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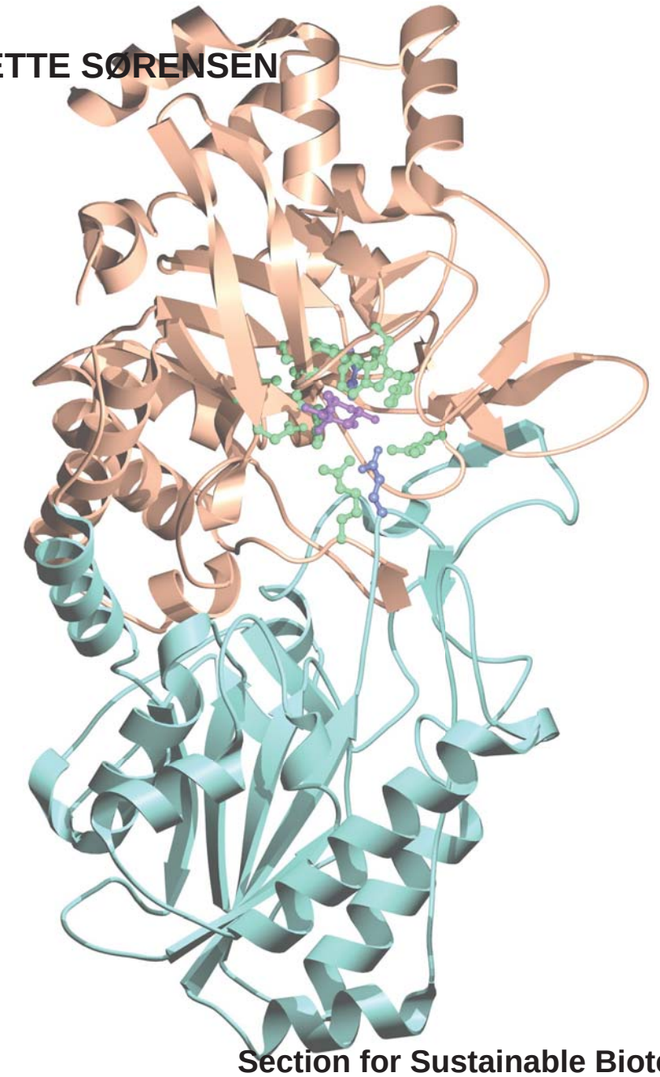

Section for Sustainable Biotechnology
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A NEW HIGHLY EFFICIENT BETA- GLUCOSIDASE FROM THE NOVEL SPECIES, *ASPERGILLUS SACCHAROLYTICUS*

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Cover:
Ribbon cartoon representation of the homology model of the catalytic module of beta-glucosidase BGL1 from *Aspergillus saccharolyticus*. The figure was provided by Dr. Wimal Ubhayasekera

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Annette Sørensen
August 2010, Denmark

THESIS STRUCTURE

Please notice that the thesis has been organized as a short summary in the beginning followed by a collection of journal manuscripts, consisting of a review paper and four research papers, ending with brief concluding remarks. The individual manuscripts are printed in a layout form consistent with the journal for which the individual manuscripts are intended. The work carried out during this Ph.D. study has resulted in a patent application, of which the summary and claims are included in the thesis.

THESIS CONTENT

SUMMARY

In a biorefinery concept, biomass polymers of cellulose and hemicellulose are converted into sugars, which can be used for production of biofuels and biochemicals, which could act as platform molecules serving as building blocks in the synthesis of chemicals and polymeric materials. This is a sustainable solution that is expected to replace today's oil refineries.

Main components of lignocellulosic biomass, primarily consisting of plant cell walls, are cellulose, hemicellulose, and lignin. Prior to enzymatic hydrolysis for generating sugar monomers, the biomass is pretreated. The pretreatment mainly opens up the cell wall structure and partly hydrolyzes hemicellulose, so that cellulose is the main target for enzyme hydrolysis. Beta-glucosidases (EC 3.2.1.21) play an essential role in efficient hydrolysis of cellulose. By hydrolysis of cellobiose, beta-glucosidases relieve inhibiting conditions, allowing for increased hydrolysis of cellulose by cellobiohydrolases and endoglucanases. Efficient hydrolysis requires high conversion rates throughout the hydrolysis. The major factors influencing this are product inhibition and temperature stability.

Traditionally, the commercial enzyme preparations Novozym 188 (mainly beta-glucosidase activity) and Celluclast 1.5L (mainly cellobiohydrolase and endoglucanase activity) (Novozymes A/S) have been used in combination for hydrolysis of pretreated biomass, and recently complete enzyme cocktails have been launched, Cellic CTec (Novozymes A/S) and AcceleraseDUET (Genencor A/S). The enzyme preparations by Novozymes A/S are used as benchmarks in the following research.

Superior enzymes can be obtained either by discovery of new enzymes through different screening strategies or by improvement of known enzymes mainly by different molecular methods.

Initially, we employed a screening strategy using different lignocellulosic materials for discovery of new enzymes to be used in an onsite enzyme production concept during bioethanol production. Different fungi were applied in this screening, mainly fungi isolated from different woody habitats. A low value stream of a cellulosic ethanol production was explored as enzyme production medium, finding *Aspergillus niger* as well as an unidentified strain AP as promising candidates for the utilization of the filter cake for growth and enzyme production. The filter cake inoculated with the respective fungi could, combined with Celluclast 1.5L, substitute the use of Novozym 188 in hydrolysis of pretreated biomass. In the wake of this, focus was placed on beta-glucosidases.

A screening for beta-glucosidase activity was conducted, using wheat bran as substrate in simple submerged fermentation, testing selected strains from the previous screening as well as new isolated strains and strains from our in-house strain collection, including *A. niger*. The strain AP showed significantly greater potential in beta-glucosidase activity than all other fungi screened. The beta-glucosidase activity of a solid state fermentation extract of strain AP was compared with Novozym 188 and Cellic CTec. In terms of cellobiose hydrolysis, the extract of strain AP was found to be a valid substitute for Novozym 188, corresponding to the previous result where filter cake inoculated with the fungus was directly used in hydrolysis of pretreated biomass. The Michaelis Menten kinetics affinity constants of strain AP extract and Novozym 188 were approximately the same, and the two preparations performed equally well in cellobiose hydrolysis with regard to product inhibition. However, the extract of strain AP showed higher specific activity (U/total protein) as well as increased thermostability. The significant thermostability of strain AP beta-glucosidases was further confirmed when compared with Cellic CTec. The extract of strain AP facilitated hydrolysis of cellodextrins with an exo-acting approach, and was, when combined with Celluclast 1.5L, able to contribute to the generation of a sugar platform from pretreated bagasse, by hydrolyzing the biomass to monomeric sugars.

Strain AP was from a polyphasic taxonomic approach identified as a yet undescribed uniseriate *Aspergillus* species belonging to section *Nigri*. Morphologically, at first glance strain AP resembles *A. japonicus*. However, in detailed phenotypic analysis, strain AP distinguished itself from the other uniseriate aspergilli, both in terms of growth characteristics on different media as well as temperature tolerance. Futhermore, the extrolite production of strain AP differed significantly from other known aspergilli in section *Nigri*, as several well-known compounds from this series were not present, and the peaks detected did not match the approximately 13500 fungal extrolites in the natural product chemist's database, Antibase2010. Genotypic analysis of the ITS region, partial beta-tubuline gene, and partial calmodulin gene placed strain AP on a separate branch in phylogenetic trees prepared with other black aspergilli, and universally primed PCR furthermore readily distinguished strain AP data from other black aspergilli. We named the novel species *A. saccharolyticus*, referring to its ability to hydrolyze cellobiose and cellodextrins, and it was deposited in the strain collection of Centraalbureau voor Schimmelcultures (CBS 127449^{T}).

Identification, isolation, and characterization of the most prominent beta-glucosidase from *A. saccharolyticus* were undertaken. The extract from this fungus, mentioned above, was fractionated by ion exchange chromatography, obtaining fractions pure enough that a specific SDS-page gel protein band of high beta-glucosidase activity could be excised and analyzed by LC-MS/MS. Using the peptide matches for design of degenerate primers, the beta-glucosidase gene, *bgl1*, of *A. saccharolyticus* was cloned. The 2919 bp genomic sequence encodes a 680 aa polypeptide which has 91% and 82% identity with BGL1 from *A. aculeatus* and BGL1 from *A. niger*, respectively. BGL1 of *A. saccharolyticus* was identified as belonging to GH family 3. A three dimentsional structure was proposed through homology modeling, finding retaining enzyme characteristics and, interestingly, a more open catalytic pocket compared to other beta-glucosidases. A cloning vector for heterologous expression of *bgl1* in *Trichoderma reesei* was constructed, combining promoter, gene, and terminator of different eukaryotic origin. Codons coding for histidine residues were included in the 3'end of the gene to assist purification. The purified BGL1 was assayed for beta-glucosidase activity, studying enzyme kinetics, temperature and pH profiles, glucose tolerance, and cellodextrin hydrolysis. A striking similarity was found when comparing the data of purified BGL1 to the data previously reported of the crude extract, indicating that we have successfully cloned and expressed the protein mainly contributing to the beta-glucosidase activity observed in the crude extract, thus the most prominent beta-glucosidase of *A. saccharolyticus*.

DANSK SAMMENFATNING

Værdien af lignocelluloseholdig biomasse bliver i et bioraffinaderikoncept maksimeret ved frembringelse af en sukkerplatform fra biomassens kulhydrater. Denne sukkerplatform kan anvendes til produktion af biobrændstoffer og biomolekyler, som kan være byggesten i syntesen af kemikalier og polymere materialer. Dette er en bæredygtig løsning, som kan erstatte nutidens olieraffinaderier.

Lignocelluloseholdig biomasse består primært af plantecellevægge. Hovedkomponenterne er cellulose, hemicellulose og lignin. For at danne en sukkerplatform fra biomasse, forbehandles biomassen og hydrolyseres derefter. Forbehandlingen åbner primært cellevægstrukturen op og hydrolyserer delvist hemicellulosen, således at cellulose er primær genstand for den efterfølgende enzymatiske hydrolyse. Beta-glucosidaser (EC 3.2.1.21) spiller en vigtig rolle i effektiv hydrolyse af cellulose. Ved at hydrolysere cellobiose fjerner beta-glucosidase de inhiberende forhold, der ellers ville begrænse cellobiohydrolase- og endoglucanase-aktiviten, og muliggør derved øget hydrolyse af cellulose. Effektiv hydrolyse kræver høj omsætningsrate under hele hydrolysen. Faktorer, der særligt kan influere på dette, er produktinhibering og temperaturstabilitet.

Traditionelt set har de kommercielle enzymprodukter Novozym 188 (primært beta-glucosidase-aktivitet) og Celluclast 1.5L (primært cellobiohydrolase- og endoglucanase-aktivitet) (Novozymes A/S) været brugt i kombination til at hydrolysere forbehandlet biomasse, og for nyligt er nye komplette enzymblandinger blevet lanceret: Cellic CTec (NovozymesA/S) og AcceleraseDUET (Genencor A/S). Enzymprodukterne fra Novozymes A/S vil løbende blive brugt som sammenligningsgrundlag i den her præsenterede forskning.

Bedre enzymer kan fremskaffes enten ved opdagelse af nye enzymer gennem forskellige screeningsstrategier eller ved forbedring af kendte enzymer primært ved forskellige molekylære teknikker.

Indledningsvist benyttede vi en screeningsstrategi, hvor forskellige lignocelluloseholdige komponenter blev anvendt til at lede efter nye enzymer til brug i et on-site enzymproduktionskoncept indenfor biobrændstofproduktion. Forskellige svampe blev testet i denne screening, hvoraf de fleste var isoleret fra naturens biomasseholdige habitater. Et restprodukt, filterkage, fra cellulose ethanolproduktion af lav kommerciel værdi blev undersøgt som enzymproduktionsmedie. *Aspergillus niger* så vel som en uidentificerbar stamme AP viste sig at være lovende kandidater for anvendelsen af filterkagen til vækst- og enzymproduktion. Filterkagen inokuleret med de respektive svampe kunne i kombination med Celluclast 1.5L erstatte brugen af Novozym 188 i hydrolyse af forbehandlet biomasse. Som følge af dette blev fokus rettet på beta-glucosidaser.

Screening for beta-glucosidase-aktivitet blev udført ved brug af hvedeklid som substrat i simpel væskefermentering. Isolater fra den tidligere screening, nyligt isolerede stammer samt isolater fra vores egen stammesamling blev testet, inkluderende *A. niger*. Stammen AP viste betydeligt større beta-glucosidase-aktivitetspotentiale end alle andre testede stammer. Beta-glucosidase-aktiviteten af et fermenteringsekstrakt af stammen AP er sammenlignet med Novozym 188 og Cellic CTec. Vi fandt, at stammen AP kan erstatte Novozym 188 inden for hydrolyse af cellobiose, hvilket stemmer over ens med de tidligere resultater, hvor filterkage inokuleret med svampen direkte blev brugt i hydrolyse af forbehandlet biomasse. Michaelis-Menten kinetik affinitetskonstanten for ekstraktet fra strammen AP og for Novozym 188 var cirka ens, og med hensyn til produktinhibering var effekten af øget glucosemængde omtrent den samme. Ekstraktet fra stammen AP viste dog højere specifik aktivitet (U/total protein) såvel som øget termostabilitet. Den signifikante termostabilitet af beta-glucosidaser fra stammen AP blev yderligere bekræftet ved sammenligning med Cellic CTec. Ekstraktet fra stammen AP kunne via exo-

aktivitet hydrolysere cellodextriner og kunne sammen med Celluclast 1.5L hydrolysere forbehandlet bagasse til sukkermonomerer og derved danne en sukkerplatform fra biomasse.

Stammen AP blev taksonomisk identificeret som en hidtil ubeskrevet uniseriat *Aspergillus* art tilhørende sektion *Nigri*. Morfologisk ligner stammen AP ved første øjekast *A. japonicus*, men ud fra detaljeret fænotypisk analyse adskilte stammen AP sig fra de andre uniseriate aspergilli, både i forhold til vækstkarakteristika på forskellige medier samt temperaturtolerance. Ydermere adskilte stammen AP sig signifikant fra andre kendte aspergilli i sektion *Nigri* ved dens sekundære metabolitproduktion, da adskillige kendte stoffer inden for denne sektion ikke kunne detekteres, og de toppe, som var til stede, matchede ikke de omtrent 13500 metabolitter fra svampe, der findes i databasen for naturprodukt kemikere, Antibase2010. Genotypisk analyse af ITS regionen, det partielle beta-tubulin gen, og det partielle calmodulin gen placerede stammen AP på en separat gren i de fylogenetiske træer, som var udarbejdet med andre sorte aspergilli. Universal-primed PCR adskilte yderligere data for stammen AP fra andre sorte aspergilli. Vi navngav den nye art *A. saccharolyticus*, hvilket refererer til dens evne til at hydrolysere cellobiose og cellodextriner. Stammen blev deponeret i stammesamling Centraalbureau voor Schimmelcultures (CBS 127449^{T}).

Identificering, isolering og karakterisering af den fremstående beta-glucosidase fra *A. saccharolyticus* blev iværksat. Ekstraktet fra svampen, beskrevet og analyseret ovenfor, blev ved ionkromatografi fraktioneret, hvorved rene nok fraktioner blev opnået, således at et specifikt proteinbånd med høj beta-glucosidase-aktivitet kunne skæres ud fra SDS-page gel og analyseres ved LC-MS/MS. Ved at benytte peptid-matchene til design af degenererede primere blev beta-glucosidase genet, *bgl1*, klonet. Den 2919 bp genomiske sekvens koder for et 680 aa polypeptid, der har hhv. 91% and 82% identitet med BGL1 fra *A. aculeatus* og BGL1 fra *A. niger*. BGL1 fra *A. saccharolyticus* blev identificeret til at tilhøre GH familie 3. En tredimensionel struktur blev foreslået ud fra homologimodellering, hvor "retaining" enzymkarakteristika blev bestemt. Det er bemærkelsesværdigt, at en mere åben katalytisk lomme blev sandsynliggjort for BGL1 relativt til andre beta-glucosidaser. En kloningsvektor blev konstrueret til benyttelse indenfor heterolog ekspression af *bgl1* i *Trichoderma reesei*, hvor promoter, gen og terminator af forskellig eukaryotisk oprindelse var kombineret. Codons, som koder for aminosyren histidin, blev inkluderet i 3'enden af genet for at lette oprensning af genet. Det oprensede BGL1 blev undersøgt for beta-glucosidase aktivitet, hvor enzymkinetik, temperatur- og pH-profil, glukosetolerance samt hydrolyse af cellodextrin blev studeret. En påfaldende ensartethed blev fundet ved sammenligning af data for det oprensede BGL1 og de data, der tidligere blev rapporteret for råekstraktet fra svampen. Dette indikerer, at vi med succes har klonet og ekspresseret det protein, som primært kontribuerer til den beta-glucosidase-aktivitet, der var observeret i råekstraktet fra *A. saccharolyticus*.

Review paper

Fungal beta-glucosidases and their applications for production of biofuels and bioproducts

Annette Sørensen, Peter S. Lübeck, Mette Lübeck, and Birgitte K. Ahring

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REVIEW ARTICLE

Fungal beta-glucosidases and their applications for production of biofuels and bioproducts

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Keywords
Beta-glucosidases; biofuels & bioproducts; biomass hydrolysis; enzyme discovery, characterization, improvement, and application

Abstract

Beta-glucosidases (EC 3.2.1.21) play an essential role in efficient hydrolysis of cellulosic biomasses. By hydrolysis of cellobiose, beta-glucosidases relieve inhibiting conditions, allowing for increased hydrolysis of cellulose by cellobiohydrolases and endoglucanases. In a biorefinery concept, polymers of cellulose and hemicellulose in the biomass materials are converted into sugars, which can be used for production of biofuels and biochemicals, which could act as platform molecules serving as building blocks in the synthesis of chemicals and polymeric materials. Biorefineries are expected to replace our day's oil refineries. This review focuses on beta-glucosidases applied for biomass hydrolysis, an application where all commercial enzymes are of fungal origin. Efficient hydrolysis requires high conversion rates throughout the hydrolysis process. The major factors influencing this are product inhibition and temperature stability. Better beta-glucosidases can be obtained either by discovery of new beta-glucosidases through different screening strategies or by improvement of known beta-glucosidases for instance by protein engineering. With the biorefinery application in mind, beta-glucosidases should be included as part of a complete enzyme cocktail, either made from a separate fungal fermentation or as part of a consolidated bioprocess.

Introduction

The ever-increasing energy consumption, the depletion of fossil resources, as well as a growing environmental concern have laid the foundation for a shift towards sustainable biofuels and bioproducts from renewable sources.

The search for renewable energy in the form of bioethanol from plant biomasses emerged in the 1970s in response to the oil crisis (NREL National Renewable Energy Laboratory, 2000). Especially second generation bioethanol production has increased in interest during this decade with focus on the use of lignocellulosic wastes for fuel production, as lignocellulosic biomass is an abundant and inexpensive renewable energy resource (Knauf & Moniruzzaman, 2004). Similarly, attention has been drawn towards production of platform molecules for new bioproducts by microbial fermentation from glucose or other simple carbohydrates. Especially organic acids are key building block chemicals that can be produced by microbial processes (Sauer *et al.*, 2008).

Today, with the recent oil disaster in the Mexican gulf, the awareness in the public has again increased towards alternatives for fossil fuels. Oil is currently the primary source of energy for the transportation sector and for production of chemicals and plastics. A biorefinery concept could replace the oil refineries, maximizing the value derived from biomass by producing fuels and platform molecules that can be used as building blocks in the synthesis of chemicals and polymeric materials (Cherubini, 2010). One of the goals of the biorefinery is the combined production of high-value low-volume products together with low-value high-volume products such as fuels (Fernando *et al.*, 2006). Sugar platform biorefineries focus on production of a platform of simple sugars extracted from biomass. These sugars can biologically be fermented into fuels (e.g. ethanol), building block chemicals (e.g. different organic acids) as specified by US Department of Energy Report (2004a), as well as other high value products.

The major constituent of plant biomass is lignocellulose. Lignocellulose consists mainly of polysaccharides such as cellulose and hemicelluloses that together with the phenolic lignin polymer form a complex and rigid structure. Proteins, wax, oils and ash make up the remaining fraction of the biomass. The biomass composition depends on the plant/crop type, with cellulose most often being the most abundant component, typically constituting 30-50% according to the Biomass Feedstock Composition database (U.S. Department of Energy, 2004b).

Cellulose, is a homogenous linear polymer of beta-D-glucosyl units linked by 1,4-beta-D-glucosidic bonds with the degree of polymerization of native cellulose being in the range of 7,000-15,000. The cellulose chains are assembled in larger rigid units held together by both intrachain and interchain hydrogen bonds and weak van der Wall's forces. Through parallel orientation, the chains form a highly ordered crystalline domain, but are interspersed with amorphous regions of more disordered structure (Beguin & Aubert, 1994; Berg *et al.*, 2002; Lynd *et al.*, 2002)

The second most abundant component, hemicellulose, is a heterogeneous, highly branched, polymer of pentoses, hexoses, and sugar acids. The degree of heterogeneity, the distribution of the different sugars, as well as the extent of branching is plant dependent. Xylan, which have a backbone of D-xylose linked by 1,4-beta bonds, is the most abundant hemicellulose. Besides of xylose, hemicellulose may contain arabinose, glucuronic acid or its 4-*O*-methyl ether, acetic acid, ferulic acid, and *p*-coumaric acid. About 80% of the xylan backbone is highly substituted with monomeric side-chains of arabinose linked to *O*-2 and/or *O*-3 of the xylose units of the backbone (Saha, 2003).

The third main component, lignin, is composed of aromatic phenyl-propanoid compounds that are either di-, mono-, or non-methoxylated, forming a three-dimensional network that is highly resistant towards biological and chemical degradation (Martinez *et al.*, 2005).

The complex structure of the cellulose fibrils embedded in the amorphous matrix of lignin and hemicellulose protects the plant against attack from microorganisms and enzymatic degradation as well as other naturally occurring environmental stress such as weather conditions through covalent cross-links that strengthen the plant cell wall. In order to produce a sugar platform from plant biomass, pretreatment is crucial as a first step for breaking this rigid structure followed by enzymatic hydrolysis. Pretreatment aims at increasing the accessibility of the polymers for the enzyme hydrolysis. Several different pretreatment strategies have been suggested over the last years, ranging from extreme temperatures, pressures, and acid/base conditions to the milder biological approaches. Several reviews on different pretreatment methods and factors influencing the following enzymatic hydrolysis exist (Sun & Cheng, 2002; Mosier *et al.*, 2005; Alvira *et al.*, 2010), with commonly used methods including (but not excluding others) alkali-, acid-, or organosolvent pretreatement, steam-, ammonia fiber- or CO_2 explosion, and wet-oxidation. The characteristics of the lignocellulosic substrate for enzyme hydrolysis, with regard to cellulose accessibility, degree of polymerization, hemicellulose content, lignin content, and other potential interfering compounds, will vary to a great extent depending on the plant material and the pretreatment applied. Therefore, the sugar yields from hydrolysis of the biomass will evidently depend on type and severity of the pretreatment (Chang & Holtzapple, 2000; Zhang *et al.*, 2006; Kabel *et al.*, 2007). Such variation in biomass characteristics will influence the composition requirements for an optimal enzyme cocktail for the breakdown of lignocellulosic biomasses (Meyer *et al.*, 2009). However, in general terms, the enzymes mentioned below are required for the breakdown of cellulose, hemicellulose, and lignin, respectively.

The three main categories of players in cellulose hydrolysis are cellobiohydrolases (or exo-1,4-beta-glucanases) (EC 3.2.1.91), endo-1,4-beta-glucanases (EC 3.2.1.4), and beta-glucosidases (EC 3.2.1.21). Cellulose polymers are through sequential and cooperative actions of these enzymes degraded to glucose. The general consensus is that cellobiohydrolases (CBHs) hydrolyze the cellulose polymer from the ends, releasing cellobiose as product in a processive fashion, with CBHIs acting on the reducing ends and CBHIIs acting on the non-reducing ends. Endo-glucanases (EGs) randomly hydrolyze the internal 1,4-beta-linkages in primarily the amorphous regions of cellulose, rapidly decreasing the degree of polymerization and creating more free ends for attack by the cellobiohydrolases (Lynd *et al.*, 2002; Zhang & Lynd, 2004; Zhang *et al.*, 2006). Such synergism of cellobiohydrolases and endoglucanases was already studied decades ago in *Trichoderma reesei* (Henrissat *et al.*, 1985). Finally, beta-glucosidases hydrolyze the cellobiose and in some cases the cellooligosaccharides to glucose. Cellobiohydrolases and endoglucanases are often inhibited by cellobiose, making beta-glucosidases important in terms of avoiding decreased hydrolysis rates of cellulose over time due to cellobiose accumulation. On the other hand beta-glucosidases are often themselves inhibited by their product, glucose (Shewale, 1982; Xiao *et al.*, 2004), and maintaining high hydrolysis rates will therefore ultimately depend on the beta-glucosidases, making them key enzymes for efficient hydrolysis of cellulose.

The general concept of polysaccharide hydrolysis has been agreed on for several years. More recently, attention has been paid to accessory enzymes that are coregulated or coexpressed by microbes during growth on cellulosic substrates. Proteins have been discovered that by themselves lack measurable hydrolytic activity, yet are able to significantly enhance the activity of cellulases on pretreated biomass. Some of these proteins have since been referred to as GH61 proteins (Harris *et al.*, 2010).

Due to the heterogeneous structure of hemicellulose, a combination of main enzymes acting on the backbone as well as different accessory enzymes acting on the side-chains must contribute to its hydrolysis. For hydrolysis of the xylan backbone, endo-1,4-beta-xylanases and beta-xylosidases are key players hydrolyzing internal 1,4-beta-linkages and xylobiose, respectively. Meanwhile accessory enzymes such as alpha-L-arabinofuranosidase, alpha-glucuronisidase acetylxylan esterase, ferulic acid esterase, and *p*-coumaric acid esterase remove substituents from the xylan backbone, which is often necessary before the backbone itself can be hydrolyzed (Saha, 2003).

Lignin depolymerization is catalyzed by unspecific oxidative enzymes that liberate unstable compounds which subsequently take part in many different oxidative reactions, a process referred to as enzymatic combustion (Kirk & Farrell, 1987). Peroxidases and laccases are the two major families involved in ligninolysis, generating aromatic radicals by oxidizing the lignin polymer. These aromatic radicals then evolve in different non-enzymatic reactions contributing to the breakdown of the lignin structure (Perez *et al.*, 2002; Martinez *et al.*, 2005).

It appears from the above mentioned enzyme systems that efficient enzymatic degradation of lignocellulose requires cooperative and synergistic interactions of enzymes responsible for cleaving different linkages in different polymers and within the same polymer. The foundation for both biofuels and bioproducts is a sugar platform of monomeric sugars such as glucose and xylose which are converted to the desired products (Cherubini, 2010). The primary challenge in making a sugar platform for bioproducts and biofuels is to get a cost-competitive product, relative to e.g. fossil-based fuels. The costs related to creating a sugar platform from lignocellulosic biomass involve biomass abundance, location and transport of the biomass to the production facility, pre-treatment strategies, and efficient hydrolytic enzymes (Knauf & Moniruzzaman, 2004). Evaluating on the overall production cost, the price of enzymes typically contributes to a large part of the total cost (Merino & Cherry, 2007). Efficient enzymes for lignocellulose degradation are, therefore, of high demand. As most of the currently used pretreatment methods remove lignin from the sugar polymers and in many cases also hydrolyze most of the hemicellulose, the main target for enzyme treatment is cellulose. As beta-glucosidases are key in terms of final cellulose hydrolysis for obtaining sugars, and commercial beta-glucosidases originate from fungi, we have chosen to focus this review on fungal beta-glucosidases.

This paper will present the characterization, discovery, production, and use of fungal beta-glucosidases for production of sugars for biofuel and bioproduct production. It focuses on some of the features that are important to consider for industrial application within production of biofuels and bioproducts as well as describes different methods to discover new beta-glucosidases and methods to improve beta-glucosidases. Besides discussing the application of beta-glucosidases for generation of monomeric sugars, integration of enzymatic saccharafication and fermentation in a single organism in a consolidated bioprocess is discussed.

Sequence, structure, and action of beta-glucosidases

Classification

Beta-glucosidases are classified as glycoside hydrolases in the IUB Enzyme Nomenclature (1984) based on the type of reaction they catalyze. Glycoside hydrolase enzymes have been assigned the number EC 3.2.1.X, which denotes their ability to hydrolyze *O*-glycosyl linkages, such as the 1,4-beta-linkage of cellobiose, with the "X" representing the substrate specificity. In the case of beta-glucosidases, the full number is EC 3.2.1.21`. This defines hydrolysis of terminal, non-reducing beta-D-glucosyl residues with release of beta-D-glucose. A wide specificity for beta-D-glucosides is found and there are examples of some beta-glucosidases hydrolyzing beta-D-galactosides, alpha-L-arabinosides, beta-D-xylosides, or beta-D-fucosides (Bairoch, 2000). Based on substrate specificity, beta-glucosidases have traditionally been divided into cellobiases (high specificity towards cellobiose), aryl- beta-glucosidases (high specificity towards substrates such as *p*-nitrophenyl-beta-D-glucopyranoside (pNPG)), or broad specificity beta-glucosidases (Shewale, 1982; Eyzaguirre *et al.*, 2005).

A classification based on substrate specificity cannot sufficiently accommodate enzymes that act on several substrates, an issue that is particularly relevant for glycoside hydrolases that frequently display broad overlapping specificities. Henrissat (1991) proposed a classification system based on sequence and structural features that could complement the IUB system for better description of the glycoside hydrolases. Through sequence alignment of the known glycoside hydrolases, GH families were defined when at least two sequences showed significant amino acid similarity or no significant similarity could be found with other families. The sequence identity of a family could be as low as 10% but with conserved sequence motifs spanning the active site being recognized. Naturally, as more sequence data of different organisms become available the total number of families is likely to increase (Henrissat, 1991; Henrissat & Bairoch, 1993; Henrissat & Bairoch, 1996) and at present (July 2010) there are 118 families. The strength of this system especially lies in the investigation of the active site of the enzymes, with significant similarity of sequences being a strong indication of similarity in the fold of the structure. Members of the same GH family, therefore, most likely share the same

folding characteristics and analysis of their primary structure can assign potential conserved active-site residues, which will be discussed in greater detail in the following paragraph. The EC and GH systems complement each other and are at the same time interlinked in the way that many of the sequence based families are polyspecific in terms of containing enzymes with different substrate specificities, implying evolutionary divergence, while at the same time enzymes with similar specificities are sometimes found in different families, implying convergent evolution (Henrissat & Davies, 1997).

The CAZY database (http://www.cazy.org/) has since 1998 been a database specifically dedicated to list the information on Carbohydrate-Active enZYmes, containing genomic, structural and biochemical information. The database comprises glycoside hydrolases (GHs), glycosyl transferases (GTs), polysaccharide lyases (PLs), and carbohydrate esterases (CEs). Searching the CAZY database, beta-glucosidases are found in families 1, 3, 9, 30, and 116; however fungal beta-glucosidases are represented only in families 1 and 3. Generally, family 1 enzymes mainly include bacterial, archaeal, plant, animal, and some fungal beta-glucosidases, while family 3 includes some bacterial, all yeast, and several fungal beta-glucosidases (Cantarel *et al.*, 2009). Most family 1 enzymes, besides of beta-glucosidase activity, also show significantly beta-galactosidase activity (Cantarel *et al.*, 2009).

For GH1 and GH3 which contain fungal beta-glucosidases, the active site consensus patterns are defined as written in Box 1, where the glutamate (E) and aspartate (D) are the active site residues (underlined), respectively. These regions are used as a signature pattern in the GH classification of the enzymes (Bairoch, 1992; Bairoch, 2000).

Structure

The topology of the active sites of all glycoside hydrolases falls into only three general classes, (i) pocket or crater, (ii) cleft or groove, and (iii) tunnel. Beta-glucosidases and non-processive exo-acting enzymes have the pocket or crater topology that is well suited for recognition of a saccharide non-reducing extremity (Davies & Henrissat, 1995), with the depth and shape of the pocket or crater reflecting the number of subsites that contribute to substrate binding and the length of the leaving group (Davies *et al.*, 1997).

Several GH1 beta-glucosidase crystal structures have been determined from different organisms, e.g. *Trifolium repens* (clover) (Barrett *et al.*, 1995), *Bacillus polymyxa* (eubacterium) (Sanz-Aparicio *et al.*, 1998), *Bacillus circulans* (Hakulinen *et al.*, 2000), *Zea mays* (maize) (Czjzek *et al.*, 2001), *Thermus nonproteolyticus* (eubacterium) (Wang *et al.*, 2003), *Triticum aestivum* (wheat) and *Secale cereale* (rye) (Sue *et al.*, 2006), *Phanerochaete chrysosporium* (white rot fungus) (Nijikken *et al.*, 2007), and *Oryza sativa* (rice) (Chuenchor *et al.*, 2008), which have helped understanding their mechanism and broad substrate specificity.

The GH3 enzymes, which are abundant in the fungal genomes, are less well characterized with only a few crystal structures having been solved: beta-glucoasidase from *Hordeum vulgare* (barley) (Varghese *et al.*, 1999), *Kluyveromyces marxianus* (a yeast), and *Thermotoga neapolitana* (a hyperthermophilic bacterium) (Pozzo *et al.*, 2010). However, at present there are no crystal structures available from filamentous fungi (Cantarel *et al.*, 2009).

Homology modeling has been the method of choice for obtaining the structural information from fungal beta-glucosidases due to unavailability of the structures. We have modeled the beta-glucosidase from the newly identified *A. saccharolyticus* in collaboration with MAX-lab, Sweden. Even though the sequence identity was relatively low, it was obvious that the residues important for substrate binding and catalysis were conserved (Research paper IV, this thesis). Similarly, Jeya *et al.* (2010) have modeled the enzyme from *Penicillium purpugenum,* where they found that the distance between the conserved catalytic residues is similar to that of the enzyme from barley, concluding a comparable function.

Structurally GH1s and GH3s differ greatly, e.g. sequence identity, fold and the active site residues as illustrated above. These structural differences alter the functional properties of the enzymes such as substrate specificity, binding and catalytic mechanism and rate. The catalytic pocket of GH1s is a tight, deep pocket like a narrow tunnel with a dead end. Meanwhile, GH3s have a shallow, open pocket. The structure of GH1s will most likely place greater constraints on the substrate conformation compared to GH3s (Harvey *et al.*, 2000; pers.com. Wimal Ubhayasekera). Evolution and environmental factors effect on these differences in structure. Henrissat & Davies (1997) proposed convergent evolution to explain the distribution of beta-glucosidases in different GH families, in other words, they have adapted towards the same kind of environment.

Box 1. Active site signatures

GH1 active site signature

[LIVMFSTC] – [LIVFYS] – [LIV] – [LIVMST] – E – N – G – [LIVMFAR] – [CSAGN]

GH3 active site signature

[LIVM](2) – [KR] – X – [EQKRD] – X(4) – G – [LIVMFTC] – [LIVT] – [LIVMF] – [ST] – D – X(2) – [SGADNIT]

Catalytic mechanism

Hydrolysis of beta-1,4-glycosidic bonds by beta-glucosidases is carried out by an overall retaining double-displacement mechanism (Sinnott, 1990). Two catalytic carboxylic acid residues at the active site separated by approximately 5Å facilitate the reaction with one carboxylic acid acting as a nucleophile and the other as an acid/base catalyst (Fig. 1). With substrate located at the active site, the departure of the aglycone is initially facilitated by general acid catalysis, where the acid/base catalyst donates a proton to the linking beta-1,4-oxygen of the scissile bond, breaking the bond, and creating an oxocarbenium-ion-like transition state with a build-up of positive charge on the anomeric carbon. The carboxylic acid nucleophile attacks the anomeric carbon, generating a covalent intermediate. This intermediate is hydrolyzed by nucleophile attack of a water molecule from the bulk solvent, which by abstraction of a proton has been activated by the deprotonated acid/base catalyst (Koshland, 1953; Mccarter & Withers, 1994; White & Rose, 1997).

The side-chain of either a glutamate or an aspartate acts as the catalytic nucleophile in GH1 and GH3 beta-glucosidases, respectively (Bairoch, 1992; Bairoch, 2000). Identification of the active site nucleophile in beta-glucosidases has been achieved through the formation of a stabilized glycosyl-enzyme intermediate using glycosidase inhibitors such as 2-fluoro-glycosides to trap the covalent intermediate (Withers *et al.*, 1987; Withers *et al.*, 1988; Withers & Aebersold, 1995). In the *Aspergillus niger* beta-glucosidase, the Asp-261 was identified as the catalytic nucleophile by the use of 2-deoxy-2-fluoroglucosyl (Dan *et al.*, 2000). The inhibitor interacts with the enzyme, where the positive charge, developed at C1 in the transition state, will be significantly destabilized due to presence of the fluorine substituent at C2 as fluorine is much more electronegative than hydroxyl. This will decrease the rate of both the glycosylation and deglycosylation. Incorporation of a good leaving group to the substrate such as 2,4-dinitrophenolate or fluoride will speed up the glycosylation step and allow for the "capture" of the inhibitor at the active site, obtaining a time-dependent inactivation via accumulation of the relative stable 2-deoxy-2-fluoroglycosyl-enzyme intermediate (Withers *et al.*, 1987; Withers & Aebersold, 1995). Dan *et al.* (2000) used this method to label the nucleophile, and compare it with a non-inhibited enzyme through peptic digestion, HPLC separation, and tandem mass spectrometric analysis, which allowed for identification of the peptide to which the inhibitor had bound. In this case, Asp-261 of the *A. niger* beta-glucosidase was the only possible nucleophile candidate present in the specified peptide.

No tagging method of the acid/base catalyst has been described, rather has the role of proposed catalysts been verified by mutagenesis and kinetic analysis (Svensson & Sogaard, 1993). Generally, several site-directed mutagenesis studies have been carried out on glycoside hydrolases in terms of understand in their mechanism better. One example is site-directed mutagenesis studies where a proposed nucleophile catalyst is replaced by other amino acids that will not change the conformation of the active site, but cannot act as a catalyst. This can give insight to the necessity and function of that particular replaced amino acid. This was done by Li *et al.* (2001) on a GH3 beta-glucosidase, providing evidence that Asp-247 plays an important role in the enzymatic reaction catalyzed by that particular beta-glucosidase and most likely functions as the nucleophile. Structural studies of beta-glucosidases give concrete evidence on active site residues, which are possible to confirm by site-directed mutagenesis. The GH3 glycosidase of *T. neapolitana* is a good example to show this process; the nucleophile Asp-242 and acid/base Glu-458 were recognized first by sequence alignment evaluation, then compared with three dimensional structure and confirmed by site-directed mutagenesis (Pozzo *et al.*, 2010).

Not only catalytic residues, but also the residues important for substrate binding can be identified from the structure of the enzyme. For example, for the GH3 beta-glucosidase from *T. neapolitana*, seven amino acids (D58, R130, K163, H164, R174, Y210, and E548) in the active site have been pointed out to be involved in hydrogen bonding with the glucopyranoside residue, where van der Walls interactions occur between atoms C4, C5, and C6 and amino acid residues W243, M207, and S307 (Pozzo *et al.*, 2010). Similarly, seven amino acids (D95, R158, K206, H207, D285, Y253, and E491) from the the barley beta-glucan exohydrolases have been reported to involve in hydrogen bonding with the substrate.

Most retaining beta-glucosidases have transglycosidic

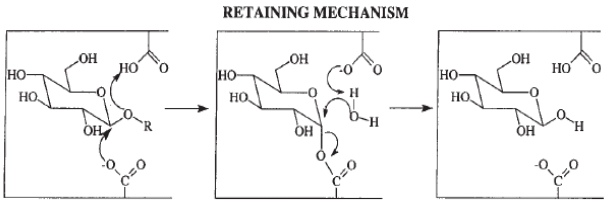

Fig. 1. Catalytic mechanism of beta-glucosidases. Reprintet from McCarter & Withers (1994).

activity. Such reaction takes place after the glycosidic bond has been cleaved and the aglycone released. Instead of a nucleophilic attack by a water molecule from bulk solvent to proceed the hydrolysis of the glycosyl-enzyme intermediate, an acceptor such as sugar or alcohol binds. The acceptor competes with water and the extent of transglycosidic reaction depends on the affinity, concentration, and tendency to react of the acceptor (Withers, 1999). In *A. niger* beta-glucosidase, Trp-262 has been found to be a key residue in terms of maintaining hydrolytic rather than transglycosidic activity. Trp-262 is located adjacent to the nucelophile catalyst, Asp-261, in the active site, and substitution of Trp-262 cause the reaction to be mainly transglycosidic (Seidle & Huber, 2005). On the contrary, Trp-49 of *A. niger* beta-glucosidase has been shown to be important for binding acceptors and thus transglycosidic activity; substitution of this residue produced an enzyme with high ratio of hydrolytic to transglycosidic activity (Seidle *et al.*, 2006).

What is a good beta-glucosidase?

In relation to industrial biomass conversion, a good beta-glucosidase facilitates efficient hydrolysis at specified operating conditions. Key points to consider when evaluating a beta-glucosidase are conversion rate, inhibitors, and stability. High conversion rates are essential, but issues such as product inhibition and thermal instability can be a restriction for maintaining high conversion rates throughout the hydrolysis.

Enzyme activity is standardly measured in units, which is equivalent to micromoles hydrolyzed per minute of the reaction under a given set of conditions. Kinetic parameters reported for beta-glucosidases used for comparison and general evaluation typically relates to Michaelis Menten kinetics.

$$Eq(1)\ \text{MM kinetics} \quad v = \frac{V_{max}[S]}{[S] + K_M}$$

where v is the initial rate of product formation, V_{max} is the maximum reaction rate, the rate observed when the enzyme is saturated with substrate, K_M is the Michaelis constant, and [S] is the substrate concentration. The kinetic constants can be determined by different rearrangements of the MM equation giving linear equation, where the constants are derived from axis intercepts or line slope, or by non-linear regression aided by different computer programs. Previous reviews on beta-glucosidases have summarized published values of kinetic properties of different microbial beta-glucosidases (Bhatia *et al.*, 2002; Eyzaguirre *et al.*, 2005) .

In terms of evaluating beta-glucosidase performance based on the kinetic parameters, V_{max} and K_M, the optimal enzyme has high V_{max} and low K_M. From the MM equation it can be derived, that in situations where substrate concentration is much greater than K_M, the velocity of the reaction, v, will equal V_{max} with no dependence on K_M. So on that note, V_{max} will be the better description in terms of enzyme evaluation. In relation to the application of beta-glucosidases for complete cellulose hydrolysis, the hydrolysis of cellobiose by the beta-glucosidases should not just be evaluated at saturated conditions in an isolated fashion. The substrate concentration for the beta-glucosidases will depend on the balance of the cellobiohydrolase, endo-glucanase, and beta-glucosidase enzyme mixture. The beta-glucosidases might efficiently hydrolyze the cellobiose product as they are formed by the cellobiohydrolases, whereby substrate saturation is never reached. The value of K_M should not be discredited in such situation.

The activity of beta-glucosidases is influenced by several factors including inhibitors and stability at process conditions. In relation to beta-glucosidase activity in hydrolysis of biomass for generating sugars the inhibition type mostly discussed and often encountered is caused by the end-product, glucose. However, compounds other than glucose are potentially present that can influence the activity of beta-glucosidases, including (but not exclusive) other simple sugars, sugar derivatives, amines, and phenols (Dale *et al.*, 1985). In case of product inhibition, the effect is naturally increased during the course of the reaction as more and more glucose is formed, and for beta-glucosidases the end-product is generally not removed during hydrolysis so the actual reaction rate will differ more and more from V_{max}. The accumulation of glucose has often been reported to decrease reaction rates of beta-glucosidases as hydrolysis proceeds, including beta-glucosidases of different origin. In terms of MM kinetics, the inhibitor constant, K_i, is frequently used to define product inhibition, a value that has also been reported for several beta-glucosidases in the review by Eyzaguirre *et al.* (2005).

A decrease in the rate of glucose formation can also be caused by transglycosylation events. Other than inhibiting the reaction by occupying the active site, glucose can also be considered to take part in transglycosylation events, thus using the active site capacity in non-hydrolyzing action which will decrease the overall rate of hydrolysis. Transglycosylation has frequently been reported for beta-glucosidases (Bhatia *et al.*, 2002). In relation to generating a sugar platform through biomass hydrolysis, transglycosylation events are unwanted. Targeted mutagenesis aiming at displacing essential amino acids involved in transglycosylation could potentially reduce this unwanted mechanism.

Stability of beta-glucosidases in relation to the physical operating conditions is required for efficient hydrolysis. Beta-glucosidases can, depending on the extremity and time of exposure, be inactivated by pH and temperature variations. Regarding influence of pH, ionic groups are involved in enzyme catalysis, such as the acid-base catalyst

in the beta-glucosidase active site. The protonation state of the carboxylic acid residue catalyst and the carboxylate nucleophile is essential for the reaction and a pH change could impair the catalytic mechanism (McIntosh *et al.*, 1996). Regarding temperature, according to the van't Hoff rule, reaction rate doubles with every 10°C increase of temperature, which applies to all chemical reactions including enzyme catalyzed reactions. However, when reaching high temperatures, protein stability will be affected, leading to denaturation, thus irreversible inacitivation, of the enzyme. Temperature and pH optimum for microbial beta-glucosidases have been reported in the reviews by Bhatia *et al.* (2002) and Eyzaguirre *et al.* (2005), but for biomass hydrolysis processes that typically run for the duration of hours or even days, the stability of the enzyme at specified temperatures is important. Thermal inactivation usually follows first order decay, with the rate of denaturation being a valid value for evaluation of the enzymes at different temperatures.

$$Eq(2)\ \text{Thermal inactivation} \quad [E]_{active} = [E]_{start} e^{-k_D t}$$

where $[E]_{active}$ is the remaining active enzymes at time=t, $[E]_{start}$ is the initial enzyme concentration at time zero, $-k_D$ is the rate of degradation, and t is the time. The enzyme stability can be reported as the half life at different temperatures, calculated from equation 2.

In practical terms, when studying the kinetics of beta-glucosidases, it is important to specify what substrate that is used as substrate specificity of beta-glucosidases varies (Riou *et al.*, 1998; Bhatia *et al.*, 2002; Eyzaguirre *et al.*, 2005; Langston *et al.*, 2006; Korotkova *et al.*, 2009) and the choice of substrate will influence the kinetic data obtained. Several different substrates with varying sensitivity and ease of use can be applied for the determination of beta-glucosidase activity. Aryl-glucosidases are often favored as substrates, as a colored or fluorescent product is released upon hydrolysis, simplifying detection of activity. In this category of substrates, pNPG is probably the most common encountered in the literature. In hydrolysis of cellulose for making sugars for production of bioproducts and biofuels, cellobiose and cellodextrins will be the major substrates for beta-glucosidases. These natural substrates should preferably be used in the evaluation of beta-glucosidases. Activity on the natural substrates is calculated by measuring release of glucose, or alternatively, decrease in substrate concentration. The latter requires high analytical precision and has been demonstrated using ion-exchange chromatography (IC) methods (Research paper II, this thesis). Detection of glucose, even in scarce amounts, can be done by the glucose oxidase/peroxidase method (Bergmeyer & Bernt, 1974; Mazura *et al.*, 2006), hexokinase/glucose-6-phosphate dehydrogenase method (Kunst *et al.*, 1984), high performance liquid chromatography (HPLC), or IC.

Beta-glucosidases for biomass hydrolysis

The two largest enzyme companies, Novozymes A/S and Genencor (a Danisco Division), have both participated in large research programs supported by the US Department of Energy, aiming at improving the performance of enzymes for biomass hydrolysis (Banerjee *et al.*, 2010b). Beta-glucosidaes are widely produced by different genera and species of the fungal kingdom including Ascomycetes and Basidiomycetes, where especially the ascomycete genus *Aspergillus* has been widely studied for beta-glucosidase production. Especially *A. niger* has been setting the standard in commercial beta-glucosidase production (Dekker, 1986).

Commercial beta-glucosidases

The primary beta-glucosidase preparation by Novozymes A/S is Novozym 188. Several publications exist where authors have used this commercial product as beta-glucosidase supplement to the hydrolysis of cellulosic material. The temperature optimum for Novozym 188 is around 65°C (Dekker, 1986; Chauve *et al.*, 2010) though with temperature stability maintained only below 60°C (Dekker, 1986; Bravo *et al.*, 2000; Calsavara *et al.*, 2001), and for prolonged incubations the stability was only upheld below 50°C (Krogh *et al.*, 2010). The pH stability range is between 4-5 (Chauve *et al.*, 2010; Krogh *et al.*, 2010; Research paper II, this thesis), with an optimum at pH 4.3-4.5 (Bravo *et al.*, 2000; Bravo *et al.*, 2001; Calsavara *et al.*, 2001; Chauve *et al.*, 2010; Research paper II, this thesis). The activity is competitively inhibited by glucose (Dekker, 1986; Chauve *et al.*, 2010), with a decrease in activity to 50% at a glucose concentration approximately 3 times that of the substrate concentration (Dekker, 1986; Research paper II, this thesis). Evidence of substrate inhibition or transglycosylation has been found using pNPG, whereas cellobiose did not appear to be inhibitory at the tested conditions (Dekker, 1986; Krogh *et al.*, 2010; Research paper II, this thesis).

The primary beta-glucosidase preparation by Genencor is AccelleraseBG. This enzyme is less frequently mentioned in the literature, but according to the manufacturer the enzyme has the best operational stability at pH 4-6 and temperatures ranging 30-55°C, with long operational times possible at the low temperatures while the higher temperatures will limit the effective period of operation. Effect of product accumulation on enzyme activity is not mentioned by the manufacturer (Danisco US Inc, 2009).

Recently both enzyme companies have launched cellulase products with sufficient beta-glucosidase activity for efficient hydrolysis of biomass without separate addition of supplementary beta-glucosidase.

Cellic CTec2 is currently the state-of-the-art enzyme from Novozymes A/S that according to the company has proven effective on a wide variety of lignocellulosic

substrates. According to its application sheet, it contains a blend of cellulases, high level of beta-glucosidase, and hemicellulases, functioning at optimal temperature of 45-50°C and pH 5.0-5.5. Some of the key elements, promised by the manufacturer, are high conversion yields, enzyme concentration, stability, and inhibitor tolerance as well as the enzyme being effective at high solid concentrations and compatible with multiple feedstocks and pretreatment methods. By containing all cellulase and beta-glucosidase components for cellulose hydrolysis, Cellic CTec2 replaces the combined use of former applied Celluclast 1.5L and Novozym 188 (Novozymes A/S, 2010).

Accellerase DUET is a newly introduced product by Genencor that, according to the company, is said to be a mile-stone in making cellulosic ethanol at a commercial profitable scale. According to its application sheet the product is produced with a genetically modified *T. reesei* strain. It contains all major activities needed for biomass hydrolysis and has enhanced hemicellulase activity. Less dosage of the product is required compared to former products. Best operational stability is at pH 4.0-5.5 and temperatures of 45-60°C, retaining 100% activity after 24 hours incubation at 50°C, but only approximately 60% at 60°C. The enzyme complex can hydrolyze a broad range of lignocellulosic carbohydrates of different feedstocks and pretreatment methods into fermentable monosaccharides.

Promising beta-glucosidases recently reported in the literature

Within the last decade, several new beta-glucosidases with different specificities have been reported in the literature. We present some of these beta-glucosidases that can potentially be important in biomass degradation.

Special attention has been paid to thermostability of beta-glucosidases, and beta-glucosidases originating from fungi belonging to different genera have been proposed as promising candidates for biotechnological processes at elevated temperatures. Thermal stability, however, is more difficult to compare as different researches use different incubation conditions, times and temperatures. As the commercial enzymes primarily are meant to operate around 50°C, only data well above this temperature is dealt with here. *Penicillium citrinum*is is reported to have a half life of 120 min at 58°C (Ng *et al.*, 2010), *Monascus purpureus* of 315 min at 60°C (Daroit *et al.*, 2008), and *A. saccharolyticus* of 365 min at 61°C (Research paper IV, this thesis). Elevating the temperature further, *A. saccharolyticus* has a half life of 61 min at 65°C (Research paper IV, this thesis), comparable to *Talaromyces emersonii* for which a half life of 62 min at 65°C has been reported (Murray *et al.*, 2004). We found *A. saccharolyticus* beta-glucosidase to be more thermostable than the commercial enzyme preparation Novozym 188 from *A. niger*, as it retained more than 90% activity at 60°C and still had approx. 10 % activity at 67°C after 2 hours of incubation, while Novozym 188 had 75% activity at 60°C, but no activity at 67°C after 2 hours of incubation (Research paper II, this thesis). After 4 hours of incubation these differences are much more pronounced as the beta-glucosidase of *A. saccharolyticus* still had more than 70 % activity while Novozym 188 drop to 40 % activity at 60°C. Rojaka *et al.* (2006) similarly found half-life of *A. niger* beta-glucosidase to be 8 hours at 55°C and 4 hours at 60°C , which is similar to our results (Research paper II, this thesis), whereas Krogh *et al.* (2010) indicate a half-life for *A. niger* beta-glucosidase of 24 hours at 60°C. This is six times longer than measured by us and Rojaka *et al.* (2006). Decker *et al.* (2000) demonstrates that *A. japonicus* and *A. tubingensis* beta-glucosidase were remarkably stable, maintaining 85% and 90% activity, respectively, after 20 hours incubation at 60°C.. However, Korotkova *et al.* (2009) found *A. japonicus* beta-glucosidase to only retain 57% of its activity after incubation for 1 hour at 50°C., contradicting the findings of Dekker *et al*. (2000). This clearly demonstrates how complex it can be to compare results by different researchers, most likely due to different conditions being used during incubation and assaying the activity. For a true comparison the enzymes in question should be tested in parallel by the same laboratory. Stability for prolonged periods at high temperatures was found for both *Aspergillus fumigatus* with a half life greater than 19 hours at 65°C (Kim *et al.*, 2007a) and *Penicillium brasilianum* with a half life of 24 hours at 65°C (Krogh *et al.*, 2010). Meanwhile, high temperature stability has sometimes been reported using a relatively short incubation time, making it difficult to compare temperature stability between different beta-glucosidases. For example, de Palma-Fermandez *et al.* (2002) have purified two beta-glucosidases from the thermophilic fungus *Thermoacus aurantiacus* and found that they retained between 75-82% activity after incubation for 1 hour at 70°C, and *Leite et al.* (2008) found that a beta-glucosidase from the yeast-like fungus, *Aureobasidum pullans*, had a thermostability up to 70°C when incubated for 1 hour, and a calculated half life of 51 min at 80°C.

High conversion rates are essential for efficient conversion of biomass. Accumulation of glucose during hydrolysis can significantly lower the rate of cellulose hydrolysis through inhibtion and another important focus for increasing overall biomass hydrolysis is, therefore, high tolerance of beta-glucosidases towards glucose accumulation. Decker *et al.* (2001) have investigated four beta-glucosidases from *Aspergillus tubingensis* of which two had high glucose tolerance, $K_{i,glu}$ of 470 and 600mM, respectively, when using pNPG as substrate. Earlier, the same lab reported high glucose tolerance from a beta-glucosidase of *Aspergillus foetidus,* with a K_i of 520mM (Decker *et al.*, 2000). A similar tolerance towards glucose was found for a beta-glucosidase from *A. niger*, K_i of 543mM (Yan *et al.*, 1998) and an even higher tolerance was reported for a beta-glucosidase of *A. oryzae*, K_i of 953mM

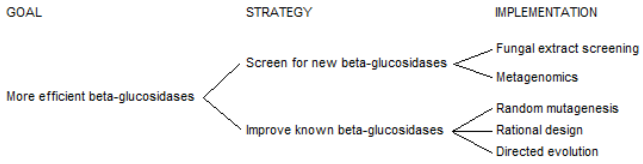

Fig. 2. Sketch of the different strategies described to obtain more efficient beta-glucosidases

(Gunata & Vallier, 1999). Recently, the activity of beta-glucosidases from *Aspergillus caespitosus* was reported by Sonia *et al*. (2008) to be stimulated in the presence of up to 300mM glucose, having 3.8 fold greater activity at these glucose concentrations than in the absence of glucose; however a sharp decline in activity was found above this concentration.

Screening for new beta-glucosidases

In order to optimize the use of different biomasses, it is important to identify new beta-glucosidases with improved abilities on the specific biomasses as well as with improved abilities such as stability and high conversion rates. Product inhibition, thermal inactivation, low product yields, and high cost of enzyme production are the main obstacles of commercial cellulose hydrolysis and therefore set the stage in the search for better alternatives to the currently available enzyme preparations. The beta-glucosidases can be arranged in three groups related to localization: intracellular, cell wall associated, and extracellular. Industrially, primarily the extracellular beta-glucosidases are of interest. With more efficient beta-glucosidases being the goal, two strategies are in play. One is to screen for new beta-glucosidases, the other is to improve known beta-glucosidases. The number of fungal species on Earth is estimated to 1.5 million of which as little as approximately 5% are known (Hawksworth, 1991; Hawksworth, 2001), a statement that calls for a more directed effort for unraveling the potential of unknown species found in nature. The question of where to look for these undescribed fungi is discussed by Hawksworth and Rossman (1997): Everywhere, including your own backyard. The identification and characterization of new fungal species are often encountered in literature. Within the black *Aspergilli*, to which the efficient beta-glucosidase producer *A. niger* belongs, several new species have been identified within the last few years (Samson *et al.*, 2004; de Vries *et al.*, 2005; Serra *et al.*, 2006; Varga *et al.*, 2007; Mares *et al.*, 2008; Noonim *et al.*, 2008a; Noonim *et al.*, 2008b; Perrone *et al.*, 2008), including *A. saccharolyticus* identified by our group (Research paper III, this thesis).

A screening strategy must be planned thoroughly because "you get what you screen for". Cheap, simple, rapid, and discriminatory detection and selection methods are sought to identify exactly those fungi harboring efficient beta-glucosidases, as screening usually involves a very large number of samples to be processed (Cheetham, 1987). Different strategies can be chosen in the search for new beta-glucosidases, including screening of fungal extracts and screening using metagenomic approaches (Fig. 2).

Fungal extract screening

In terms of screening fungal extracts for beta-glucosidase activity the secreted proteins are assayed. The outcome of such screening strategy builds on two key elements: the choice of growth conditions and beta-glucosidase assays.

The composition of a fungal extract will to a certain degree depend on the growth medium used. A rich medium supporting good growth can be chosen to screen general expressed proteins. However, in several cases specifically media with lignocellulose as sole carbon source have been chosen to induce expression of enzymes for biomass degradation. The screening is thereby directed towards lignocellulose degrading fungi which would include beta-glucosidases for complete hydrolysis to glucose that the fungus can use in its metabolism.

Assaying for proteins with beta-glucosidase activity can be done using several different substrates, a few of which have been mentioned previously. Measuring beta-glucosidase activity on its natural substrate, cellobiose and short cellooligomers, is usually tedious compared to chromogenic or flourogenic substrates that react to give colored or fluorescent products and are therefore often preferred as an easy first step in identification of the presence of beta-glucosidase activity. Zhang *et al.* (2006) suggest the use of chromogenic p-nitrophenyl-beta-D-1,4-glucopyranoside (yellow), and flourogenic beta-naphthyl-beta-D-glucopyranoside (blue) or 4-methylumbelliferyl-beta-D-glucopyranoside (blue) for beta-glucosidase screening, while Perry *et al.* (2007) investigated the use of several other chromogenic beta-glucosidase substrates, that can be used in plate assays with the various color precipitates formed remaining tightly localized to the place of the hydrolytic reaction.

To our knowledge, broad screenings of fungal extracts for beta-glucosidase activity have not frequently been published. Sternberg *et al.* (1977) searched amongst 200 fungal strains for strains producing large quantities of beta-glucosidases that could supplement the *Trichoderma viride* cellulases for cellulose saccharification. Generally, they found black *Aspergilli* to be superior in terms of beta-

glucosidase production. Another screening has been published by our lab (Research paper II, this thesis), using pNPG to screen 86 fungal extracts from submerged fermentation of a broad range of in-house fungal strains. We found a black *Aspergillus* strain that we identified as a novel species, *A. saccharolyticus* (Research paper III, this thesis), which is very promising in terms of beta-glucosidase production (Research paper II, this thesis). Another study focused on identification of acid- and thermotolerant extracellular beta-glucosidase activities in Zygomycetes fungi (Tako *et al.*, 2010). They also used pNPG for screening of fungal extracts from 94 strains that had been cultivated in either solid state or submerged fermentation, with the finding that especially *Rhizomucor miehei* could be of industrial interest. *Jeya et al.* (2010) have used plates containing 4-methylumbelliferyl-beta-D-glucoside as an initial screening for beta-glucosidase producing fungi in soil samples, from which a smaller number of strains were selected based on observed fluorescence and thereafter assayed more thoroughly in liquid culture with pNPG with the finding that a *P. purpurogenum* strain was especially efficient in producing beta-glucosidase.

Zymogram techniques have been employed for the identification of beta-glucosidases, where gel-electrophoresis of protein mixes is employed for separation, followed by incubation with for example 4-methylumbelliferyl-beta-D-glucoside and in situ visualization of the reaction in the gel facilitated by the fluorescent product formation (Murray *et al.*, 2004). This substrate is preferred over pNPG, as pNP diffuse more quickly and will not stay in the gel at its place of production. A proteomics strategy using tandem mass spectrometry has by *Kim et al.* (2007a) been coupled with zymography to discover beta-glucosidases from *A. fumigatus*, where the activity spots were cut from the gel, digested by trypsin, and analyzed by LC/MS/MS.

Following an initial screening using the simple chromogenic or flourogenic substrates, a more detailed screening of a selected number of strains can be performed on cellobiose, short cellooligomers, followed by evaluation using pretreated lignocellulosic biomasses in combination with known efficient cellulases.

Metagenomic screening

Other strategies for obtaining beta-glucosidases have included screening of environmental DNA for beta-glucosidase using a metagenomics approach (Kim *et al.*, 2007b; Jiang *et al.*, 2009; Jiang *et al.*, 2010). Many microbial species are difficult to cultivate in the laboratory because of specialized growth requirements. The metagenomic approach of directly cloning environmental DNA and screen this unidentified pool for beta-glucosidase activity will widen the screening to include such organisms. The libraries will serve as basis for screening, either using hybridization (DNA probes) or PCR techniques on the basis of sequence similarity or conserved motifs, directly screening the library at genomic level. Such prospecting will facilitate identification of novel variants of distinct protein families or of known functional classes of proteins. Another approach is to express the library in appropriate host strains and thereafter perform activity screening (Lorenz & Schleper, 2002). As pointed out by Hawksworth and Rossman (1997), you can search anywhere for new fungi, but searching for novel beta-glucosidases it would seem most appropriate to explore lignocellulosic environments. Enzyme discovery in lignocellulosic ecosystems harboring relevant enzymes can easily be too complex for metagenomic sequencing technologies, therefore DeAngelis *et al.* (2010) recently proposed a strategy of cultivating the microbes from these ecosystems under defined conditions to obtain feedstock-adapted communities with reduced diversity that can be screened. Kim *et al.* (2007b) were the first to report the isolation of a family 1 beta-glucosidase from a metagenome library originating from Korean soil samples, using 4-methylumbelliferyl-beta-D-cellobioside in activity screening, an enzyme of which the crystal structure was later derived (Nam *et al.*, 2008). Jiang *et al.* (2009) have likewise identified a novel beta-glucosidase from soil metagenome of which the amino acid sequence did not show homology with other beta-glucosidases, yet the purified recombinant protein efficiently hydrolyzed D-glycosyl-beta-1,4-D-glucose, and later the same research group have through a similar approach identified another novel beta-glucosidase from sludge samples from a biogas reactor (Jiang *et al.*, 2010).

Improving beta-glucosidases

The features aimed for when improving beta-glucosidases are the same as the goals in screening for novel beta-glucosidases, such as improve enzyme activity, fine-tune specificity, e.g. by eliminating transgycosylation activity and glucose inhibition, create novel specificities and activities, change substrate specificity, change stereo-specificity, and/or improve thermo stability.

Classical random mutagenesis

Mutagenesis by UV light or chemical treatment followed by screening for improved activity or stability has been carried out for decades. One good example of developing a host strain with improved total protein production and activity is the work of Montenecourt and Eceleigh (1979). Using a combination of UV irradiation and nitrosomethyl guanidine treatment resulted in strain RutC30, one of the best existing *T. reesei* cellulase mutants. The increased activity obtained from such classical mutagenesis is most often due to changes at the regulatory level of enzyme expression leading to increased production of the gene of interest or decreased expression of conflicting genes and is therefore

minded on production strain improvements, rather than changes to the enzyme itself for improved activity.

Gamma ray irradiation of *Cellulomonas biazotea* has resulted in a mutant with increased beta-glucosidase productivity (Rajoka *et al.*, 1998). Analysis of purified beta-glucosidases of this organism showed difference in pKa compared to the beta-glucosidases of the wild type, indicating that the hydrophobic micro-environment in the vicinity of the active site has been changed. Improvements in both K_M and thermo stability were found (Rajoka *et al.*, 2005), thus showing that the radiation has influenced the actual beta-glucosidase gene, not just its expression mechanism; however, no investigation of the specific changes caused by the irradiation was undertaken.

Replacement of single amino acids through random mutagenesis can have great effects. Stability of the *Bacillus polymyxa* beta-glucosidase was enhanced using hydroxylamine for random mutagenesis, switching the nucleotide base pairs from A to G or from C to T (LopezCamacho & Polaina, 1993). Especially two mutations, E96K and M416I, caused an increase in thermal and pH stability (LopezCamacho *et al.*, 1996). The E96K mutation caused a change in the secondary structure (identified by crystallography) most likely due to an ion pair involving the new Lys96, introducing a salt bridge linking two distant segments together, advancing the stability of the tertiary structure (Sanz-Aparicio *et al.*, 1998). Meanwhile, the effect of the M416I is likely due to increased resistance to oxidation of isoleusine, correlating well with the finding that beta-glucosidase of thermostable microorganisms generally have a low content of methionine (LopezCamacho *et al.*, 1996).

Rational design

More advanced mutagenesis, as rational design, e.g. site directed mutagenesis, is used for improvements, but also carried out to understand and characterize the function of different regions of the beta-glucosidases (Antikainen & Martin, 2005). To improve a function through rational design, detailed knowledge of the protein structure, and function related to structure, is required (Zhang *et al.*, 2006). In rational design, a commonly used technique for changing specific amino acids is the overlap extension method, where the mutation is introduced through primers containing a mutant codon with a mismatched sequence (Reikofski & Tao, 1992). Specific amino acid sites to be changed are chosen, for example by taking advantage of known 3D structures. Bioinformatics is a prerequisite for rational design. A great amount of knowledge is available on for example the protein engineering possibilities for improving thermo stability, stabilizing the protein through increasing the number of disulfide and hydrogen bonds, increasing the internal hydrophobicity, or substituting for certain amino acids as protection against chemical destabilization (Nosoh & Sekiguchi, 1990). However, rational design can in practice be hindered by the complexity of the protein function (Tao & Cornish, 2002).

Generally, site-directed mutagenesis has in several cases been used to confirm the identity of the amino acid catalysts of beta-glucosidases. A combination of site-directed and random mutagenesis was carried out on the active site region of an *Agrobacterium faecalis* beta-glucosidase, evaluating the importance of different amino acids related to enzyme activity. The essential role of E358 as nucleophile in catalysis was confirmed, D374 was suggested as acid-base catalyst candidate, and four other conserved residues in the active site region were found to have different degrees of significance in terms of activity (Trimbur *et al.*, 1992). The mechanism of transglycosylation could preferably be altered by site-directed mutagenesis if specific amino acids known to be important for this function can be identified (Seidle *et al.*, 2006). This would favor the formation of glucose monomers by beta-glucosidase and be of great value for the generation of a sugar platform for biofuels and bioproducts.

Directed evolution

Directed evolution seems to be the method of choice in terms of improving beta-glucosidases. Rather than classical mutagenenis where the change could occur in any part of the genome, directed evolution targets the specific gene of choice, but opposed to rational design you need no knowledge of enzyme structure and specific interactions between enzyme and substrate, as random changes are performed delimited to the gene of choice, followed by evaluation of the mutants (Antikainen & Martin, 2005). Mutation, recombination, and selection set the stage for functional evolution in nature. Directed evolution mimics natural evolution by combining reiterative random mutagenesis and recombination with screening or selection for enzyme variants with improved properties (Tobin *et al.*, 2000; Chirumamilla *et al.*, 2001; Cherry & Fidantsef, 2003). Reviews have been published by both Kaur and Sharma (2007) and Sen *et al* (2006), mentioning several different directed evolution strategies and discussing the advantages and disadvantage of the different techniques. In 1994 Stemmer introduced the research community to gene shuffling, which is generally said to be the beginning of the modern era of directed evolution. DNA shuffling resembles natural sexual recombination and through shuffling multiple related DNA sequences, evolution can radically be accelerated. The tools for performing directed evolution are there, however, the key is how to correctly evaluate the performance of mutants generated by these recombinant DNA techniques (Cherry & Fidantsef, 2003; Antikainen & Martin, 2005; Zhang *et al.*, 2006).

Enhanced thermo-resistance has been obtained through error prone PCR of the *Paenibacillus polymyxa* beta-glucosidase (Gonzalez-Blasco *et al.*, 2000), expanding the range of mutations to this gene previously obtained by

chemical mutagenesis mentioned above (LopezCamacho & Polaina, 1993; LopezCamacho *et al.*, 1996). Investigation of the same gene randomly mutated by error prone PCR and DNA shuffling-mediated recombination was published. Several single amino acid substitutions generated through error prone PCR were found to contribute to increased thermal resistance. The mutants with advantageous substitutions were recombined by gene shuffling, with the result that the clone that exhibited the greatest thermal resistance was a triple mutant. Again, the improvements are attributed to formation of salt bridges and amino acids less prone to oxidation (Arrizubieta & Polaina, 2000). A similar approach of combining error prone PCR and gene shuffling was performed on *Pyrococcus furiosus* beta-glucosidase, where the calculated average mutation frequency was 2.3 base pairs per gene (equals approximately 1 or 2 amino acid substitutions per enzyme). In this case, the beta-glucosidase of the organism is hyperthermostable and it was sought to improve low temperature hydrolysis. The hydrolysis at low temperature of cellobiose was increased up to two fold and the substrate specificity towards pNP-glucopyranoside compared to pNP-galactopyranoside was increased 7.5 fold (Lebbink *et al.*, 2000). Shuffling combined with gene truncation of two different family 3 beta-glucosidases has given information on the importance of different regions in relation to enzyme activity and folding (Singh & Hayashi, 1995; Hayashi *et al.*, 2001). The homologous C-terminal regions of the beta-glucosidase genes play an important role in determining enzyme characteristics including pH/temperature activity, stability, and specificity (Singh & Hayashi, 1995), while the N-terminal catalytic domain showed the greatest importance in determining enzyme folding (Hayashi *et al.*, 2001).

Random drift mutagenesis has been performed on *Caldicellulosiruptor saccharolyticus* beta-glucosidase, where the screening only focused on retained ability to hydrolyze beta-glucosidase substrates. Whether improved, unchanged, or reduced, the mutations were carried along and pooled for use as template in another round of mutagenesis, allowing multiple adaptive, neutral, and harmful (but not inactivating) mutations to occur (Bergquist *et al.*, 2005; Hardiman *et al.*, 2010). Hardiman *et al.* (2010) performed iterative mutagenesis procedures followed by screening using a high throughput method to screen for retained abilities. Fluorescence activated cell sorting was combined with in vitro compartmentalization that allows the substrate to remain associated with individual cells expressing the different mutants. Finally the final library was screened for recombinants with improved activity, and an improved beta-glucosidase was identified.

Production and implementation related to application

Production of enzymes in industrial scale needs to be economically favorable. Commercial development of an enzyme from its natural source comprises different fundamental tasks and strategies: Screening for microorganism, culture selection, fermentation studies, isolation, purification, characterization, evaluation, toxicology study, improvement of fermentation, recovery process development, product formulation, and finally, marketing (Saha & Bothast, 1997). This can be very costly and time consuming and, therefore, to maximize product purity and economy, more than 90% of industrial enzymes are produced recombinantly in hosts that have been modified in such a way as to decrease unwanted product and increase expression of the introduced genes (Cherry & Fidantsef, 2003).

Complete enzyme cocktail

A complete enzyme cocktail can be obtained by co-culturing fungi that separately are known for efficient production of single important enzymes, especially fungi from the genera *Trichoderma* and *Aspergillus* have frequently been used in mixed fermentations to obtain efficient cellulose hydrolysis where the beta-glucosidase contribution is mainly from *Aspergillus* (Duff *et al.*, 1985; Madamwar & Patel, 1992; Wen *et al.*, 2005). With the strategy of producing and selling enzymes on a protein mass basis, the goal is to increase the specific activity of the mixture, thereby obtaining high sugar yields with low enzyme loadings (Banerjee *et al.*, 2010a). It is therefore valuable to have a single organism expressing all enzymes required for making the sugars. However, most fungal strains do not produce significant amounts of all enzymes needed for complete hydrolysis of lignocellulosic biomasses and supplementation by other enzyme extracts is needed. Such an example is the cellulase product of *T. reesei* which is widely used and has for a long period set the stage in industrial production of enzymes for biomass hydrolysis. Beta-glucosidase activity is found in the product of *T. reesei*, but only roughly constitutes 0.5% of the secreted protein mix (Kubicek, 1992; Merino & Cherry, 2007). This is too low amounts for an efficient hydrolysis of cellulose. Enhancement of *T. reesei* beta-glucosidase has been achieved through displacement of the promoter by homologous recombination with xylanase and cellulase promoters obtaining a 4-7.5 fold increase in beta-glucosidase activity (Rahman *et al.*, 2009). Other ways of increasing the beta-glucosidase activity of *T. reesei* include heterologous expression of beta-glucosidase from other fungi, e.g. *Talaromyces emersonii* (Murray *et al.*, 2004), *Aspergillus oryzae* (Merino & Cherry, 2007), or *A. saccharolyticus* (Research paper IV, this thesis), thus creating a single expression host for the production of all

relevant enzymes for converting biomass into monomeric sugars. The opposite strategy of expressing endo-glucanases and/or cellobiohydrolases in a strain efficient in beta-glucosidase production can, of course, also be pursued.

Application wise, completing the value chain of a biorefinery concept has been considered in terms of reusing low value stream as fungal growth medium for enzyme production and directly using this product (enzymes, fungus, and medium) in hydrolysis of biomass. This on-site production could apply to both co-culturing as well as culturing an optimized production strain expressing all relevant enzymes. We have shown, that when cultured in the filter cake that is left after hydrolysis and fermentation in a bioethanol process, both *A. niger* and *A. saccharolyticus* can produce sufficient amounts of beta.glucosidase to substitute Novozym 188 in hydrolysis of pretreated wheat straw (Research paper I, this thesis).

Consolidated bioprocess

Two strategies can be sought for a consolidated process where one organism is responsible for both producing the enzymes required for saccharification as well as performing the fermentation of the saccharified sugars into fuels or platform molecules for bioproducts. The first strategy is to engineer an efficient enzyme producer, making it able to ferment sugars into desired products. The second strategy is to engineer an organism already capable of fermenting sugars into desired fermentation products, making it able to produce the array of enzymes needed for hydrolysis of polysaccharides (Xu *et al.*, 2009).

In terms of bioethanol production, most research on consolidated bioprocessing has been carried out using *Saccharomyces cerevisiae*. *S. cerevisiae* is the most frequently used microorganism for fermenting ethanol from glucose; it has GRAS status, high glucose fermentation rates, and high ethanol tolerance (van Zyl *et al.*, 2007). Additionally, xylose fermentation by yeast has been acquired to improve the economy of industrial lignocellulosic biomass conversion by efficiently directing both main monomeric sugars to ethanol fermentation (Chu & Lee, 2007). Fungal beta-glucosidases have successfully been heterologously expressed in *S. cerevisiae* enabling the strain to produce ethanol from growth on cellobiose (van Rooyen *et al.*, 2005). Further beta-glucosidase, endo-glucanase, and cellobiohydrolase of *Aspergillus aculeatus* have successfully been expressed by *S. cerevisiae* (Ooi *et al.*, 1994; Takada *et al.*, 1998), setting the stage for combined expression of all enzymes, making a strain with all three important enzymes. Fujita *et al.* (2004) and Yanase *et al.* (2010) have reported such engineered *S. cerevisiae* strain expressing all three enzymes: *T. reesei* endoglucanase and cellobiohydrolase, and *A. aculeatus* beta-glucosidase. This stain was able to directly ferment amorphous cellulose into ethanol. In a consolidated process, where yeast both produces enzymes for the hydrolysis of the lignocellulosic polysaccharides to sugar monomers, and ferments the monomeric sugars to ethanol, the issues of glucose accumulation inhibiting enzyme hydrolysis will be diminished. The main drawback of a yeast consolidated process is that a compromise in hydrolysis and fermentation must be made in terms of process conditions; especially the temperature must be reduced compared to the temperatures usually used for hydrolysis, and efficient enzymes with lower temperature optima should, therefore, be screened for to optimize consolidated bioprocessing with yeast.

The use of fungi for ethanol production through consolidated bioprocessing has recently been discussed by Xu *et al.* (2009). *T. reesei* possess the pathways for conversion of biomass into ethanol. However, the yield of ethanol, rate of production, and ethanol tolerance of the organism are low. Improvements needed for a successful *T. reesei* consolidated bioprocess would, therefore, include identification and modification of genes involved in ethanol tolerance, introduction of heterologous genes to enhance the ethanol pathway, and knockout of genes responsible for interfering byproducts (Xu *et al.*, 2009). With reference to the previous discussion of the insufficiency of *T. reesei* beta-glucosidases additional heterologous expression of better beta-glucosidases should be part of the improvements. In terms of producing platform molecules for bioproducts, several fungi naturally produce different organic acid, either as natural products or at least as intermediates in major metabolic pathways; examples include citric acid, oxalic acid, and gluconic acid by *A. niger*, itaconic acid by *A. terreus*, fumaric acid and malic acid by *Rhizopus oryzae*, and succinic acid by *Fusaruim* spp., *Aspergillus* spp., and *Penicillium simplicissium* (Magnuson & Lasure, 2004). A consolidated bioprocess using fungi to make platform molecules for bioproducts, therefore, seems appealing. *A. niger* is especially known in regards to its beta-glucosidase activity but this fungus actually possess the full array of hydrolytic enzymes for biomass degradation (Pel *et al.*, 2007) and could be a valid candidate for further improvements towards a consolidated bioprocessing organism. The issue related to compromise in process conditions of hydrolysis and fermentation will further apply for the use of fungal strains as was the case for yeast. Overcoming this could potentially be done by looking into the use of thermophilic rather than mesophilic fungi as the consolidated bioprocess organism.

Conclusions

Fungal beta-glucosidases are important enzymes in efficient hydrolysis of cellulosic biomass, as they relieve the inhibition of the cellobiohydrolases and endoglucanases by reducing cellobiose accumulation. They are key enzymes in the final part for creating the necessary sugars for production of biofuels and platform molecules that can

serve as building blocks in the synthesis of chemicals and polymeric materials. The biorefinery concept is a sustainable solution that could replace today's oil refineries. Important features for maintaining efficient hydrolysis of cellobiose and cellodextrins by beta-glucosidases are high glucose tolerance as well as high temperature stability. Heterologous expression of efficient beta-glucosidases allows for production of a complete enzyme cocktail by a suitable host. As of current, in the process of making sugars, enzymes are mainly added as a fermentation product of a separate process. To increase the value chain of a biorefinery concept, alternatives should be considered such as on-site enzyme production using low value streams, or the use of hosts expressing the enzyme cocktail and producing the products in a consolidated bioprocess, facilitating combined biomass hydrolysis and fermentation.

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Research paper I

On-site enzyme production during bioethanol production from biomass: screening for suitable fungal strains

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On-site enzyme production during bioethanol production from biomass: screening for suitable fungal strains

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ABSTRACT

Several process steps are used for production of cellulosic ethanol from biomass raw materials such as pre-treatment, enzymatic hydrolysis, fermentation, and distillation. Use of streams within cellulosic ethanol production was explored for on-site enzyme production as part of a biorefinery concept. In order to discover the most suitable fungal strain with efficient hydrolytic enzymes for lignocellulose conversion, a screening of 64 fungal isolates from environmental samples was carried out. These strains were examined in a plate assay for lignocellulolytic activities. Of the 64 isolates, 25 were selected for further enzyme activity studies using a stream derived from the bioethanol process. The filter cake that is left after hydrolysis and fermentation was the substrate chosen as the medium for enzyme production. Five of the 25 isolates were further selected for synergy studies with commercial enzymes, Celluclast 1.5L and Novozym 188. Finally, two strains, IBT25747 (*Aspergillus niger*) and AP (unidentified), were found as promising candidates for on-site enzyme production where the filter cake was inoculated with the respective fungus and in combination with Celluclast 1.5L used for hydrolysis of pretreated biomass.

1. Introduction

The need for purchasing commercial enzymes is an economical boundary for feasible second generation bioethanol production (NREL National Renewable Energy Laboratory, 2009). Therefore, the production of enzymes on-site using low cost substrates or even process waste streams as a production substrate should be considered.

Evaluating on the overall production cost of bioethanol the price of commercial enzymes is of outmost importance as it typically contributes to a large part of the final bioethanol cost. Other costs related to second generation ethanol production involve biomass raw material costs as well as transport of the biomass to the ethanol production facility. Operational costs are mainly linked to pre-treatment strategies of the material, enzymes, microorganisms for the fermentation, distillation method, other process options such as discharge or reuse of waste streams, and distribution of the product to the consumer (Knauf and Moniruzzaman, 2004)

The bioethanol process in focus is based on the Maxifuel concept, where the main process steps are pretreatment, hydrolysis, C6-sugar fermentation, separation, C5-sugar fermentation, and anaerobic digestion (Ahring and Westermann, 2007). The separation step between C6- and C5-sugar fermentation results in a "filter cake" that represents a waste stream that could be used as substrate for enzyme production by solid state fermentation. The advantages of solid state fermentation include high volumetric productivity, relatively higher concentration of the products, less cell mass generation, as well as lower energy consumption (Pandey et al., 1999; Pandey, 2003).

For efficient hydrolysis of cellulose, a complete set of cellulase enzymes is needed (Mansfield et al., 1999). Celluclast 1.5L and Novozym 188 represent two commercial enzyme preparations from Novozymes A/S often used in combination for hydrolysis of lignocellulosic biomasses. In order to reduce the cost of commercial enzymes for the hydrolysis of pre-treated lignocellulosic biomasses introduction of enzymes produced on-site using a slip stream from the bioethanol

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process as part of the fermentation medium could be an attractive alternative.

For this purpose a screening strategy was used, using different lignocellulosic, cellulosic, and hemicellulosic substrates to identify fungi able to degrade lignocelluloses, with primary focus on the cellulolytic activities. A predictive cellulase assay or screening is particularly difficult to develop because of the complex nature of the plant biomasses that the enzymes should hydrolyze; the pre-treated substrates are not pure and possible inhibitory compounds may influence the hydrolysis (Zhang et al., 2006). In this study, pre-treated wheat straw that had been processed in a pilot plant and the filter cake obtained after C6-sugar fermentation were used to screen for and to test potential on-site fungal enzyme producers. Fungal isolates collected for this work were screened for their cellulase activity and compared with the activity of two reference strains, *Aspergillus niger* and *Trichoderma reesei* that are commonly known as good enzyme producers (Mathew et al., 2008). The aim was to identify fungal strains that efficiently can utilize the filter cake stream of the second generation bioethanol production for enzyme production and to investigate the potential of such on-site enzyme production compared to the use of commercially available enzymes.

2. Materials and methods

2.1 Biomass and biomass characterization

Wet oxidized wheat straw (WO) and the filter cake (FC) obtained after C6-sugar fermentation of wet oxidized straw were kindly provided by Biogasol ApS, Denmark. Total solids (TS), volatile solids (VS), and ash contents were determined according to the NREL procedures "Determination of Total Solids in Biomass version 2005" and "Determination of Ash in Biomass version 2005", while structural carbohydrates and lignin was determined according to NREL procedure "Determination of Structural Carbohydrates and lignin in Biomass version 2006" (NREL National Renewable Energy Laboratory, 2010).

2.2 Media

Potato dextrose agar (PDA) was prepared by finely dicing 200 g peeled potatoes, boiling them in one litre of water for one hour, and letting this mixture pass through a sieve. 1.5% agar, 2% dextrose, and 1 ml trace metals (TM: 1 g $ZnSO_47H_2O$, 0.5 g $CuSO_45H_2O$ in 100 ml water) per litre were added, followed by autoclaving at 121°C for 20 minutes.

Agar plates of filter cake (FC) (Biogasol ApS), wet oxidized wheat straw (WO) (Biogasol ApS), birch wood xylan (Sigma), avicel (Sigma), and carboxymethyl cellulose (CMC) (Sigma) were prepared as follows. The FC was washed to decrease the amount of free sugars by adjusting the FC to a TS (total solids) of 4%, autoclave at 121°C for 30 minutes, followed by settling for one hour, removing of top liquid, and addition of new water. The procedure was repeated, ending up with a TS of approximately 4%. 2% agar was added to the washed FC, autoclaved at 121°C for 15 minutes, and pH adjusted to 4.8. The other agar plates were prepared by adding 2% w/v of either WO, birch wood xylan, avicel, or CMC to the basic Czapek (CZ) agar media (3 g/l $NaNO_3$, 1 g/l K_2HPO4, 0.5 g/l KCl, 0.5 g/l $MgSO_47H_2O$, 0.01 g/l $FeSO_47H_2O$, 15 g/l agar) (Samson et al., 2004) and adjusting the pH to 4.8.

2.3 Fungal collection

Fungal isolates were obtained from various sources. Samples were isolated from wooden biomass such as decomposed wood in a local swamp, treated hardwood, and rye bread. Additionally, fungal strains were provided from IBT's Culture Collection of Fungi by selection of Professor Jens C. Frisvad, Centre for Microbiology, Depertment of Systems Biology, Technical University of Denmark. These included the following strains: IBT25747 (*Aspergillus niger*), IBT3016 (*A. "massa"*), IBT3945 (*Penicillium "pseudofuniculosum"*), IBT14668 (*P. "rapidoviride"*), IBT15094 (*P. "rapidoviride"*), IBT16756 (*A. pseudofumigatus*), IBT18366 (*P. "pseudoverruculosum"*), IBT26808 (*P. pulvillorum*), and IBT7612 (*Trichiderma reesei*), where *A. niger* and *T. reesei* are used as reference strains.

2.4 Fungal isolation

Initially a small piece of e.g. wood was placed on PDA plates and incubated at room temperature. Several rounds of culture transfers onto new PDA plates were carried out in order to obtain pure single isolates.

2.5 Screening of fungal growth on agar plates

Five different substrates were used for the initial screening on agar plates: Filter cake (FC), wet oxidized wheat straw (WO), birch wood xylan, avicel, and carboxymethyl cellulose (CMC), prepared as described above. Spores of seven day old fungal cultures were transferred to the centre of the different plates using a 1 µl inoculation loop. The plates were incubated at 25°C for 7 days, and growth was graded on a subjective scale 1-5 by visually determining the colony diameters. On the xylan plates an additional subjective grade was introduced: 0-3z, which relates to the clearing zone observed in the medium, often having a larger diameter than the growth of the fungus itself. The screening on different agar media was carried out in single determination.

2.6 Activity studies using AZCL-plates

Agar plates with CZ-medium pH 4.8 containing 0.1% azurine cross-linked substrates: AZCL-HE-Cellulose (HE=hydroxyethyl) and 0.1% AZCL-arabinoxylan (Megazyme), were used for cellulase and xylanase activity studies, respectively. Inoculation was done by placing 1 µl of 10^6/ml spore suspension in the centre of the plates. The activity was indicated by blue color zones resulting from hydrolysis of the substrate. The progress of the blue zones was followed for 7 days at room temperature and the final coloration was used to categorize the strains on a subjective scale from 1 to 5. The activity study on AZCL-plates was carried out in double determination.

2.7 Activity studies by solid state fermentation on filter cake and hydrolysis of wet oxidized biomass

FC was autoclaved and freeze dried in order to stabilize the biomass. A mixture of 4.15 ml CZ medium and 0.85 g freeze dried FC (TS = 17%), in total 5 grams, was used as growth medium in solid state fermentation. The medium was inoculated with a spore solution of approximately 10^6 spores per gram DM in each fermentation and the beakers were incubated at 25°C in high humidity for 7 days. Growth set-up was conducted in triplicates. After incubation, 0.1 M Na-citrate buffer pH 4.8 was added directly to the beaker to give a TS of 3%, followed by blending with a coffee mixer for 30 seconds to disrupt the fungus/medium complex created by the growth of the fungus. This homogenized fermentation broth (0.85 g DM FC) was then added to wet oxidised straw (containing 2.8 g DM WO) pH 4.8, and the total mixture was brought to a final TS of 2% by addition of 0.1 M Na-citrate buffer pH 4.8. The FC to WO ratio was approximately 1:3 on dry matter basis. Incubation was done in 50 ml vials at 50°C for 4 days with shaking at 160 rpm, and 1 ml samples were taken before and after hydrolysis. The samples were centrifuged and the supernatants were analysed using HPLC and the reducing sugar assay (see below).

2.8 Synergistic test with commercial enzymes

Solid state fermentation was used for the purpose of evaluating synergistic effects of the fungi and Celluclast 1.5L and Novozym 188 (Novozymes A/S, Denmark). The set-up was as described above, but with commercial enzyme preparations added after the FC with fungus and WO had been mixed. One setup contained the fungus as the only source of enzymatic activity; a second setup contained the fungus and 1 FPU (filter paper unit) Celluclast 1.5L added; while a third contained the fungus, 1 FPU Celluclast 1.5L added, and Novozym 188 (in a ratio of 4:1, respectively).

2.9 Activity assay of fungal extracts

Endo-glucanase activity was determined by the use of AZO-CMC, assayed according to the manufacturer's description (Megazyme) (Wood and Bhat, 1988). The enzyme solution was assayed in various dilutions at the conditions stated by the manufacturer. A standard curve was obtained by activity measurements of a pure endoglucanase from *Aspergillus niger* (Megazymes), with an activity of 322 U/ml at 40 °C, pH 4.6, reported by the manufacturer.

Beta-glucosidase activity was assayed with 5 mM *p*-nitrophenyl-beta-D-glucopyranoside (Sigma) in 50 mM sodium citrate buffer pH 4.8 as substrate. The assay was carried out as described by Flachner et al. (1999). The activity was expressed in units (µmoles substrate converted per minute).

2.10 Sugar analysis

All samples to be analyzed by HPLC (Hewlet Packard 1100 series) were run on a 300·7.8 mm Aminex HPX-87H Column (BioRad) at 60°C with sulfuric acid as eluent at a flowrate of 0.6 ml/min and an injection volume of 10 µl. The components were detected refractometrically on a RI detector.

The reducing sugar assay was carried out according to Ghose (1987) and Miller (1959). One ml milliQ water and 0.5 ml sample solution were mixed before 3.0 ml dinitrosalicylic acid (DNS) was added. The mixture was boiled for 5 minutes; then diluted with MilliQ water, followed by spectrophotometrical absorbance measurements at 540 nm (Milton Roy spectronic 301), using MilliQ water as standard. A standard curve was obtained with different concentrations of glucose, thereby expressing absorbance as glucose equivalents.

3. Results and discussion

3.1 Initial screening and biomass evaluation

A screening program for different new fungal isolates from environmental samples was established in order to identify strains with high hydrolytic activity compared to wellknown reference strains. A total of 64 strains were included in the initial screening that comprised grading of growth on the different substrates: filter cake (FC), wet oxidized wheat straw (WO), xylan, avicel, and carboxymethyl cellulose (CMC) (Table 1). The grading was based on colony size (morphology), as it was expected that the better the fungi grew, the better they utilized the substrate. In general, FC and WO were the media that resulted in the best growth of the fungi tested. 25% of the strains were graded with 3 or above on FC, while 35% of the strains were graded 3 or above on WO, yet WO was at the

same time the substrate having the greatest number of strains that did not grow at all (Table 1).

FC of hydrolyzed wet oxidized wheat straw represents a fraction from the bioethanol process with low commercial value and could be of great interest to utilize as a substrate for enzyme production for the bioethanol process. The reuse of FC would loop back the un-hydrolyzed biomass into the process and thereby make good value of this fraction. The main components of the FC are relatively high levels of lignin followed by cellulose (Table 2). The cellulose of FC is regarded as poorly available as it remained part of the solid product and thus was not initially hydrolyzed. It is speculated that fungi capable of growing on FC potentially have new interesting enzymes that can facilitate further hydrolysis of FC. Overall, this favors the use of FC for on-site enzyme production and FC was therefore included in the screening. Adding FC as fermentate directly in the hydrolysis will increase the overall lignin concentration in the hydrolysis; this could potentially have a negative impact on the enzyme hydrolysis as lignin is known to enhance enzyme adsorption (Berlin et al., 2005). However, FC is likely to already be associated with proteins from the previous hydrolysis (Jorgensen et al., 2007). This would reduce both the non-productive and productive binding of new enzymes due to low accessibility. During fermentation the close association of the growing fungus with the FC could result in proteolytic metabolic activities that to a certain extent could remove some of the enzymes bound to the FC. This would increase the accessibility of the remaining cellulose in the FC (Berlin et al., 2006).

WO represents another fraction in the ethanol process and could be a potential substrate for enzyme production as it is for ethanol production. Wet oxidized material has previously been reported to contain compounds inhibitory towards enzyme hydrolysis and fermentation (Klinke et al., 2004).

Table 1. Grading of growth on substrates on a scale 0-5 (0=poor growth, 5=very good growth).
*z=zone of clearance, graded on a scale 0-3 (0=no clearance, 3=large zone of clearance)

	1.1	1.1.1	1.2	1.2.2	1.3	1.4	1.5.1	1.6	1.7	1.8.1	1.8.2	2.1	2.2	2.3	2.4	2.5	2.6	2.7	2.8	2.9	3.1	3.2
FC	2	2	2	1	2	2	4	2	1	3	2	1	1	4	0	2	0	0	5	0	1	4
WO	2	3	0	0	4	4	4	2	0	2	3	0	2	3	0	3	0	2	3	2	0	2
Xylan*	3 z2	5 z1	5 z3	1 z0	5 z3	5 z3	2 z0	4 z3	1 z1	5 z3	3 z2	2 z0	3 z0	5 z0	2 z1	2 z1	2 z0	2 z0	4 z1	2 z0	4 z1	4 z1
Avicel	1	1	2	0	1	1	1	2	1	5	1	3	1	1	0	1	1	1	3	1	1	1
CMC	2	3	1	0	2	1	1	3	0	5	1	1	1	3	0	2	1	1	1	2	0	5

	3.3	4.1	4.2	5.1	7.1	7.2	7.3	9.1	9.2	9.3.1	9.3.2	9.4	9.4.1	9.5	10.1	10.2	10.3	10.4	10.5	11.1	11.2	11.3
FC	1	3	2	4	2	2	1	1	1	2	3	1	2	1	1	1	1	1	1	1	2	1
WO	0	3	3	3	1	1	1	2	3	2	3	2	2	2	3	2	2	0	3	0	0	0
Xylan*	2 z1	1 z0	1 z0	4 z0	3 z0	2 z1	3 z0	2 z1	2 z2	2 z2	1 z0	2 z2	2 z3	2 z2	1 z0	1 z0	1 z0	2 z2	1 z0	1 z0	1 z0	2 z3
Avicel	0	1	1	3	2	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
CMC	1	1	2	5	1	1	2	1	3	1	2	1	2	1	1	1	1	1	2	0	2	0

	11.4	11.5	13	14	15	Hj1	Hj3	AP	A1	A2	A3	IBT3016	IBT3945	IBT14668	IBT15094	IBT16756	IBT18366	IBT26808	Ref.strain IBT25747	Ref.strain IBT7612
FC	1	3	1	3	3	1	3	1	1	1	0	3	1	3	3	0	3	3	1	4
WO	3	2	3	3	2	3	0	3	0	0	0	0	2	3	3	2	4	4	2	3
Xylan*	3 z1	3 z2	2 z2	2 z2	2 z2	1 z0	3 z3	1 z0	2 z2	1 z0	1 z0	3 z1	1 z1	3 z3	3 z3	3 z1	1 z0	3 z2	4 z3	2 z0
Avicel	2	1	1	2	1	0	3	1	2	1	0	1	1	2	2	1	1	1	1	1
CMC	3	2	1	1	2	2	1	3	1	1	0	3	2	3	2	1	2	2	2	0

Table 2. Biomass composition of pre-treated wheat straw (WO) and filter cake (FC). The cellulose, hemicelluloses, and lignin content are calculated using NREL procedures, where glucose and xylose amounts after acid hydrolysis are calculated into cellulose and hemicellulose, respectively.

	Cellulose (% of DM)	Glucose (g/gDM)	Hemicellulose (% of DM)	Xylose (g/gDM)	Lignin (% of DM)
WO	41.0 ± 0.3%	0.46 ± 0.003 g/gDM	16.0 ± 0.6%	0.18 ± 0.007 g/gDM	27.94 ± 0.1%
FC	28.3 ± 0.4%	0.31 ± 0.004 g/gDM	6.3 ± 0.2%	0.07 ± 0.002 g/gDM	46.61 ± 0.2%

However, poor growth of enzyme producers on this medium does not necessarily relate to a lack of cellulolytic enzymes produced by the fungus. Amongst those strains that did grow on WO, an increased number of strains received good growth grades indicating that the greater levels of free sugars in the WO media facilitated increased growth compared to the FC where washing had removed the free sugars. Free sugars will help initiate fungal growth and perhaps thereby boost the fungi that have the right combination of enzymes to degrade the substrate to continue growth. The lower lignin content of the WO (Table 2) compared to FC might also explain the increased growth on WO.

Of the commercial hemicellulose and cellulose substrates, xylan was the substrate supporting good growth of the greatest number of strains (grades of 3 or above), and was the only substrate that supported growth of all strains (Table 1). This clearly shows that xylanases are widespread amongst fungi in general. In contrary, the strains did not grow well on the cellulose substrates as less than 10 and 20% were given grades of 3 or above on avicel and CMC, respectively (Table 1), thus making the strains that did succeed in utilizing this carbon source outstanding compared to the most strains tested.

Xylan was included in the screening to determine the ability of the fungus to utilize a hemicellulosic carbon source, as the enzymatic degradation of xylan to xylose could add value to the process in terms of the pentoses later being fermented into ethanol by C5 fermenting microbes. Avicel and CMC represented the cellulose fraction of the biomass, each having different degree of crystalline and amorphous regions, where avicel primarily requires cellobiohydrolase activity and CMC requires endoglucanase activity for their hydrolysis (Zhang et al. 2006).

Of the 64 strains included in the screening, 25 were chosen for further studies (grey colored in Table 1), with the grey colored grades representing the primary reasons for choosing these particular strains. The selection was based on good growth grades (≥3) on FC, WO, avicel, and CMC. *A. niger* IBT25747and *T. reesei* IBT7612 were included as reference strains.

3.2 Enzyme activity studies of strains selected from screening

Two activity studies were carried out to evaluate the enzyme activity of the 25 strains chosen from the initial screening: one using AZCL medium for simple measurements of cellulase and xylanase activity, and one using the complex filter cake as substrate for enzyme production followed by its use in hydrolysis of WO to determine activity on this complex substrate. AZCL media are commonly used for broad enzyme screening with focus on endo-activites (Pedersen et al., 2007; Pedersen et al., 2009). The use of pretreated biomass (including wheat straw, wheat bran, rice straw, groundnut shells, corn fiber) and wastes (including paper pulp, municipal refuse, stillage of sugar cane bagasse and spruce wood hydrolysates) to support fungal growth and enzyme production has previously been reported (Alriksson et al., 2009; Dien et al., 2006; Doppelbauer et al., 1987; Gupte and Madamwar, 1997; Thygesen et al., 2003). However, it has not previously been reported to use the waste stream after C6 fermentation and where C5 sugars are removed with the

Table 3. Grading of blue color formation from growth on AZCL-Polysaccharides plates. Blue colour zone and intensity graded on a scale 0-5

	1.1.1	1.5.1	1.6	1.8.1	2.1	2.3	2.8	3.2	4.1	5.1	9.2	9.3.2	11.4	11.5	14	15	Hj3	AP	IBT3016	IBT14668	IBT15094	IBT18366	IBT26808	Ref.strain IBT25747	Ref.strain IBT7612
AZCL-cellulose	4	1	3	5	2	3	1	4	0	5	3	3	4	3	1	2	3	4	2	3	4	2	1	3	0
AZCL-arabinoxylan	4	2	3	5	3	3	2	0	2	5	3	3	4	4	2	2	3	4	4	3	4	2	4	4	4

soluble phase. That is, to use the filter cake of the wheat straw bioethanol process for enzyme production and reuse it directly for hydrolysis without separating out the enzymes, thereby adding value to the overall process.

The activity study using AZCL-substrates was subjectively graded based on degree of blue color zone and its intensity (Table 3). Comparing these results with the initial growth screening (Table 1), it was found that the grades on CMC and AZCL-cellulose correlate well. Therefore, the ability to grow well on CMC was indeed an indication that the fungi had enzymes for hydrolysis of CMC, shown by the dye-release on AZCL-cellulose. The growth grades on xylan (Table 1) and color grades on AZCL-arabinoxylan (Table 3) were for the most part comparable. However, the clearing zone grades on xylan only partially correspond with the color grades on AZCL-arabinoxylan. This might be due to that the color grades on AZCL-arabinoxylan are based on both color intensity as well as zone diameter, while it for the xylan plates was difficult to judge clearing zone intensity.

The enzyme activities produced by the fungi growing in solid state on FC were evaluated by analysis of the soluble sugars after hydrolysis of WO. Increase in reducing ends (measured as glucose equivalents) as well as final C6 and C5 sugars present after hydrolysis of wet oxidized biomass were compared (Table 4). The increase in monomeric sugar concentration only partly correlates with the measured increase in reducing ends of the samples. Two good examples are the strains 11.4 and IBT15094; these are in the top 5 of the reducing ends measurements, but do not even get in top 10 of the monomeric sugar measurements. A high score was, however, given on both AZCL-cellulose and –arabinoxylan, indicating high endo-activity. Great endo-activity will increase the number of reducing ends, but if the fungus does not have good beta-glucosidase/-xylosidase activity, no great monomeric concentration will be detected. To reach a high concentration of sugar monomers, a full enzyme cocktail must work together, consisting of exo-, endo-glucanases/xylanases, and beta-glucosidases/-xylosidases (Zhang et al., 2006). Accumulation of cellobiose was detected for strain 1.8.1 and 2.3 (data not shown), indicating high cellobiohydrolase activity, but lack of sufficient beta-glucosidase activity. These different observations highlight the importance of multiple screening methods to be able to map the different activities of each fungus.

Table 4. Increase in concentration of reducing ends (measured as glucose equivalents) and monomeric sugars after hydrolysis of wet exploded wheat straw by fungal enzymes produced on filter cake. The number in the parenthesis assigns the ranking of the fungus among all fungi examined.
*Here, an accumulation of cellobiose was found.

	1.1.1	**1.5.1**	**1.6**	**1.8.1**	**2.1**	**2.3**	**2.8**	**3.2**	**4.1**	**5.1**	**9.2**	**9.3.2**	**11.4**
Reducing ends (g/l)	1.27	0.58	1.33 (8)	0.75	1.11	0.91	1.24	0.66	1.23	1.27	1.37 (7)	1.16	1.42 (5)
Glucose (g/l)	0.41	0.68 (4)	0.18	0.52*	0.58 (9)	0.65* (5)	0.4	0.32	0.28	0.73 (3)	0.61 (7)	0.16	0.27
Xylose (g/l)	1.49 (3)	0.09	0.41	0.35	1.39 (7)	0.41	1.16	0.06	1.32	1.29	1.46 (5)	0.73	1.17

	11.5	**14**	**15**	**Hj3**	**AP**	**IBT3016**	**IBT14668**	**IBT15094**	**IBT18366**	**IBT26808**	**Ref.strain IBT25747**	**Ref.strain IBT7612**
Reducing ends (g/l)	0.91	0.76	1.49 (3)	1.25	1.68 (2)	0.53	1.32 (9)	1.48 (4)	1.38 (6)	1.24	2.23 (1)	1.29
Glucose (g/l)	0.6 (8)	0.23	0.51	0.55	0.9 (2)	0.15	0.45	0.52	0.45	0.33	1.48 (1)	0.63 (6)
Xylose (g/l)	0.36	0.11	1.46 (4)	1.42 (6)	1.57 (2)	0.07	1.1	0.85	1.37 (8)	0.57	1.81 (1)	1.35 (9)

Of the 25 strains, five were selected for further studies with commercial enzymes. We chose to continue with the strains that had generally performed best in terms of total cellulose degrading abilities in multiple of the assays (ref strain IBT25747, AP, and 5.1), and also to include some that are thought to specifically possess high endo-activity (IBT15094) or exo-glucanase acitivity (1.8.1).

3.3 Synergies with commercial enzymes

The five strains were grown in FC for enzyme production, followed by hydrolysis of WO in combination with the commercial enzymes Celluclast 1.5L and Novozym 188. Low filter paper unit (FPU) loadings of Celluclast 1.5L and a 4:1 ratio of Novozym 188 were applied to visualize the synergistic effects of the fungi that were tested and the commercial enzymes. The beta-glucosidase and endo-glucanase activities were measured for each fungal or commercial enzyme addition (Table 5).

Of the two commercial enzyme preparations, the beta-glycosidase activity of Novozym 188 helps increase the glucose yields and decrease the cellobiose concentration created by the cellobiohydrolase activity of Celluclast 1.5L that can be self-inhibiting (Beguin and Aubert, 1994; Bhat and Bhat, 1997). The glucose concentration after hydrolysis with Celluclast 1.5L and Novozym 188, was slightly higher than the sum of glucose and cellobiose after hydrolysis using just Celluclast 1.5L (data not shown). This could be explained by the higher cellobiose concentration inhibiting the endoglucanases and cellobiohydrolases resulting in a lower total hydrolysis in the case of just using Celluclast 1.5L (Mansfield et al. 1999).

Such synergies were examined in our five selected strains, using their enzymes produced on FC to hydrolyze WO alone, WO in combination with Celluclast1.5L, and WO in combination with Celluclast 1.5L and Novozym 188 (Figure 1). Evaluations were primarily based on the cellobiose concentration at the end of the hydrolysis. On this basis, synergies were found with the strain AP and IBT25747, as there was no accumulation of cellobiose and therefore sufficient synergies with the enzyme activities of Celluclast 1.5L. With strain 1.8.1, 5.1, and IBT15094, accumulation of cellobiose was seen after hydrolysis when hydrolysis was performed with the test fungus and Celluclast 1.5L and here it is obvious that synergetic activities resulting from growth on FC were insufficient. This correlates well with the measured activities (Table 5), where AP and IBT25747 showed high beta-glucosidase activity while the beta-glucosidase activity of 1.8.1, 5.1, and IBT15094 was low. It was further confirmed by the addition of Novozym 188 that in all cases resulted in a drop in cellobiose concentrations.

The profiles of the strains AP and IBT25747 are very similar with regards to beta-glucosidase activity, while IBT25747 has far greater endo-glucanase acitivity (Table 5). Strain IBT25747 gives the highest glucose yield when hydrolysis was performed with only this fungus, while AP gives the second highest (Figure 1). Combining either IBT25747 or AP with Celluclast 1.5L for hydrolysis showed that the beta-glucosidase activity of IBT25747 and AP has

Table 5. Units of beta-glucosidase (BG) and endoglucanase (EG) added by each fungus or commercial enzyme prep to the WO hydrolysis

	Celluclast 1.5L	Novozym 188	1.8.1	5.1	AP	IBT15094	Ref strain IBT25747
BG	0.4	1.5	low	low	7.24	low	7.85
EG	41	low	0.99	1.05	8.09	2.92	85.37

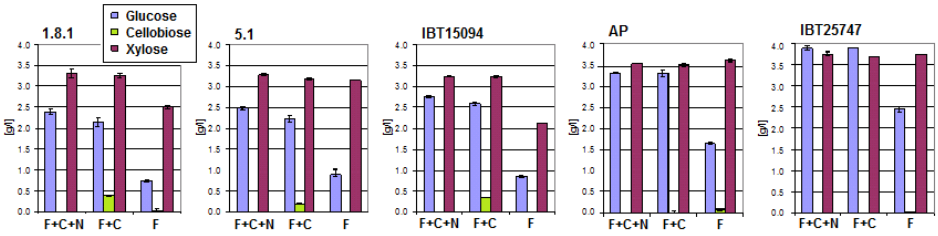

Figure 1. Synergies with commercial enzymes. Increase in glucose, cellobiose, and xylose concentrations after hydrolysis of WO. F= fungus; C = Celluclast 1.5L; N = Novozyme 188; error bars represent the double determinations of the hydrolysis.

synergies with the cellobiohydrolase activity of Celluclast 1.5L, and the endoglucanase activities add to the total hydrolysis. Addition of Novozym 188 has no significant effect on total hydrolysis evaluated by glucose yields, with the enzyme concentrations used here, likely explained by the fact that the fungus contributes with about 5 times the amount of beta-glucosidase activity compared to the Novozym 188 added (Table 5).

Xylose is readily released by all strains (Figure 1), supporting the fact that xylose is more accessible due to the nature of the hemicellulose structure (Saha, 2003). It is therefore speculated that enzymes with relatively high xylanase activity are likely to be "part of the package" when using organisms with cellulolytic activities.

Evaluating the enzyme activities (Table 5) together with the hydrolysis results (Figure 1), it is clear that greater activity results in a higher degree of hydrolysis. The endoglucanase activity of IBT25747 is approximately 10 times higher than the endoglucanase activity of AP, while the beta-glucosidase activity is approximately the same. This resulted in a difference in glucose yield amongst the two strains of 46% when hydrolysis was performed with only the fungus, but only a difference of 17% when hydrolysis was performed with the fungus and Celluclast 1.5L combined. The additional endo-glucanase contribution from Celluclast 1.5L is the most likely reason for this decreased difference in yield.

Strain IBT25747 is *A. niger* and it is, therefore, not surprising that its enzymes in combination with Celluclast 1.5L are good at hydrolyzing WO. However, besides of this known strain, one of our own unknown isolates, strain AP, showed a very promising profile in terms of on-site enzyme production using FC as growth medium. Stain AP was able to compete with the reference strain and was sufficient for hydrolysis of WO in combination with Celluclast 1.5L. Addition of Novozym 188 had no extra effect on sugar yields when FC pregrown with strain AP was added. Eliminating the need for beta-glucosidase addition during hydrolysis will significantly lower the cost of enzyme addition so our results have importance for practical applications.

4. Conclusion

This work demonstrates the possibility of using a low value stream of the biofuel production, FC, for enzyme production, where the fungus is grown in the FC and the FC with the fungus is used directly during hydrolysis of WO to obtain monomeric sugars for biofuel production. Such on-site enzyme production is valuable in terms of obtaining a complete value chain of the biofuel production. Through a broad screening for on-site enzyme producers as well as testing for synergistic effects, promising candidates were selected. From the use of reference enzymes, e.g. Celluclast 1.5L, that is known to lack important enzyme activities for complete hydrolysis, identification of enzymes that can contribute to synergy and thereby more efficient hydrolysis were found. Here, ref strain IBT25747 and own strain AP were found as promising candidates for on-site enzyme production with FC as growth and production medium. It was showed that the FC grown with these fungi can substitute Novozym 188 in the hydrolysis of WO..

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Research paper II

Screening for beta-glucosidase activity amongst different fungi capable of degrading lignocellulosic biomasses: discovery of a new prominent beta-glucosidase producing *Aspergillus* sp.

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Screening for beta-glucosidase activity amongst different fungi capable of degrading lignocellulosic biomasses: discovery of a new prominent beta-glucosidase producing *Aspergillus* sp.

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ABSTRACT

Through a broad fungal screening program for beta-glucosidase activity using wheat bran as substrate in submerged fermentation, a new prominent beta-glucosidase producing strain was identified amongst 86 screened strains. This uncharacterized strain (AP) showed significantly better beta-glucosidase potential than all other fungi screened, with *Aspergillus niger* showing the second greatest activity. Strain AP was from its ITS1 sequence identified as an *Aspergillus* sp., and phylogenetic analysis indicated it was a new species. The potential of a solid state fermentation extract of strain AP was compared with the commercial beta-glucosidase containing enzyme preparations: Novozym 188 and Cellic CTec. The extract of strain AP was found to be a valid substitute for Novozym 188 in terms of better cellobiose hydrolysis, by having the same Michaelis Menten kinetics affinity constant, and by performing equally well during hydrolysis with regard to product inhibition. Furthermore, the extract from the strain had higher specific activity (U/total protein) and also increased thermostability compared with Novozym 188. The significant thermostability of strain AP beta-glucosidases was further confirmed when compared with Cellic CTec. The beta-glucosidases of strain AP were able to degrade cellodextrins with an exo-acting approach, and they were also found capable of hydrolyzing pretreated bagasse to monomeric sugars when combined with Celluclast 1.5L.

1. Introduction

Exploitation of lignocellulosic biomasses for production of biofuels, biochemicals, and pharmaceuticals comprises a promising alternative to the world's limited fossil energy resources. Lignocellulosic biomasses mainly consist of cellulose, hemicellulose, and lignin, with different distribution of each component depending on the specific plant species. Cellulose is of great interest in terms of producing sugars for biofuels and chemicals as its hydrolysis product, glucose, can readily be fermented into ethanol or converted into high value chemicals. The hydrolysis of cellulose involves the synergistic action of cellobiohydrolases (EC 3.2.1.91), endoglucanases (EC 3.2.1.4), and beta-glucosidases (EC 3.2.1.21) [1]. The first two act on the solid substrate, where the cellobiohydrolases are capable of degrading the crystalline parts of cellulose by cleaving off cellobiose molecules from the ends of the cellulose chains. The endoglucanases hydrolyze glucosidic bonds of the more amorphous regions of the cellulose, decreasing the degree of polymerization and creating more ends for substrate-enzyme association by cellobiohydrolases. Finally, the beta-glucosidases act in the liquid phase hydrolyzing mainly cellobiose to glucose, but also to some extent cellodextrins, sugars with a low degree of polymerization [2].

Historically, enzymes from *Trichoderma reesei* and *Aspergillus niger* are known as a good match for the hydrolysis of cellulose; *T. reesei* enzymes mainly contribute with cellobiohydrolase and endoglucanase activity and *A. niger* enzymes with beta-glucosidase activity [3]. Beta-glucosidases are of key importance as they are needed to supplement the cellobiohydrolase and endoglucanase activities for ensuring final glucose release and at the same time decreasing the accumulation of cellobiose and shorter cellooligmers, which

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are known as product inhibitors for the cellobiohydrolases [1]. Especially efficient beta-glucosidases, that are not themselves easily inhibited by their product, glucose, are of great interest. Most commercial cellulase preparations are produced by *T. reesei*, e.g. Celluclast 1.5L (Novozymes A/S), which has to be supplemented with extra beta-glucosidase activity from another source, e.g. Novozym 188 (Novozymes A/S), in order to improve cellulose hydrolysis. Recently, Cellic CTec (Novozymes A/S) has been launched, which has all activities needed for hydrolysis of cellulose in one single preparation. The commercially available beta-glucosidases have relatively low long-term temperature stability. Robustness, thermostability and substrate specificity are very important characteristics for enzymes to be applied in industrial processes.

The aim of the present work was to search for beta-glucosidase producing fungi using a screening strategy based on wheat bran as substrate and to compare the enzymes from the best strain(s) with commercial enzyme preparations based on enzyme kinetics, including Michaelis-Menten studies, thermostability, pH optimum, glucose tolerance, and ability to hydrolyze cellodextrins and pretreated lignocellulose. The hypothesis is that new and better beta-glucosidase enzyme producers can be found through a broad screening of lignocellulose degrading fungi. The fungi selected for the screening originated from several different countries and was partly collected by ourselves for this study and partly donated by other researchers.

2. Materials and methods

2.1 Fungal samples

This study comprises fungal samples from many different sources, including new isolations and fungi from our "in house" fungal collection. Table 1 specifies the stain numbers, identity, identification method, origin, and reference of each sample. New fungal isolates were from soil and decaying wood samples, isolated by multiple transfers on potato broth agar (PDA) plates supplemented with 50 ppm chloramphenicol and 50 ppm kanamycin, incubated at room temperature. Samples isolated in this work were all identified by ITS sequencing, using the method described below. All fungi were grown on potato dextrose agar (PDA, Sigma) at room temperature and maintained in 10 % glycerol at –80 ˚C.

The following aspergilli reference strains were kindly donated by Professor Jens C. Frisvad, Technical University of Denmark (DTU): *A. niger* CBS 554.65^T, *A. homomorphus* CBS 101889^T, *A. aculeatinus* CBS 121060^T, *A. aculeatus* CBS 172.66^T, *A. uvarum* CBS 121591^T, and A. *japonicus* CBS 114.51^T.

2.2 Beta-glucosidase screening

For beta-glucosidase activity screening was carried out in a Falcon tube set-up where three 0.5x0.5cm squares were cut from PDA plates with 7 days old single fungal strains and incubated in 10 ml of media shaking (180 rpm) at room temperature for another 7 days. The media was composed of 20g/l wheat bran (Finax), 20g/l corn steep liquor (Sigma), 3g/l $NaNO_3$, 1g/l K_2HPO_4, 0.5 g/l KCl, 0.5 g/l $MgSO_47H_2O$, 0.01 g/l $FeSO_47H_2O$. The samples were centrifuged at 10,000 rpm for 10 minutes and the supernatants were subsequently assayed for beta-glucosidase activity and protein content.

Beta-glucosidase activity was measured using 5 mM p-nitrophenyl-beta-D-glucopyranoside (pNPG) in 50 mM Na-Citrate buffer pH 4.8 as substrate. 15 µl sample and 150 µl substrate were incubated at 50°C for 10 minutes in 200 µl PCR tubes in a thermocycler (Biorad); 30 µl of the reaction was transferred to a microtiter plate already containing 50 µl 1M Na_2CO_3 for termination of the reaction. Absorbance was read at 405 nm in a plate reader (Dynex Technologies Inc.). pNP was used to prepare a standard curve. One unit (U) of enzyme activity was defined as the amount of enzyme needed to hydrolyze 1 µmol pNPG in 1 minute.

Protein quantification was done using the Pierce BCA protein assay kit microplate procedure according to manufacturer's instructions (Pierce Biotechnology).

2.3 Identification of fungi using sequencing of ITS1 region

DNA extraction was carried out by the method of Dellaporta *et al.* [11] using bead beating (2x 20sec) of fungal biomass in extraction buffer (500 mM NaCl, 100 mM Tris pH8, 50 mM EDTA, 1m M DTT) and 1x 20sec with SDS added to final concentration of 2%. Protein and cell debris was precipitated with potassium acetate at a final concentration of 1.4 M. DNA was precipitated with equal volumes of sample and 2-propanol, followed by washing with 70% ethanol, and finally resuspended in water.

Two fungal primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS2 (5' GCTGCGTTCTTCATCGATGC 3') that match the conserved 18S and 5.8S rRNA genes, respectively, were used for the amplification of the non-coding IST1 region [12, 13]. Approx 100 ng genomic DNA was used as template in a polymerase chain reaction with 1 U proof reading WALK polymerase (A&A Biotechnology), PCR buffer (50 mM Tris pH 8, 0.23 mg/ml BSA, 0.5% Ficoll, 0.1 mM cresol red, 2.5 mM $MgCl_2$), 0.2 mM of dNTP, 0.4 µM of each primer ITS1 and ITS2. Using a thermocycler (BioRad), an initial denaturation step (94°C, 2min) was followed by 35 cycles of denaturation (94°C, 30 sec), annealing (60°C, 30 sec), and elongation (72°C, 1 min), and a final elongation step (72°C, 2 min) following the last cycle. All products were checked by gel electrophoresis. Depending on the purity of the sample, either GelOut or CleanUp was performed (EZNA kits from Promega) according to the manufacturer's instructions.

DNA sequencing was performed by either MWG Eurofins, Germany or Starseq, Germany, directly sequencing the PCR products with the ITS1 or ITS2 primer. The sequence data was submitted to the GenBank NCBI nucleotide blast search database for fungal identification.

2.4 Molecular phylogeny

Phylogenetic analysis of the ITS1 region of the fungus AP and different aspergilli was carried out as described by Varga *et al.* [14] and Samson *et al.* [15]. ClustalW multiple alignment was used for sequence alignment and manual improvement of the alignment was performed using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The PHYLIP program package version 3.69 [16] was used for preparation of phylogenetic trees. The distance matrix of the data set was calculated based on the Kimura method [17] using the program "Dnadist". The phylogenetic tree was prepared by running the program "Neighbor" using the neighbor-joining method [18] to obtain an unrooted trees. *Talaromyces emersonii* was defined as the outgroup in the program "Retree", and finally the tree was visualized using the program TreeView (win32) [19]. Bootstrap values [20] were calculated by running the program "Seqboot" to produce 1000 bootstrapped data sets from the original data set. Again, "Dnadist" with the Kimura method was used to prepare distance matrices of the multiple data sets, and ""Neighbor" with the neighbor-joining method to obtain unrooted trees of the multiple data sets. Finally, the bootstrap values were obtained from the consensus tree which was identified by the majority-rule consensus method by running the program "Consense".

2.5 Strain AP, culture conditions, and enzyme extract preparation

The fungal strain AP was grown on potato dextrose agar (Sigma) for sporulation. Spores from one petri dish were harvested after 7 days of growth using sterile water. The heavy spore suspension was filtered through Miracloth

(Andwin Scientific). Two ml of the spore solution was inoculated into 200 ml seed medium (2.0 g/l wheat bran, 5 g/l corn steep powder, 0.25 g/l yeast extract, 0.75 g/l peptone, 1.4 g/l $(NH_4)_2SO_4$, 2.0 g/l KH_2PO_4, 0.4 g/l $CaCl_2$ $2H_2O$, 0.3 g/l $MgSO_4$ H_2O, 5.0 mg/l $FeSO_4$, 1.6 mg/l $MnSO_4$ $7H_2O$, 1.4 mg/l $ZnSO_4$ $7H_2O$, 2.0 mg/l $CoCl_2$ $6H_2O$) in a 500 ml Erlenmeyer flask, and incubated at 30°C for 2 days, shaking at 160 rpm. Solid state fermentation at approximately 30% TS was carried out adding 100 ml of the cultivated seed medium to 1 l of a solid state fermentation medium comprising of 343 g wheat bran (TS of 87.4%), 9 g corn steep powder, 557 ml Czapek liquid (3 g/l $NaNO_3$, 1 g/l K_2HPO_4, 0.5 g/l KCl, 0.5 g/l $MgSO_4$ $7H_2O$, 0.01 g/l $FeSO_4$ $7H_2O$) [21]. Incubation was carried out in large flat boxes (20 cm x 20 cm x 5 cm) in order to allow a large surface area where the fermentation medium had a height of approx. 2 cm. The samples were incubated at 30°C without shaking. After 7 days incubation, liquid was extracted from the medium by pressing the medium by hand using gloves. The extract was centrifuged at 10,000 g, and the supernatant filtered through Whatman filter paper.

2.6 Beta-glucosidase activity assays

In this work, specific activity (U/mg) is defined as units per amount of total protein. Specific beta-glucosidase activity was measured using two different substrates: pNPG and cellobiose. The assay using 5 mM pNPG in Na-Citrate buffer pH 4.8 was performed as previously described; enzyme samples were assayed at different concentrations in triple determination to ensure substrate saturation in the assay. The assay using 6 mM cellobiose in 50 mM Na-Citrate buffer pH 4.8 was performed as follows: 15 µl sample and 150 µl substrate was incubated at 50°C for 10 minutes in PCR tubes in a thermocycler; 50 µl of the reaction was transferred to a HPLC vial already containing 1 ml 100 mM NaOH for termination of the reaction. The glucose concentration was measured at Dionex ICS3000 using gradient elution: 0-20% eluent B (0.5 M Na-Acetate in 100 mM NaOH) in 13 minutes followed by 2 minutes washing with 50% eluent B and 5 minutes re-equilibrating with 100% eluent A (100 mM NaOH). Samples were assayed at different concentrations in triple determination to ensure substrate saturation in the assay.

2.7 Kinetic studies

For performing Michaelis-Menten kinetics beta-glucosidase activity was measured as described above, but using different substrate concentrations (pNPG 0.1-10 mM, cellobiose 0.2-18 mM), and with an enzyme dilution that ensured substrate saturation was reached within this range. Triple determinations were performed. A substrate saturation curve was prepared by plotting substrate concentration [S] vs. reaction rate, v. The Michaelis-Menten constants K_M and V_{max} were determined from Hanes-Wolf plots where substrate concentration [S] is plotted against substrate concentration over reaction rate [S]/v, and the linear relationship of the data gives a slope of $1/V_{max}$, a y-intercept of K_M/V_{max}, and an x-intercept of $-K_M$.

2.8 Glucose tolerance

For testing glucose tolerance, 5 mM pNPG in Na-Citrate buffer pH 4.8 was used as substrate with different glucose amounts added, ranging final glucose concentrations of 0-280 mM. The remaining activity (glucose tolerance) was measured spectrophotometrically by release of pNP at 50°C reaction conditions, as described earlier. Triple determinations were performed. Using 20 mM cellobiose in Na-Citrate buffer pH 4.8 as substrate and glucose concentrations ranging from 0-120 mM, 15 µl sample and 150 µl substrate with the different glucose concentrations were incubated at 50°C for 10 minutes in PCR tubes in a thermocycler; 100 µl of the reaction was transferred to a tube already containing 100 µl 200 mM NaOH for termination of the reaction. The reactions were further diluted 512 times and final cellobiose concentration was measured at Dionex ICS3000 using gradient elution: 0-20% eluent B in 13 minutes followed by 2 minutes washing with 50% eluent B (0.5 M Na-Acetate in 100 mM NaOH) and 5 minutes re-equilibrating with 100% eluent A (100 mM NaOH). The activity was calculated by the amount of cellobiose being hydrolyzed. Triple determinations were performed.

2.9 pH and temperature profile

For testing the thermostability of the enzyme extract, aliquots of the extracts were incubated in PCR tubes in a thermocycler with temperature gradient option at 12 different temperatures from 48.5 to 67.0°C for different time periods (0-4 hours) followed by assaying the activity at 50°C with 5 mM pNPG in Na-Citrate buffer pH 4.8 as substrate. The rate of denaturation, k_D, was calculated as the slope of a semi-logaritmic plot of remaining activity vs. incubation time. The half life was calculated as: $T_{½} = \ln(2)/k_D$.

For testing the pH optimum of the enzyme extracts, they were assayed at 50°C with 5 mM pNPG in Citrate Phosphate buffer at different pH ranging from 2.65 to 7.25. Endoglucanase activity of Celluclast 1.5L was assayed with AZO-CMC as described by the manufacturer (Megazyme), testing the same pH range 2.65-7.25 as for the pNPG assay.

2.10 Hydrolysis of cellodextrins

Hydrolysis of cellohexaose was carried out by mixing, in the ratio 1:1, 0.2 mM cellohexaose in 50 mM NaCitrate buffer pH 4.8 and enzyme diluted in 50 mM NaCitrate buffer pH 4.8 to a concentration of 3.7 µg/ml. The reaction was incubated at 50°C and for a period of 30 minutes, 100 µl sample was placed on ice every 5 minutes. 100 µl 200 mM NaOH was added to terminate the reaction, and after another 1 fold dilution with 100 mM NaOH, the samples were analyzed at Dionex ICS3000 using gradient elution: 0-30% eluent B in 26 minutes followed by 2 minutes washing with 50% eluent B (0.5 M Na-Acetate in 100 mM NaOH) and 5 minutes re-equilibrating with 100% eluent A (100 mM NaOH).

2.11 Hydrolysis of pretreated bagasse

Pretreated bagasse was kindly provided by BioGasol ApS, Denmark. The bagasse was pretreated using a steam explosion method with addition of oxygen (pers. comm. with BioGasol ApS). Bagasse hydrolysis was carried out in 2 ml Eppendorf tubes in thermoshaker heating blocks. The pretreated bagasse was hydrolyzed at 5% dry matter (DM) with a total enzyme load of 10 mg protein per g DM. The ratio amount of Celluclast 1.5L vs. extract from strain AP or Novozym 188 was varied, ranging 0-100% of one compared to the other. The hydrolysis was carried out at 50°C for 24 hours, using triple determinations. The samples were centrifuged and supernatants filtered through 0.45 µm filters before sugar analysis using the Ultimate 3000 HPLC (see below).

2.12 Analytical equipment

Dionex ICS3000 equipped with an amperometric detector using a gold working electrode and an Ag/AgCl pH reference electrode was used for measuring glucose, cellobiose, and cellodextrins by ion exchange chromatography, acquiring and interpreting data with the Chromeleon software (Dionex). 10 µl samples were run on a CarboPac PA1 column with 100 mM NaOH as eluent A and 0.5 M Na-Acetate in 100 mM NaOH as eluent B. Gradient runs were performed as described in the different assays, all at a flow rate of 1 ml/min. Standards of glucose, cellobiose, -triose, -tetraose, -pentaose, and –hexaose were run at concentrations 3.125 µM - 0.1 mM.

Ultimate 3000 HPLC equipped with RI-101 detector (Shodex) was used for measuring glucose and cellobiose by high pressure liquid chromatography, acquiring and interpreting data with the Chromeleon software (Dionex). 10 µl samples were run on a BIORAD aminex HPX-87H ion exclusion column, heated to 60°C, run with 4 mM H_2SO_4 as eluent at flow rate 0.6 ml/min. Standards of glucose and cellobiose were run at concentration 0.5-20 g/l.

Table 1. Fungi included in the beta-glucosidase screening

Identity (strain number)	ID method[1]	Origin	Reference[2]
Alternaria radicina (R27)	M	Poland	K. Tylkowska
A. radicina (R28)	M	Poland	K. Tylkowska
Alternaria sp.(AS1-2)	ITS	Jamaica	This work
Amorphothea resinae (Anja)	ITS	Denmark	This work
Aspergillus sp (AP)	ITS	Denmark	This work
Aspergillus sp. (1259)	M	Costa Rica	[4]
A. fumigates (AS3-1)	ITS	Jamaica	This work
A. fumigatus (AS11-2)	ITS	Jamaica	This work
A. fumigatus (AS11-3)	ITS	Jamaica	This work
A. fumigatus (AS12)	ITS	Jamaica	This work
A. fumigatus (AS2-1)	ITS	Jamaica	This work
A. fumigatus (AS2-3)	ITS	Jamaica	This work
A. fumigatus (AS9-7)	ITS	Jamaica	This work
A. fumigatus (AS11-4)	ITS	Jamaica	This work
A. niger (Hj1)	ITS	Denmark	This work
*A. niger (*IBT25747)	ITS	Not known	This work
A. terreus (AS4-1)	ITS	Jamaica	This work
A. terreus (AS9-2)	ITS	Jamaica	This work
Chaetomium aureum (1165)	M	Costa Rica	[4]
C. globosum (11.4kont)	ITS	Denmark	This work
Cladosporium sp.(1160)	M	Costa Rica	[4]
Cladosporium sp.(1209)	M	Costa Rica	[4]
Cladosporium sp. (1195)	M	Costa Rica	[4]
Cladosporium sp. (1208)	M	Costa Rica	[4]
C. cladosporioides(2.1)	ITS	Denmark	This work
Clonostachys rosea (IBT9371)	M, UP-PCR	Denmark	[5]
C. rosea (Gr3)	M, UP-PCR	Denmark	[5]
C. rosea (Gr5)	M, UP-PCR	Denmark	[5]
Colletotrichum acutatum (9955)	ITS	Denmark	T. Sundelin
C. acutatum (F5-3)	ITS	Costa Rica	[6]
C. acutatum (F7-1)	ITS	Costa Rica	[6]
C. acutatum (Lupin1A)	ITS	Not known	T. Sundelin
C. acutatum (SA2-2)	ITS	Denmark	[7]
C. gloeosporioides (2133A)	ITS	Denmark	T. Sundelin
Coprinopsis cinerea (AS2-2)	ITS	Jamaica	This work
Dreshlera sp. (1178)	M	Costa Rica	[4]
Fusarium sp. (3.012)	M	Denmark	I. Weiergang
Fusarium sp. (3.015)	M	Denmark	I. Weiergang
F. avenaceum/trincinctum (1.8.1)	ITS	Denmark	This work
F. culmorum (IBT9615)	M	Norway	[8]
F. equiseti (1236)	M	Costa Rica	[4]
F. graminearum (1237)	M	Costa Rica	[4]
F. graminearum (NRRL31084)	M	USA	[8]

Table 1. continued

Identity (strain number)	ID method[1]	Origin	Reference[2]
F. graminearum (IBT9203)	M	Costa Rica	[8]
F. moniliforme (1247)	M	Costa Rica	[4]
F. moniliforme (1258)	M	Costa Rica	[4]
F. oxysporum (1244)	M	Costa Rica	[4]
F. oxysporum f.sp. pisi (88.001)	M	Denmark	I. Weiergang
F. semitectum (1232)	M	Costa Rica	[4]
F. semitectum (1242)	M	Costa Rica	[4]
Nigrospora sp. (1168)	M	Costa Rica	[4]
Penicillium sp. (1219)	M	Costa Rica	[4]
P. chrysogenum or P. commune (11.5)	ITS	Denmark	This work
P. chrysogenum or P. commune (2.3A)	ITS	Denmark	This work
P. paneum (14)	ITS	Denmark	This work
P. paneum (2.8)	ITS	Denmark	This work
P. spinolosum (1.6)	ITS	Denmark	This work
P. spinolosum (2.3B)	ITS	Denmark	This work
P. spinolosum (9.3.2)	ITS	Denmark	This work
P. spinolosum (9.4.2)	ITS	Denmark	This work
P. swiecickii or P. raistrickii (11.4)	ITS	Denmark	This work
Pestalotiopsis sp. (1220)	M	Costa Rica	[4]
Pestalotiopsis sp. (1226)	M	Costa Rica	[4]
Rhizoctonia solani (CS96)	M, UP-PCR	Japan	[9]
R. solani (ST-11-6)	M, UP-PCR	Japan	[9]
R. solani (AH-1)	M, UP-PCR	Japan	[9]
R. solani (RH165)	M	Japan	[9]
R. solani (GM10)	M, UP-PCR	Japan	[9]
Binuclear Rhizoctonia (S21)	M	USA	[9]
Binuclear Rhizoctonia (SN-1-2)	M	Japan	[9]
Rhizopus microsporum (AS1-1A)	ITS	Jamaica	This work
R. microsporum (AS1-1B)	ITS	Jamaica	This work
R. microsporum (AS2-4)	ITS	Jamaica	This work
Spaeropsidales (1190)	M	Costa Rica	[4]
Stenocarpella sp. (1198)	M	Costa Rica	[4]
Stenocarpella sp. (1214)	M	Costa Rica	[4]
Stenocarpella sp. (1239)	M	Costa Rica	[4]
Thielavia sp. (AS11-1)	ITS	Jamaica	This work
Trichoderma harzianum (5.1)	ITS	Denmark	This work
T. harzianum (O7)	M	Costa Rica	[4]
T. harzianum (IBT9385)	M, UP-PCR	Sweden	[5]
T. koningii (1211)	M	Costa Rica	[4]
T. koningii (CBS850.68)	M, UP-PCR	Germany	[5]
T. virens (I10)	ITS	Italy	[10]
T. viride (IBT8186)	M, UP-PCR	Denmark	[5]
T. viridescens (7.1)	ITS	Denmark	This work

[1]Identification method: M=morphology, ITS=ITS and NCBI blast search, UP-PCR= PCR finger printing

[2]Where names are found instead of reference numbers, the fungal strains have not previously been published, but identified by the person specified.

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Inge Weiergang, Maribo Seed, Nordzucker AG

3. Results

3.1 Beta-glucosidase activities in broad screening

Eighty six filamentous fungal strains, covering 19 different fungal genera, mainly belonging to the Ascomycota phylum, were screened for extracellular beta-glucosidase activity using pNPG as substrate (Table 1). The screening showed a great variety in activity levels, with a few strains showing remarkably better results (Fig. 1). All produced extracellular beta-glucosidase, though for about 35% of the assayed fungi the activity was negligible (<0.1 U/ml). Some genus tendencies were found, with *Aspergillus,* a few *Fusarium, Penicillium*, and *Trichoderma* showing greatest beta-glucosidase activity at the assayed conditions. Where several strains belonging to same species were assayed, the variation at species level was in most cases insignificant, except for *A. niger* where a great variation was observed within the two strains, the stain Hj1 showing approximately two times the activity of strain F1.

Strain AP (identified as an *Aspergillus* sp.) and strain Hj1 (identified as an *A. niger*) showed significantly greater activity than all other strains assayed at these conditions, with strain AP reaching more than ten times greater activity than the average of all the stains assayed.

3.2 Identity of the prominent beta-glucosidase producing Aspergillus sp.

Primers matching the conserved 18S and 5.8S rRNA genes were used for the amplification of the non-coding ITS1 region. The best hits obtained in a GenBank NCBI nucleotide blast of the ITS1 sequence of strain AP were different black aspergilli. These best hits, together with other selected aspergilli in the section *Nigri* based on the work by Samson *et al.* [15], were used to prepare a phylogenetic tree (Fig. 2B). Strain AP showed to be phylogenetically placed on its own branch far from the other aspergilli, almost as far from them as the outgroup *Talaromyces emersonii*. The percentage identity of strain AP ITS1 sequence compared to selected aspergilli in the tree confirm that strain AP is significantly different from the others (Fig. 2A). This, and the location of strain AP on a separate branch in the phylogenetic tree, indicated that the strain AP might belong to an unknown species.

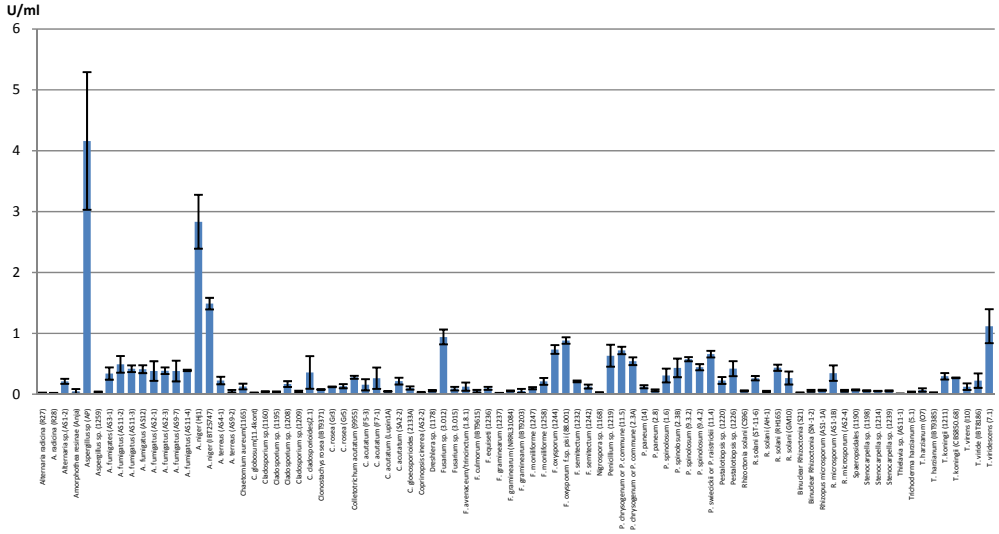

Fig. 1 Extracellular beta-glucosidase activity of screened fungi grown in simple submerged fermentaion. pNPG was used as substrate in the assays, with one unit (U) of enzyme activity defined as the amount of enzyme needed to hydrolyze 1umol pNPG in 1 minute.

	Strain AP (HM853552)	A. japonicus (AJ279985)	A. aculeatus (AJ280005)	A. homomorphus (EF166063)	A. elipticus (AJ280014)	A. carbonarius (DQ900605)	A. niger (AJ223852)	A. brasiliensis (AJ280010)	A.cervinus (EF661270)	T. emersonii (FJ389922)
Strain AP (HM853552)	100	81	82	81	78	76	77	77	81	68
A. japonicus (AJ279985)	81	100	99	95	85	82	84	83	83	70
A. aculeatus (AJ280005)	82	99	100	95	84	82	84	82	82	70
A. homomorphus (EF166063)	81	95	95	100	86	85	86	86	83	70
A. elipticus (AJ280014)	78	85	84	86	100	92	95	95	84	76
A. carbonarius (DQ900605)	76	82	82	85	92	100	94	95	83	75
A. niger (AJ223852)	77	84	84	86	95	94	100	98	84	75
A. brasiliensis (AJ280010)	77	83	82	86	95	95	98	100	83	74
A.cervinus (EF661270)	81	83	82	83	84	83	84	83	100	72
T. emersonii (FJ389922)	68	70	70	70	76	75	75	74	72	100

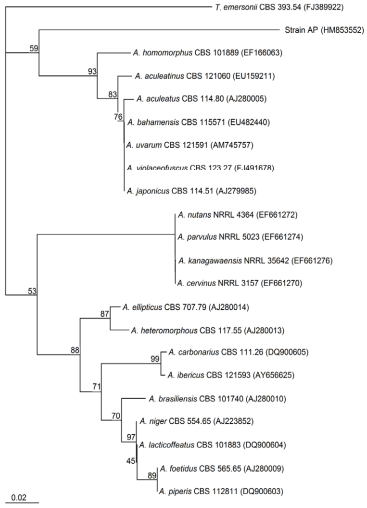

Fig. 2. Neighbor-joining phylogenetic tree (bottom) and homology matrix (top) of ITS1 region sequence data of black aspergilli, including strain AP, using *T. emersonii* as out group. Numbers above the branches in tree are bootstrap values. Bar, 0.02 substitutions per nucleotide. Numbers in matrix are percentage identity between the ITS1 region of the different strains.

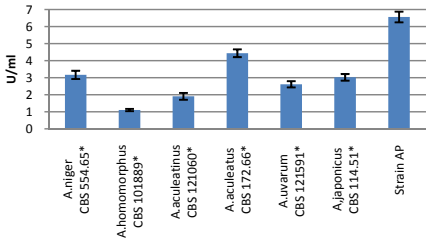

Fig. 3. Beta-glucosidase activity of extracts from selected aspergilli grown in simple submerged fermentation (*=type strain). One unit (U) of enzyme activity is defined as the amount of enzyme needed to hydrolyze 1umol pNPG in 1 minute.

3.3 Aspergillus screening

The beta-glucosidase activity of the prominent *Aspergillus* sp. (strain AP) was compared to neighbor aspergilli in the submerged fermentation set up using wheat bran as growth medium as described in the screening. *A. niger* was specifically included as this fungus is a known and industrially used beta-glucosidase producer [22]. *A. niger* data is placed as the column furthest from strain AP as it is the most distantly related strain in this *Aspergillus* screening. At the conditions tested, AP produces significantly greater amount of beta-glucosidase activity (Fig. 3). The protein levels (data not shown) in the assayed extracts did not vary much compared to the difference seen in enzyme activity. Relative to the other aspergilli tested, strain AP is therefore more specialized towards beta-glucosidase productions at the tested conditions.

3.4 Potential of strain AP enzyme extract compared with commercial enzymes

A solid state fermentation extract of the strain AP was compared to the commercially available Novozym 188, Celluclast 1.5L, and Cellic CTec (Novozymes AS, Denmark). Solid state fermentation was chosen to obtain as concentrated an extract as possible. In the previous screening, pNPG activity of 6.6 U/ml (Fig. 3) and specific activity of 3.1 U/mg total protein (data not shown) were obtained for the submerged fermentation of strain AP. With the solid state fermentation, a pNPG activity of 105 U/ml and a specific activity of 5.7 U/mg total protein were obtained. The volume based activity is naturally increased as the water content of solid state is severely reduced compared to submerged fermentation. However, there is no definite conclusion to whether the difference in specific activity (U/mg protein) is due to the solid state fermentation favoring the expression of specifically beta-glucosidase proteins.

As the enzyme extract of strain AP is intended for use in combination with Celluclast 1.5L for complete hydrolysis of cellulosic biomasses, the working pH must match the pH profile of Celluclast 1.5L cellulase activity. Within pH 4.5-6 Celluclast 1.5L activity stays above 90% of maximum activity measured (Fig. 4). The pH span of strain AP beta-glucosidase was examined using pNPG as substrate. Its profile is very similar to Novozym 188, with an optimum around pH 4.2 (Fig. 4). Within the pH range 3.8-4.8 the activity stays above 85% of maximum. pH 4.8 generally used in hydrolysis experiments with Celluclast 1.5L and Novozym 188 is therefore also valid for hydrolysis with AP extract beta-glucosidases.

Enzyme kinetics are preferably carried out on pure enzyme preparations, but were in this study used for the comparison of the beta-glucosidases of the crude enzyme

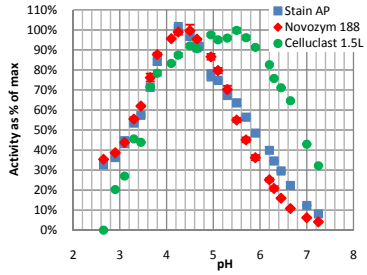

Fig. 4. pH profile. Beta-glucosidase activity of Strain AP and Novoym 188 at different pH measured on pNPG. Endoglucanase activity of Celluclast 1.5L at different pH measured on AZO-CMC.

extract of strain AP and the commercially available enzyme preparations Novozym 188 and Cellic CTec. Any parameter expressed per amount of protein was always total protein content in the extract or commercial enzyme preparation, with no specific knowledge of how large a fraction that was beta-glucosidase proteins.

Kinetic analysis was performed on both pNPG and cellobiose, measuring the specific activity at different substrate concentrations. By plotting reaction rate vs. substrate concentration, it was found that for all three samples, strain AP, Novozym 188, and Cellic CTec, the hydrolysis of pNPG only followed MM kinetics at low substrate concentrations, while evidence of substrate inhibition or transglycosylation was found at higher concentrations, seen by a decrease in reaction rate with increased substrate concentration (data no shown). With regards to cellobiose, no substrate inhibition was observed within the substrate concentrations tested. The MM kinetics parameters, V_{max} and K_M, were therefore only determined for cellobiose. The enzyme extract from strain AP and the commercial preparation Novozym 188 had similar affinity for cellobiose and values being slightly better than Cellic CTec (Table 2), the lower the K_M values the better the affinity. The maximum activity, V_{max}, was, however, highest for Cellic CTec, but with strain AP being significantly better than Novozym 188.

Table 2 Kinetic properties of strain AP, Novozym 188, and Cellic CTec with cellobiose as substrate for MM kinetics study.

	V_{max} (U/mg)	K_M (mM)
Strain AP	11.3	1.09
Novozym 188	7.5	1.06
Cellic CTec	22.9	1.69

Product inhibition was found to be substrate dependent, especially for strain AP beta-glucosidases (Fig. 5). Using pNPG as substrate, the beta-glucosidase activity of strain AP remained greater than 80% at product concentrations 12 times higher than the substrate concentration. Cellic CTec was slightly lower (approx 75%), while the activity of Novozym 188 at this product-substrate ratio had dropped to just below 40%. The activities of strain AP, Cellic CTec, and Novozym 188 was calculated to reach half the maximum activity at concentrations 180, 115, and 60 mM glucose (equal to 36x, 23x, and 12x the substrate concentration), respectively. With regards to cellobiose, an activity drop to around 80% was found for both strain AP and Novozym 188 when the product and substrate occured in equal concentrations. Overall, the profile of substrate inhibition was identical for strain AP and Novozym 188 when using cellobiose as substrate, while with pNPG, strain AP beta-glucosidases performed much better at high glucose concentrations than Novozym 188. This glucose inhibition study demonstrated the importance of testing the true substrate, cellobiose, and not just relying on pNPG data.

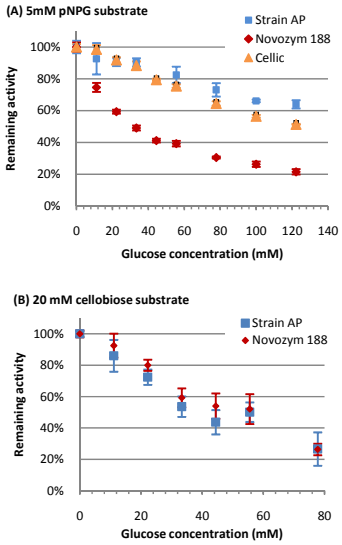

Fig. 5. Product inhibition; remaining beta-glucosidase activity at different inhibitor (glucose) concentrations relative to activity measured without inhibitor. A: pNPG as substrate, activity measured by release of pNP. B: cellobiose as substrate, activity measured by decrease in cellobiose concentration.

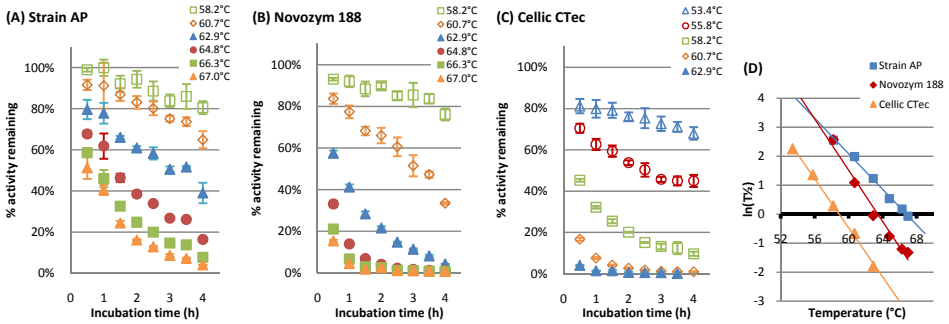

Fig. 6. (A), (B), and (C): Time course of thermal inactivation of the beta-glucosidases of (A) strain AP, (B) Novozym 188, and (C) Cellic CTec. Thermostability is evaluated based on the remaining activity after 0-4 hours of incubation at different temperatures relative to the activity without incubation. (D) Semi-logarithmic plot of calculated half life at different temperatures. The x-axis intercept indicates the thermal activity number, temperature at which the half life is one hour.

The thermostability of the enzymes was examined at temperatures ranging from 48.5 to 67.0°C using pNPG as substrate. At temperatures up to 58°C there was no significant difference between strain AP and Novozym 188 in terms of stability; both were fairly stable throughout the four hours of incubation (data not shown). Meanwhile, the beta-glucosidases of Cellic CTec were much more sensitive to temperature increases. At temperatures ≥60°C, strain AP beta-glucosidases were clearly more stable than Novozym 188, and Cellic CTec was showing a lack of performance as it was severely inactivated even within the first half hour (Fig. 6A-C). At 60.7°C, 65% of the activity remained for strain AP beta-glucosidases after 4 hours of incubation, while only 33% activity remained for Novozym 188. The inactivation roughly followed first order kinetics, with the rate constants of denaturation, k_D, defined by the slopes of the lines in a semi-logaritmic plot of the remaining activity vs. time for the different temperatures, and the half-life was calculated as $T_{½} = \ln(2)/k_D$. The calculated half-life of strain AP at 60.7°C was 440 min vs. 180 min for Novozym 188. To reach a half-life of 180 min for Cellic CTec, the temperature should be lowered to around the tested 55.8°C, while for strain AP the temperature should be raised to around the tested 62.9°C (Fig. 6A-C). The calculated half-lives at different temperatures were plotted in a semi-logarithmic plot vs. temperature (Fig. 6D). The data for each enzyme preparation form a straight line, and the thermal activity number, the temperature that gave a half-life of 1 hour, was 66.8°C, 63.4°C, and 58.9°C for strain AP, Novozym 188, and Cellic CTec, respectively.

Cellodextrins were used to make a hydrolytic time course study of strain AP extract, Novozym 188, Celluclast 1.5L, and Cellic CTec (Fig. 7). Strain AP enzyme extract showed clear exo-activity, with a cellopentaose and glucose concentration increase as the cellohexaose concentration decreased. Less rapidly, the cellotetraose and cellotriose concentrations increased too. Evidence of endo-activity or cellobiohydrolase activity was found, as the cellobiose concentration went up relatively fast compared to the cellotetraose and cellotriose. On the contrary, Novozym 188 only showd beta-glucosidase exo-activity, with the only significant change over time being glucose and cellopentaose increase as cellohexaose decrease. The results suggested that the beta-glucosidases act by capturing the substrate, cleave the glycosidic bond, and release the products. They do not continuously cleave one bond after another upon capturing the substrate. Celluclast 1.5L mainly possess cellobiohydrolase and endoglucanase activity, seen by the immediate increase in cellobiose and cellotriose, and lacking sufficient beta-glucosidase activity as the glucose concentration did not increase, but the cellobiose concentration increases continually. As the only sample, Cellic CTec showed continuously increase in both cellobiose and glucose, indicating a combination of cellobiohydrolase and beta-glucosidase activity. Endoglucanase activity was most likely present too, identified by the formation of cellotriose.

Pretreated bagasse was hydrolyzed by strain AP beta-glucosidases combined with Celluclast 1.5L to investigate its capabilities on a lignocellulosic substrate. This was compared with hydrolysis data of Novozym 188 and Celluclast 1.5L. Strain AP beta-glucosidases and Novozym 188 beta-glucosidases are compared on total protein amount basis. A dosage-response plot of hydrolysis of 5% DM pretreated bagasse showed a leveling off in glucose yields at total enzyme dosages greater than 10mg/gDM (data not shown). This total enzyme dosage was used for optimal enzyme ratio

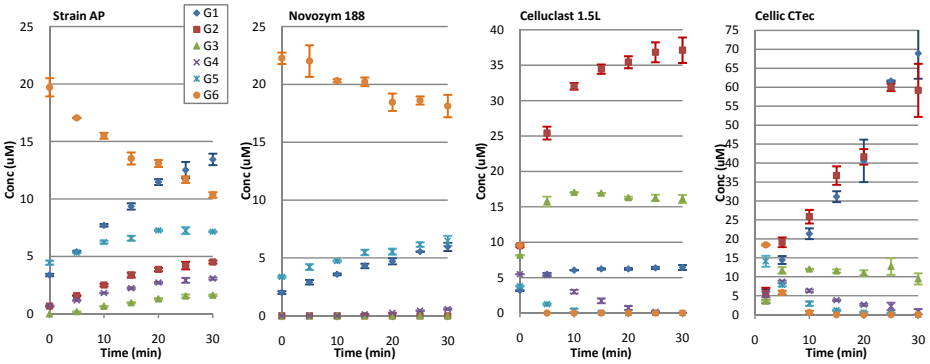

Fig. 7. Hydrolysis af cellohexaose by Strain AP extract, Novozym 188, Celluclast 1.5L, and Cellic CTec, showing a clear difference in the mode of action. G1=glucose, G2=cellobiose, G3=cellotriose, G4=cellotetraose, G5=cellopentaose, G6=cellohexaose.

determination; the ratio of Celluclast 1.5L and Strain AP extract or Novozym 188. The greatest yields were found with approximately 20% Novozym 188 (80% Celluclast 1.5L) and 15% strain AP extract (85% Celluclast 1.5L) (Fig. 8). Generally, the glucose yields were higher when using strain AP extract compared with Novozym 188, illustrating the possibility of substituting the commercial enzyme preparation with an extract from our newly isolated *Aspergillus* strain AP. These results for bagasse hydrolysis correlated well with the fact that the beta-glucosidases of strain AP extract had a higher reaction rate on cellobiose (V_{max}, Table 2) compared to Novozym 188.

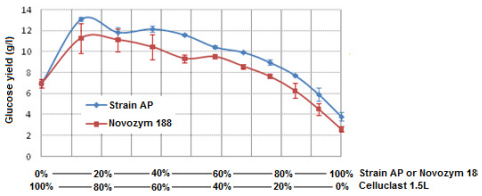

Fig. 8. Hydrolysis of bagasse using different enzyme ratios of Strain AP or Novozym 188 relative to Celluclast 1.5L.

4. Discussion

Traditionally, two commonly used enzyme preparations that supplement each other in the hydrolysis of cellulosic biomasses are Novozym 188 and Celluclast 1.5L (Novozymes A/S), contributing with beta-glucosidase activity, and endoglucanase and cellobiohydrolase activity, respectively [23]. Recently, an enzyme preparation containing all three components has been released into the market, Cellic CTec (Novozymes AS, Denmark). Costs related to enzymatic hydrolysis make this step a bottle neck in the process of creating a sugar plat form for biofuels, chemicals, and pharmaceuticals; therefore there is a need for more efficient enzymes, both in terms of reaction rates and stability [23]. Beta-glucosidases are widely distributed in nature, with especially fungi known to be industrial producers of these enzymes for cellulose hydrolysis. In this study, we present a prominent fungal beta-glucosidase producer naturally producing an enzyme cocktail with better beta-glucosidases compared to the commercial preparation Novozym 188 and markedly better thermostabily than the commercial beta-glucosidase containing preparations, Novozym 188 and Cellic CTec.

The present work included a broad screening of 87 fungal strains collected from soil and decaying wood samples as well as was from an in house collection of fungi kindly donated by various scientists. To our knowledge, there are not many examples on screening of fungal strains belonging to different species for beta-glucosidase activity. Sternberg et al. [3] screened 200 fungal strains, thus found beta-glucosidase that

could supplement the *Trichoderma viride* cellulases for cellulose saccharification. Generally, in accordance with or results, black *Aspergilli* were found to be superior in terms of beta-glucosidase production [3]. Later, a study focusing on identification of acid- and thermotolerant extracellular beta-glucosidase activities in zygomycetes fungi was carried out where *Rhizomucor miehei* performed best [24]. Screening for general cellulase activities have included beta-glucosidase activities in a few cases [25-28], and other strategies for obtaining beta-glucosidases have been employed such as screening environmental DNA for beta-glucosidase activity rather than collecting microbial samples [29], and a proteomics strategy to discover beta-glucosidases from *Aspergillus fumigatus* has been reported [30]. In this work, wheat bran was used as substrate in a submerged fermentation, as it is generally known as a good substrate for cellulase and beta-glucosidase production [31, 32], being rich in carbohydrates and protein [33], and submerged fermentation allows for easy assaying of the extracellular enzymes of the fungi. The supernatants were tested for beta-glucosidase activity using pNPG at 50°C pH 4.8; which are optimal conditions for Celluclast 1.5L, thus aiming at finding enzyme activities supplementing this enzyme preparation.

It was found that especially strains from the genera *Aspergillus, Fusarium, Penicillium*, and *Trichoderma* had the highest beta-glucosidase activity, with strains of *Aspergillus* being the best. These fungal genera have also been found in other screening programs for discovery of cellulolytic enzymes [26, 34]. Aspergilli in general have a high capacity for producing and secreting extracellular enzymes [35, 36], especially *A. niger*, with all classes of enzymes essential for cellulose degradation having been found [36]. Within the *A. fumigati* strains, the expression levels of beta-glucosidase at the tested conditions were very consistent, while great strain variation was found in *A. niger* (Fig. 1). This variation was not surprising as it correlates well with publications on citric acid, antioxidant, and urease production in *A. niger*, which is also very strain dependent [37-39].

Several studies have been published on the kinetics of Novozym 188 and *A. niger* beta-glucosidases, and it is apparent that substrate affinity, K_M, does vary amongst strains within this species [31, 40-42]. However, most common amongst the *A. niger* beta-glucosidases is that they have greater affinity for pNPG than for cellobiose. Jäger et al. [31] report similar findings for other *Aspergillus* strain beta-glucosidases. We have, however, chosen to only calculate MM kinetics parameters related to hydrolysis of cellobiose as hydrolysis data of pNPG did not fit the MM equation. pNPG might actually be a poor substitute in terms of assaying for beta-glucosidase activity, which was also concluded by e.g. Khan et al. [43], and especially in this study it was additionally evident in relation to product inhibition. To compare activities, it is always desired to perform measurements at substrate saturation; however, with pNPG the saturation point could not be determined as substrate inhibition was the dominating factor at high substrate concentrations which correlates with studies carried out by Dekker [22] and Eyzaguirre et al. [41]. As transglycosylation activity has been reported in several cases for different beta-glucosidases [44] it was speculated if the proposed substrate inhibition was rather a transglycosylation reaction at high product concentrations. However, the option of the enzyme carrying out transglycosylation by coupling the glucose product to a new pNPG at high pNPG concentrations was not investigated, which could potentially mimic substrate inhibition in data evaluation. The effect of pNPG substrate inhibition or transglycosylation reaction on the measured reaction rates in the beta-glucosidase screening strategy was a factor that was not taken into account.

Product inhibition is a common phenomenon with beta-glucosidases; glucose being the main inhibitor, which can have a significant influence on process reaction in industrial applications [23]. The importance of testing such inhibitory effects on the true substrate, cellobiose, rather than the substitute, pNPG, was demonstrated in this study. The beta-glucosidases of strain AP compared to Novozym 188 only showed low inhibition by glucose using pNPG as substrate, but when using cellobiose, the inhibition patterns of the two enzyme preparations were similar, with the activities reaching only 50% when twice the concentration of glucose is present compared to cellobiose concentration (Fig. 5). pNPG is an easy-to-use substrate, but can be misguiding in terms of beta-glucosidase performance in true cellulosic hydrolysis conditions.

The extract of strain AP showed greater specific beta-glucosidase activity than Novozym 188, while that of Cellic CTec was found to be even greater (Table 2). The enzyme preparations were evaluated on basis of total extracellular proteins, which, however, also comprise proteins originating from the growth medium. It is therefore unknown how much of the measured protein is actually fungal proteins. Furthermore *aspergilli* strains are known to possess several beta-glucosidases that can have different relative activities and specificities, e.g. three beta-glucosidases from *A. aculeatus* have been assayed with the findings that one has very weak and the two other very high activities towards cellobiose relative to pNPG [45]. The beta-glucosidase activity of the screened extract is therefore very likely the combined activity of several beta-glucosidases of the strain AP. Without further optimization, the specific activity of the solid state fermentation extract of strain AP was able to compete with Novozym 188 in hydrolysis of cellobiose, and in the case of

cellohexaose the rate by which the cellohexaose concentration decreased and the glucose and cellopentaose increased was greater for strain AP than Novozym 188. Increasing the degree of complexity and potential amount of inhibitors, etc, bagasse is one of many cellulose containing biomasses of interest for bioethanol purposes. Bagasse is a lignocellulosic waste product from the sugar cane industry produced in great quantities in countries such as Brazil and other tropical places [46]. Its utilization for fuel production is value contributing to the current processes [47]. Hydrolysis of bagasse was here used to show that our enzyme extract from strain AP supplemented with Celluclast (Fig. 8) did work on actual lignocellulosic material and was competitive with Novozym 188.

Thermostability and temperature optima presented in different publications are difficult to compare as the incubation time, reaction time, and temperatures tested vary. Generally, the dependence of temperature resembles a bell-shaped curve, with a maximum where the enzyme is actually not at its optimum as the maximum indicates the beginning of the irreversible denaturation process [48]. This method of directly assaying at different temperatures to determine the temperature profile is of no real use in terms of industrial hydrolysis as hydrolysis reactions are usually run for several hours and time dependent enzyme degradation will play a role. By pre-incubating the enzymes at distinct temperatures and assaying after different time intervals at normal assay temperatures, the beta-glucosidases of strain AP were found to have excellent temperature stability compared to Novozym 188 and Cellic CTec (Fig. 6A-C). Novozym 188 has previously been reported to only maintain stability at temperatures at or below 50°C [41]. Cellic CTec was found to be very unstable at elevated temperatures observed by the poor performance when assaying for activity after incubation above 50°C, which relates to the manufactures instructions of best performance at temperatures 40-50°C [49]. The time course of the inactivation of all enzymes approximately followed a first order reaction from which the denaturation rates and half-lives at the different temperatures were calculated, and the thermal activity number confirmed the dominating status of strain AP beta-glucosidases in terms of thermostability (Fig. 6D).

To conclude, a new yet unidentified species has been identified: strain AP, belonging to the *Aspergillus nigri* group. This stain had significantly greater beta-glucosidase potential than all other fungi screened and was shown to be a valid substitute for Novozym 188, even performed better than Novozym 188 in some aspects, and definitely out-competed Cellic CTec in terms of thermostability.

Acknowledgements

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Research paper III

Aspergillus saccharolyticus sp. nov., a new black *Aspergillus* species isolated from treated oak wood in Denmark

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Aspergillus saccharolyticus sp. nov., a new black *Aspergillus* species isolated from treated oak wood in Denmark

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A novel species, *Aspergillus saccharolyticus* sp. nov., is described within the black *Aspergillus* section *Nigri* species. This species was isolated in Denmark from treated hardwood. Its taxonomic status was determined using a polyphasic taxonomic approach with phenotypic (morphology and extrolite profiles) and molecular (beta-tubulin, internal transcribed spacer and calmodulin gene sequences, and universally-primed PCR fingerprinting) characteristics. These features clearly distinguished this species from other black aspergilli. *A. saccharolyticus* is a uniseriate black *Aspergillus* with a similar morphology to *A. japonicus* and *A. aculeatus,* but with a totally different extrolite profile as compared to any known *Aspergillus*. The type strain is CBS 127449^T (= IBT 28509^T).

INTRODUCTION

The black aspergilli (*Aspergillus* section *Nigri*) (Gams *et al.*, 1985) are important industrial working horses as they are frequently used in the biotech industry for production of hydrolytic enzymes and organic acids (Pel *et al.*, 2007, Goldberg *et al.*, 2006, Pariza & Foster, 1983).

Black aspergilli are some of the most common fungi being responsible for postharvest decay of fruit (Pitt & Hocking, 2009). Of these *A. carbonarius* is especially problematic due to its high production of the carcinogenic ochratoxin A, which is an important mycotoxin in wine and raisins (Abarca *et al.*, 2003). Recently, also *A. niger* was shown to contaminate wine with the carcinogenic mycotoxin fumonisin B_2 (Mogensen *et al.*, 2010). In addition to these toxins, the black aspergilli are well known for their prolific production of many secondary metabolites. In the biseriate species a diverse array of polyketides and several alkaloids are known, while the uniseriate species, *A. aculeatinus, A. aculeatus, A. japonicus*, and *A. uvarum* predominantly produce alkaloids and few polyketides (Nielsen *et al.*, 2009).

Aspergillus section *Nigri* is one of the more taxonomically difficult groups, but the uniseriate subgroup differ significantly in morphology and physiology from the biseriate subgroup (Samson & Varga, 2009, Samson *et al.*, 2007, Perrone *et al.*, 2008, Noonim *et al.*, 2008). The species concept of black aspergilli has been discussed by several researches within the *Aspergillus* research community, and it is generally agreed that it is important to delimit a species in the genus by combining molecular, morphological, and physiological characteristics using a polyphasic approach (Samson & Varga, 2009, Samson *et al.*, 2007).

During a broad screening of different fungal strains collected in Denmark for prominent beta-glucosidase producing fungi (Research paper II, this thesis), we discovered a uniseriate *Aspergillus*, morphologically

Abbreviations: ITS, internal transcribed spacer region; UP-PCR, universally primed PCR.

The GenBank accession numbers for the beta-tubulin, ITS, and calmodulin gene sequenced of strains examined are shown on the phylogenetic trees.

The Mycobank (http://www.mycobank.org) accession number for *A. saccharolyticus* sp. nov. is MB 158695

similar to *A. japonicus*. However, both molecular data and extrolite profile showed that this fungus differed significantly from known aspergilli from section *Nigri*. In this paper we describe the relationship of this strain to other black aspergilli using the polyphasic approach with studies of ITS, calmodulin, and beta-tubulin sequence phylogeny, UP-PCR finger printing, macro- and micro-morphology, temperature tolerance, and extrolite production.

METHODS

A strain of a novel species, *Aspergillus saccharolyticus*, was isolated indoor from treated oak wood in Denmark. The isolate was maintained on potato dextrose agar at room temperature. All reference strains and accession numbers used for comparison are listed in Supplemetary Table 1.

Molecular analysis. Fungal biomass for DNA extraction was obtained by scraping the surface of a PDA plate with a seven day old colony. DNA extraction was carried out as described by (Yu & Mohn, 1999), using bead beating for cell disruption. The two fungal primers Bt2a (5' GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (5' ACCCTCAGTGTAGTGACCCTTGGC) were used to amplify a fragment of the beta-tubulin gene (Glass & Donaldson, 1995), while the primers Cmd5 (5' CCGAGTACAAGGAGGCCTTC) and Cmd6 (5' CCGATAGAGGTCATAACGTGG) were used to amplify a segment of the calmodulin gene (Hong *et al.*, 2006), and the primers ITS1 (5' TCCGTAGGTGAACCTGCGG) and ITS4 (5' TCCTCCGCTTATTGATATG) were used to amplify the ribosomal rDNA spacers, ITS1 and ITS2 (White *et al.*, 1990).

Phylogenetic analysis of the beta-tubulin, calmodulin, and internal transcribed spacer region of rRNA (ITS1 and ITS2) sequences of the novel isolate was carried out as described by Varga *et al*. (2007), using the beta-tubulin, calmodulin, and ITS region sequences of the aspergilli presented in the article by Samson *et al.* (2007). ClustalW multiple alignment was used for sequence alignment and manual improvement of the alignment was performed using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The PHYLIP program package version 3.69 was used for preparation of phylogenetic trees (Felsenstein, 2004). The distance matrix of the data set was calculated based on the Kimura method (Kimura, 1983) using the program "Dnadist". The phylogenetic tree was prepared by running the program "Neighbor" using the neighbor-joining method (Saitou & Nei, 1987) to obtain unrooted trees. *A. flavus* was defined as the outgroup in the program "Retree", and finally the tree was visualized using the program TreeView (win32) (Page, 1996). Bootstrap values (Felsenstein, 1985) were calculated by running the program "Seqboot" to produce 1000 bootstrapped data sets from the original data set. Again, "Dnadist" with the Kimura method was used to prepare distance matrices of the multiple data sets, and "Neighbor" with the neighbor-joining method to obtain unrooted trees of the multiple data sets. Finally, the bootstrap values were obtained from the consensus tree which was identified by the majority-rule consensus method by running the program "Consense".

UP-PCR fingerprinting was carried out using two different UP primers, L45 (5' GTAAAACGACGGCCAGT) and L15/AS19 (5' GAGGGTGGCGGCTAG) (Lubeck *et al.*, 1999) for DNA amplification in separate reactions. The amplification was performed as described in Lübeck *et al.* (1999) except that the reactions were carried out in a 25 µl volume containing 50 mM Tris pH 8, 0.23 mg/ml BSA, 0.5 % Ficoll, 2.5 mM $MgCl_2$, 0.2 mM of dNTP, 0.4 µM of primer and 1 U RUN polymerase (A&A Biotechnology).

Morphological analysis. For microscopic analysis, microscopic mounts were made in lactophenol from colonies grown on MEA (malt extract autolysate) and OA (oat meal agar).

For investigation of morphological characteristics, a dense spore suspension of *A. saccharolyticus* was three-point inoculated on the following media: CREA (creatine sucrose), CYA (Czapek yeast autolysate), CY20S (CYA with 20% sucrose), CY40S (CYA with 40% sucrose), CYAS (CYA with 50g/l NaCl), MEA (malt extract autolysate), OA (oat meal agar) and YES (yeast extract sucrose) agar (Samson *et al.*, 2004a), and incubated 7 days in the dark at 25°C. For temperature tolerance analysis, three-point inoculating was performed on CYA and incubated 7 days in the dark at different temperatures: room temp, 30°C, 33°C, 36°C, and 40°C.

Extrolite analysis. Three 6 mm diameter plugs were taken from each strain grown as three-point inoculations in the dark at 25 °C for 7 and 14 days on YES, CYA20, CYA40, PDA, CYA media (Nielsen *et al.*, 2009, Samson *et al.*, 2004a). The plugs were transferred to a 2 ml vial and 1.4 ml of ethyl acetate containing 1% formic acid was added. The plugs were placed in an ultra-sonication bath for 60 min. The ethyl acetate was transferred to a new vial in which the organic phase was evaporated to dryness by applying nitrogen airflow at 30 °C. The residues were re-dissolved by ultrasonication for 10 min in 150 µl ACN/H_2O (1:1, v/v) mixture.

HPLC-UV/VIS-high resolution mass spectrometry (LC-HRMS) analysis was performed with an Agilent 1100 system (Waldbronn, Germany) equipped with a diode array detector and coupled to a Micromass LCT (Micromass, Manchester, U.K.) equipped with an electrospray (ESI) (Nielsen *et al.*, 2009, Nielsen & Smedsgaard, 2003). Separations of 2 µl samples was performed on a 50 × 2 mm inner diameter, 3 µm Luna C_{18} II column (Phenomenex, Torrance, CA) using a linear water-ACN gradient at a flow of 0.300 ml/min with 15-100% ACN in 20 min followed by a plateau at 100 % ACN for 3 min (Nielsen *et al.*, 2009). Both solvents contained 20 mM formic acid. Samples were analyzed both in ESI^- and ESI^+ mode.

For compound identification, each peak was matched against an internal reference standard database (~800 compounds) (Nielsen *et al.*, 2009, Nielsen & Smedsgaard, 2003). Other peaks were tentatively identified by matching data from previous studies in our lab and searching the accurate mass in the ~13500 fungal metabolites reported in Antibase 2010 (Laatsch, 2010).

RESULTS AND DISCUSSION

In a screening program, fungal strains were obtained from different environmental habitats, Danish as well as international, and tested for beta-glucosidase activity (Research paper II, this thesis). Some of the strains were found indoor in Denmark on treated oak wood, and one of

these strains showed an extraordinary good beta-glucosidase activity. In this work, a thorough characterization was carried out in order to identify the strain.

Morphological data showed that the strain was related to *A. japonicus* or *A. aculeatus*, but extrolite profiles and DNA sequencing data showed that the strain clearly was different from all known species. The genetic relatedness of this novel species, *A. saccharolyticus,* to other black aspergilli was investigated by comparing sequence data of parts of the beta-tubulin and calmodulin genes as well as the ITS region, using *A. flavus* as the out group. The black aspergilli chosen for comparison are the same as the ones presented by Samson *et al.* (2007). Phylogetic trees were prepared for *A. saccharolyticus* based on these sequence data and data obtained in this work, with especially the ITS and calmodulin sequence trees showing similar topology (Fig 1 and Supplementary Fig S1 and S2). Based on the phylogenetic analysis of the ITS and calmodulin gene sequence data, *A. saccharolyticus* was with high bootstrap values found to belong to the clade with *A. homomorphus, A. aculeatinus*, *A. uvarum*, *A. japonicus*, and both *A. aculeatus* strains, while for the beta-tubulin gene

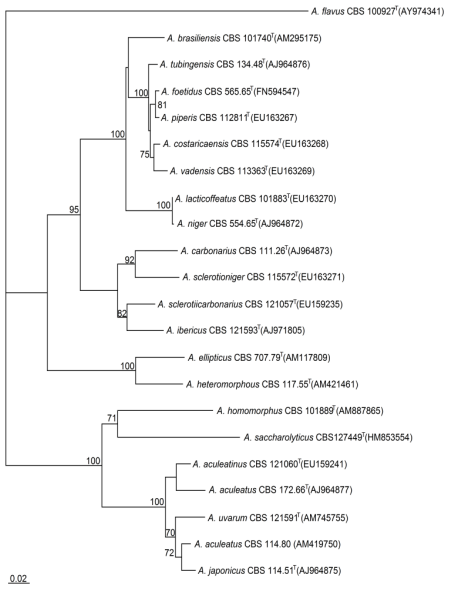

Fig. 1. Neighbor-joining phylogenetic tree based on partial calmodulin gene sequence data for *Aspergillus* section *Nigri*. Numbers above the branches are bootstrap values. Only values above 70% are indicated. Bar, 0.02 substitutions per nucleotide.

sequence data *A. saccharolyticus* clustered with *A. homomorphus*, *A. aculeatinus*, *A. uvarum*, and *A. aculeatus* CBS 114.80. The separate grouping in the beta-tubulin tree of *A. japonicus* and *A. aculeatus* CBS 172.66^T has consistently been shown in other publications (Noonim *et al.*, 2008, Samson *et al.*, 2007, Varga *et al.*, 2007, de Vries *et al.*, 2005, Samson *et al.*, 2004b). For all three loci, *A. saccharolyticus* is placed on its own branch far from the other species in the clade supported by the majority-rule consensus analysis for all three loci and high bootstrap values for the beta-tubulin and calmodulin loci, but low bootstrap value (51%) for the ITS locus. Sequence alignment revealed that amongst the species from section *Aculeati* that are in clade with *A. saccharolyticus*, interspecific sequence divergences are ≤0.7%, 7.1%, and 5.7% for the ITS, calmodulin, and beta-tubulin regions, respectively. Meanwhile, the interspecific sequence divergences in the ITS, calmodulin, and beta-tubulin region between *A. saccharolyticus* and the other species in the clade are on average 12.9±0.6%, 20±0.5%, and 15.4±1.2%, respectively. The variation in sequence data observed between *A. saccharolyticus* and *A. homomorphus* is the same as the variation between *A. homomorphus* and the smaller clade(s) of *A. aculeatinus*, *A. uvarum*, *A. japonicus*, and both *A. aculeatus* strains. Searching the NCBI database does not give any closer genetic match. Based on this, there is a clear genetic foundation for proposing the new species, *A. saccharolyticus*.

Furthermore, this strain could readily be distinguished from other black aspergilli by Universally Primed-PCR analysis using each of the two UP primers, L45 and L15/AS19 (Supplementary Fig S3). UP-PCR is a PCR fingerprinting method that has demonstrated its applicability in different aspects of mycology. These applications constitute analysis of genome structures, identification of species, analysis of population and species diversity, revealing of genetic relatedness at infra- and inter-species level, and identification of UP-PCR markers at different taxonomic levels (strain, group and/or species) (Lübeck & Lübeck, 2005). Each of the analyzed aspergilli, *A. saccharolyticus, A. aculeatinus*, *A. ellipticus*, *A. homomorphus*, *A. niger*, *A. uvarum*, *A. aculeatus* and *A. japonicus*, produced a unique banding profile, and did not share any bands (Supplementary Fig S3). This is an indication of clearly separated species, as strains within a species should at least have some similarities in their banding profiles (Lübeck & Lübeck, 2005).

The extrolite profiles further showed that *A. saccharolyticus* produced the largest chemical diversity on YES agar (25°C), whereas CYA (25 and 30°C), and CYAS, CY20S, CY20, CY40S, and PDA (all at 25°C) yielded fewer peaks. The results further showed that it is

Table 1. Physiological features and extrolite production[1] by the strains of uniseriate species in *Aspergillus* section *Nigri*.

Species	Growth on CYAS (diam, mm)	Growth at 37°C on CYA (diam, mm)	Extrolites
A saccharolyticus sp. nov. (CBS 127449^T)	11-14	7-14	12 compounds not described in the literature* including ACU-1** and ACU-2**
A aculeatinus (CBS 121060^T, CBS 121875, IBT 29275)	37-54	18-52	Aculeasins, neoxaline, secalonic acid D & F
A aculeatus (CBS 172.66^T)	0-4	15-26	Secalonic acid D & F, ACU-1** and ACU-2**
A japonicus (CBS 114.51^T, IBT 29329, IBT 26338, ITEM 4497)	0	8-25	Cycloclavine, festuclavine
A uvarum (CBS 121591^T, ITEM 4834; ITEM 4856; ITEM 5024)	54-73	11-14	Asterric acid, dihydrogeodin, erdin, geodin, secalonic acid D & F

[1]Extrolite production described in (Parenicova *et al.*, 2001) and (Noonim *et al.*, 2008), updated here.
* No matches found among the 13 500 fungal metabolites listed in Anitbase2010.
** ACU-1 and ACU-2 unidentified compounds with UV max 242 nm (100%) and 346 (88%) with mono isotopic masses of 315.1799 and 218.1268 Da respectively.

probably a new species since it does not share any metabolites with other species in the *Nigri* section where e.g. the naphto-γ-pyrones are consistently produced (Nielsen *et al.*, 2009) and only two compounds, ACU-1 and ACU-2, with series *Aculeati* (Table 1 and Supplementary Fig S4) whereas the well-known compounds from the series: neooxaline, secalonic acids, cycloclavine and aculeasins were not detected (Parenicova *et al.*, 2001). In addition, none of the 12 detected peaks matched with the approx. 13500 fungal extrolites in Antibase2010 (Laatsch, 2010) indicating that the species has not been investigated by natural products chemists.

Morphologically, *A. saccharolyticus* is most closely related to *A. japonicus* (Fig 2), but with larger conidia of 5-6 µm and vesicle size in the high margin of *A. japonicus*. Based on physiological features, differences between *A. saccharolyticus* and other uniseriate species in the *Nigri* section were found. Growth on CREA resembled that of *A. aculeatinus*, as moderate growth and medium acid

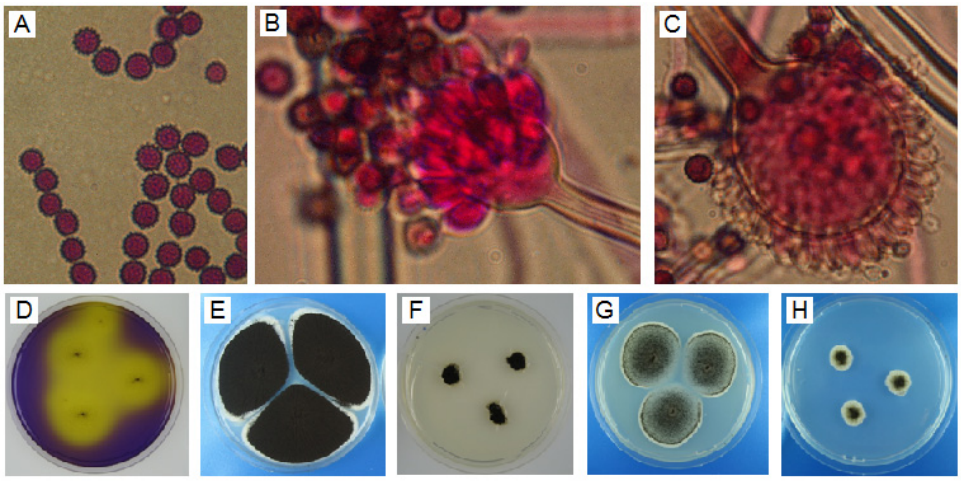

Fig. 2. *A. saccharolyticus* sp. nov. CBS 127449^T. A) conidia, B-C) conidial heads, D-H) Three-point inoculation on CREA, CYA, CYA 37°C, MEA, and CYAS, respectively, incubated 7 days

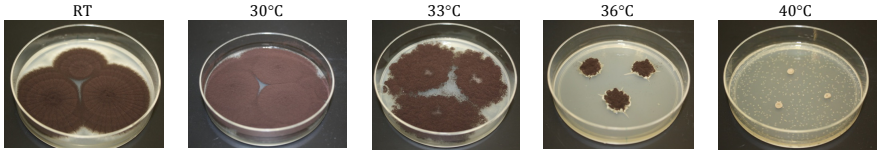

Fig. 3. *A. saccharolyticus* sp. nov. CBS 127449^T three point inoculation on CYA, incubation at different temperatures, growth observation day 7.

production was observed, while growth on CYA mostly resembled that of *A. aculeatus*, however, the reverse side of *A. saccharolyticus* is olive-green/brownish with sulcate structure, while that of *A. aculeatus* is curry-yellowish/brown (Fig 2 compared with Samson *et al.* (2007)). MEA was a medium where colony size was clearly different, with *A. saccharolyticus* being smaller than the other uniseriate aspergilli. *A. saccharolyticus* grew better on CYAS than *A. aculeatus* and *A. japonicus*, but growth was limited compared to *A. aculeatinus* and *A. uvarum*. Growth diameter of *A. saccharolyticus* on CYA at 37°C was approximately the same as for *A. uvarum*, while *A. aculeatus* and *A. japonicus* were less inhibited, and *A. aculeatinus* even less inhibited measuring the largest diameter of all uniseriate at this elevated temperature (Table 1).

With regards to temperature tolerance, growth was examined on CYA at 30°C, 33°C, 36°C, and 40°C. The maximum temperature *A. saccharolyticus* was able to grow at was 36°C, but growth at this temperature was restricted compared to the lower temperatures, which is generally the case for the other uniseriate aspergilli as well (Samson *et al.*, 2007). *A. saccharolyticus* showed a distinct change in morphology on CYA from 30°C to 33°C, but maintained good growth at both temperatures (Fig 3). The same tendency has been observed for *A. aculeatinus* grown on MEA, while *A. japonicus*, and *A. aculeatus* showed no change in morphology at these temperatures, while growth of *A. uvarum* was inhibited at 33°C (Samson *et al.*, 2007).

Our conclusion, that we have identified a novel species is based on a polyphasic approach combining phylogenetic analysis of three genes and UP-PCR data for characterizing the genotype, and morphological, physiological, and chemotaxonomical characteristics for phenotype analysis. Because the strain was unique in its genetic phylogeny, UP-PCR profile, extrolite profile, morphological, and physiological characteristics, we propose it as a novel species, *Aspergillus saccharolyticus*. The novel species is an efficient producer of beta-glucosidases (Research paper II, this thesis) and the name refers to its great ability to hydrolyze cellobiose and cellodextrins.

Latin diagnosis of *Aspergillus saccharolyticus* Sørensen, Lübeck *et* Frisvad sp. nov.

Coloniae post 7 *dies* 58-62 mm *diam in agaro* CYA*, in* CYA, 37 °C: 7-14 *mm; in* MEA 35-37, *in* YES 75-80 mm*, in agaro farina avenacea confecto* 39-42 mm*, in* CREA 30-34 mm*. Coloniae primum albae, deinde obscure brunneae vel atrae, reversum cremeum vel dilute brunneum. Conidiorum capitula primum globosa, stipes* 200-850 x 5-7 µm*, crassitunicatus, levis, vesiculae* 25-40 µm *diam, fere globosae; capitula uniseriata; phialides lageniformes, collulis brevis,* 5.5-7 µm*; conidia globosa vel subglobosa,* 5-6.2 µm*, echinulata. Sclerotia haud visa.*

Typus CBS 127449^T (= IBT 28509^T), *isolatus e lignore Quercetorum in* Gentofte, Dania.

Description of *Aspergillus saccharolyticus* Sørensen, Lübeck *et* Frisvad sp. nov.

Aspergillus saccharolyticus (sac.ca'ro.ly'ti.cus. N.L. masc. adj. *saccharolyticus*, being able to degrade cellobiose and cellodextins).

Colony diameter at 7 days: CYA at 25°C: 58-62 mm, at 37°C: 7-14 mm; CYAS: 11-14 mm; YES: 75-80 mm; OA: 39-42 mm; CY20S: 42-54 mm; CY40S: 43-54 mm; MEA: 35-37 mm; CREA 30-34 mm, poor growth, good acid production, colony first white then dark brown to black (Fig 2). Exudates absent, reverse cream-coloured to light greyish olive brown on CYA and light brown on YES. Conidial heads globose; stipes 200-850 x 5-7 µm, walls thick, smooth; vesicles 25-40 µm diam, globose; uniseriate, phialides flask shaped with a short broad collulum, 5.5-7 µm; conidia mostly globose, but some are subglobose, 5-6.2 µm, distinctly echinulate, with long sharp discrete spines, the spines being 0.6-0.8 µm long. Sclerotia have not been observed.

The type strain CBS 127449^T (= IBT 28509^T) was isolated from under a toilet seat made of treated oak wood, Gentofte, Denmark

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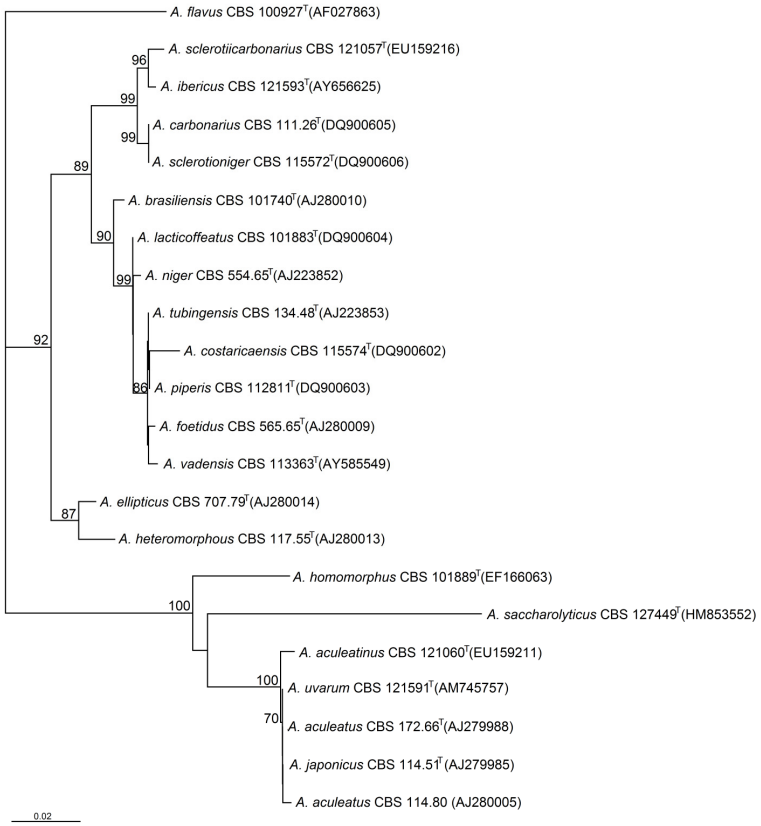
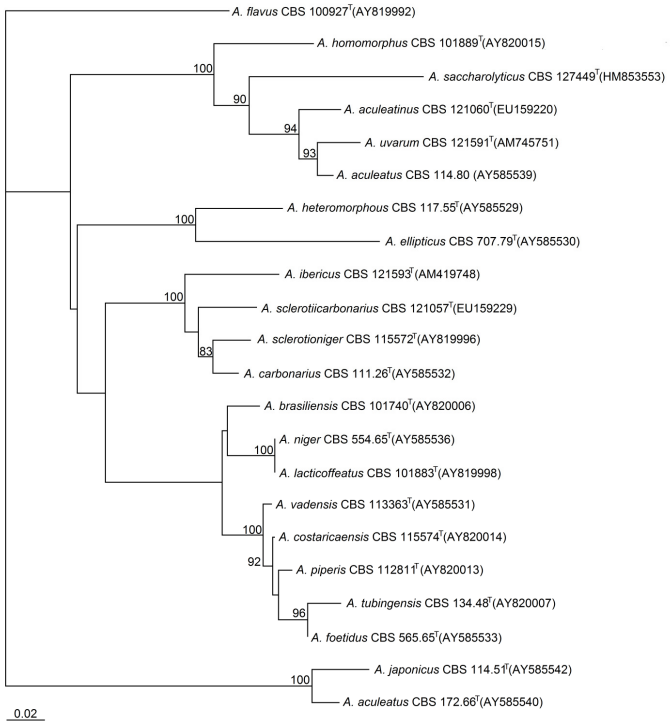
Supplementary Table 1. GenBank accession numbers of sequence data used to prepare the phylogenetic trees

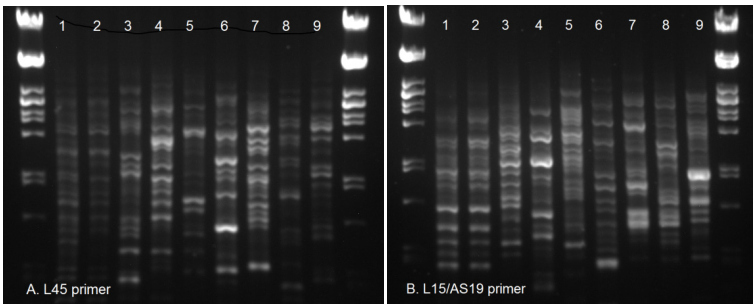
	ITS	Beta-tubulin	Calmodulin
A. niger CBS 554.65^{T}	AJ223852	AY585536	AJ964872
A. tubingensis CBS 134.48^{T}	AJ223853	AY820007	AJ964876
A. japonicus CBS 114.51^{T}	AJ279985	AY585542	AJ964875
A. aculeatus CBS 172.66^{T}	AJ279988	AY585540	AJ964877
A. foetidus CBS 565.65	AJ280009	AY585533	FN594547
A. brasiliensis CBS 101740^{T}	AJ280010	AY820006	AM295175
A. heteromorphous CBS 117.55^{T}	AJ280013	AY585529	AM421461
A. ellipticus CBS 707.79^{T}	AJ280014	AY585530	AM117809
A. vadensis CBS 113363^{T}	AY585549	AY585531	EU163269
A. ibericus CBS 121593^{T}	AY656625	AM419748	AJ971805
A. costaricaensis CBS 115574^{T}	DQ900602	AY820014	EU163268
A. piperis CBS 112811^{T}	DQ900603	AY820013	EU163267
A. lacticoffeatus CBS 101883^{T}	DQ900604	AY819998	EU163270
A. carbonarius CBS 111.26^{T}	DQ900605	AY585532	AJ964873
A. sclerotioniger CBS 115572^{T}	DQ900606	AY819996	EU163271
A. homomorphus CBS 101889^{T}	EF166063	AY820015	AM887865
A. aculeatinus CBS 121060^{T}	EU159211	EU159220	EU159241
A. sclerotiicarbonarius CBS 121057^{T}	EU159216	EU159229	EU159235
A. uvarum CBS 121591^{T}	AM745757	AM745751	AM745755
A. aculeatus CBS 114.80	AJ280005	AY585539	AM419750
A. saccharolyticus CBS 127449^{T}	HM853552	HM853553	HM853554
A. flavus CBS 100927^{T}	AF027863	AY819992	AY974341



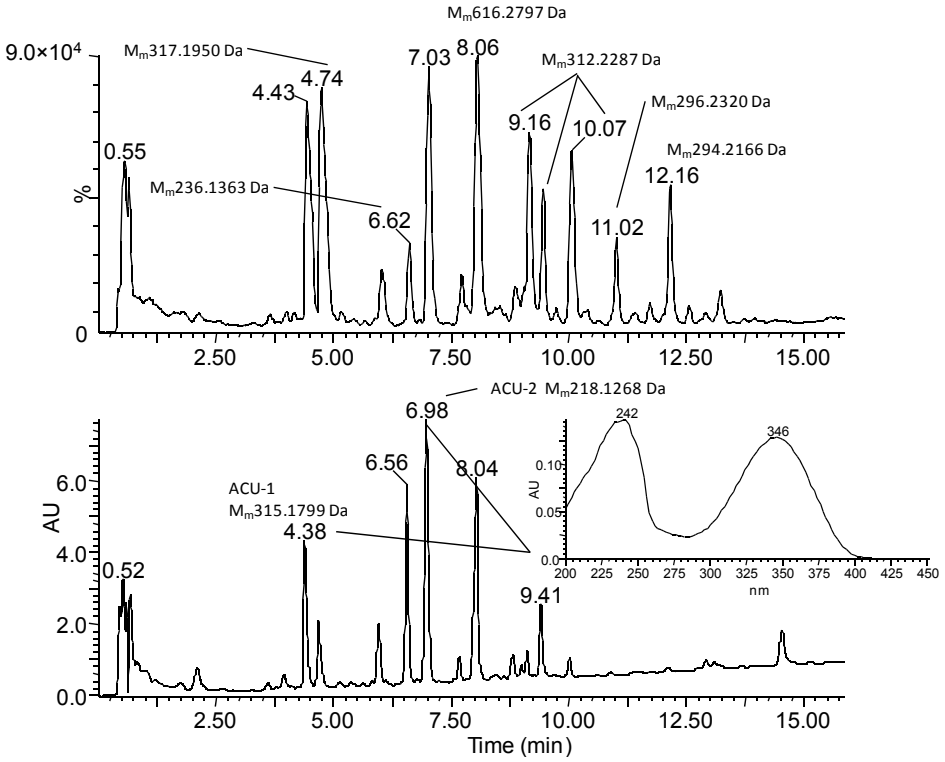
Supplementary Fig. S1. Neighbor-joining phylogenetic tree based on ITS sequence data for *Aspergillus* section *Nigri*. Numbers above the branches are bootstrap values. Only values above 70% are indicated. Bar, 0.02 substitutions per nucleotide.



Supplementary Fig. S2. Neighbor-joining phylogenetic tree based on partial beta-tubulin gene sequence data for *Aspergillus* section *Nigri*. Numbers above the branches are bootstrap values. Only values above 70% are indicated. Bar, 0.02 substitutions per nucleotide.



Supplementary Fig. S3. Universally Primed-PCR analysis using each of the two UP primers, L45 and L15/AS19. Loaded in lanes: 1&2) *A. saccharolyticus* sp. nov. CBS 127449^{T} , 3) *A. aculeatinus* CBS 121060^{T}, 4) *A. ellipticus* CBS 707.79^{T}, 5) *A. homomorphus* CBS 101889^{T}, 6) *A. niger* CBS 554.65^{T}, 7) *A. uvarum* CBS 121591^{T}, 8) *A., auleatus* CBS 172.66^{T}, 9) *A. japonicus* CBS 114.51^{T}



Supplementary Fig. S4. Extrolite profile from YES agar (14 days 25°C) of *A. saccharolyticus* sp. nov. CBS 127449^{T}. Above is the ESI^{+} trace (m/z 100-900) and below the UV trace (200-700 nm, 0.05 min ahead of ESI^{+}). Mono isotopic masses (M_m) of major peaks are inserted. The UV spectrum of two related compounds, ACU-1 and ACU-2, with identical UV spectra is also inserted.

Research paper IV

Cloning, expression, and characterization of a novel highly efficient beta-glucosidase from *Aspergillus saccharolyticus*

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Kenneth S. Bruno, David E. Culley, and Peter S. Lübeck

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Cloning, expression, and characterization of a novel highly efficient beta-glucosidase from *Aspergillus saccharolyticus*

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A novel beta-glucosidase, BGL1, was identified in the solid state enzyme extract from the new species *Aspergillus saccharolyticus*. Ion exchange chromatography was used to fractionate the extract, yielding fractions with high beta-glucosidase activity and only one visible band on SDS-page gel. LC-MS/MS analysis of this band gave peptide matches of aspergilli beta-glucosidases. The beta-glucosidase gene, *bgl1*, of *Aspergillus saccharolyticus* was cloned by PCR using degenerate primers followed by genome walking, obtaining a 2919 bp genomic sequence coding for the BGL1 polypeptide. The genomic sequence includes 6 introns and 7 exons resulting in a 860 aa polypeptide, BGL1, which has 91% and 82% identity with BGL1 from *Aspergillus aculeatus* and BGL1 from *Aspergillus niger*, respectively. The *bgl1* gene was heterologously expressed in *Trichoderma reesei* QM6a, purified, and characterized by enzyme kinetics studies. The enzyme was able to hydrolyze cellobiose, pNPG, and cellodextrins. V_{max} and K_M for cellobiose were determined to be 45 U/mg and 1.9 mM, respectively. Using pNPG as substrate, the enzyme was inhibited by glucose with a 50% reduction in activity at glucose concentrations 30 times greater than the pNPG concentration. The pH optimum was 4.2, the enzyme was stable at 50°C and at 60°C it had a halflife of approximately 6 hours. Generally the kinetics observed for the pure BGL1 enzyme resembled those of raw extract of *A. saccharolyticus* previously reported by our lab. BGL1 was identified as belonging to GH family 3. Through homology modeling, a 3D structure was proposed, finding retaining enzyme characteristics and, interestingly, a more open catalytic pocket compared to other beta-glucosidases.

Cellulose is the most abundant renewable biomass available on earth. Three main players are involved in the hydrolysis of cellulose: cellobiohydrolases (EC 3.2.1.74), endo-glucanases (EC 3.2.1.4), and beta-glucosidases (EC 3.2.1.21). Hydrolysis of cellulose is completed through their synergistic actions. Cellobiohydrolases processively hydrolyze cellulose from the ends releasing cellobiose, while endo-glucanases hydrolyze the amorphous regions thereby creating more ends for the cellobiohydrolases. Finally, beta-glucosidases hydrolyze the short cellodextrins and cellobiose to glucose (3, 55), hereby lowering the concentration of cellobiose and cellodextrins that are inhibitors of cellobiohydrolases and endo-glucanases (3, 7, 12, 15, 16) .

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Beta-glucosidases are of great industrial interest in relation to efficient hydrolysis of lignocellulosic biomass into glucose. Aspergilli are known to be good beta-glucosidase producers (56), and *Aspergillus niger* which has GRAS status is used industrially (46). It has been known for a long time that the combination of *A. niger* beta-glucosidases and *Trichoderma reesei* cellulases perform well in hydrolysis of cellulose, with increased rates of glucose production compared to the use of any one of the components alone (51).

Several beta-glucosidases have been purified and/or cloned, from aspergilli and other filamentous fungi (13, 22, 25, 33, 35). A number of these beta-glucosidases have been expressed in heterologous hosts, including *T. reesei*, which has been used extensively for industrial enzyme production. Characterization of different purified beta-glucosidases is part of the search for new and more efficient enzymes for the biotech industry, where especially enzymes with high specificity towards cellobiose and enzymes that function at high temperatures are

of interest. In addition to being indispensable for an efficient cellulase system, beta-glucosidases themselves are also of great interest as versatile industrial biocatalysts for their ability to activate glucosidic bonds (transglycosylation activity) facilitating synthesis of stereo/region-specific glycosides or oligosaccharides. These may be useful as functional materials, nutraceuticals or pharmaceuticals because of their biosignaling, recognition, or antibiotic properties (48). Fungal beta-glucosidases are classified into glycosyl hydrolase (GH) family 1 and 3. The GH3 enzymes, which are most abundant in the fungal genomes (11), are less well characterized than their GH1 homologues and only a few crystal structures have been solved, one from barley (53), and preliminary structures from the hyperthermophilic bacterium *Thermotoga neapolitana* (40) and the yeast *Kluyveromyces marxianus* (59), and at present there is no structure available from filamentous fungi (11).

In a recent study, *Aspergillus saccharolyticus* was found to produce beta-glucosidases with more efficient hydrolytic activity compared to commercial beta-glucosidase containing preparations, especially with regard to thermostability (Research article II, this thesis). The aim of this work was to identify, isolate, and characterize the most prominent beta-glucosidase from *A. saccharolyticus*. We report the molecular cloning of the novel beta-glucosidase gene, *bgl1*, and a model prediction of its structure. The novel beta-glucosidase was expressed in *T. reesei* for purification and the enzyme was then characterized by Michaelis-Menten kinetic studies, thermostability, pH optimum, glucose tolerance, and ability to hydrolyze cellodextrins.

MATERIALS AND METHODS

Fungal strain and enzyme extract preparation. *A. saccharolyticus* CBS 127449^T was initially isolated from treated hard wood (Research article III, this thesis) and routinely maintained on potato dextrose agar. A solid state fermentation enzyme extract of *A. saccharolyticus* was prepared as described in research article II, this thesis.

Fractionation by ion exchange chromatography. The enzyme extract of *A. saccharolyticus* was fractionated by ion exchange chromatography using an ÄKTApurifier system with UNICORN software. HiTrap Q XL 1 ml anion column (GE Healthcare) was run at a flow rate of 1 ml/min, 5 CV of buffer A (Tris buffer pH 8) was used to equilibrate the column, 5 CV sample (approx 0.5 mg protein/ml) was loaded onto the column, followed by a 2 CV wash with buffer A. Gradient elution was carried out over 30 CV with buffer B (Tris buffer pH 8, 1M NaCl) reaching 70% of the total volume. The column was finally washed with 5 CV buffer B and reequilibrated for the next run with buffer A. Aliquots of 1ml were collected and assayed for beta-glucosidase activity as well as quantified in terms of protein content, as described below.

Assays for beta-glucosidase activity and protein quantification. Beta-glucosidase activity was assayed using using 5mM p-nitrophenyl-beta-D-glucopyranoside (pNPG) (Sigma) in 50mM Na-Citrate buffer pH 4.8 as described in research article II, this thesis.

Protein quantification was done using the Pierce BCA protein assay kit microplate procedure according to manufacturer's instructions (Pierce Biotechnology), using bovine serum albumin as standard.

Electrophoresis. Sample preparation and electrophoresis was performed using ClearPAGE precast gels and accessories (C.B.S. Scientific Company, Inc). Samples were prepared by mixing 65 vol% protein, 25% 4xLDS sample buffer (40% glycerol, 4% Ficoll-400, 0.8M Triethano amine pH 7.6, 6N HCl, 4% Lithium dodecyl sulphate, 2mM EDTA di-sodium, 0.025% Brilliant blue G250, 0.025% Phenol red), and 10% 10x reducing agent (20mM DTT) and heating for 10 min at 70°C. 25 µl of each sample were loaded on a ClearPAGE 4-12% SDS-gel, using ClearPAGE two-color SDS marker for band size approximation. The gel was stained with ClearPAGE Instant Blue stain by placing the gel in a small container, adding the Instant Blue stain till the gel was covered, followed by shaking the gel gently for 10-30 minutes till desired band intensity was achieved. No destaining was performed, but the gels were washed a few times in ultrapure water, with gently shaking.

Mass Spectrometry and Protein Identification. Bands of interest were excised from the gel, and in-gel digestion was performed as described by Kinter and Sherman, 2000 (29). The trypsin digestion, and sample analysis was carried out by The Laboratory for Biotechnology and Bioanalysis 2 (LBB2), Washington State University, Pullman, Washington, USA, where analysis was performed by LC-MS/MS using LC Packings Ultimate Nano high-performance liquid chromatography system (with LC Packings monolithic column PS-DVB) and Esquire HCT electrospray ion trap (Bruker Daltonics, Billerica, MA) as described in their former publication (37). The Mascot search engine (www.matrixscience.com) was used to search the peptide finger prints against predicted peptides in the NCBI database with the significance threshold $p<0.05$.

Isolation and cloning of beta-glucosidase gene. Based on the *Aspergillus aculeatus* peptide match found in the LC-MS/MS analysis, the corresponding full length beta-glucosidase protein (GenBank: BAA10968) was submitted to a NCBI blast search in the protein entries of GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify similar beta-glucosidases. An alignment of these sequences was made with BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) to identify conserved regions. Degenerate primers were designed using the CODEHOP strategy (44): forward primer: 5'CACGAAATGTACCTCtggcccttygc and reverse primer 5'CCTTGATCACGTTGTCGccrttcykcca. Genomic DNA of *A. saccharolyticus* was isolated as described in research article II, this thesis. The primers were used in polymerase chain reaction (PCR) with genomic DNA and RUN polymerase (A&A Biotechnology), obtaining a fragment of approximately 950 bp. The band was excised from the gel and sequenced using the sequencing service at MWG, Germany, and was by NCBI blast found to be related to other aspergilli beta-glucosidase fragments. From several rounds of genome walking (18), the flanking regions were characterized, thereby obtaining the full genomic sequence of the gene. The start and stop codon was predicted by NCBI blast comparison and the GenScan Web server (http://genes.mit.edu/GENSCAN.html).

RNA was prepared from 4 day old fungal spores and mycelium grown on plates containing 20 g/l wheat bran, 20 g/l corn steep liquor, 3 g/l $NaNO_3$, 1 g/l K_2HPO_4, 0.5 g/l KCl, 0.5 g/l $MgSO_47H_2O$, 0.01 g/l $FeSO_47H_2O$, 15g/l agar. The cells were disrupted by bead beating (2x 20sec) in Fenozol supplied with the Total RNA kit (A&A Biotechnology) and RNA purified following the kit protocol. cDNA was prepared from total RNA using First strand cDNA synthesis kit and random hexamer primers (Fermentas),

A cassette comprising *Magnaporthe grisea* ribosomal promoter RP27, the beta-glucosidase genomic DNA gene, six histidine residues, and *Neurospora crassa* beta-tubulin terminator was constructed using PCR cloning techniques and was cloned into the *PciI* site of pAN7-1. The plasmid pAN7-1 containing the *E. coli hyg*B resistance gene was donated by Peter Punt (University of Leiden, The Netherlands) (41). The promoter and terminator were from plasmid pSM565 (10) and the beta-glucosidase gene was from *A. saccharolyticus* genomic DNA. PCR was performed using proofreading WALK polymerase (A&A Biotechnology), while restriction enzymes (fast digest *PciI*), alkaline phosphatase (fastAP), and ligase (T4 DNA ligase) were from Fermentas. The construct pAS3-gBGL1 was transformed into *E. coli* Top 10 competent cells (prepared using $CaCl_2$ (45)) and plated on LB plates (10 g/l bacto tryptone, 5 g/l yeast extract, 10 g/l NaCl, 15 g/l agar, pH 7.5) with ampicillin selection (100ppm). Correct transformants were checked for by colony PCR using several different promoter, beta-glucosidase, and terminator specific primers. An overnight culture with a correct transformant was prepared and the constructed cloning vector purified the following day (E.Z.N.A. Plasmid Midi Kit, Omega Biotech). The final cloning vector, pAS3-gBGL1, is sketched in Fig. 1.

Transformation and identification of active recombinant beta-glucosidase in *T. reesei.* Protoplast preparation of *T. reesei* QM6a was carried out similarly to the procedure described by Pentillä *et al.*, 1987 (39). 100 ml complete medium (10 g/l glucose, 2 g/l peptone, 1 g/l yeast extract, 1 g/l casamino acids, 6 g/l $NaNO_3$, 0.52 g/l KCl, 0.52 g/l $MgSO_4{\cdot}7H_2O$, 1.52 g/l KH_2PO_4, 22mg/l $ZnSO_4{\cdot}7H_2O$, 11mg/l H_3BO_3, 5mg/l $MnCl_2{\cdot}4H_2O$, 5mg/l $FeSO_4{\cdot}7H_2O$, 1.7mg/l $CoCl_2{\cdot}6H_2O$, 1.6mg/l $CuSO_4{\cdot}5H_2O$, 1.5mg/l $Na_2MoO_4{\cdot}2H_2O$, and 50mg/l Na_2EDTA) in a 500 ml baffled flask was inoculated with fresh *T. reesei* conidia reaching a concentration of 10^6 spores per ml and incubated for 16-22 hours at 30°C, 120rpm. The mycelium was collected on double folded Miracloth (Andwin scientific) and washed with sterile water. The mycelium was suspended in 20 ml protoplasting solution (1.2 M $MgSO_4$, 50 mM $NaPO_4$ pH 5.8) with 60mg VinoTaste Pro (Novozymes A/S) enzyme per ml,

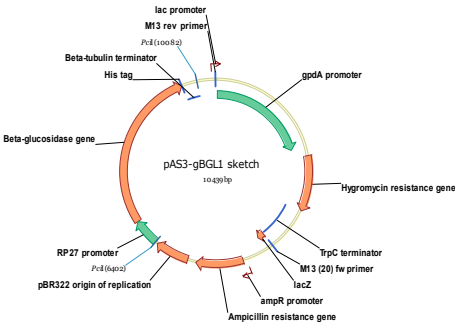

FIG. 1. Sketch of the cloning vector, pAS3-gBGL1: pAN7-1 modified with a cassette of RP27 promoter, beta-glucosidase gene *bgl1*, his-tail, and beta-tubulin terminator inserted at the *PciI* restriction site.

incubated for 2-4 hours at 30°C, 65 rpm, then filtered through double folded Miracloth, and washed with a few ml protoplasting solution. The protoplasts were overlaid with ST buffer (0.6 M sorbitol, 0.1M Tris-HCL pH 7.0) (approx. 20% the volume of protoplasting solution) and centrifuged at 1000g for 10 min. Protoplasts were collected from the interphase and washed twice with STC buffer (1.0 M sorbitol, 10mM $CaCl_2$ $2H_2O$, 10mM Tris-HCl pH 7.5), finally resuspending in STC buffer at a concentration of approximately $5x10^7$ for immediate use in transformation.

For transformation, 200 µl protoplasts, 10 µl plasmid DNA (>1µg), and 50 µl PEG1 (25% PEG 6000 in STC buffer) were mixed gently and incubated on ice for 20 min. 2 ml PEG2 (25% PEG 6000, 50mM $CaCl_2$, 10mM Tris-HCl pH7.5) was added and incubated 5 min at room temperature, followed by addition of 4 ml STC buffer. Aliquots of 1 ml were plates in recovery agar (1 g/l $MgSO_47H_2O$, 10 g/l KH_2PO_4, 6 g/l $(NH_4)_2SO_4$, 3 g/l NaCitrate $2H_2O$, 10 g/l glucose, 182 g/l sorbitol (final 1M), 5 mg/l $FeSO_47H_2O$, 1.6 mg/l $MnSO_4H_2O$, 1.4 mg/l $ZnSO_4H_2O$, 2 mg/l $CaCl_22H_2O$, 15 g/l agar) with 100ppm hygromycin as selection, and incubated at 28°C over night. The following day, a top layer of the above described agar, again with 100ppm hygromycin, but without sorbitol, was added, and the plates were incubated for another 2-4 days before colonies that had surfaced were picked and carried through multiple retransfers and streaking on selective medium to obtain pure colonies.

Identification of positive transformants was done by a simple pNPG activity screen, where three 0.5x0.5cm agar plugs of the transformants were added to 10 ml growth medium (20 g/l wheat bran, 20 g/l corn steep liquor, 3g/l $NaNO_3$, 1g/l K_2HPO_4, 0.5 g/l KCl, 0.5 g/l $MgSO_47H_2O$, 0.01 g/l $FeSO_47H_2O$) in 50 ml Falcon tubes and incubated at 30°C, 180rpm for five days. The supernatant was collected, centrifuged at 10,000 rpm for 10 min and assayed for beta-glucosidase activity using the pNPG assay described earlier. At these conditions, the wild type QM6a showed no significant activity, so positive transformants were identified by the presence of beta-glucosidase activity.

Purification of expressed beta-glucosidases. Using the HisSpin Trap kit (GE Healthcare), following the protocol supplied with the kit, the optimal imidazole concentration for purification of the histidine tagged beta-glucosidases was found to be 0 mM imidazole.

The transformant having shown the greatest beta-glucosidase activity was cultured in 150 ml growth medium (specified above) in a 500 ml baffled flask, incubated at 30°C, 160 rpm for 6 days. The supernatant was centrifuged at 10,000 rpm for 10 min, filtered through a 0.22 µm filter (Millipore) and pH adjusted to 7.4.

The his-tagged beta-glucosidases were purified on the ÄKTApurifier system with UNICORN software, using a HisTrap HP 5 ml anion column (GE Healthcare), run at a flow rate of 5 ml/min. The column was equilibrated with 5 CV of binding buffer (20mM sodium phosphate, 0.5M NaCl, pH 7.4), 100 ml sample was loaded onto the column, followed by washing with binding buffer till the absorbance reached the baseline. The his-tagged beta-glucosidases were eluted with 3 CV elution buffer (20mM sodium phosphate, 0.5M NaCl, 500mM imidazole, pH 7.4) and the peak (1 CV) collected by monitoring the absorbance.

Assays for characterization of purified beta-glucosidases. Michaelis Menten kinetics, glucose tolerance, termostability, pH optimum, and cellodextrin hydrolysis were carried out as described in research article II, this thesis.

Sequence comparisons and homology modeling. Sequences similar to BGL1 were located by BLAST (1) in the protein entries of GenBank (5) and aligned using hidden Markov models (26) and CLUSTAL W (52). Similar beta-glucosidase catalytic domain structures were obtained from the Protein Data Bank (PDB; (6)), then superimposed and compared with the program O (24). Multiple sequence alignments were used to generate the best pair-wise alignment of the *A. saccharolyticus* beta-glucosidase with that of the *T. neapolitana* beta-glucosidase 3B. This pair-wise alignment was the basis of creating a homology model, with PDB entry 2X40 (40) as the template in the program SOD (30). The model was adjusted in O, using rotamers that would improve packing in the interior of the protein. The model is available upon request from the authors. The figure was prepared using O, MOLSCRIPT (32) and Molray (19).

RESULTS

Identification of beta-glucosidase in *A. saccharolyticus* extract

The enzyme extract of *A. saccharolyticus* produced by solid state fermentation on wheat bran, was fractionated by ion exchange chromatography, investigating the beta-glucosidase activity and protein content of each fraction (Fig. 2). Protein content could be measured in all fractions. Approximately 25% of the proteins did not bind to the column, but passed through prior to the start of the gradient elution. No beta-glucosidase activity was found in this initial flow-through. The fractions displaying the greatest beta-glucosidase activity were the fractions #15-17 (Fig. 2). These fractions with eluted beta-glucosidase were calculated to have a NaCl concentration of approximately 0.14-0.23M. From SDS-page, one dominating band of approximately 130 kDa was discovered in these fractions (Fig. 2). The intensity of this band in the different fractions followed the measured beta-glucosidase activity. The proteins in this band were found to be highly expressed relative to other proteins in the raw *A. saccharolyticus* extract (Fig. 3). The band of fraction 16 was excised from the gel, trypsin digested, analyzed by LC-MS/MS, and searched against the NCBI database for peptide matches using the Mascot program. The sample was identified as a beta-glucosidase, having peptides identical to several aspergilli species, including *A. aculeatus* (Swiss-Prot: P48825), *A. terreus* (NCBI ref seq XP_001212225), *A. niger* (GenBank CAB75696), and *A. fumigatus* (NCBI ref seq XP_750327). The best match was the beta-glucosidase of *A. aculeatus*, with five peptide matches. Besides of beta-glucosidase peptides, also peptide matches of beta-galactosidase from different *Aspergillus* species were found suggesting that the analyzed band contained more than one protein. Only the results for the beta-glucosidase peptides were used for degenerate primer design to obtain the homologous beta-glucosidase of *A. saccharolyticus*.

Characterization of beta-glucosidase gene, *bgl1*, and predicted protein, BGL1

By the use of degenerate primers and genome walking, the genomic coding sequence of *bgl1* of 2919 base pairs (incl stop coden) was obtained (GenBank HM853555). The sequence comprises seven exons, intercepted by six introns located at 58-

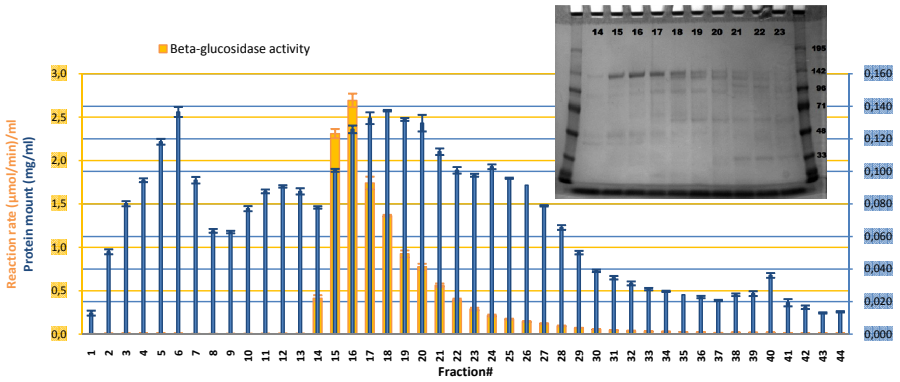

FIG. 2. Beta-glucosidase activity and protein content of the different fractions from ion exchange. Fractions 2-6 are flow through from the load, fractions 7-8 are wash prior to gradient elution, fractions 9-38 are gradient elution, and fractions 39-45 are final column stripping. Top right corner: SDS-page 4-12% of fractions 14-23 with high beta-glucosidase activity.

119, 263-313, 359-414, 468-523, 1716-1776, 2636-2685 bp, which all followed the GT-AG rule at the intron/exon junctions. The gene encodes a 860 amino acid polypeptide, BGL1, predicted by NetAspGene 1.0 (54) and confirmed by mRNA isolation and sequencing of the derived cDNA. A signal peptide with a cleavage site between amino acid 19 and 20 was predicted by SignalP server (4, 36). A TATA-like sequence at position -138 bp, a CCAAT box at position –695 bp, and several CreI sites at positions -145, -619, -1186, -1294, and -1313 were identified upstream of the start codon. Analysis of the predicted cDNA gene sequence revealed 85% identity with *bgl1* from *A. aculeatus* (GenBank D64088.1) (27) and 75% identity with *bgl1* *A.* niger (NCBI ref seq XM_001398779) (38).

The pI of BGL1 was calculated to 4.96 and the molecular mass was calculated to 91 kDa using the ExPASy Proteomics server (17). This prediction does not relate to the size of the band in fraction 16 (Fig. 3), but from the NetNGlyc 1.0 server (9) BGL1 has 12 asparagines that are potential N-linked glycosylation sites. The molecular weight of 130 kDa observed by SDSpage therefore probably reflects extensive glycosylation.

The previous MS/MS data of the band in faction 16 was by use of Mascot searched against possible trypsin fragments and expected MS/MS patterns of the BGL1 sequence. The observed MS/MS data matched the expected data, thus confirming that the cloned *bgl1* gene codes for the protein present in fraction 16.

Analysis of the amino acid sequence of BGL1 resulted in 91% identity with beta-glucosidase BGL1 from *A. aculeatus* (GenBank BAA10968) (27) and 82% identity with beta-glucosidase BGL1 from *A. niger* (NCBI ref seq XP_001398816) (38). Alignment of the amino acid sequence of BGL1 from *A. saccharolyticus* with several aspergilli glycosyl hydrolase (GH) family 3 beta-glucosidases revealed a high degree of homology in highly conserved regions including the catalytic sites (Fig. 4), and PROSITE scan (49) confirmed the presence of a GH family 3 active site in BGL1, predicting the signature sequence to be between amino acids 248-264 (LLKSELGFQGFVMSDWGA) in the mature protein. The putative nucleophile, Asp261, of the mature BGL1 of *A.*

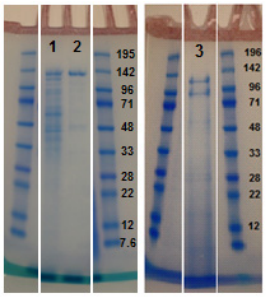

FIG. 3. SDS-page 12-20%, lane 1) *A. saccharolyticus* raw extract, lane 2) fraction #16 from the ion exchange fractionation, lane 3) His-tag purified BGL1

```
A. saccharolyticus (HM853555)    (241) SYTLNKLLKSELGFQGFVMSDWGAHHSGVG
A. aculeatus (BAA10968)          (241) SYTLNKLLKAELGFQGFVMSDWGAHHSGVG
A. niger (CAK48740)              (241) SYTLNKLLKAELGFQGFVMSDWAAHHAGVS
A. avenaceus (AAX39011)          (242) SLTLNKLLKAELGFQGFVMSDWSAHHSGVG
A. kawachii (BAA19913)           (241) SYTLNKLLKAELGFQGFVMSDWAAHHAGVS
A. terreus (ACY03273)            (242) SLTLNKLLKAELGFQGFVMSDWSAHHSGVG
A. oryzae (BAE54829)             (242) SETLNKLLKAELGFQGFVMSDWTAHHSGVG
A. fumigatus (EAL88289)          (252) SQTLNKLLKAELGFQGFVMSDWSAHHSGVG
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FIG. 4. Alignment of proposed active site region (boxed) of different aspergilli GH3 beta-glucosidases, the GenBank accession number is given in parenthesis.

saccharolyticus is located in this region (20).

Homology modeling studies show that *A. saccharolyticus* beta-glucosidase, BGL1, catalytic module possesses a fold similar to that of beta-glucosidase 3B from *T. neapolitana* with 5 deletions and 8 insertions compared to it (Fig. 5). Although the sequence identity is relatively low (35%) it is obvious that the residues important for substrate binding and catalysis are conserved (Fig. 5A).

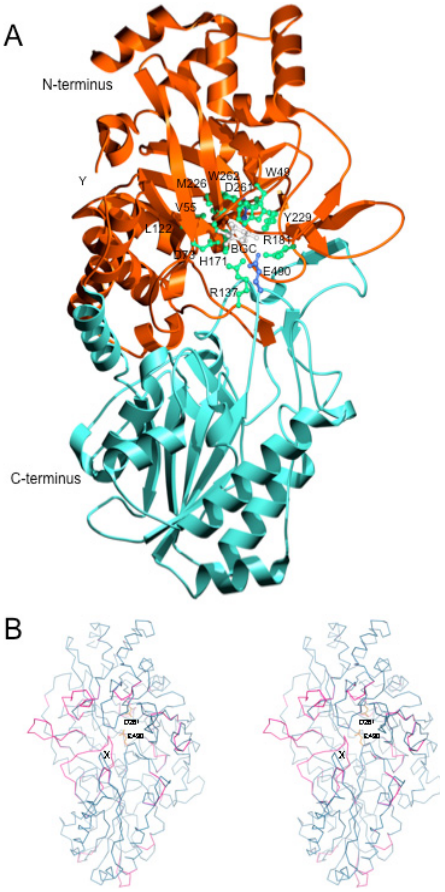

FIG 5. Homology model of the catalytic module of beta-glucosidase, BGL1, from *Aspergillus saccharolyticus*. A. Ribbon cartoon representation of the catalytic module showing the important residues for catalysis (in royal-blue) and substrate/product binding (in spring-green). Glucose (in gray) is modeled into the catalytic site. The part of the loop marked 'Y' is not modeled. B. Stereo diagram illustrating the comparison of beta-glucosidase homology model of *A. saccharolyticus* (in steel-blue) with the template structure from *T. neapolitana* (PDB entry 2X41) (in orange-red). The catalytic nucleophile (D261) and the acid/base (E490) are shown in gold.

These results imply that BGL1 is a novel beta-glucosidase belonging to GH family 3.

Heterologous expression of *bgl1* by *T. reesei*

The *bgl1* gene was heterologously expressed in *T. reesei* from the constitutive RP27 ribosomal promoter. Positive transformants were selected by simple pNPG assay, where the negative control, wild type QM6a, showed no beta-glucosidase activity at the culture and assay conditions used. The transformant identified from the screening to have the highest beta-glucosidase activity was confirmed by PCR to have the expression cassette incorporated into its genomic DNA. Using a Ni-sepharose column, the his-tagged proteins were purified from an extract of the transformant. An SDS-page gel of the eluent showed two bands (Fig. 3), one correlating in size with the band found in fractions 15-17 in the initial fractionation of the *A. saccharolyticus* extract (approximately 130 kDa) and another that was smaller (approximately 90 kDa) correlating with the predicted size of the protein. 3.8 mg purified protein was obtained from 100 ml filtered culture extract, which corresponded to about 2.7% of the total amount of protein in the culture extract.

Characterization of the purified BGL1

The purified BGL1 was characterized by its activity on pNPG and cellobiose.

A substrate saturation plot of BGL1 revealed inhibition of the enzyme reaction, when pNPG in high concentrations was used as substrate. This was observed by a decrease in reaction rate with increasing substrate concentration rather than a leveling off toward a maximum velocity (Fig. 6A). Meanwhile, a MM kinetics relationship was found for cellobiose, where the reaction rate tends towards the maximum velocity (Fig. 6A). A Hanes plot of the cellobiose data gave a good distribution of the data points for preparation of a straight trendline from which V_{max} and K_M were determined to be 45 U/mg and 1.9 mM, respectively. Glucose inhibition was investigated with pNPG as substrate, showing a reduction in activity to 50% at a product concentration 30 times greater than the substrate concentration (Fig. 6B).

BGL1 was incubated at different temperatures for different time periods to investigate the thermostability and half-life of the enzyme. At regularly used hydrolysis temperature of 50°C, the enzyme was stable throughout the incubation period (data not shown). The enzyme is fairly stable at temperatures up to 58°C at 4 hour incubation (Fig. 7A), and with temperatures around 60°C the calculated half-life is approximately 6 hours (Fig. 7A). From 62 °C and up to 65 °C there is a gradual decrease in the half-life to less than 2.5 hours. The calculated half-lives at different temperatures were plotted in a semi-logarithmic plot vs. temperature (Fig. 7B). The data forms a straight line, and the thermal activity number of BGL1, the temperature that gives a half-life of 1 hour, was 65.3°C. The pH span of BGL1 was examined at 50°C using pNPG as substrate. Its profile gives the typical bell-shape curve with an optimum around pH 4.2, and within the pH range 3.8-4.8 the

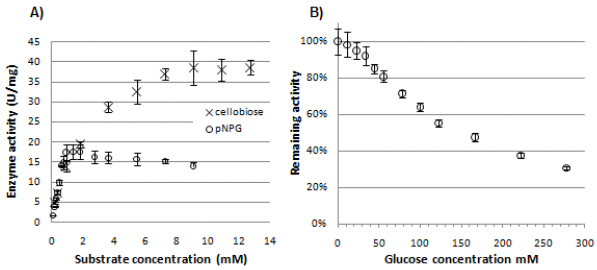

FIG. 6. A) Substrate saturation plot where enzyme activity is related to substrate concentration with the two substrates, pNPG and cellobiose. B) Relative beta-glucosidase activity at different inhibitor concentrations with a substrate concentration of 5mM pNPG.

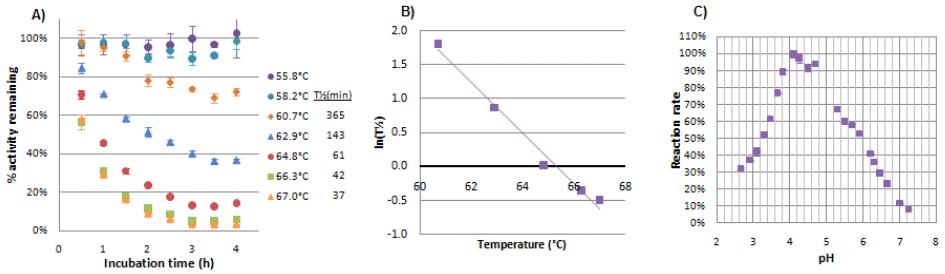

FIG. 7. A) Thermostability of BGL1 incubated at different temperatures for different time period followed by assaying at 50°C, pH 4.8, 10 min reactions. $T_{½}$= half-life, calculated for temperatures above 60°C. B) Semi-logarithmic plot of calculated half life at different temperatures. The x-axis intercept indicates the thermal activity number, temperature at which the half life is one hour. C) pH profile of BGL1 assayed at 50°C, varying pH, 10 min reactions.

activity stayed above 90% of maximum. At alkaline conditions, activity was below 10%, showing that acidic pH values are better suited for enzyme activity (Fig. 7C).

The ability of BGL1 to hydrolyze short chains of glucose units was studied with cellohexaose, -pentaose, -tetraose, and -triose, where only data for cellohexaose hydrolysis is shown here (Fig. 8). Initially, as the level of cellohexaose decreases, the concentration of primarily cellopentaose and glucose increase. Later, as the concentration of cellopentaose has increased, an increase in cellotetraose is observed, indicating that the enzyme hydrolyzes the different cellodextrins depending on the concentration in which they occur. Similar results were obtained using cellopentaose, -tetraose, and –triose as initial substrates. These observations of the different cellodextrins increasing in concentration over time related to their length suggests that the enzyme hydrolyzes the different cellodextrins through exohydrolase action, removing one glucose unit at the time releasing glucose and the one unit shorter product before it associates with another substrate, rather than processively cleaving off glucose units.

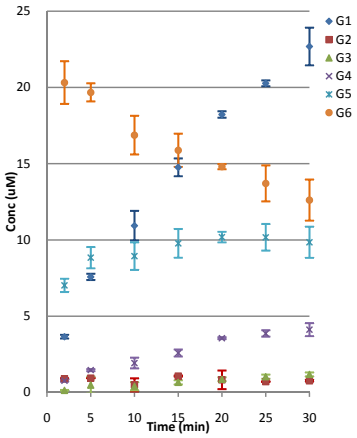

FIG. 8. Snap shot at different time points of the hydrolysis of cellohexaose for analysis of the degradation pattern. G1= glucose, G2= cellobiose, G3= cellotriose, G4= cellotetraose, G5= cellopentaose, G6= cellohexaose.

DISCUSSION

We have cloned a beta-glucosidase, BGL1, from the novel species *A. saccharolyticus* (Research article III, this thesis) and expressed it in *T. reesei* in order to purify the enzyme for a specific characterization.

Initially, we used ionexchange fractionation of the raw enzyme extract of *A. saccharolyticus* followed by LC-MS/MS analysis of the dominating protein band in the fractions with high beta-glucosidase activity. This was applied for the identification of active beta-glucosidases from *A. saccharolyticus*. Aspergilli are known to possess several beta-glucosidases in their genomes, e.g. *A. niger* has 11 GH3 beta-glucosidases predicted (38) of which 6 were identified as extracellular proteins by the SignalP 1.0 server (4, 36). With this approach we intended to identify the key beta-glucosidase player amongst the potential several expressed beta-glucosidases of *A. saccharolyticus*.

Ion exchange separates molecules on the basis of differences in their net surface charge. The net surface charge of proteins will change gradually as pH of the environment changes (2). The isoelectric point (pI) of the 6 predicted *A. niger* secreted beta-glucosidases (38) were, using ExPASY proteomics server (17), calculated to around pH 5. Assuming the pI of the secreted *A. saccharolyticus* beta-glucosidases are in the same range, an anion column was chosen for ion exchange using Tris buffer pH 8, as proteins will bind to an anion exchanger at pH above its isoelectric point (2). The beta-glucosidases of *A. saccharolyticus* did bind to the column at these conditions, with no activity found in the initial flow through fractions, and analysis of the later deducted amino acid sequence of BGL1 was calculated to have a pI of 4.96, correlating well with the above.

Proteomics is useful for the identification of secreted proteins and have been used for the identification of beta-glucosidases of *A. fumigatus* (28). MS/MS peptide analysis followed by molecular techniques were here employed for the identification and cloning of beta-glucosidases from *A. saccharolyticus*,. From LC-MS/MS analysis, peptides of a beta-glucosidase and a beta-galactosidase were identified in the protein band that was dominating in the protein fractions with high beta-glucosidase activity, indicating that it had not been a pure band, but rather had it contained both a beta-glucosidase and a beta-galactosidase of *A. saccharolyticus*. Only the identification of the beta-glucosidase was further pursued.

By genome walking, the beta-glucosidase was successfully cloned, and the size of its cDNA corresponded well with the beta-glucosidases of *A. aculeatus* (GenBank: BAA10968) and *A. niger* (GenBank: XP_001398816) to which it is most closely related. However, the predicted polypeptide size of the cloned beta-glucosidase was only 91 kDa compared to the approximately 130 kDa band seen in the SDS-page gel. Glycosylation of beta-glucosidases is common (13, 14, 23, 33, 35) and it was therefore assumed that the SDS-page gel size estimation of the protein was mislead by glycosylation that makes the protein run slower in the gel, which has also been seen with beta-glucosidases from *Talaromyces emersonii* expressed in *T. reesei* (35). Several potential N-glycosylation sites were identified for BGL1, supporting this assumption. Based on interest in obtaining knowledge of regulation of *bgl1* expression in *A. saccharolyticus*, putative binding sites for the cellulose regulatory protein, CREI, were searched for upstream of the *bgl1* gene. Five putative CreI sites were found within the 1350 pb sequence upstream of the gene obtained by genome walking. CREI is known to be involved in carbon catabolite repression of many fungal cellulase genes.

BGL1 was based on its amino acid sequence characterized as belonging to the GH family 3, matching the active site signature (11, 20). Several GH3 beta-glucosidases have been cloned and characterized, but few studies have been published on heterologous expression by *T. reesei*, while several have been expressed by *E. coli*, and some by Yeast (8). One example of expression by *T. reesei* is the beta-glucosidase, *cel3a* cDNA, of *T. emersonii* where the *chb1* promoter and terminator of *T. reesei* were used (35). Another example is the *T. reesei* production strain by Novozymes A/S expressing *A. oryzae* beta-glucosidase for improved cellulose conversion (34). We here present heterologous expression of *bgl1* from *A, saccharolyticus* by *T. reesei* QM6a using the constitutive *M. grisea* ribosomal promoter RP27 and the *N. crassa* beta-tubulin terminator to control expression of the gDNA clone of *bgl1*, thereby successfully combining host, promoter, gene, and terminator from different eukaryotes. The host strain was transformed with the non-linearized plasmid for random insertion, giving recombinant protein yields of 3.8mg/100ml from 6 days cultivation of the best transformant. This is significantly greater than the expression levels reached with *T. emersonii* beta-glucosidase (35), but still low compared to the secretion capacity of *T. reesei* (34).

Interestingly, it appeared that *T. reesei* secreted the heterologously expressed BGL1 in two different forms represented by the two bands on the SDS-page gel of the histidine-tag purified proteins. It is speculated that these two different bands represent different degrees of glycolysation, the large band being glycosylated to the same extent as found in the *A. saccharolyticus* secreted BGL1, and the smaller band correlating with the predicted molecular mass thus not being glycosylated. Postsecretional modification of glycosylated proteins expressed by *T. reesei* is medium dependent, with the effect on extracellular hydrolases being most dominating in enriched medium (50), possibly explaining the different forms of the recombinant BGL1 beta-glucosidases.

BGL1 is classified as a broad specificity beta-glucosidase as it can hydrolyze both aryl-beta-glycosides, cellobiose, and cellooligo-saccharides (8). Comparing the properties of *A. saccharolyticus* BGL1 to other *Aspergillus* beta-glucosidases, the observed inhibition at high pNPG substrate concentrations has also been reported for *A. niger* (33, 47, 58), *A. aculeatus* and *A. japonicus* (14). Whether the inhibition of *A. saccharolyticus* BGL1 is due to regular substrate inhibition

Table 1. Comparison of V_{max} values reported in the literature for hydrolysis of cellobiose by purified beta-glucosidases, either heterologously expressed or directly purified from extract of origin.

Organism ID	V_{max} (U/mg)	Assay conditions (°C, pH)	Reference
A. saccharolyticus CBS 127449	49	50, 4.8	This work
A. niger NIAB280	36.5	50, 5.0	(42)
A. niger CCRC31494	5.27	40, 4.0	(58)
A. niger BKMF-1305	38.8	50, 4.0	(22)
A. carbonarius KLU-93	15.4	50, 4.0	(22)
A. phoenicis QM329	27.3	50, 4.0	(22)

kinetics with an additional pNPG binding to the substrate-enzyme complex hindering release of product, or if transglycosylation occurs with pNPG playing the role of the nucleophile competing with a water molecule in breaking the enzyme-product complex, is not known. BGL1 had a K_M value of cellobiose comparable with other reported values for *Aspergillus* beta-glucosidases, with K_M values of 2-3 mM for *A. phoenicis*, *A. niger*, and *A. carbonarius* beta-glucosidases (22), 1 mM for *A. japonicus* (31), and a general literature search by Jäger *et al.*, 2001, showing K_M varying from 1.5-5.6 mM for *A. niger* (22). Meanwhile, the specific activity, V_{max}, of *A. saccharolyticus* BGL1 was significantly higher than values reported for other purified *Aspergillus* beta-glucosidases, with cellobiose as substrate in hydrolysis (Table 1) (22, 42, 57).

Hydrolysis of cellodextrins was facilitated by BGL1, as has also been found with *A. niger* beta-glucosidase, that similarly in exo-fashion removes one glucose unit at the time from the end of cellodextrins, so that products released are subsequently used as substrates to be shortened by another glucose (47).

Acidic pH being best suited for beta-glucosidase activity was also found for beta-glucosidases from *A. oryzae*, *A. phoenicis*, *A. carbonarius*, *A. aculeatus*, *A. foetidus*, *A. japonicus*, *A. niger*, and *A. tubingensis* with optima ranging pH 4-5, and close to no activity at alkaline conditions (14, 22, 31, 43). Thermal stability, however, is more difficult to compare as different researches use different incubation conditions, times and temperatures. We found *A. saccharolyticus* BGL1 to be more thermostable compared with Novozym 188 from *A. niger*, as it retained more than 90% activity at 60°C and still had approx. 10 % activity at 67°C after 2 hours of incubation, while Novozym 188 had 75% activity at 60°C but no activity at 67°C after 2 hours of incubation (Research article II, this thesis). After 4 hours of incubation these differences is much more pronounced as our BGL1 still had more than 70 % activity while Novozym 188 drop to 40 % activity at 60°C. Jäger *et al.*, 2001, studied beta-glucosidases from *A. phoenicis*, *A. niger*, *A. carbonarius*, finding them all to be stable at 2 hours incubation at 50°C, while activities of 87%, 64%, and 53%, respectively, remained after 2 hours incubation at 60°C, and total inactivation was observed after 2 hours at 70°C (22). Compared to this, *A. saccharolyticus* BGL1 showed approximately the same stability as *A. phoenicis* expect for the total inactivation at 70°C. Rojaka *et al.*, 2006, similarly find half-life of *A. niger* beta-glucosidase to be 8 hours at 55°C and 4 hours at 60°C (42), which is similar to our results (Research article II, this thesis), whereas Krogh *et al.*, 2010, indicate a half-life for *A. niger* BGL of 24 hours at 60°C. This is six times longer than measured by us and Rojaka *et al.,* 2006. Decker *et al.*, 2000, demonstrates that an *A. japonicus* and *A. tubingensis* beta-glucosidase were remarkably stable, maintaining 85% and 90% activity, respectively, after 20 hours incubation at 60°C (14). However, Korotkova *et al.*, 2009, found *A. japonicus* beta-glucosidase to only retain 57% of its activity after incubation for 1 hour at 50°C (31), contradicting the findings of Dekker *et al*., 2000.

The crude extract of *A. saccharolyticus*, from which BGL1 was identified, has previously been characterized by its beta-glucosidase activity and evaluated against two commercial enzyme preparations (Research article II, this thesis). Comparing the enzyme kinetics, temperature and pH profiles, glucose tolerance, and cellodextrin hydrolysis, a striking similarity was found for the crude extract and the purified BGL1. Substrate inhibition (or transglycosylation activity) with pNPG was found in both cases, while none was observed for cellobiose within the tested concentrations. There was therefore no foundation for the calculation of V_{max} and K_M for pNPG as no tendency of the activity approaching a maximum was seen rather the activity decreased with higher substrate concentrations. Therefore V_{max} and K_M were only calculated for cellobiose where the data correlated well with MM kinetics and a straight Hanes plot could be obtained for determination of the kinetic parameters. Calculated K_M values for cellobiose were similar, 1.9 mM for the purified BGL1 vs. 1.09 mM for the crude extract, while the V_{max} value expectedly increased for the purified BGL1 compared to the crude extract. The pure enzyme was inhibited by glucose to the same extent as the crude extract, the pH and temperature profiles were very similar, and the mode of hydrolysis of cellodextrins was consistent. This all together indicate that BGL1 is the main contributor to the beta-glucosidase activity observed in the crude extract of *A. saccharolyticus*.

The crystal structure of a beta-glucosidase from barley has recently been used as template to construct a homology model of a beta-glucosidase from *Penicillium purpurogenum* where superimposition of the modeled structure on the true structure from barley showed similar orientation and location of the conserved catalytic residues (23). We chose to use the recently resolved crystal structure of a *T. neopolitana* beta-glucosidase to construct a homology model of the *A. saccharolyticus* BGL1 and found that the conserved catalytically important residues show that the enzyme possesses beta-glucosidase activity (Fig. 5A). The deletion of loop X (Fig. 5B), having Ser370 described to have weak H-bonds with glucose in -1 subsite in *T. neapolitana* structure, makes the catalytic pocket wider where this may be important for substrate accessibility as well as to remove the product fast from the enzyme. The insertions and deletions lining the catalytic pocket (Fig. 5B) may play a major role in the dynamics of the enzyme. The motif KHFV, Lys163, His164, Phe165, Val166, in the *T. neapolitana* structure is considered to be important for substrate recognition (40). However, this motif in the *A. saccharolyticus* enzyme is slightly different, KHYI, Lys170, His171, Tyr172 and Ile173.

The homology modeling revealed that the catalytic pocket of *A. saccharolyticus* beta-glucosidase is open compared to those of barley (PDB entry 1LQ2) (21), *Pseudoalteromonas* sp. BB1 (PDB entry 3F94) and *T. neapolitana* (40) indicating possible high activity. The distance between the putative nucleophile (D261) and the acid/base (E490) is approximately 5.8Å displaying the general characteristic of a retaining enzyme.

In conclusion, we have successfully identified and expressed a novel highly efficient beta-glucosidase from the newly discovered species *A. saccharolyticus.* The enzyme has a great potential for use in industrial bioconversion processes due to its high degree of thermostability compared to the commercial beta-glucosidase from *A. niger* (Novozym 188) as well as a high specific activity.

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Patent

PA 2010 7034

Beta-glucosidases and nucleic acids encoding same

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PA 2010 7034 – Beta-glucosidases and nucleic acids encoding same

SUMMARY OF THE INVENTION

The present invention relates to the identification of a novel and improved beta-glucosidase producing strain of the fungus *Aspergillus*, namely *Aspergillus saccharolyticus*, which is efficient in the degradation of lignocellulosic biomasses into glucose for production of biofuels, biochemicals and pharmaceuticals. Several enzymes of the newly identified strain are efficient in degradation of lignocellulosic biomasses. In particular one enzyme has been identified and characterised as having improved beta-glucosidase activity. The identified beta-glucosidase has improved thermal stability, while maintaining its activity at a high level for a prolonged period of time compared to other fungal beta-glucosidases. This makes it a superior choice for degradation of lignocellulosic material.

In one aspect, the present invention relates to An isolated polypeptide comprising

a. an amino acids consisting of the SEQ NO:1,
b. a biologically active sequence variant of SEQ NO:1, wherein the variant has at least 92% sequence identity to said SEQ NO:1, or
c. a biologically active fragment of at least 30 consecutive amino acids of any of a) through b), wherein said fragment is a fragment of SEQ ID NO:1.

In a preferred embodiment the polypeptide is purified from *Aspergillus saccharolyticus*, such as deposit no.: CBS 127449. The polypeptide is capable of degrading or converting lignocellulosic material that may be obtained from various sources. In a preferred embodiment the polypeptide of the present invention is capable of hydrolyzing a beta 1-4 glucose-glucose linkage.

A second aspect of the invention relates to an isolated polynucleotide comprising a nucleic acid or its complementary sequence being selected from the group consisting of

a. a polynucleotide sequence encoding a polypeptide consisting of an amino acid sequence SEQ ID NO:1,
b. a biologicaly active sequence variant of the amino acid sequence, wherein the variant has at least 92% sequence identity to said SEQ ID NO 1, and
c. a biologically active fragment of at least 30 contigous consecutive amino acids of any of a) through b), wherein said fragment is a fragment of SEQ ID NO 1 or
d. SEQ ID NO.: 3 or 4 or
e. a polynucleotide comprising a nucleic acid sequence having at least 70% identity to SEQ ID NO: 3 or 4 or
f. a polynucleotide hybridising to SEQ ID NO.: 3 or 4 and
g. a polynucleotide complementary to any of a) to f).

The polynucleotide may be used for cloning purposes and for production of the polypeptide of the invention. Thus, in a third aspect, the present invention also relates to a recombinant nucleic acid vector comprising a polynucleotide of the invention.

It is appreciated that the polynucleotide and/or the recombinant nucleic acid vector of the present invention may be introduced into host cells. Accordingly, the invention in a fourth aspect pertains to a recombinant host cell comprising a polynucleotide of the present invention and/or a nucleic acid vector of the invention.

A fifth aspect of the invention relates to an isolated microorganism comprising a polypeptide of the invention, a polynucleotide of the invention and/or a recombinant nucleic acid vector of the invention. The isolated microorganism is in one preferred embodiment the newly discovered strain *Aspergillus saccharolyticcus* of the invention or progeny thereof.

A sixth aspect relates to a method of producing a polypeptide as disclosed in the present invention comprising

a. cultivating a microorganism, where said microorganism produces said polypeptide,
b. recovering the polypeptide from said microorganism.

The microorganism may thus comprise a polynucleotide of the invention and/or a recombinant nucleic acid vector of the invention. The microorganism may be any microorganism suitable for the purpose. In a preferred embodiment, wherein microorganism is Aspergillus, and particularly *Aspergillus saccharolyticcus or progeny thereof*.

According to the invention the polypeptide, recombinant host cell and/or microorganism may be used in a composition. Thus, a seventh aspect related to a composition comprising at least one polypeptide of the invention, at least one recombinant host cell of the invention and/or at least one microorganism of the invention.

It is further appreciated that the polypeptide, recombinant nucleic acid vector, recombinant host cell, microorganism and/ or composition of the present invention may be combined with other components. Thus, a further aspect pertains to a kit-of parts comprising at least one polypeptide of the invention, at least one recombinant nucleic acid vector of the invention, at least one recombinant host cell of the invention, at least one isolated microorganism of the invention and/or at least one composition of the invention, and at least one additional component. An additional component is typically enzymes that aid in the degradation or conversion of biomass for example cellulases, endogluconase, cellobiohydrolase, beta-glucosidase, hemicellulase, esterase, laccase, protease and/or peroxidise.

The invention in yet a further aspect relates to a method for degrading or converting a lignocellulosic material, said method comprising a) incubating said lignocellulosic material with at least one polypeptide of the invention, at least one microorganism of the invention, at least one recombinant host of the invention, at least one composition of the invention and/or at least one kit-of parts of the invention and b) recovering the degraded lignocellolosic material.

In a final aspect the invention pertains to a method for a method for fermenting a cellulosic material, said method comprising

a. treating the cellulosic material with at least one polypeptide of the invention, at least one recombinant host cell of the invention, at least one microorganism of the invention, at least one composition of the invention, at least one kit-of parts of the invention, and

b. incubating the treated cellulosic material with one or more fermenting microorganisms.

c. obtaining at least one fermentation product .

PA 2010 7034 – Beta-glucosidases and nucleic acids encoding same

CLAIMS

1. An isolated polypeptide comprising

    a. an amino acids consisting of the SEQ NO:1,
    b. a biologically active sequence variant of SEQ NO:1, wherein the variant has at least 92% sequence identity to said SEQ NO:1, or
    c. a biologically active fragment of at least 30 consecutive amino acids of any of a) through b), wherein said fragment is a fragment of SEQ ID NO:1.

2. The polypeptide according to any of the preceding claims, wherein said polypeptide is purified from Aspergillus saccharolyticus, such as deposit no.: CBS 127449

3. The polypeptide according to any of the preceding claims, wherein said isolated polypeptide is capable of degrading or converting lignocellulosic material.

4. The polypeptide according to any of the preceding claims, wherein said lignocellulosic material is obtained from agricultural residues such as straw, maize stems, corn fibers and husk, forestry waste such as sawdust and/or wood-chips, and/or from energy crops such as willow, yellow poplar and/or switch grass.

5. The polypeptide according to any of the preceding claims, wherein said polypeptide is capable of hydrolyzing a β1-4 glucose-glucose linkage.

6. An isolated polynucleotide comprising a nucleic acid or its complementary sequence being selected from the group consisting of

    a. a polynucleotide sequence encoding a polypeptide consisting of an amino acid sequence SEQ ID NO:1,

    b. a biologicaly active sequence variant of the amino acid sequence, wherein the variant has at least 92% sequence identity to said SEQ ID NO 1, and

    c. a biologically active fragment of at least 30 consecutive amino acids of any of a) through b), wherein said fragment is a fragment of SEQ ID NO 1 or

    d. SEQ ID NO.: 3 or 4 or

    e. a polynucleotide comprising a nucleic acid sequence having at least 70% identity to SEQ ID NO: 3 or 4 or

    f. a polynucleotide hybridising to SEQ ID NO.: 3 or 4 and

    g. a polynucleotide complementary to any of a) to f).

7. The polynucleotide according to claim 6, wherein said polynucleotide is selected from the group consisting of

    a. a polynucleotide encoding an amino acid sequence consisting of SEQ ID NO.: 1 or

    b. a biologically active sequence variant of the amino acid sequence, wherein the variant has at least 92% sequence identity to said SEQ ID NO.: 1 and

    c. a biologically active fragment of at least 30 consecutive amino acid of any of a) trough b), wherein said fragment is a fragment of SEQ ID NO.: 1.

8. A recombinant nucleic acid vector comprising a polynucleotide sequence as defined on any of claims 6 to 7
9. A recombinant host cell comprising a polynucleotide as defined in any of claims 6 to 7 and/or a recombinant nucleic acid vector as defined in claim 8
10. An isolated microorganism comprising a polypeptide as defined in any of claims 1-5, a polynucleotide as defined in any of claims 6 to 7 and/or a recombinant nucleic acid vector as defined in claim 8
11. The microorganism according to claim 10, wherein said microorganism is Aspergillus.
12. The microorganism according to any of claims 10 to 11, wherein said microorganism is deposited in CBS under accession no.: CBS 127449, or progeny thereof.
13. A method of producing a polypeptide as defined in any of claims 1 to 5 comprising
    a. cultivating a microorganism, where said microorganism produces said polypeptide,
    b. recovering the polypeptide from said microorganism.
14. The method according to claim 13, wherein said microorganism comprises a polynucleotide as defined in any of claims 6 to 8 and/or a recombinant nucleic acid vector as defined in claim 8
15. The method according to any of claims 13 to 14, wherein microorganism is Aspergillus.
16. The method according to any of claims 13 to 15, wherein said microorganism is deposited in CBS under accession no.: CBS 127449, or progeny thereof.
17. A composition comprising at least one polypeptide as defined in any of claims 1 to 5, at least one recombinant host cell as defined in claim 9 and/or at least one microorganism as defined in any of claims 10 to 12
18. A kit-of parts comprising at least one polypeptide as defined in any of claims 1 to 5, at least one recombinant nucleic acid vector as defined in claim 8, at least one recombinant host cell as defined in claim 9, at least one isolated microorganism as defined in any of claims 10 to 12 and/or at least one composition as defined in claim 17, and at least one additional component.
19. The kit-of parts according to claim 18, wherein said additional component is selected from the group consisting of cellulases, endogluconase, cellobiohydrolase, beta-glucosidase, hemicellulase, esterase, laccase, protease and peroxidise
20. A method for degrading or converting a lignocellulosic material, said method comprising a) incubating said lignocellulosic material with at least one polypeptide as defined in any of claims 1 to 5, at least one microorganism as defined in any one of claims 10 to 12, at least one recombinant host cell as defined in claim 9, at least one composition as defined in claim 17 and/or at least one kit-of parts as defined in any of claims 18 to 19 and b) recovering the degraded lignocellolosic material.
21. The method according to claim 20, wherein said lignocellulosic material is obtained from straw, maize stems, forestry waste, sawdust and/or wood-chips.
22. The method according to any of claims 20 to 21, said method comprising treating said lignocellulosic material with at least one additional component.
23. The method according to any of claims 20 to 22, wherein said at least one additional component for treating said lignocellulosic material is selected from the group consisting of cellulase, endogluconase, cellobiohydrolase, beta-glucosidase, hemicellulase, esterase, laccase, protease and peroxidise.

24. The method according to any of claims 20 to 23, wherein said lignocellulosic material is at least partly converted or degraded to monosaccharide glucose units.

25. The method according to any of claims 20 to 24, wherein at least 50% of said lignocellulosic material is degraded or converted to monosaccharide glucose units.

26. A method for a method for fermenting a cellulosic material, said method comprising

    a. treating the cellulosic material with at least one polypeptide as defined in any of claims 1 to 5, at least one recombinant host cell as defined in claim 9, at least one microorganism as defined in any of claims 10 to 12, at least one composition as defined in claim 17, at least one kit-of parts as defined in any of claims 18 to 19, and

    b. incubating the treated cellulosic material with one or more fermenting microorganisms.

    c. obtaining at least one fermentation product .

27. The method of claim 26 wherein said at least one fermentation product is at least one alcohol, inorganic acid, organic acid, hydrocarbon, ketone, amino acid, and/or gas

Concluding remarks

Annette Sørensen

CONCLUDING REMARKS

The work presented in the four research papers shows a successful screening event leading to the discovery of a new efficient beta-glucosidase from a novel fungal species. As stated by Hawsworth and Rossman (1997), you can look everywhere, including your own backyard, when searching for undescribed fungi. This, indeed, is true, as the novel fungus *A. saccharolyticus* was found in the bathroom of my own home. The extract from this fungus could in biomass hydrolysis compete with beta-glucosidases from commercial enzyme preparations, Novozym 188 and Cellic CTec, and was superior especially in terms of thermostability. The key beta-glucosidase, BGL1, in the extract of *A. saccharolyticus* was identified and heterologously expressed for purification. The novelty of *A. saccharolyticus* and its beta-glucosidase, BGL1, as well as the surprising elevated thermostability of the enzyme was the foundation for the patent PA 2010 70347. We see the potential of BGL1 as a key enzyme in a biorefinery concept. This can be in an onsite enzyme production strategy, where BGL1 is expressed by *A. saccharolyticus* that has been modified for improved endoglucanase and cellobiohydrolase expression or co-cultured with an efficient producer of these enzymes. The other option could be to heterologously express BGL1 in an organism of choice for production of a complete enzyme cocktail or even be part of a consolidated bioprocess.

One of the most important features concerning BGL, that would be relevant to investigate further, is prolonged thermostability. Hydrolysis of biomass is usually carried out for a duration of several hours or even days. It is therefore important to test the stability of the enzyme at different conditions for longer periods of time. Kinetically, it would be of great value to perform more studies with cellobiose as substrate, especially assays related to product inhibition, where for example prolonged duration of hydrolysis can be used to evaluate performance related to glucose accumulation. Other inhibition studies to test the influence of different compounds typically present in pretreated biomass should as well be performed. Another aspect of kinetics that would be of interest related to the use of BGL for making a sugar platform, is to investigate the degree of transglycosylation activity the enzyme possesses. We are further more very interested in expressing the *bgl* gene in *E. coli* to compare the non-glycosylated protein with the glycosylated one to test how glycosylation affects the function and stability of the enzyme. From the non-glycosylated protein, a crystal of the protein can be made and the three dimensional structure thereby determined. This could give valuable information as to what amino acid residues that could potentially be targeted in engineering for enzyme improvement. Finally, we have in preliminary studies identified additional beta-glucosidase genes in the genome of *A. saccharolyticus*. It could be interesting to clone, express, and purify these to explore and compare their potential as well as study the expression pattern of the genes when *A. saccharolyticus* is grown in different conditions, including media containing different carbon sources ranging from simple saccharides such as cellobiose to complete sources such as lignocellulosic biomass. This will enhance the basic understanding of the function of the different beta-glucosidases biomass degradation and utilization.