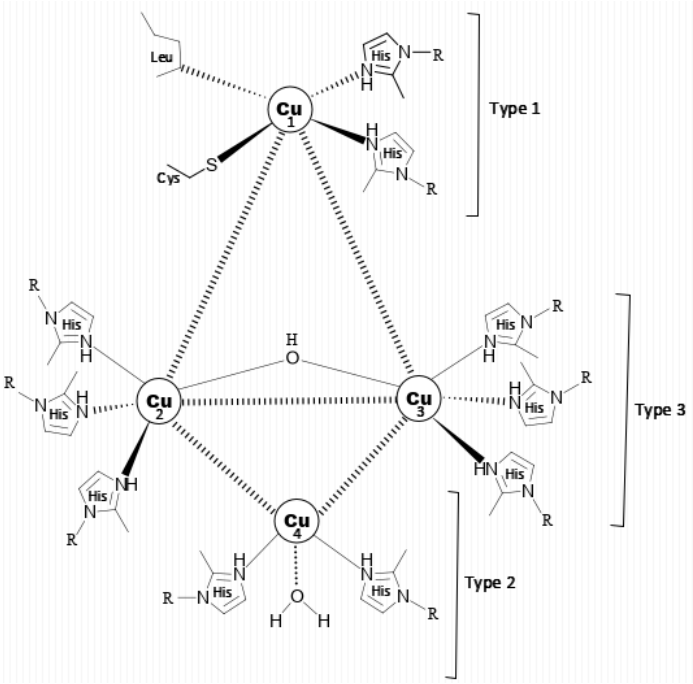

Figure 4: The four copper centres of Laccases.

1.4.2 Esterases

Another important enzyme concerning degradation of materials like cereal wastes, plastics or other toxic chemicals, are esterases. They are very similar to the enzyme group called lipases. They differ only in substrate specificity, more precise lipases degrade triglycerides of long chain fatty acids, and esterases affects general and specific type of esters. The second difference concerns their binding pocket, lipases exhibit a hydrophobic active domain while esterases have an acyl binding site [24].

Esterases have several applications in food industry, as transesterification processes like the conversion of low value fats to more valuable fats. In this field of industry not only the main food item but also the packaging material is of great importance. Pollutants like plastics, polyester, polyurethane, polyethylene, etc. can be present in the packaging materials but can be also introduced to the main item via the manufacturing process. Cholesterol esterases can be utilized for the degradation of such components [25]. Esterases also have a wide area of application in pharmaceutical industries. Studies have revealed that esterases play an important role in the synthesis of chiral drugs. Interestingly esterases produced from *Pseudomonas sp*. are involved in the production of the commercially available and well known drug Ibuprofen by the synthesis of the very pure (S)-ketoprofen [26]. Not only laccases but also esterases are of main importance in pulp and paper, textile, leather and baking industries. They play a significant role in the pitch removal during paper pulp manufacturing which is accomplished by hydrolysis of sterol esters [27]. Ignoring the pitch problem and not counteracting against it would lead to a negative impact on the production machines itself as well as on the paper quality [24].

There would still be so many promising applications for esterases and not all esterases have been yet identified or characterized. The goal to render more and more information about microorganisms, proteins and enzymes should not be blocked in any way throughout scientific research. Microorganisms should be characterized in a way to understand their metabolic pathways, to learn about their behavior to environmental changes and to study their protein or enzymes expressed. Those procedures and outcomes can lead to well defined and optimized enzyme productions in large scales in very specialized fields in all kinds of industry.

1.4.3 Cellulases

The most plenteous polysaccharide in our environment is the main component of plant cell walls, cellulose. Before continuing with the enzymes using cellulose as substrate, short description is provided about cellulose. β–1,4 glycosidic bonds connect individual glucose molecules and lead to the formation of cellulose, which is shown in the upper part of Figure 2. Compared to cellobiose, which is formed by the connection of exactly two glucose subunits, cellulose can be built up from hundred to thousand glucose subunits [28]. A wide variety of fungi and bacteria, growing on cellulosic material, can be induced to produce cellulases, which enzymes catalyze the hydrolysis of β–1,4 linked glucose units, releasing glucose, cellobiose or oligosaccharides. Those enzymes have

a broad application in different fields of industry as they can be used in pulp and paper, food, bioconversion, agriculture, fermentation, detergents and textile industry [29]. Cellulase systems are dependent on the availability and later the synergy of three different enzymes, endoglucanases, exoglucanase, and β-glucosidase. As billions of tons cellulose wastes are produced every year from e.g. agricultural procedures or industrial food processing steps, focus is on developing solutions for recycling, biodegradation or bioconversion of this abundant polymer leading to the release of sugars, which can be raw material for the production of bioethanol, organic acids, sugars and animal feed [30]. Concerning pulp and paper industry, they are used for deinking processes, reducing energy needed for mechanical pulping and improving drainage procedures [29].

Most recent topic is the increase of environmental pollution and the trend to avoid usage of fossil fuels leading to the release of toxic gases like greenhouse gases. Lignocellulosic materials are available in huge amount, cheap and easy to get. After the main three steps of physiochemical pretreatment to release cellulose and hemicellulose content, enzymatical hydrolysis of the biomass by the cellulase systems into fermentable sugars and the followed fermentation process itself, substances like ethanol can be gained [31].

1.5 GLYCOSIDE HYDROLASES

The main source of energy for microorganisms are carbohydrates. Monosaccharides like glucose, fructose or galactose are building blocks for series condensation reactions leading to the formation of large molecules called polysaccharides like starch, cellulose, chitin or glycogen. Connection of the monosaccharides is obtained by the formation of a glycosidic bond, which is the connection of an aldehyde or ketone group with a hydroxyl group from another sugar leading to the removal of water. Back reaction or hydrolysis of this linkage is performed by special enzymes called glycoside hydrolases/glycosidases [32].

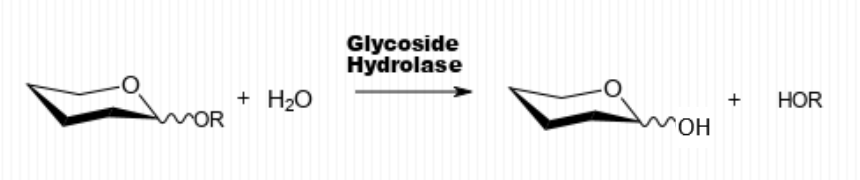

Figure 5: Retaining reaction mechanism of glycoside hydrolases.

Glycoside hydrolases can be classified in different ways. They can be either classified due to their ability to cleave substrate at the endo- or exo- site, due to the enzyme commission (EC) number, or due to their reaction mechanisms (retaining or inverting glycoside hydrolases). Latter can be performed based on sequence. Subsequently they are assigned to one of already 100 existing glycoside hydrolase families (GH family). Proteins in each different family are similar in sequence allowing some predictions in concern to catalytic machinery and molecular mechanism [33].
Glycoside hydrolases included in family 16 act on β-1,4 or β-1,3 glycosidic bonds.
Enzymes belonging to this group are:

- xyloglucosyltransferase
- keratan-sulfa te endo-1,4-β-galactosidase
- Endo-1,3-β-glucanase / laminarinase
- endo-1,3(4)-β-glucanase
- endo-β-1,3-galactanase
- chitin β-1,6-glucanosyltransferase
- β-transglycosidase
- β-glycosidase
- licheninase
- β-agarase
- xyloglucanase
- hyaluronidase
- endo-β-1,4-galactosidase β-carrageenase
- endo-β-1,3-galactanase
- β-porphyranase

1.6 DIFFERENT CARBON SOURCES FOR FERMENTATION

Polysaccharides constitute the major biomolecules that comprise biomass on this planet. Moreover, they provide energy storage and serve structural rigidity [34]. As there is still high nutrition in agricultural industrial waste products, the materials can be further used throughout different fermentation strategies as carbohydrate source in order to produce different classes and amounts of proteins. Most of the obtained waste is of lignocellulosic nature such as sugar beet pulp, paper waste pulp or brewers' spent grain. Different substrates in the fermentation media can open different metabolic pathways and therefore induce production of different enzymes [35].
Last experiments of this work were the evaluation if the fungi *A. glauca* can use waste products like sugar beet pulp, waste paper bulk, and brewers' grain for growth or if the microorganism can carry out the breakdown of saccharides like N-acetylglucosamine, xylan, carrageenan or cellobiose and use it as energy source.

1.6.1 Sugar beet pulp

After the extraction of sucrose from sugar beets the dry weight of pulp still contains about 75% of polysaccharides like cellulose or hemicellulose [36]. Therefore it may be a potential carbon source in fermentation media and would aim the complete usage of material from sucrose production.

1.6.2 Brewerys spent grain

273 breweries are present in Austria and the consumption of beer per capita per year is 105 L [37] Malt is collected in a silo and after the milling process mashing is followed. With the addition of hot water the lauter process is continued where the insoluble part, the brewers' spent grain (BSG), is then separated from the wort, which is then boiled with hops. Different kinds of yeast are added afterwards and the main fermentation process can start [38].

BSG is the major by-product of brewing industry and consists of husk-pericarp-seed coat layers which are coated with original barley grain. This lignocellulosic product mainly comprise around 17% cellulose, 28% non-cellulosic polysaccharides, and 28% lignin. But also minerals, vitamins and amino acids are present in low quantities. Due to that very attractive chemical composition and high nutritional value, BSG is proposed to be a promising substrate for the cultivation of microorganisms and would circumvent the wastage of nutritional resources [39]. Studies showed that BSG can be used as substrate for the fungus *Trichoderma reesei* for the production of the enzyme group cellulases [40].

1.6.3 Waste paper sludge

Restrictive parameters such as expensive nutrient sources set limitations in the production of microbial enzymes. To overcome these issues, waste for example from paper and pulp industry can be used as carbon sources for enzyme production. Studies show that waste paper sludge can be used as carbon source for *Acremonium cellulolyticus* using cellulose [41]. The dry paper sludge used for those experiments contained 30% clay and 60% organic material, meaning cellulose and hemicellulose [42].

1.6.4 N-acetyl glucosamine

As the β-1-4 linked polymer N-acetyl-glucosamine is the chemical structure of chitin, we investigated if the fungi expressed enzymes enabling the breakdown of the component and hence utilizing it as energy source.

1.6.5 Xylan

Xylan is a diverse plant polysaccharide with a backbone consisting of β-1,4 linked xylose residues and a main chain comprising of β-xylopyranose residues. Xylan presents the most common hemicellulosic polysaccharide in cell walls of land plants [43]. Hydrolysis of the xyloglucan into xyloglucan oligosaccharides is performed by the enzyme called xyloglucanase which is also included in the glycoside hydrolase enzyme class.

1.6.6 Cellobiose

Two glucose subunits β-1-4 glycosidic connected leads to the formation of cellobiose. In contrast with maltose, which consists also of two glucose molecules but differ in configuration of the glycosidic bond. Cellulose is therefore the polysaccharide composed of cellobiose disaccharide units and is broken down by β glucosidases[44].

1.6.7 Carrageenan

Carrageenan can be extracted from red seaweeds and is widely used as thickener and gelling agent in food industry [45]. The linear polysaccharide is defined by a repeating sequence consisting of 1,3 linked α-D-galactopyranose and a 1,4 linked β-D-galactopyranose. Carrageenan can be put on a structural functional level to cellulose in plants. The 3-linked units arise as unsulfated or 2-and 4-sulfate, while the 4-linked unit on the other hand occurs as 2- sulfate, as 2,6-disulfate, 3,6-anhdride and 3,6-anhydride—2-sulfate [46].

2 MATERIALS

2.1 CHEMICALS, BUFFERS AND SOLUTIONS

Table 1: List of chemicals used during this work.

Chemical	Company
2,2'-Azino-di(3-ethylbenzthiazolin-6-sulfonsäure) (ABTS)	Roche
2-Methyl 2-Butanol	Sigma-Aldrich
Acetic Acid	Merck
Aceton	
Ammonium sulfate (NH_4SO_4)	Sigma-Aldrich
Bovine Serum Albumin Standards (2mg/mL)	Sigma-Aldrich
Bromophenolblue	Sigma-Aldrich
Calciumchlorid dihydrate ($CaCl * 2 H_2O$)	Sigma-Aldrich
Commassie Briliant Blue R250	Sigma-Aldrich
D(+)-Glucose	Roth
Dipotassiumhydrogenphosphat (K_2HPO_4)	Sigma-Aldrich
Dithiothreitol (DTT)	
EndoH	New england BioLabs
Ethanol	Sigma-Aldrich
Glycerol	Sigma-Aldrich
HCl	Sigma-Aldrich
Magnesium sulfate heptahydrate ($MgSO_4 * 7 H_2O$)	Sigma-Aldrich
Maltextract	Roth
Nutrientagar	Merck
p-Nitrophenol	Sigma-Aldrich
p-Nitrophenolbutyrat (p-NPB)	
Potassium nitrate	Merck
Potassiumdihydrogenphosphat (KH_2PO_4)	Broth
Potato glucose Agar	Sigma-Aldrich
Protein Assay Dye Reagent Concentrate 5x	Bio Rad
Protein Marker peqGold IV	PEQLAB
Sodium acetate (NaAc)	
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich
Sodiumchlorid (NaCl)	Roth
ß-Mercaptoethanol	Merck
Trizma®base	Sigma-Aldrich
Urea	Sigma-Aldrich

Table 2: List of buffers and solution.

Solution	Components	Used for
100 mM Phosphate Buffer pH 7	• K_2HPO_4 • KH_2PO_4	Activity assays
50 mM Tris-HCl Buffer pH 7.5 and 9	• Trizma®base • HCl	Protein purification and activity assays
Lämmli-Buffer 2x	• dH2O • 0,5M Tris pH 6,8 • Glycerol • 20 % SDS • ß-Mercaptoethanol • 1 % Bromophenol blue	SDS-Gelelectrophoresis
Running buffer 10x	• 25 mM Tris • 192 mM Glycine • 0,1 % SDS	
Staining solution	• 600 mL ddH_2O • 300 mL Ethanol • 100 mL Acetic acid • + 0,125 % Coomassie	
Destaining Solution	• 600 mL ddH_2O • 300 mL Ethanol • 100 mL Acetic acid	
Denaturation buffer 10x	• 5 % SDS • 0,4 M DTT	Deglycosylation
G5 Reaction Buffer 10x	• 500 mM NaAc	Deglycosylation
C1	• $K_4[Fe(CH)_6]$ * 3 H_2O	HPLC sample preparation
C2	• $ZnSO_4$ * 7 H_2O	

2.2 SOFTWARE AND DEVICES

Table 3: List of devices used during this work.

Usage	Describtion
Autoklave	• Bench-top Autoclave-CERTOCLAV
Centrifuge	• Eppendorf Centrifuge 5417 R (F45-30-11, max. 14000 rpm, max. 30x3.75g) • Eppendorf Centrifuge 5920 R (S-4x1000, max 3700 rpm, max. 4x2.1kg),
Chromatographic system	• ÄKTA pure; Amersham pharmacia biotech, Sweden • Agilent technologies 1260 Infinity II HPLC • Ion exchange column ION 300 (Transgenomic Omaha, USA) with refractive index detection (1100 series, Agilent Technologies, Santa Clara, USA).
Electrophoresis Equipment	• BioRad Mini-Protean® Tetra System • BioRad PowerPac® HV • BioRad ChemiDoc MP imaging system
Incubator	• Heraeus instruments
Lamina	• LaminAir® HB 2472
Magnetic stirrer with heating plate	• IKA® RH basic 2
Microscope	• OLYMPUS BX43
PD-10 desalting column	• GE Healthcare
pH Meter	• Mettler Toledo Five Easy
Plate Reader	• Tecan Infinite M200 Pro
Reactor	• INFORS HT with Iris V6.0.lnk
Thermomix	• Eppendorf Thermomix comfort
Freeze drier	• CHRIST; Alpha 2-4 LSCplus
Vivaspin® 20 mL	• GE Healthcare (Molecular cut-off: 10;30;50 kDa)
Lyophilizer	• Christ; Alph 2-4 LSCplus
Bioreactor	• Infors
FT-IR	• FT-IR Perkin Elmer Spectrum 2000 instrument

3 METHODS

3.1 CULTIVATION OF *ABSIDIA GLAUCA*

Absidia glauca isolates are obtained from The University of Natural Resources and Life Sciences from the Institute of Environmental Biotechnology. They are cultivated in Erlenmeyer flasks containing potato-glucose medium. Depending on the growth of the fungi and the size of the visible mycelium respectively, the flasks are incubated 3–4 days while shaking at 24 °C and serve as preculture for the preparation of sterile 30 % glycerol stock solutions. Those stocks are stored at –80 °C and serve as inoculum for all different fermentations performed according to this work.

3.2 SOLID STATE FERMENTATION

To gain acknowledge about the optimal growth parameters for the batch fermentations different solid-state fermentations are accomplished. Based on the combination of salts, on different nitrogen sources and on temperature, growth of the fungi is observed and documented. Three-point inoculation with 10 µL per each point is performed and measured in mm once every day. The pH is set to 4.4 on the one hand due to the outcome from previous pH-dependent growth experiments and to avoid contaminations in the later performed bigger fermentations on the other hand. The different components of the media including concentrations are presented in Figure 6 below. Corresponding to those outputs, plans of the following fermentations are adapted and performed dependently.

	Ingredients [g/L]												
Medium	Peptone	Maltextract	Glucose	Agar	$(NH_4)SO_4$	K_2HPO_4	$MgSO_4*7H_2O$	$CaCl*2H_2O$	NaCl	KNO_3	Urea	pH value	used acid
M1	5	2.5	2	20								4.4	H_2SO_4
M2	5	2.5	2	20	5	1	5	1	1			4.4	H_2SO_4
M3	5	2.5		20								4.4	H_2SO_4
M4	5	2.5		20	5	1	5	1	1			4.4	H_2SO_4
M5	5	2.5	2	20						5		4.4	H_2SO_4
M6	5	2.5	2	20	5	1	5	1	1	5		4.4	H_2SO_4
M7	5	2.5	2	20							7.2	4.4	H_2SO_4
M8	5	2.5	2	20	5	1	5	1	1		7.2	4.4	H_2SO_4

Figure 6: Media components for the fermentations, investigating optimal growth conditions.

3.3 SMALL SCALE FERMENTATION IN BAFFLED FLASKS

3.3.1 Determination of growth dependency on salts:

Revealed by previous performed batch fermentations, expression of big amount of proteins is observed in accordance with the availability of different salts. For the determination whether one particular salt or the combination is crucial for the induction, fermentations are carried out in 1 L flasks in triplicates containing medium presented in Figure 7. 1L baffled flasks are filled with 400 mL corresponding medium and closed with cotton and special caps to prevent the distribution of the spores. The medium and the flasks are autoclaved and inoculated with a one-week old potato-glucose containing liquid *A. glauca* preculture. Samples are taken twice a day and stored in the -20 °C freezer. The fermentation is stopped after one week and subsequent glucose content is determined with HPLC (Carrez-precipitation) and SDS PAGE is prepared according to descriptions below.

	Ingredients [g/L]							
Medium	Peptone	Maltextract	Glucose	$(NH_4)SO_4$	K_2HPO_4	$MgSO_4*7H_2O$	$CaCl*2H_2O$	NaCl
M1	5	2.5	2	5	1	5	1	1
M2	5	2.5	2					
M3	5	2.5	2	5				
M4	5	2.5	2		1			
M5	5	2.5	2			5		
M6	5	2.5	2				1	
M7	5	2.5	2					1

Figure 7: Media containing different salts.

3.3.2 Influence of different carbon sources on growth:

Flasks and media are prepared as already described above and Figure 8 offers insights into the distinguishable medium. Important to mention are the carbon sources. Relying on the results obtained from the mass spectroscopy sequencing, sources like brewers' grain, sugar beets, xylan, cellobiose, N-acetylglucosamine, carrageenan and waste paper sludge are utilized. The aim was to investigate whether the fungi can use other sources than glucose as energy source or not, implying that the microorganism is capable of the breakdown of other saccharides. Dependent on the growth, samples are collected in duplicates and used for SDS and HPLC analysis.

Non - Varying Ingredients [g/L]						
Peptone	Maltextract	$(NH_4)SO_4$	K_2HPO_4	$MgSO_4*7H_2O$	$CaCl*2H_2O$	NaCl
5	2.5	5	1	5	1	1

	Different Carbon sources [g/L]							
Medium	Glucose	N-Acetylglucosamin	Cellobiose	Waste paper	Brewers grain	Sugar beets	Xylan	Carragen
M1G	2							
M2N		2						
M3C			2					
M4WP				2				
M5T					2			
M6Z						2		
M7X							2	
M8C								2

Figure 8: Media containing the different carbon sources. M1-Glucose, M2N-Aceylglucoasmine, M3-Cellobiose, M4-WastePaperSudge, M5-BrewersGrain, M6-Sugarbeets, M7-Xylan, M8-Carrageenan.

3.4 Batch Fermentation with Bioreactors

Different media for all four reactors are prepared. Stock solutions of glucose and all five salt components are made and the salts are added to the main components already solved in water, peptone and malt extract. The glucose stock solution is autoclaved separately, the other components are however poured together before. 1-2 weeks prior to each fermentation with the bioreactors, *A. glauca* containing stock solutions are transferred in a T-flask containing potato-glucose agar and incubated at room temperature. An invariably amount of spores/L throughout all fermentations are consistent with the development of a reproducible process. Therefore, sterilized physiological NaCl solution is previously poured onto the preculture and diffused for 1 hour. The spores/L in the received solution are determined with Thomakammer and need to be in the range of 10^{10} spores/L. Before starting the fermentation by adding 40 mL of the inoculum, glucose is added aseptically to the reactor by support of a syringe.

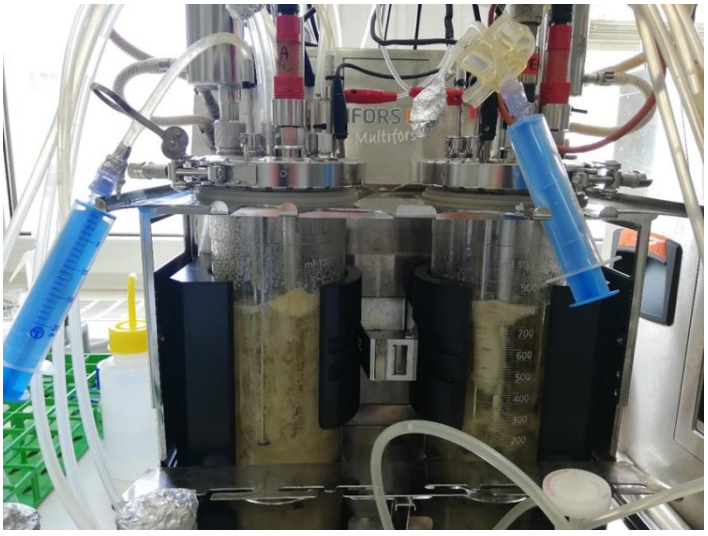

Figure 9: Setup of the bioreactor.

Parameters like pH, pO_2, temperature, antifoam and stirrer velocity are recorded throughout the fermentation via the program Iris and compared by the system software to a defined set point enabling a continuous control. A constant pH of 4 is defined for every fermentation. Counteraction against a decrease or increase of the environmental pH is achieved by addition of either 3 molar H_2SO_4 or 3 molar NaOH. Temperature is adjusted to 24 °C and the air flow is minimum 0.3 mL/min and maximum 2 mL/min. Samples are taken twice a day and stored at - 20 °C. The supernatant is collected after each fermentation and used for further analysis. In addition, the mycelium is frozen at - 80 °C and lyophilized at 20 °C and 0.5 mbar. The first fermentations, including F230419, are carried out to detect the expression of enzymes at all. Protein concentrations are determined with Bradford and according to those results concentration and desalting steps are accomplished. Laccase, esterase and cellulase activity assays are executed according to descriptions below. Electrophoretic separation is carried out and main bands are cut out with a scalpel and sent to Graz for sequencing.

Concerning F290719, 40 mL of supernatant are concentrated to 1 mL directly after fermentation and enzyme activity and pH optimum is determined.

Parameters	
Fermentation Volume	800 mL
pH	4.0 ± 0.1
Temperature	24 ± 2°
Aeration pO_2	40 % O_2 saturation
Inoculum	40 mL ($1*10^{10}$ spores/L)
Minimum flow	0.300 mL/min
Maximum flow	2 mL/min

Figure 10: Parameters of each bioreactor fermentation.

3.5 HARVESTING OF BIOMASS AND EXTRACTION OF FUNGAL CHITOSAN

The mycelium of each fermentation is sieved and harvested in small plastic bags. The biomass is frozen at - 80 °C overnight and freeze dried with parameter already described in the section before. The biomass is collected and weight to obtain the whole biomass of the 800 mL fermentation volume.

The biomass is subsequent treated under strong alkaline conditions with 5 M NaOH at 60 °C. After the alkaline treatment the insoluble material (AIM) is separated by centrifugation and washed with

96 % ethanol, double distillated water and freeze dried. The AIM is afterwards treated with 1 % acetic acid solution at 60 °C with a ratio of 1:100. The impurities are afterwards removed by centrifugation and chitosan is preserved by re-precipitation. The resulting chitosan is analyzed with FT-IR, which is described below.

3.6 PROTEIN PURIFICATION AND IDENTIFICATION

3.6.1 PD-10 Column

The high salt concentration in the fermentation media leads to several disturbing background reactions. Therefore, a PD-10 column is applied resulting in a desalting process. Molecules with a small molecular weight will penetrate the pores in contrast to the molecules of interest, which are incapable of that interaction due to the large molecular weight, and consequently elute first [47].

25 mL of the according buffer are applied to the column before loading of 2.5 mL protein solution is carried out. After the addition of again 3.5 mL final buffer the solution dropping out of the column is collected and contains the protein of interest in a desalted solution.

3.6.2 Deglycosylation with Endo H

Deglycosylation of the protein sample is necessary to get straight clear bands instead of smeared bands during SDS PAGE procedure. Therefore, 500 µL sample are mixed with 60 µL denaturation buffer and incubated for 10 minutes at 99 °C. The tubes are cooled down on ice and 68 µL reaction buffer (10 x G5) and 2 µL Endo H are added and incubated at 37 °C for 3 hours at 300 rpm.

3.6.3 Sodium dodecyl sulfate gel electrophoresis

The protein solution or pellet is resolved in Laemmli buffer (1:1), which contains sodium dodecyl sulfate (SDS), β-Mercaptoethanol, for breaking the disulfide bonds, and glycerol, in order to get the sample hold in the stacking gel. The proteins are denatured at 99 °C for 10 minutes and 10 µL of the sample and 5 µL of the PEQ GOLD IV (VWR) marker are loaded on the polyacrylamide gel with 4-15 % gel percentage. An electrical field of 150 V is applied, and electrophoresis performed in a chamber filled with TGS buffer (1X) for around 45 minutes. The gel is afterwards stained with Coomassie blue for 40 min, destained twice with destaining solution for 1 h and again distained overnight in milli-Q water before capturing the picture.

3.6.4 Sequencing of protein band with mass spectroscopy

Bands of interest are cut out with a scalpel, put in a labelled tube and sent to Graz for sequencing. With the obtained accession number further information about the whole sequence and function of the protein is collected with the database Uniprot. The amino acid sequence is used to align it with a protein sequence with Blast. Based on that information, especially the pI of the protein, further purification steps are planned, and ion exchange chromatography is finally settled.

3.6.5 Ion exchange chromatography

With the purpose of separating one specific protein expressed from *A. Glauca* the chromatographic method depending on ion exchange is used. Since the proteins charge is dependent on the environmental pH the experimental conditions can be selected in a way that the protein is either loaded positive or negative. Due to prior sequencing experiments suggesting the pI of the different protein bands to be 9.8, 50 mM Tris-HCl buffer pH 7.5 and 9 is selected in two different ion exchange experiments. Knowing that the protein is loaded positive at environmental buffer pH under the individual pI a cation exchange is used.

The main chromatographic part is preceded with several concentration steps with viva spin. A total amount of 80 mL supernatant is concentrated and washed with a 30 kDa molecular cut off viva spin at 4 °C with 3700 rpm and the Tris-HCL buffer pH 7.5 and 9. Meantime, the column is applied and after equilibration of the stationary phase with 50 mM Tris-HCl binding buffer, the sample application is accomplished. During this step the protein of interest binds to the stationary phase and the other proteins present in the sample are washed away. The third main step is the elution of desired protein. Taking place by a switch of binding buffer to an increased gradient of 50 mM Tris-HCl elution buffer to 100 %. It is important to mention that the elution buffer contains 1 M NaCl. These ions compete with the bound proteins, resulting in the elution of the protein of interest. Different fraction from binding but also from the elution are collected automatically with the system sampler. The obtained chromatogram reveals the fractions, supposed to contain the molecules of interest, which are then mixed with three times the amount of acetone. Overnight precipitation is carried out at 4 °C. The solution is centrifuged with 14000 rpm at 4 °C and the pellets are air dried to avoid any residual acetone left in the sample. Subsequently, 20 µL Laemmli

buffer are added and the proteins are denatured at 99 °C for 10 minutes prior to applying to the SDS PAGE.

3.7 ANALYSIS OF PROTEIN ACTIVITY

3.7.1 Laccase activity assay

During this work, the supernatant is characterized regarding enzyme activities, including laccases. The most prominent aromatic substrate for laccases is the ABTS 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonate). The progression of ABTS oxidation can be spectrophotometrically measured at 420 nm. Therefore, 150 µL of 100 mM potassium phosphate buffer with either pH 5, 6 and 7 are mixed with 20 µL fermentation supernatant in the well. Directly before measuring with the plate reader 50 µL ABTS are added to the wells, mixed carefully and the absorbance is monitored for 15 minutes in 20 cycles. The enzyme activity is calculated in katal (kat) and correlates with laccase converting 1 mol of ABTS per second. Experiments are carried out in triplicates. Calculations are performed in accordance with the equation below.

Equation 1: Calculation of the enzymatic activity.

$$A = \frac{k * DF * V_{total}}{V_{enzyme} * \varepsilon_{420} * d}$$

A…… Enzymatic activity

k…… Slope

V_{total}..... Volume total

V_{enzyme}…. Volume enzyme solution

ε_{420}…… Extinction coefficient

d…… Path length

DF……. Dilution factor enzyme solution

3.7.2 Esterase activity assay

Two solutions are required to perform the esterase assay. Solution A contains 86 µL p-NPB (para-nitrophenyl butyrate) and 1 mL 2-Methyl-2-Butanol. Due to light sensitivity of this solution A it is necessary to use light impermeable tubes or aluminum foil. The solutions B has to be freshly prepared before each measurement and consists of 40 µL solution A and 1 mL of the corresponding 100 mM potassium phosphate buffer. The assay is performed by pipetting 200 µL solution B and 20 µL of 40 x viva spin concentrated enzyme solution in a well and measuring the kinetic absorbance with the tecan plate reader at 405 nm for 15 minutes. 53 cycles of 18 seconds at 30 °C. The activity is calculated the same as described already above in Equation 1.

3.7.3 Cellulase activity assay

The enzyme assay is performed in 10 mL Pyrex tubes. 1 mL of 40 x concentrated enzyme supernatant is transferred in a tube and one previously prepared filter paper roll is added and the tubes closed. The tubes are incubated for 60 minutes in a tempered water bath and afterwards put on ice before the addition of 1 mL 3.5-Dinitrosalicylic acid (DNS) reagent is performed. The samples are put for 5 minutes into a boiling water and cooled down. 8 mL water are added, and absorbance is directly measured in a 96 well plate at 540 nm.

3.8 ANALYTICS

3.8.1 Analytical analysis of glucose content

Metabolism of the fungi is dependent on the availability of a carbon source like glucose or maltose. The most effortless way for the fungi to grow is simply to get energy as a result from the oxidation of glucose. If the free available C-source as glucose is completely depleted, the fungi needs to alter the strategies for growth and need to express other proteins facilitating proliferation, meaning that the microorganism is capable using other components present in the media for the generation of energy.

In order to gain information since which time point glucose is complete exhausted, the content after each sampling is determined with high performance liquid chromatography (HPLC). Prior to the main analysis 1 mL of fermentation broth is centrifuged for 20 minutes at 14000 rpm and 500

µL of supernatant are transferred in a fresh Eppendorf tube. Those solutions are diluted with 460 µL H_2SO_4 and the precipitation is carried out by the addition of 20 µL C_1, carefully vortexing and another addition of 20 µL C_2 solution. The tubes are centrifuged for 20 minutes at 4 °C at 14000 rpm to remove the precipitate. The supernatants are transferred to HPLC vials through a 0.45 µm nylon filter to be analyzed later by means of HPLC. HPLC analysis is performed using an ion exchange column ION 300 (Transgenomic Omaha, USA) with refractive index detection (1100 series, Agilent Technologies, Santa Clara, USA). A 5 mM sulfuric acid is used as mobile phase with a flow rate of 0.325 mL per minute at 45 °C. 40 µL of each sample are injected an analyzed for 70 minutes.

3.8.2 FT-IR

Conversion of big amount of data by a computer can be carried out by a mathematical process called “Fourier transform”. With FTIR it is possible to get an infrared spectrum of absorption, emission, photoconductivity or Raman scattering of a solid, liquid or gaseous sample. Concerning this spectroscopic method a beam, containing the full spectrum of wavelengths, is created by a broadband light source. The aim is to determine how much of the beam at each different wavelength is absorbed by the sample [50].

The sample used for the analysis are freeze dried and ground to a fine powder. The pulverized samples are then measured with a FT-IR in the wavenumber range of 550 to 4000 $\frac{1}{cm}$ with 2 $\frac{1}{cm}$ resolution and 25 scans per sample. The degree of deacetylation (amino group percentage) of chitosan is determined by FTIR analysis and calculated using the baselines and equation according to Roberts [51].

$$\text{DA (\%)} = \frac{\left[\left(\frac{A_{1655}}{A_{3450}}\right)*100\right]}{1.33}$$

A1655 is the intensity of the absorption of amide I, used as the specific band for N – acetylation. A3450 is the intensity of the absorption of the hydroxyl group band, used as the reference band, 1.33 is the ration of the absorbance at 1655 $\frac{1}{cm}$ to that of the absorbance at 3450 $\frac{1}{cm}$ for fully N – acetylated chitosan. DD was then calculated using the following equation:

$$DD\ (\%) = 100 - DA\ \ (\%)$$

A characteristic FT-IR spectrum of chitosan extracted from shrimp shell opposing chitosan extracted from fungal CTS is presented below (Figure 11). Representative for the polysaccharide is the absorption peak at a wavelength of 1000 cm^{-1} whereas the degree of acetylation influences the absorbance at 1500-1800 $cm^{-1.}$

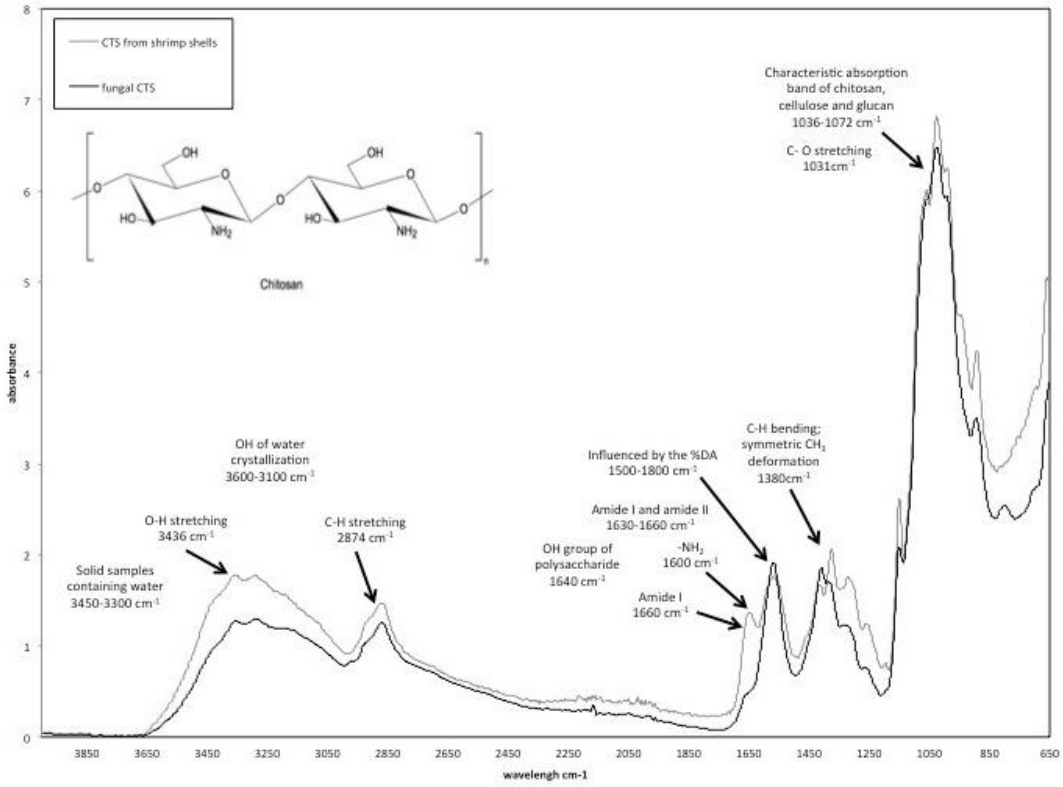

Figure 11: Comparison of a FT-IR spectrum from shrimp shell CTS and fungal CTS.

4 RESULTS AND DISCUSSION

4.1 SOLID STATE FERMENTATION

Absidia glauca plates were inoculated and incubated at room temperature. The different media used for this experiment are shown in Figure 6. As shown in Figure 12, a clearly measurable growth is already visible after the first day. Evaluation on the third day revealed that the plates were completely overgrown, and the form of the mycelium was not able to be measured any more. Thus, the results are not illustrated in the graph. The different nitrogen sources in the media seem to play an important role for the fungi. Medium containing urea (M7 and M8) revealed far lower growth compared to all the others and also to the medium without any salt (M1), implying that urea has inhibitory impact in some metabolic pathways in *A. glauca* and therefore on the growth. Additionally, M2 was observed to be the most favoured one for the fungi containing all salts except urea. In media M5 and M6 additionally potassium nitrate is present, leading neither to a better nor to a worse growth behaviour. Interestingly, the fungi is also growing good on the substrate without glucose, M3 and M4. But again, the medium containing additional salts (M4) is favoured. M9 indicates potato dextrose agar containing 20 g/L glucose and was only determined to have a comparable value for strong growth. It was never considered to be taken for the main fermentation due to the high amount of glucose.

A. glauca plates with the same media illustrated below were also incubated at 30 °C for 1 week. Far less growth was observed compared to the incubation at room temperature and therefore no graph is presented.

Those acquired data served as basic knowledge for the choice of media used in the following fermentations. Specialization was put on M2 and M1 and on subsequent evaluation if there is a correlation between the expression pattern of proteins and the addition of salts, eventually serving as cofactors.

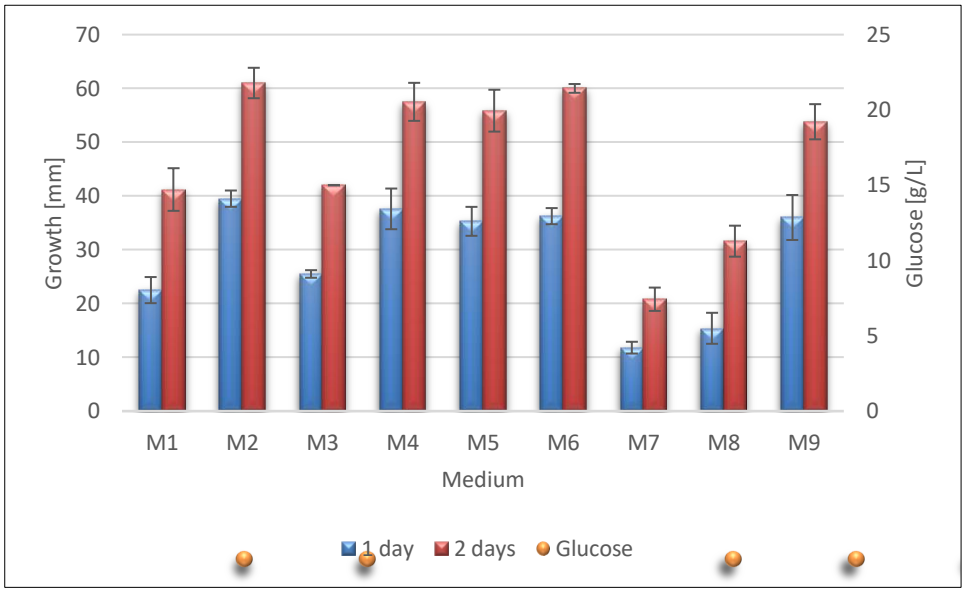

Figure 12: Measurement of the growth of A. glauca at room temperature in dependency of different media (Figure 6) and glucose content. On the primary vertical axis, the growth of the fungi in mm is displayed. On the secondary vertical axis, the glucose content in g/L is depicted and is indicated by the orange dots. The medium components are described in Figure 6 and the different media are indicated on the x-axis. Growth after one day is displayed in blue and after two days in red color.

4.2 DEPENDENCE ON DIFFERENT SALTS

Fermentation 190819S

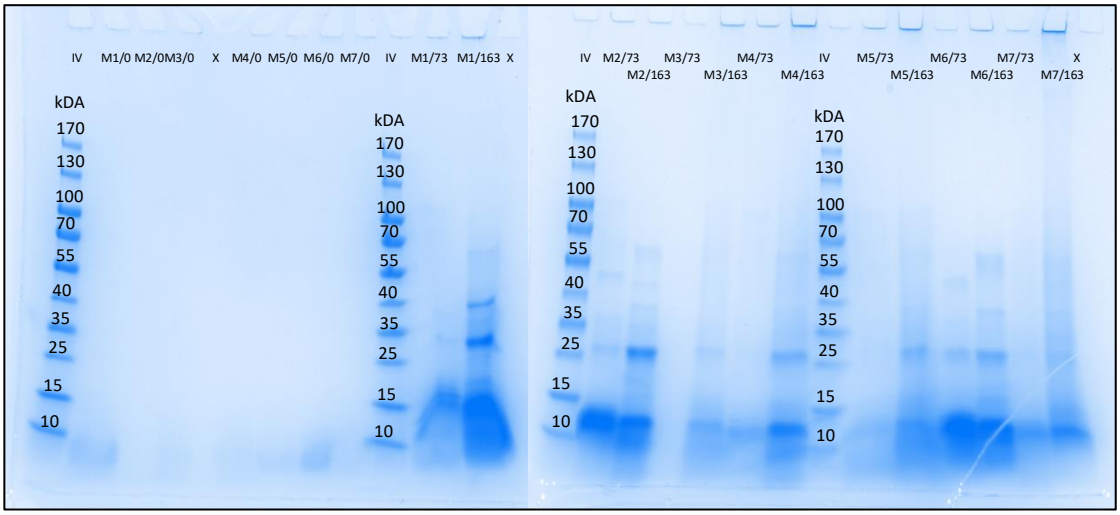

Figure 13: SDS PAGE from culture supernatants containing different salts. Mx indicates the different media and /x the different time points in hours. Time point 0 is directly after inoculation with the fungi, and after 163 hours the fermentation was stopped.

The purpose of SDS PAGE is to separate different proteins in a solution according to their relative molecular weight [49]. As indicated in the name, the separation technique is carried out with a polyacrylamide gel and by heating the protein mixture, denaturation of the proteins is obtained. The role of SDS is to change the tertiary structure of the protein to gain a linear sequence and to evenly load the protein negatively. If the biological activity of a native protein must be determined during subsequent functional testing, SDS PAGE cannot be applied.

Time points 0 were taken exactly after inoculation of the flasks and therefore should not show any bands, like observable in Figure 13 (Mx/0). There are already bands occurring slightly at time point after 73 hours (Mx/73) but not as intense as after 163 hours (Mx/163). The main point to mention is that the medium containing all five salts (M1) lead to the best results and quantitative to the best expression of proteins after 163 hours. There is a main band between 25 and 35 kDa and another one occurring at around 40 kDa. Description of the media is present in Figure 7. Medium only containing additionally CaCl * 2 H_2O (M6) lead almost to the same output than medium containing the constant nutrients peptone, malt extract and glucose (M2). If comparing with M3, M4 and M5 it can be stated that either those additional ions alone have inhibitory effects on the synthesis or one of the other salts is missing. Studying time points seven from M2 and M6 assumption of available proteases or instability properties can be met due to the occurrence of a band at around 50 kDa, which is already fading away at time point 8.

This experiment did not reveal any specific assumptions about the cofactors of the protein, or which special salt is needed for the expression. Only with the combination of all salts, medium 1, the best results for secretion of the proteins into the supernatant are obtained.

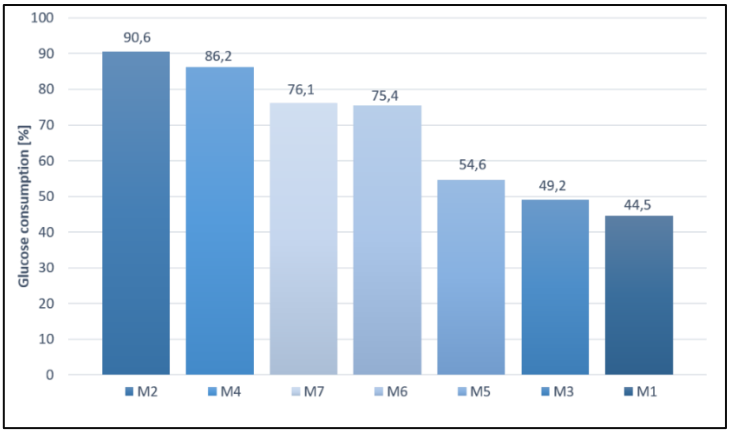

Figure 14: Consumption of the glucose content in percentage after 73 hours of fermentation dependent on different media.

The glucose content of the samples after 73h and 163h was determined with HPLC. Figure 14 outlines the consumption of the initial 2 g/L glucose in percentage from *A. glauca* after 73h. With medium 1 containing all salts, the fungi consumed not even half of the glucose content after 73h whereas with medium containing only peptone and malt extract, the fungi obviously consumed 90.6 % of the whole glucose concentration (M2). After 173 hours of fermentation the results from the high performance liquid chromatogram did not show a peak at the specific retention time of the carbohydrate glucose at all, leading to the assumption that all glucose was completely used up.

4.3 BATCH FERMENTATION IN THE BIOREACTOR

4.3.1 First Fermentation of *A.glauca* in the Bioreactor: F230419

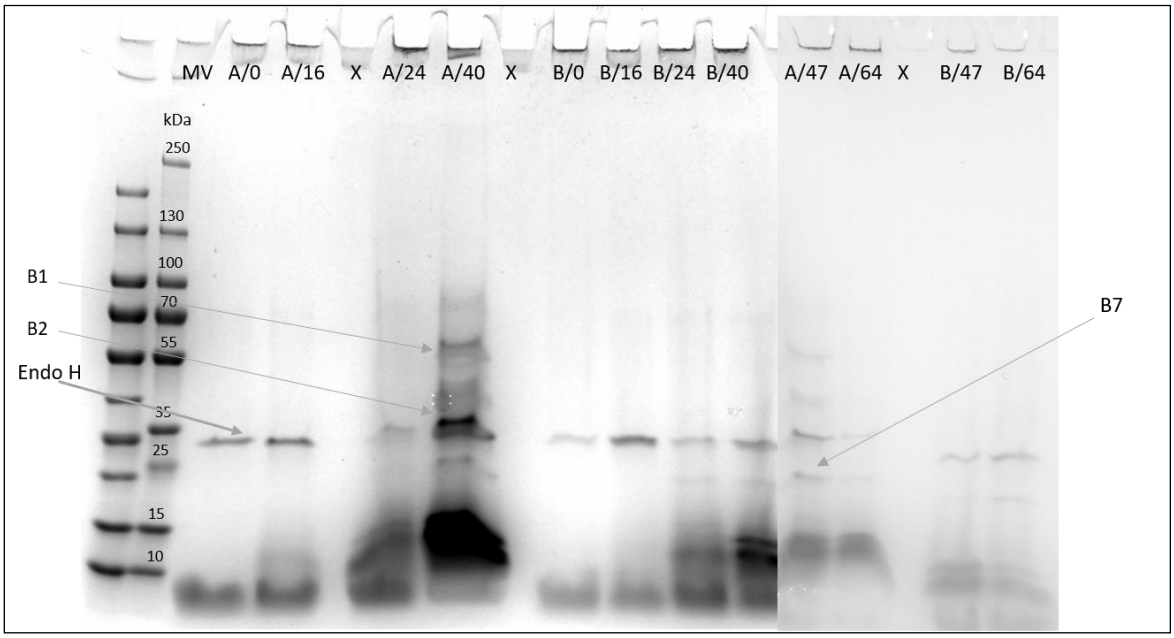

Figure 15: SDS PAGE of deglycosylated culture supernatant from fermentation 230419 with marked bands for sequencing. A indicate samples from reactor A and B the samples from reactor B, respectively. Different sampling time point are indicated in hours, A/x. Time point 0 is directly after inoculation and the fermentation was stopped after 40 hours. Endo H is visible at 29 kDa.

Figure 15 show the SDS PAGE of reactors A and B containing ammonium sulfate as nitrogen source. Reactors C and D were supplied with potassium nitrate instead (Table 4). The protein band occurring constantly at around 30 kDa represents the enzyme Endo H, which is present due to the deglycosylation procedure of the protein before loading to the gel. Adding sugar residues to the amino acid sequence is common in fungi and other organism in the evolutionary tree in order to modify the proteins in a way to specify localization, secretion, function and stability [48].

Relying on the results from reactors A and B it can be stated, that after 24 hours the expression of proteins begin to emerge and at the next time point after 40 hours a distinct pattern is visible. Comparing the SDS PAGE of reactor A and B it is obvious that glucose is needed for this synthesis because no promising bands are visible except Endo H from the samples of reactor B. The gels from reactors C and D are not presented above because no production of proteins was detected (Appendix Figure 29). Hence, the production of those distinct proteins of *A. glauca* is evidently dependent on different ammonium sources and either ammonium sulfate is needed for the production of the different enzymes or potassium nitrate inhibits some pathways leading to no expression at all.

Notable is also the bigger arising band above the Endo H at around 35 kDa. The band is very strong after 40 hours, but it is already slightly fading away after 47 hours of fermentation and totally gone after 64 hours, indicating either the presence of a protease in the supernatant or the protein shows very unstable characteristics.

Based on those first results, several further fermentations with medium 1, purification experiments and enzymatic assays were performed. As indicated by the grey arrows, bands B1, B2 and B7 were sent in for sequencing and results are presented below. Macroscopic considerations of the biomasses from the four different reactors reveal that biomass of reactor A exhibit the most differences compared to the biomasses in the other three reactors (Figure 16). It is interesting that not only the biomass of the fungi looks different but also the protein expression is induced in compliance with this special media.

Table 4: Medium contained in the 4 different reactors of F230419.

Medium	Reactor A	Reactor B	Reactor C	Reactor D
Peptone from casein (Roth)	5 g/L	5 g/L	5 g/L	5 g/L
Malt extract (Roth)	2,5 g/L	2,5 g/L	2,5 g/L	2,5 g/L
D(+) Glucose (Sigma Aldrich)	2 g/L	-	2 g/L	2 g/L
$(NH_4)SO_4$	5 g/L	5 g/L	-	5 g/L
K_2HPO_4	1 g/L	1 g/L	-	1 g/L
$MgSO_4*7H_2O$	5 g/L	5 g/L	-	5 g/L
$CaCl*2H_2O$	1 g/L	1 g/L	-	1 g/L
NaCl	1 g/L	1 g/L	-	1 g/L
KNO_3	-	-	5 g/L	5 g/L

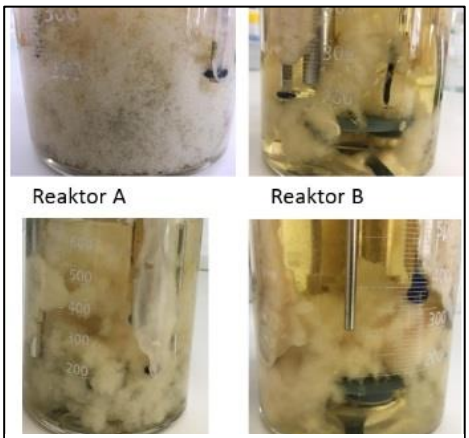

Figure 16: Biomass of the different fermentations.

Concerning the fermentation diagram of reactor B (Figure 17) it can be stated that the fungi consumes maltose until around 1 g/L. Simultaneously the pO_2 decreases and the flow increases. But after that point, maltose is not used up anymore and consequently the flow and pO_2 stay constant. This implies that the fungi is not growing any more after approximately 16 hours and as indicated by the gel does not secrete proteins or enzymes in the supernatant at all.

The fermentation diagram of reactor A looks quite comprehensible. Almost all glucose is consumed already after 22 hours and the pO_2 is correspondingly decreasing. Growth of the fungi reaches its maximum at around 40 h according to the maximum of the flow. After 40 hours maltose and glucose is completely depleted and the flow starts to decrease again indicating a discontinued growth.

Fermentation diagram of reactor C and D is attached in appendix Figure 28.

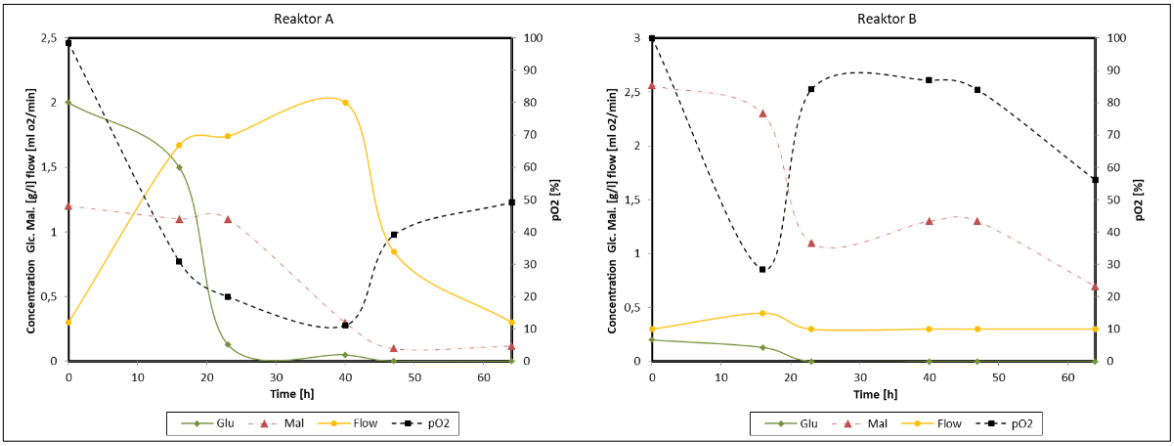

Figure 17: Fermentation diagram of F230419 of reactors A and B. On the primary vertical axis, the course of glucose/ maltose consumption and flow in mL O_2/min is displayed. On the secondary vertical axis, pO_2 is shown and on the x-axis the duration of the fermentation in hours is depicted.

4.3.2 **F290419**

Next step was the repetition of the fermentation with medium containing all five salts like indicated in reactor A below in Table 5. Furthermore, the dependence of the protein expression on the different nitrogen sources was investigated focusing on comparison of ammonium sulfate and potassium nitrate.

Concerning reactor A, SDS PAGE revealed that the proteins present after 40 hours are not that intense compared to reactor A from the previous fermentation 230419 at the same time point.

The protein band begin to get stronger after another 7 hours but decline again after 64 hours of fermentation. As already discussed during fermentation 230419, there is no need to perform a 64 hour fermentation.

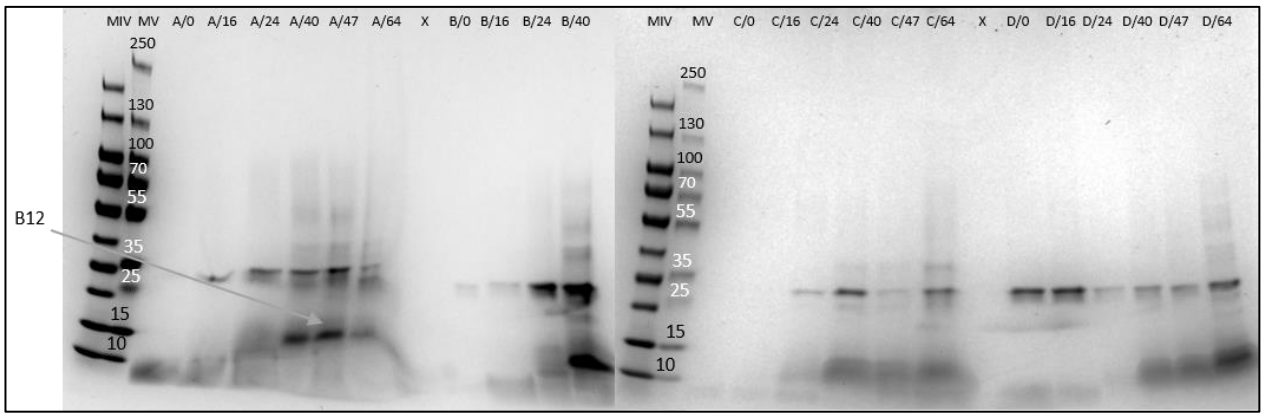

Figure 18: SDS PAGE of deglycosylated culture supernatant from all 4 reactors. Time point 0 is directly after inoculation. The fermentation was stopped after 64 hours.

Regarding reactor B, samples were only taken for 40 hours, since we had to abort the fermentation because of INFORs related issues. Nonetheless, protein expression and secretion into the supernatant is observed till that time point.

Referring to the SDS PAGE from reactors C and D it is exposed that after 64 hours, little concentration of protein seem to be present in both reactors. Potassium nitrate does not inhibit the expression but it takes the fungus more time for the production and secretion. As after 40 h the flow in the fermentation diagrams (Appendix Figure 30) stabilizes again around the starting point of 0.3 mL/min, the pO_2 increases, and proteins are already visible on SDS PAGE, the decision to carry out all next fermentation for 40 h was met. Labeled band 12 was also used for sequencing.

Table 5: Medium contained in the 4 different reactors of F290419.

Medium	Reaktor A	Reaktor B	Reaktor C	Reaktor D
Peptone from casein (Roth)	5 g/L	5 g/L	5 g/L	5 g/L
Malt extract (Roth)	2,5 g/L	2,5 g/L	2,5 g/L	2,5 g/L
D(+) Glucose (Sigma Aldrich)	2 g/L	2 g/L	2 g/L	2 g/L
$(NH_4)SO_4$	5 g/L	5 g/L	-	-
K_2HPO_4	1 g/L	-	1 g/L	-
$MgSO_4*7H_2O$	5 g/L	-	5 g/L	-
$CaCl*2H_2O$	1 g/L	-	1 g/L	-
NaCl	1 g/L	-	1 g/L	-
KNO_3	-	-	5 g/L	5 g/L

4.3.3 **F290719**

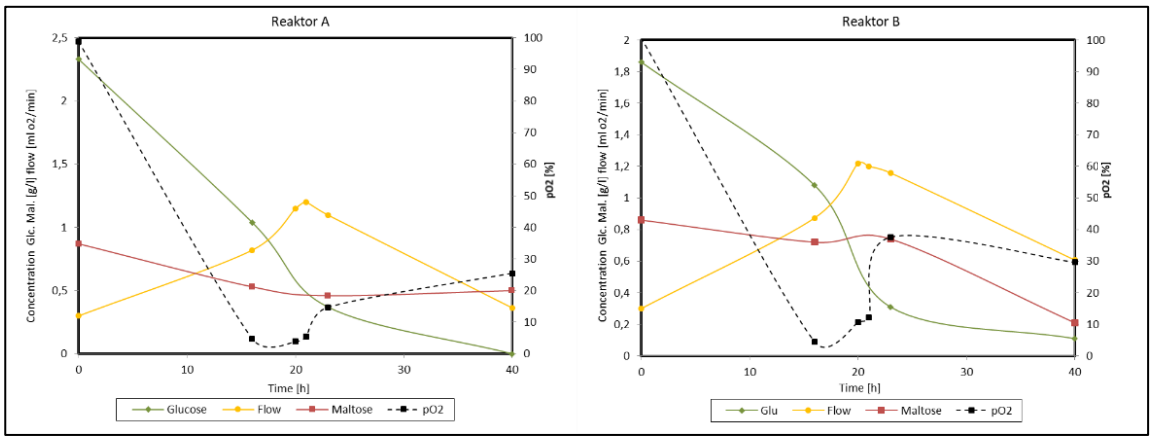

Figure 19: 290719 fermentation diagram of reactors A and B. On the primary vertical axis, the course of glucose/maltose consumption and flow in mL o2/min is displayed. On the secondary vertical axis, pO_2 is shown and on the x-axis the duration of the fermentation in hours is depicted.

Those two fermentations, with a duration of 40 hours, were now with medium containing the defined five salts (M2 in Figure 6). Intention of performing this experiment was to obtain more supernatant for further identifying steps and to confirm that the process time of 40 hours is enough in order to receive a satisfied amount of protein. As seen from the fermentation diagram the pO_2 decreased very quickly during the first 15 hours while the flow increased in the same time, correlating with a quick growth of the fungi. After around 20 hours the flow decreased and the pO_2 increased again, resulting from growth discontinuation of the fungi. The glucose was depleted completely after 40 hours but the maltose doesn't seem to be the first choice for the fungi and decreases therefore very slowly. Comparing the diagrams of F230419 RA, F290419 RA and of the discussed fermentations in this sector it can be ascertained that they don't vary significantly, meaning that it is fairly reproducible process.

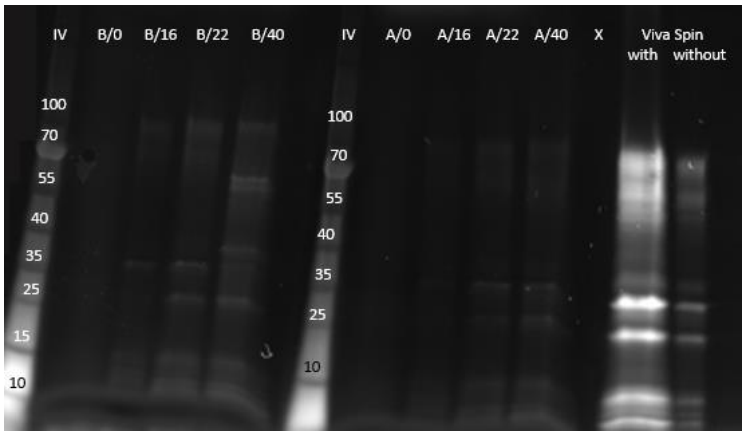

Figure 20: SDS PAGE representing the different time points of reactor A and B. Time points were taken after 0, 16, 22 and 40 hours. The second last band represents a viva spin concentrated (40x) sample of reactor A after 40 hours with overnight acetone precipitation, and the last band displays the same concentrated sample but without acetone precipitation.

Results from the SDS-Gels showed again exactly the same protein expression pattern like from F230419 in Figure 15. It is clear to see the synthesis over the course of time. After 22 hours not all proteins are yet expressed or secreted extracellular to the medium compared to the samples taken after 40 hours where you can see the expression pattern from *A. glauca*. By comparison of reactor A with reactor B it is observable that the pattern is almost the same. Eventually reactor A would have required more time, but taking into account the fermentation diagram, glucose was already depleted, the flow rate already decreased again, and the fungi completely overgrew the reactor, completing the fermentation was a transparent decision.

Without knowing on which enzyme expression to specialize on and how to drive the expression in a defined direction, the enzyme concentration in the supernatant is quite low. Without concentration steps before the different assays and purification experiments, no successful outputs can be expected. Therefore, an ultrafiltration method using viva spin was performed. Viva spins are available for very different volume amounts and different molecular weight cut offs (MWCO). The two bands right on the SDS PAGE show with viva spin 30 kDa cut off concentrated samples. The left one with an overnight acetone precipitation and the right one without any precipitation.

The 40 times concentrated sample was tested on laccase, cellulase and esterase activity whereby only the esterase activity reveled positive results. The presence of laccase was tested at pH 5, 6, 7 and 8. But within none of these conditions ABTS was oxidized and no increase of the signal was

observed. However, it can be concluded that the enzymes are not specific for ABTS but potentially laccases with other specificities are present. The concentrated sample was also tested on cellulase presence, whereas again negative results were received.

Only esterase activity was confirmed with p-NPB as substrate. The pH optimum was determined at pH 7 (Table 6), whereby in view Figure 21 no strict distinction can be discovered between those to environmental changes.

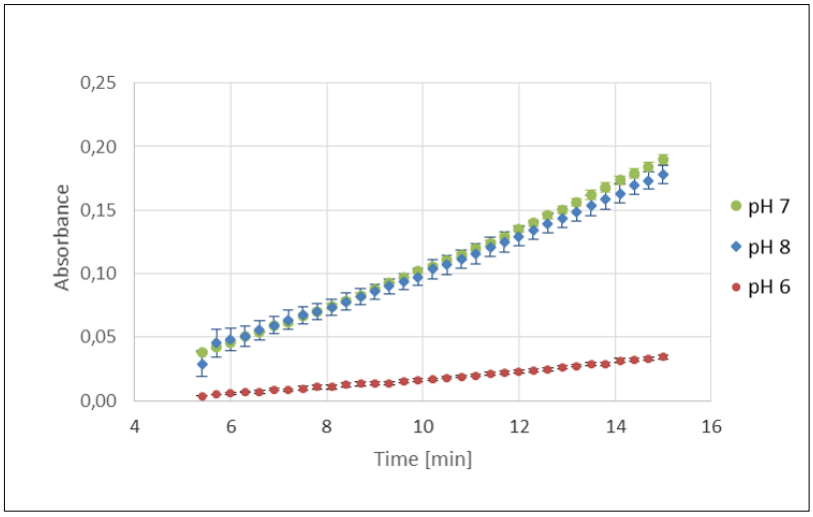

Figure 21: Esterase activity of the concentrated culture supernatant at pH 6, 7 and 8 with displayed error indication. The Enzyme solution was 40 x concentrated after harvesting of the supernatant with 30 kDa viva spin and resolved in 100 mM potassium phosphate buffer. Potassium phosphate buffer was also used for the main activity determination according to the different pH.

Table 6: pH-dependence evaluation of esterase activity.

pH	Average [U/mL]	Activity [mU/mL]	Standard deviation
6	0.013	12.59	0.001
7	0.063	63.08	0.002
8	0.058	58.39	0.002

4.4 SEQUENCING RESULTS

Characterization of fungi correlates with gathering acknowledge about the metabolism in the cells. Concerning this work, focus was put on the secretion of extracellular proteins. The supernatants of the different fermentations are collected and analyzed in different ways. With the interest of specifying the assays, the main bands obtained from the SDS-Gel are assigned to a specific protein.

Labelled bands in Figure 15 and Figure 18 were sent to Medical University of Graz for sequencing by mass spectrometry and following results were obtained. The outcome from the sequencing (Appendix Table 10) revealed a possible protein for band 1 and three different protein possibilities for band 2, on which special focus was laid on during this work. Taking the score into account (Table 7), it is most likely for B2 to be identified as the protein with the accession number A0A163KAJ3 and therefore with a pI of 9.8. Using the program Uniprot it was revealed that the enzyme exhibit hydrolase and o-glycosyl compounds hydrolyzing activity and is involved in carbohydrate metabolic processes. Sequence alignments with Blastp resulted in compliance with glycoside hydrolase (GH) family 16 proteins with a percentage identity of 51.06 %, E-value of 3e-69 and a query number of 99 % (Figure 22).

Table 7: Summary of the MS analysis results.

Band	Row	Accession number	pI	Scores
B1	1	A0A163MPU6	5.2	5977.2
B2	**8**	**A0A163KAJ3**	**9.8**	**894.6**
	9	A0A168NZT3	5.1	687.4
	10	A0A163MPU6	5.2	641.8

Sequence of B2 (A0A163KAJ3)

```
10         20         30         40         50         60
MKTDFRKSHK GWKALDSSKT YSFTKNGLEL KLLKPKKYVR KTDKASNLPY NVYPGEGATF
70         80         90         100        110        120
NYTTYLQYGS FSATMKPSTV GGAVTAFIGI GNGADEIDFE FLGGNSQNIQ SNYFWGKNIV
130        140        150        160        170        180
YGANGGVHAL PNKALPGSAY HKYTINWTPS QIQWSIDGKV IRTKTRASTK NKKGQYEYPT
190        200        210        220
QPLRIQLGLW DASTAPYTAQ WANGPINWSK QKASVTAYIR DVTITCPK
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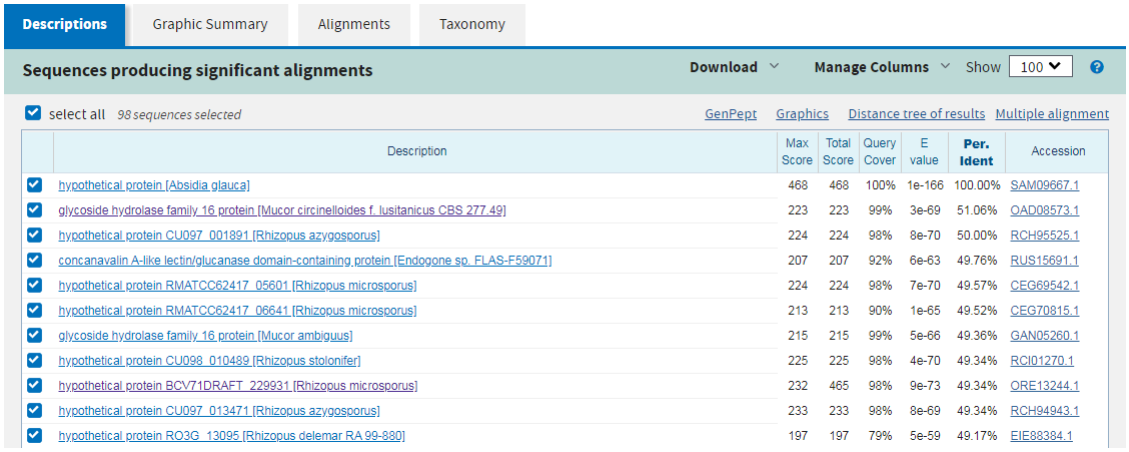

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download Manage Columns Show 100

select all *98 sequences selected* GenPept Graphics Distance tree of results Multiple alignment

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
hypothetical protein [Absidia glauca]	468	468	100%	1e-166	100.00%	SAM09667.1
glycoside hydrolase family 16 protein [Mucor circinelloides f. lusitanicus CBS 277.49]	223	223	99%	3e-69	51.06%	OAD08573.1
hypothetical protein CU097_001891 [Rhizopus azygosporus]	224	224	98%	8e-70	50.00%	RCH95525.1
concanavalin A-like lectin/glucanase domain-containing protein [Endogone sp. FLAS-F59071]	207	207	92%	6e-63	49.76%	RUS15691.1
hypothetical protein RMATCC62417_05601 [Rhizopus microsporus]	224	224	98%	7e-70	49.57%	CEG69542.1
hypothetical protein RMATCC62417_06641 [Rhizopus microsporus]	213	213	90%	1e-65	49.52%	CEG70815.1
glycoside hydrolase family 16 protein [Mucor ambiguus]	215	215	99%	5e-66	49.36%	GAN05260.1
hypothetical protein CU098_010489 [Rhizopus stolonifer]	225	225	98%	4e-70	49.34%	RCI01270.1
hypothetical protein BCV71DRAFT_229931 [Rhizopus microsporus]	232	465	98%	9e-73	49.34%	ORE13244.1
hypothetical protein CU097_013471 [Rhizopus azygosporus]	233	233	98%	8e-69	49.34%	RCH94943.1
hypothetical protein RO3G_13095 [Rhizopus delemar RA 99-880]	197	197	79%	5e-59	49.17%	EIE88384.1

Figure 22: Results from sequence alignment with Blastp. Displaying a percentage identity of the protein of interest with a glycoside hydrolase family 16 protein of 51.06 %. And a query cover of 99 %.

Based on those results two different paths were taken. On the one hand flask fermentations were carried out in order to reveal the possible existing glycoside hydrolase specificity and on the other hand purification of this obviously as GH family 16 member identified protein was tried to be achieved with ion exchange chromatography.

4.5 ION EXCHANGE CHROMATOGRAPHY

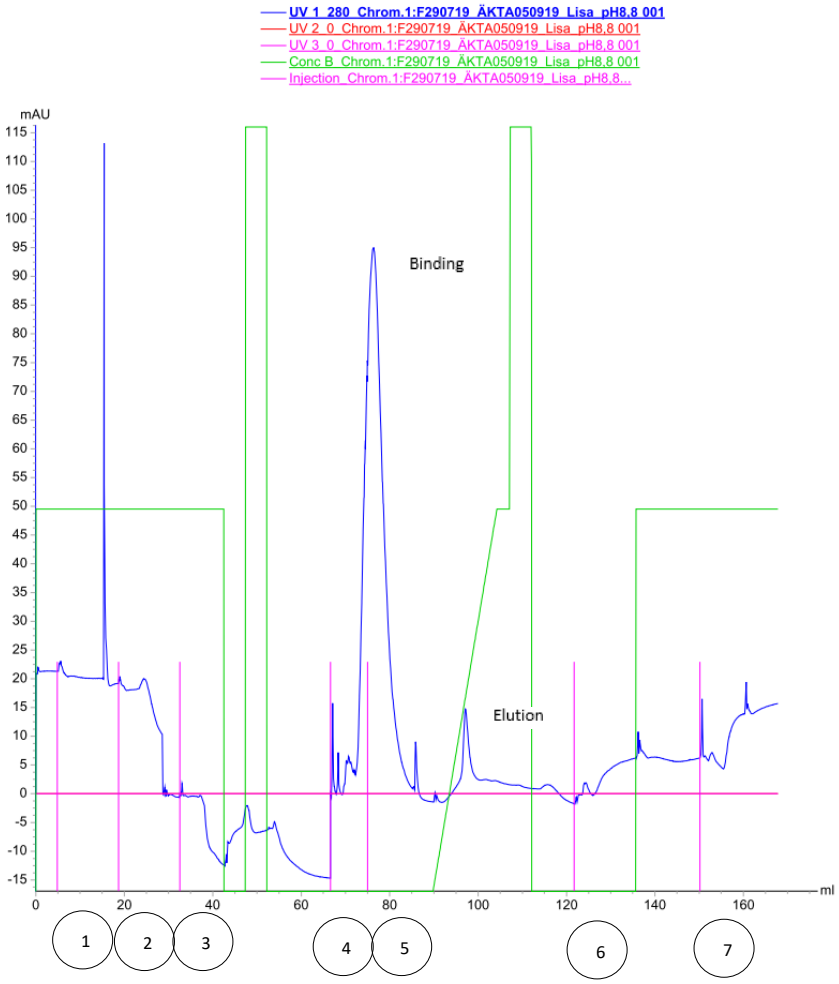

Figure 23: Ion exchange chromatogram with buffer pH 8.0 and labelled injections. The binding of the protein of interest is observed at around 75 mL with a peak maximum of 95 mAU, which means that the signal is due to the elution of the proteins not bound to the cationic column. The elution of the protein of interest itself is at around 95 mL with a peak maximum of approximately 15 mAU.

The chromatogram from the IEX chromatography with pH 9 is presented above. The pink injection lines indicate the different steps in the course of separation.

1) Washing the hose with ethanol
2) Washing the hose with water
3) Equilibration of the stationary phase with binding buffer Tris HCl pH9
4) Application of 2 mL sample
5) Changing the flow rate from elution buffer to 100 %

6) Washing the column with water
7) Washing the column with ethanol

The UV signal (blue line) increase at around 75 mL and the peak has its maximum at around 95 milli absorbance units (mAU). That peak represents the proteins not binding to the cationic stationary phase, which are eluting and collected by the auto sampler. After the signal reaches again the base line, the elution buffer (indicated by the green line) is applied and increases to 100 %. Regarding the UV-signal, it is noticeable that there is again a smaller peak at around 95 mL with a maximum absorbance at around 15 mAU. Correlating with the elution of the protein of interest with a pI of 9.8.

The different fractions were collected and after the overnight acetone precipitation the SDS PAGE was prepared and is presented below. Relying on the sequencing results (appendix Table 10) the proteins from band B1, B7 and B12 are supposed to have a pI below 9.0. Considering the buffer pH to be 9.0 these proteins, which are loaded negatively, aren't able to bind to the negative stationary phase and elute therefore directly after sample application.

As observable in the Figure 24 below, the protein of interest (band B2) with the pI of 9.8 is quantitatively most present in fraction B8. Comparing the fractions from elution with the ones from binding it can be argued that the protein with supposed glycoside hydrolase activity has considerably high affinity to the stationary phase as almost no band is visible in fractions A4-A7 at a molecular weight comparable to the proteins in fractions B. Next steps could be either the determination of the pI of the bands with lower molecular weight, adjustment of the buffer's pH and repetition of the chromatographic procedure. Or directly connection of the IEX chromatography with a hydrophobic interaction chromatography. Conceivable a higher separation can be achieved.

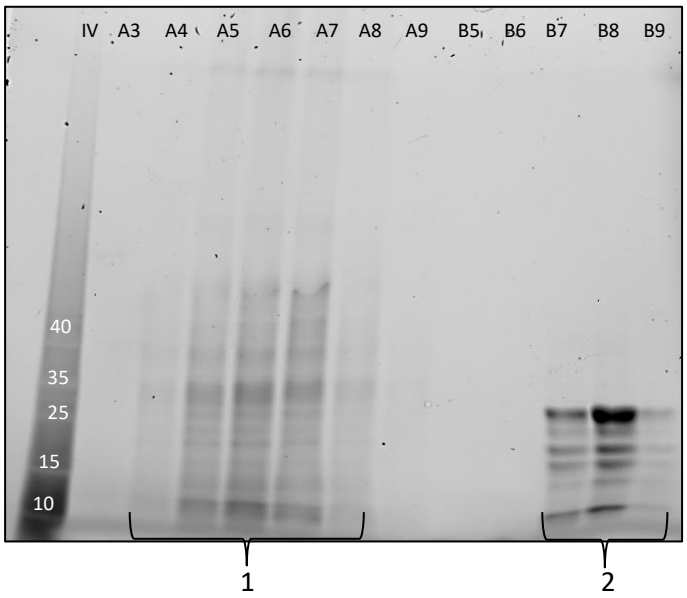

Figure 24: SDS electrophoretic protein separation of the different fractions obtained from IEX chromatography. Labels of the different bands indicate the different fractions from the ion exchange chromatography. Bracket 1 is the elution of the proteins not bound to the column and bracket 2 indicate the elution of the protein of interest.

4.6 FERMENTATIONS WITH DIFFERENT CARBON SOURCES

4.6.1 Shaking flask fermentation

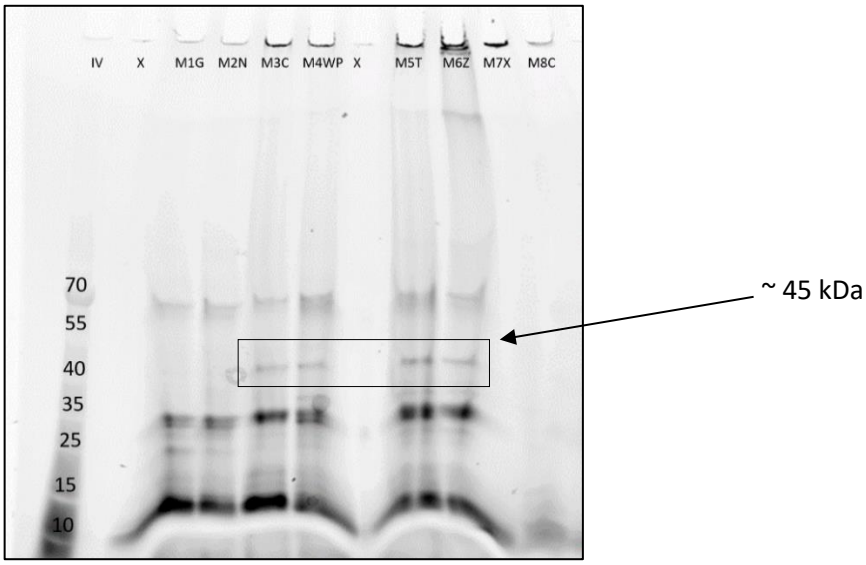

Figure 25: SDS PAGE results from fermentations with different carbon sources. Media contained peptone, malt extract, salts and the different carbon source. M1glucose, M2N-acetyl glucosamine, M3cellobiose, M4waste paper, M5brewerys grain, M6sugar beet pulp, M7xylan and M8 carragenan.

The results of the SDS PAGE, preceded an acetone precipitation, are presented in Figure 25. The different bands refer to fermentations with different carbon sources. The band occurring at around 45 kDa is induced with sources like cellobiose, waste paper, brewers' grain and sugar beets. It is not present in the fermentations with carbon sources like glucose, N-acetyl-glucosamine, xylan or carrageenan. The microorganism manages to grow with those carbon sources as well, but do not express that specific enzyme. Additionally, it is striking that at the row with glucose as carbon source (M1G), there is one band occurring below the supposed GH16 family protein. That one is not occurring at any other row that strong. Another point to mention is the bands are slightly smeared, especially in the lower part of the figure due to the high salt concentration in each sample, which negatively influences the electrophoretic separation. It was not possible to circumvent that issue for the preparation of this gel.

Those results initiated planning and performance of the next fermentation with the 1 L bioreactor containing brewers' grain as the only carbon source.

4.6.2 Bioreactor fermentation with brewer's grain as the only carbon source

F290919:

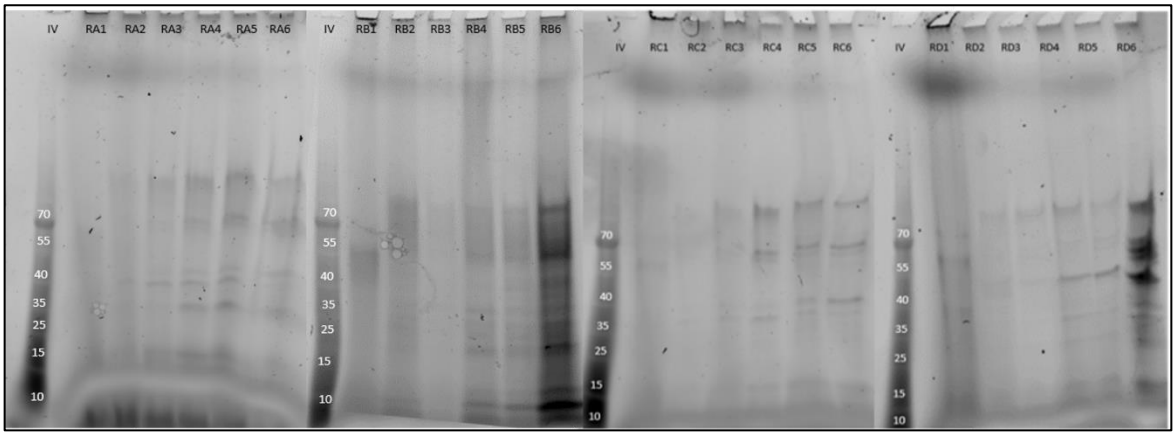

Figure 26: SDS PAGE of different bioreactor fermentation with the waste product brewers' grain as carbon source.

Four different fermentations were performed for 64 hours (Time point 6). Reactor A and C contain M1 and reactor B and D M2 from Figure 7, whereby the waste product brewers' grain is used instead of glucose. The environmental pH of RA and RB was controlled by the system and counteracted with the pumps, whereas RC and RD where continued without any control. Thus, the pH is also displayed in the fermentation diagram (Appendix Figure 31). The separation of the protein with gel electrophoresis was performed twice but it was hard to obtain an appropriate

separation and make any interpretations, because the bands are all shifted. The protocol for the sample preparation before loading to the gel was the same as with all the other fermentations, that's why no explanation can be given at this point.

4.7 EVALUATION OF CHITOSAN PRODUCTION FROM DIFFERENT FERMENTATIONS

Table 8: Overview of obtained biomass and chitosan yield. The grey background indicates four times performance of the same fermentation supplemented with the four different salts.

	Glucose (g/l)	K2HPO4 (g/l)	MgSO4 (g/l)	CaCl (g/l)	NaCl (g/l)	KNO3 (g/l)	NH4SO4 (g/l)	pH	CDW (g/L)	Yield CTS (%)	DA(%)
F230419											
A	2	1	5	1	1	-	5	4	7.94	0.31%	81.52
B	0	1	5	1	1	-	5	4	8.75	2.23%	51.71
C	2	-	-	-	-	5	-	4	8.60	4.33%	78.72
D	2	1	5	1	1	5	5	4	14.42	0.02%	75.49
F290419											
A	2	1	5	1	1	-	5	4	10.58	1.60%	52.08
C	2	1	5	1	1	5	-	4	13.62	0.66%	78.07
D	2	-	-	-	-	5	-	4	9.69	5.68%	87.89
F290719											
A	2	1	5	1	1	-	5	4	7.89	2.45%	55.99
B	2	1	5	1	1	-	5	4	8.38	1.93%	68.74

Results displayed in Table 8 show the summary of three fermentations and its biomass and chitosan yield. Concerning F230419 it can be stated that the fungi is able to produce the best yield of chitosan (4.33 %) in accordance with the absence of salts ($(NH_4)SO_4$, K_2HPO_4, $MgSO_4*7H_2O$, $CaCl*2H_2O$, NaCl, KNO_3). On the other hand, no proteins were secreted into the supernatant as shown in Figure 15. Additionally, concerning the fermentation without glucose but with maltose as carbon source instead (Reactor B), the fungi seems to produce chitosan (2.23 %). Figure 17 indicates that *A. glauca* does not completely utilize all maltose available in the medium and still 0.7 g/L of the carbohydrate are left. However, no proteins were determined by SDS PAGE separation. Noticeable, using both nitrogen sources (reactor D) during the fermentation the fungi is not able to build up high yields of chitosan (0.02 %) and express proteins.

Comparing all three fermentations in figure 24, it can be noted that the highest yield of chitosan is obtained during F290419 in reactor D, where the supplements included only potassium nitrate. In contrast to RD, RC shows a very low yield of chitosan (0.66 %). Only in reactor A proteins were found in the SDS PAGE Figure 18 with a chitosan yield of about 1.60 %.

Application of different media leads to variations in the degree of acetylation of chitosan. The presence of potassium nitrate correlates with a high degree of acetylation, not including reactor A from F230419, which is considered as outlier. Throughout the next fermentations using different waste products as carbon source different chitosan contents and deacetylation degrees were determined (Appendix Figure 35). As discussed previously, due to the outcome from SDS PAGE (Figure 25) the decision was taken to perform 1 L fermentations in the INFORS with brewers' grain. As presented in Table 9 , the chitosan content and DA was monitored with a rather high chitosan yield relating to the fermentations without any salts at all (reactor B and D). As already mentioned above, it is striking that not only the yield of chitosan varies significantly but also the degree of acetylation is far lower looking at the media without salts.

Table 9: Overview of chitosan yield and degree of acetylation of the fermentations including 2g/L brewers' grain as the only carbon source for the fungi.

	Brewers grain (g/l)	K2HPO4 (g/l)	MgSO4 (g/l)	CaCl (g/l)	NaCl (g/l)	KNO3 (g/l)	NH4SO4 (g/l)	pH	CDW (g/L)	Yield CTS (%)	DA(%)
F280919											
A	2	1	5	1	1	-	5	4	4.77	1.05%	81.96
B	2	-	-	-	-	-	-	4	4.95	3.94%	58.48
C	2	1	5	1	1	-	5	-	4.64	1.02%	89.89
D	2	-	-	-	-	-	-	-	2.88	4.95%	56.05

To sum it up, extraction of chitosan from *Absidia glauca* mycelium can be accomplished resulting in different outcomes depending on different media components. The focus of this work was primarily on the expression of proteins. Interpretations of the biomass and chitosan yield results led to the recognition that best yields of chitosan do not correlate with the expression and secretion of proteins into the supernatant. But by using the media M1 in Figure 7 the induction of protein expression and a reasonable yield of 1.99 ± 0.01 % chitosan and DA of 58.94 ± 7.11 % was achieved.

5 CONCLUSION AND OUTLOOK

In this thesis, the expression and secretion of different proteins from the chitosan producing fungi *Absidia glauca* into the culture supernatant was investigated. It is hard to state any comparison of the obtained results with literature values. Not much is yet known about any characteristic of the fungus or about expression of proteins. The main promising and already known characteristic of *A. glauca* is the production of chitosan. There is already published data from chitosan extraction using a two-step extraction method yielding in a higher quality chitosan compared to a single step alkali extraction [52]. This feature of the microorganism was also the main background idea for choosing it for the experiment. As results show, we accomplished to obtain a yield of 1.99 ± 0.01 % chitosan with a degree of acetylation of 58.94 ± 7.11 %.

For the production of large amounts of *A. glauca* biomass and later on chitosan, great volume of supernatant is obtained. To implement a closed process where all products are utilized, the identification of secreted enzymes was carried out. Testing the supernatant on esterase activity with p-NPB ((p-nitrophenyl butyrate) led to a positive result with pH optimum at 7. Sequencing results revealed that induction of protein expression can be established by using a medium containing ammonium sulfate, dipotassium phosphate, magnesium sulfate heptahydrate, calcium chloride dihydrate and sodium chloride. With ion exchange chromatography, separation of the protein bands was obtained.

Fermentation with different carbon sources showed that this microorganism is able to grow on industrial waste product as good as with glucose as energy source. In connection with further experiments for production of chitosan those waste substances, in special brewers' grain, can be used instead of glucose in order to obtain high amounts of biomass with high yield of chitosan.

In conclusion, the microorganism *A. glauca* secretes a moderate amount of enzymes to the supernatant with batch fermentation technique by using a defined media containing 5 different salts. Further investigation i.e. characterization of proposed GH family 16 protein or improvement of the chitosan yield in accordance with protein expression is necessary.

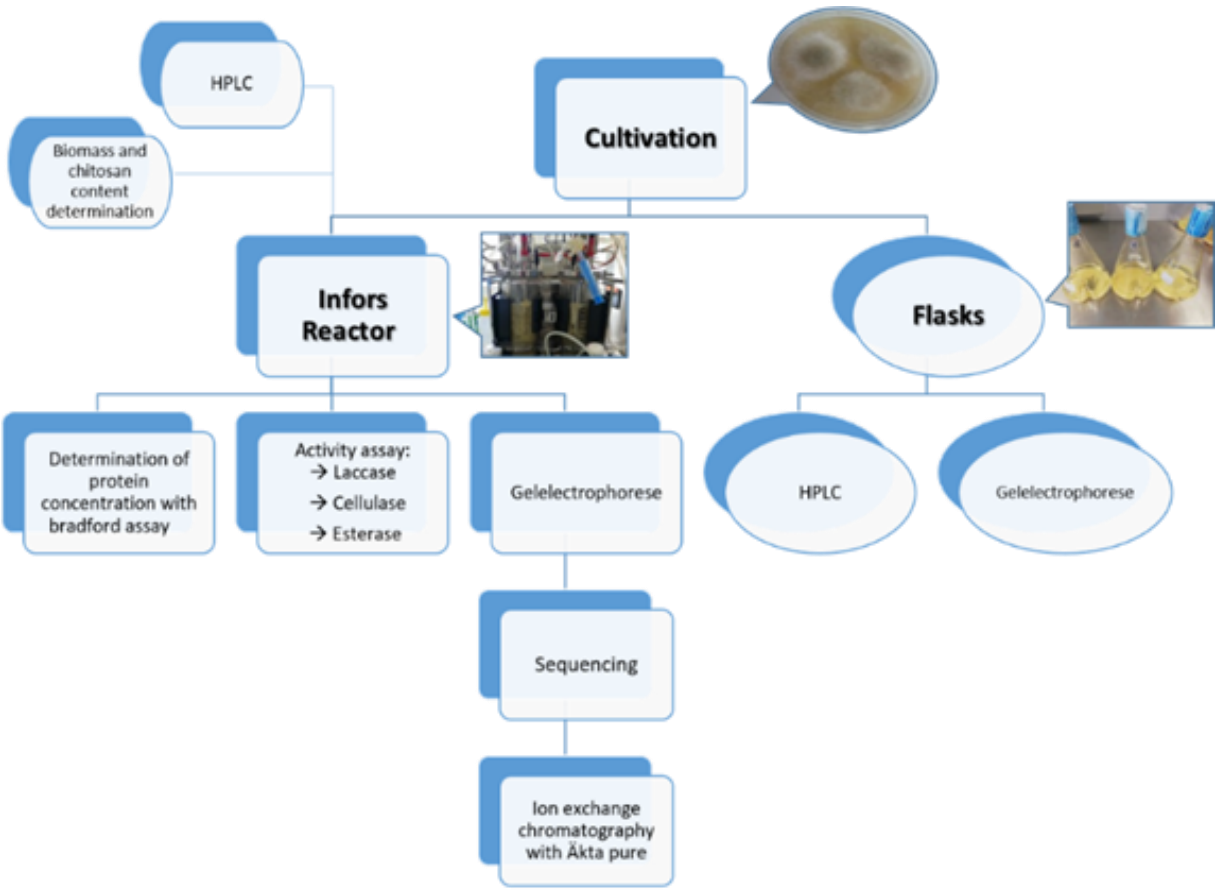

Figure 27: Overview of the performed steps according to this work.

6 APPENDIX

6.1 F230419

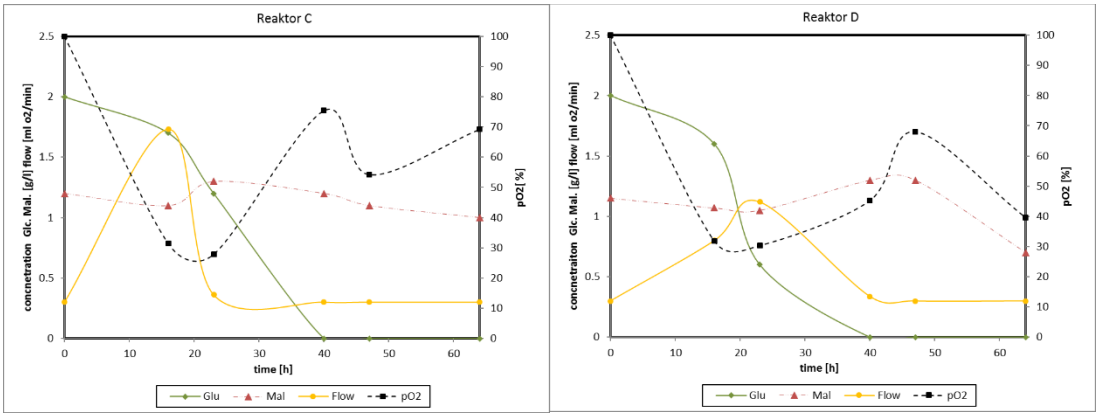

Figure 28: Fermentation diagram of F230419 from reactor C and Reactor D.

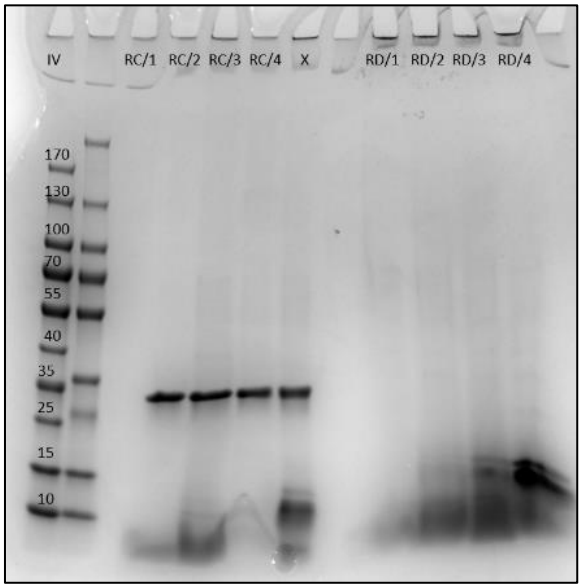

Figure 29: SDS PAGE from different time points of reactor C and D. Bands between 25 and 35 kDa concerning RC represents Endo H which was not added to the different time points from reactor D.

6.2 F290419

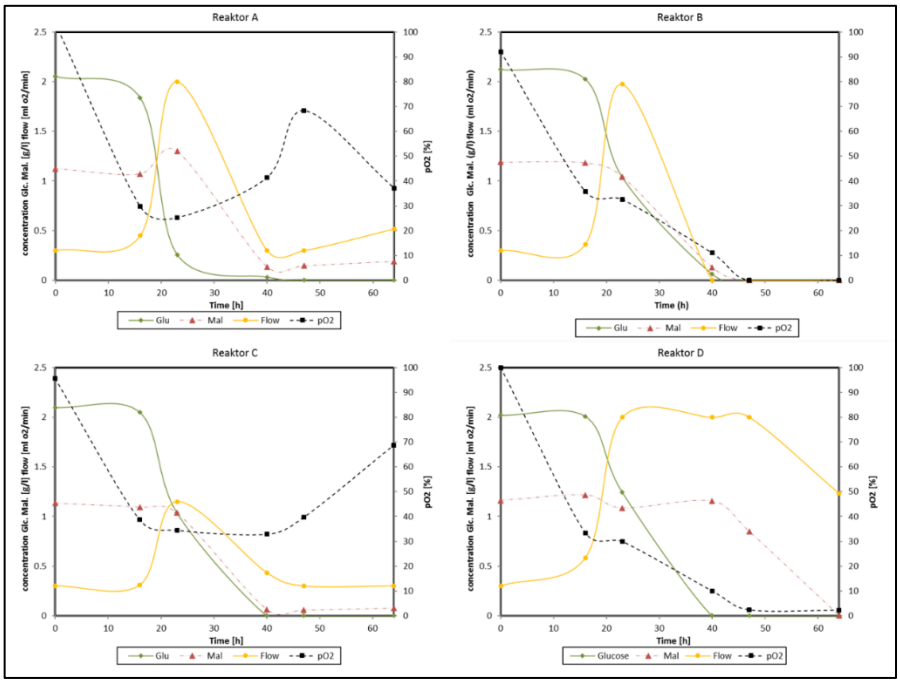

Figure 30: Fermentation diagram of F290419.

6.3 FERMENTATION WITH BREWERS GRAIN AS CARBON SOURCE

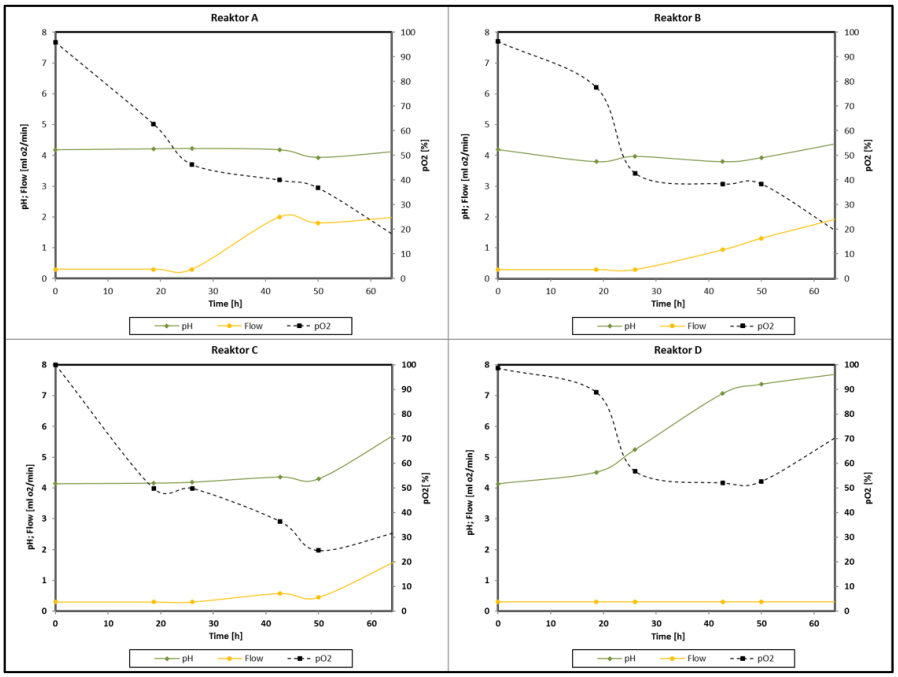

Figure 31: Fermentation diagram of bioreactor fermentation with brewers' grain as carbon source.

6.4 SEQUENCING RESULTS

Table 10: Sequencing results from Graz.

Row	User Flag	OK	Accession	Protein	MW [kDa]	pI	#Alt. Proteins	Scores	#Peptides	SC [%]	RMS90 [ppm]	Rank
B1												
1		WAHR	A0A163MPU6	Uncharacterized protein OS=Absidia glauca OX=4829 GN=ABSGL_13012.1 scaffold 13554 PE=4 SV=1	51,3228976	5,20017242	1	5977.2 (M.expect:0.0,M.score:5977.2,M.siglimit:16.0)	53	69,3277311	0,704435312	1
B2												
8		WAHR	A0A163KAJ3	Uncharacterized protein OS=Absidia glauca OX=4829 GN=ABSGL_15368.1 scaffold 16614 PE=4 SV=1	25,3609729	9,80065155	2	894.6 (M.expect:0.0,M.score:894.6,M.siglimit:13.0)	13	42,9824561	1,039049758	11
9		WAHR	A0A168NZT3	Uncharacterized protein OS=Absidia glauca OX=4829 GN=ABSGL_07259.1 scaffold 8717 PE=3 SV=1	39,7178876	5,11205292	2	687.4 (M.expect:0.0,M.score:687.4,M.siglimit:13.0)	16	39,84375	0,962972335	1
10		WAHR	A0A163MPU6	Uncharacterized protein OS=Absidia glauca OX=4829 GN=ABSGL_13012.1 scaffold 13554 PE=4 SV=1	51,3228976	5,20017242	2	641.8 (M.expect:0.0,M.score:641.8,M.siglimit:13.0)	26	49,1596639	0,901849962	2
B7												
4		WAHR	A0A163J7L3	Uncharacterized protein OS=Absidia glauca OX=4829 GN=ABSGL_04146.1 scaffold 5122 PE=4 SV=1	21,407659	8,31266022	2	300.8 (M.expect:0.0,M.score:300.8,M.siglimit:13.0)	16	66,6666667	1,315480649	7
10		WAHR	A0A163JPE9	Uncharacterized protein OS=Absidia glauca OX=4829 GN=ABSGL_06569.1 scaffold 8461 PE=3 SV=1	39,7729046	4,80464935	1	61.5 (M.expect:0.0,M.score:61.5,M.siglimit:13.0)	4	16,4921466	0,651571545	11
11		WAHR	A0A163JV01	Uncharacterized protein OS=Absidia glauca OX=4829 GN=ABSGL_09099.1 scaffold 10677 PE=4 SV=1	30,8217113	9,11716461	1	41.1 (M.expect:1.2,M.score:41.1,M.siglimit:13.0)	3	8,57142857	0,851617415	12
B12												
7		WAHR	A0A163JTP8	Uncharacterized protein OS=Absidia glauca OX=4829 GN=ABSGL_07633.1 scaffold 8929 PE=4 SV=1	15,3676608	8,96324921	2	147.3 (M.expect:0.0,M.score:147.3,M.siglimit:13.0)	7	57,2413793	1,068684394	10
8		WAHR	A0A168NIR9	Uncharacterized protein OS=Absidia glauca OX=4829 GN=ABSGL_06331.1 scaffold 8126 PE=4 SV=1	14,033892	7,60652924	1	141.3 (M.expect:0.0,M.score:141.3,M.siglimit:13.0)	3	28,125	1,863391837	7
9		WAHR	A0A163JBR0	Uncharacterized protein OS=Absidia glauca OX=4829 GN=ABSGL_03823.1 scaffold 4673 PE=4 SV=1	15,3191224	8,12146759	1	119.1 (M.expect:0.0,M.score:119.1,M.siglimit:13.0)	10	48,951049	2,095510548	8

6.5 OVERVIEW OF MEDIA FOR ALL DESCRIBED FERMENTATIONS

	F230419 Ingredients [g/L]										
Medium	Peptone	Maltextract	Glucose	(NH4)SO4	K2HPO4	MgSO4*7H2O	CaCl*2H2O	NaCl	KNO3	Urea	pH value
M1	5	2.5	2	5	1	5	1	1			4
M2	5	2.5		5	1	5	1	1			4
M3	5	2.5	2						5		4
M4	5	2.5	2	5	1	5	1	1	5		4

	F290419 Ingredients [g/L]										
Medium	Peptone	Maltextract	Glucose	(NH4)SO4	K2HPO4	MgSO4*7H2O	CaCl*2H2O	NaCl	KNO3	Urea	pH value
M1	5	2.5	2	5	1	5	1	1			4
M2	5	2.5	2	5							
M3	5	2.5	2		1	5	1	1	5		4
M4	5	2.5	2						5		4

	F290719 Ingredients [g/L]										
Medium	Peptone	Maltextract	Glucose	(NH4)SO4	K2HPO4	MgSO4*7H2O	CaCl*2H2O	NaCl	KNO3	Urea	pH value
M1	5	2.5	2	5	1	5	1	1			4
M2	5	2.5	2	5	1	5	1	1			4

	Treber Fermentation Ingredients [g/L]										
Medium	Peptone	Maltextract	Treber	(NH4)SO4	K2HPO4	MgSO4*7H2O	CaCl*2H2O	NaCl	KNO3	Urea	starting pH value
M1	5	2.5	2	5	1	5	1	1			4
M2	5	2.5	2								4
M3	5	2.5	2	5	1	5	1	1			4
M4	5	2.5	2								4

	Solid State Fermentation Ingredients [g/L]											
Medium	Peptone	Maltextract	Glucose	Agar	(NH4)SO4	K2HPO4	MgSO4*7H2O	CaCl*2H2O	NaCl	KNO3	Urea	pH value
M1	5	2.5	2	20								4.4
M2	5	2.5	2	20	5	1	5	1	1			4.4
M3	5	2.5	0	20								4.4
M4		2.5	0	20	5	1	5	1	1			4.4
M5	5	2.5	2	20						5		4.4
M6	5	2.5	2	20	5	1	5	1	1	5		4.4
M7	5	2.5	2	20							7.2	4.4
M8	5	2.5	2	20	5	1	5	1	1		7.2	4.4
Potato glucose M9			20									4.4

	Different salt ingredients [g/L]							
Medium	Peptone	Maltextract	Glucose	(NH4)SO4	K2HPO4	MgSO4*7H2O	CaCl*2H2O	NaCl
M1	5	2.5	2	5	1	5	1	1
M2	5	2.5	2					
M3	5	2.5	2	5				
M4	5	2.5	2		1			
M5	5	2.5	2			5		
M6	5	2.5	2				1	
M7	5	2.5	2					1

	Non - Varying Ingredients [g/L]							**Different Carbon sources [g/L]**							
Medium	Peptone	Maltextract	(NH4)SO4	K2HPO4	MgSO4*7H2O	CaCl*2H2O	NaCl	Glucose	N-Acetylglucosamin	Cellobiose	Waste paper	Brewers grain	Sugar beets	Xylan	Carragen
M1G	5	2.5	5	1	5	1	1	2							
M2N	5	2.5	5	1	5	1	1		2						
M3C	5	2.5	5	1	5	1	1			2					
M4WP	5	2.5	5	1	5	1	1				2				
M5T	5	2.5	5	1	5	1	1					2			
M6Z	5	2.5	5	1	5	1	1						2		
M7X	5	2.5	5	1	5	1	1							2	
M8Carrageenan	5	2.5	5	1	5	1	1								2

6.6 FT-IR OF CHITOSAN EXTRACTED FROM A. GLAUCA MYCELIUM

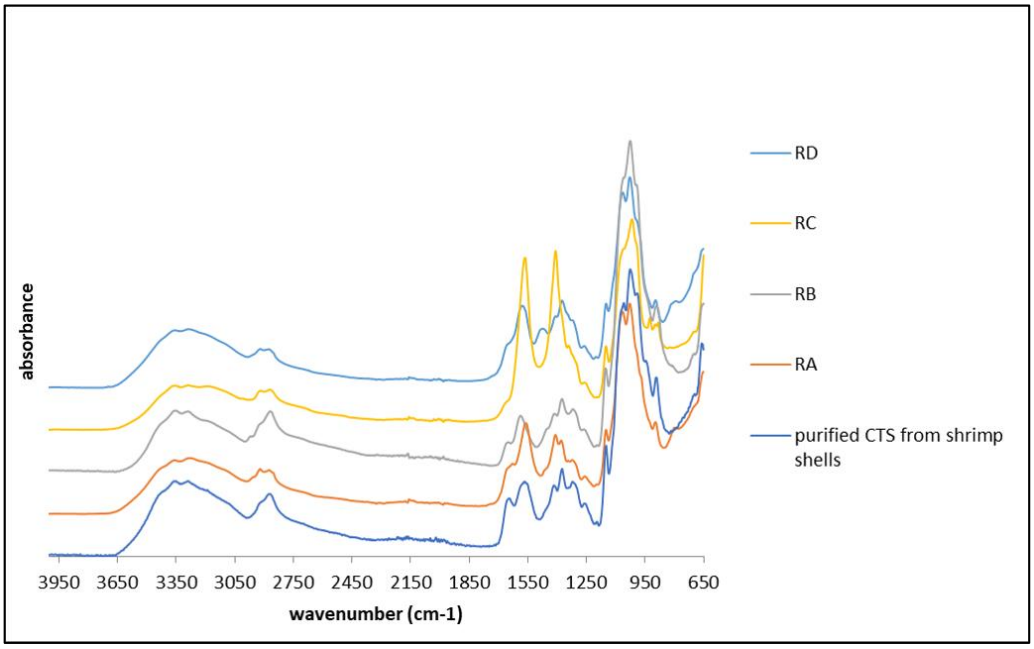

Figure 32: FT-IR from F230419.

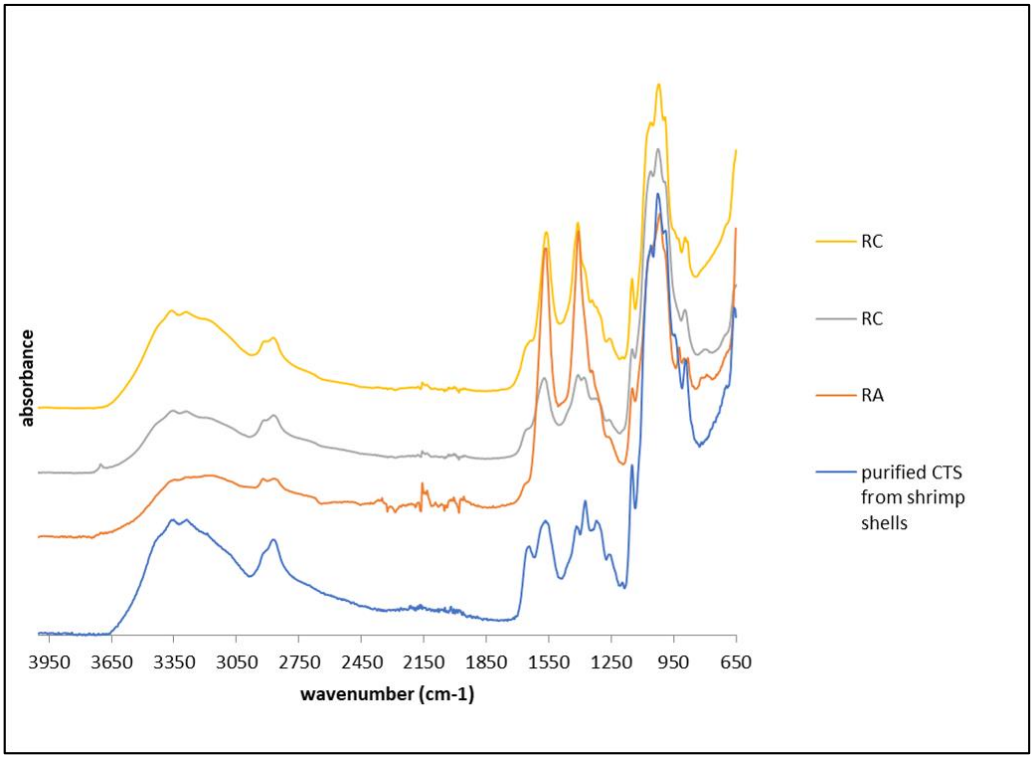

Figure 33: FT-IR from F290419.

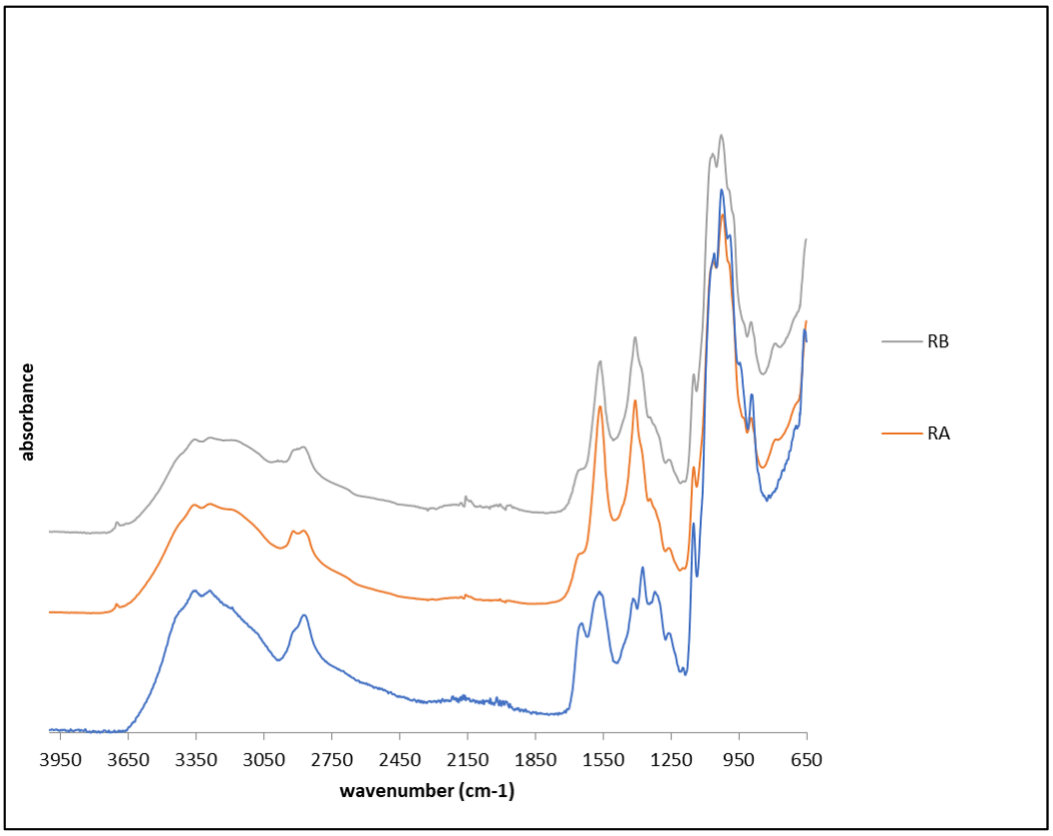

Figure 34: FT-IR from F290719.

Table 11: Chitosan yields and its degree of acetylation from A. glauca mycelium after fermentation with 8 different energy sources.

	CDW (g/L)	yield CTS (%)	DA
M1 Glucose	10.56 +/- 1.42	1.06 +/- 0.01	88.94 +/- 0.00
M2 N-acetylglucosamine	11.27 +/- 1.57	2.56 +/- 0.01	49.525 +/- 10.49
M3 Cellobiose	10.86 +/- 0.46	2.12 +/- 0.01	86.75 +/- 0.00
M4 waste paper	11.53 +/- 0.81	5.79 +/- 0.01	73.85 +/- 5.70
M5 Treber	12.16 +/- 0.55	1.96 +/- 0.47	71.39 +/- 6.50
M6 Zuckerrüben	12.45 +/- 0.05	3.82 +/- 0.30	59.71 +/- 26.47
M7 Xylane	10.32 +/- 1.22	2.98 +/- 0.12	88.28 +/- 16.57
M8 Carrageenan	11.23 +/- 2.25	2.88 +/- 0.12	67.85 +/- 20.91

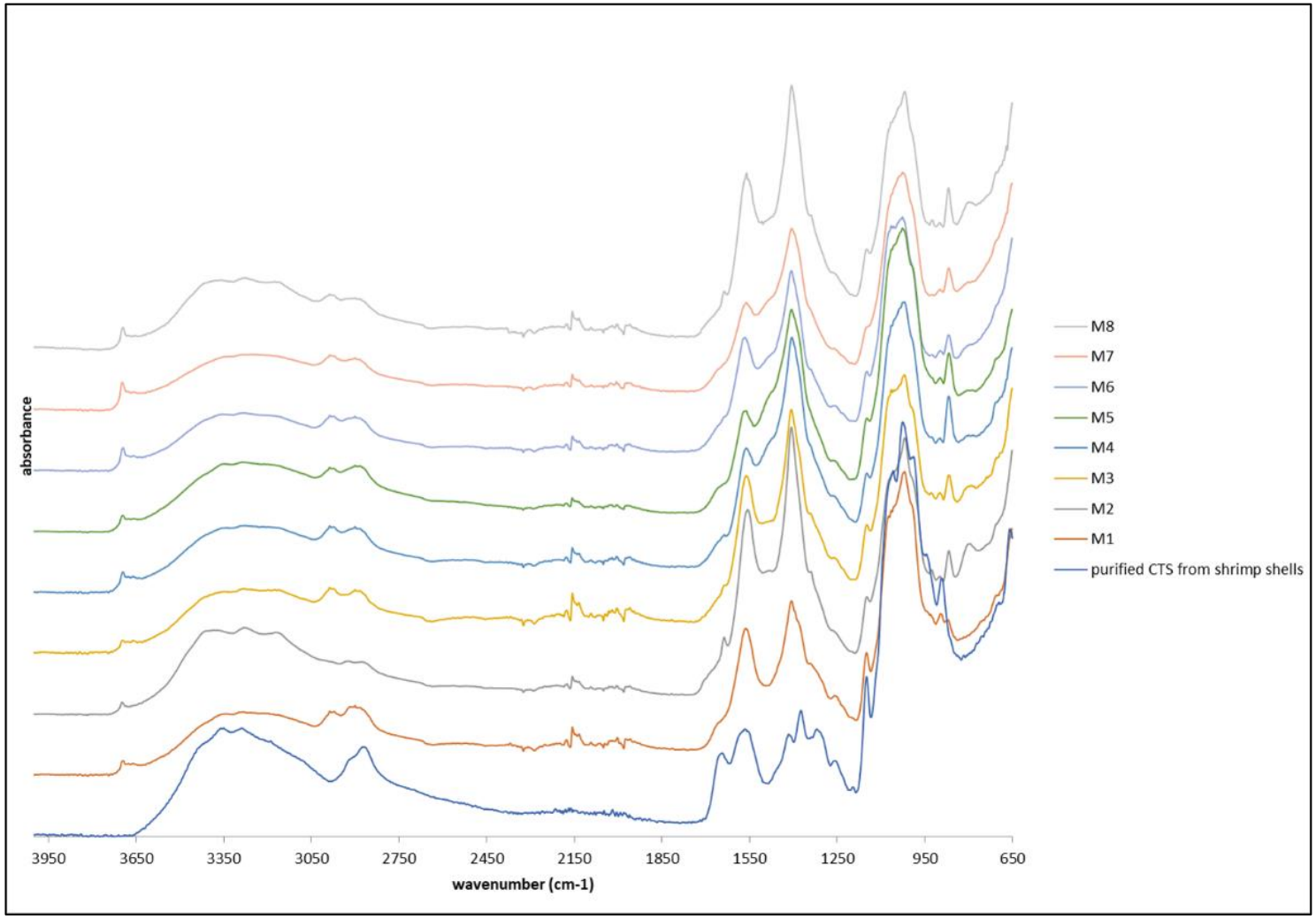

Figure 35: FT-IR from shaking flasks containing different waste products as energy source.

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