Biomass degrading enzymes from Penicillium – cloning and characterization

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Ph.D. Thesis
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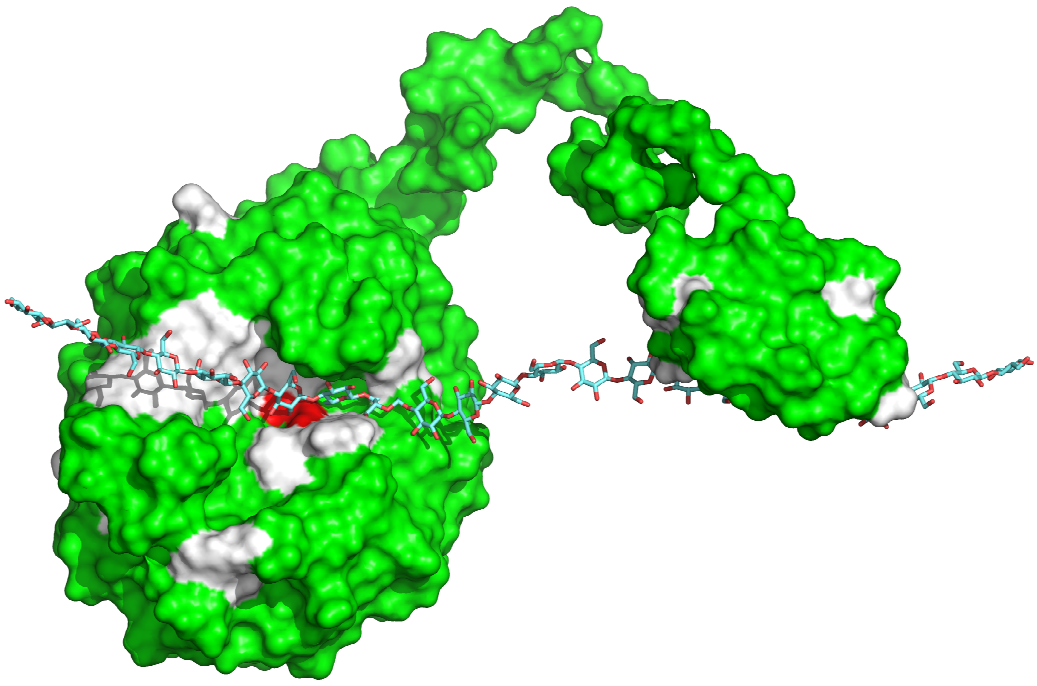

Department of Systems Biology
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Biomass degrading enzymes from *Penicillium* – cloning and characterization

Ph.D. Thesis

Kristian B. R. Mørkeberg Krogh

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We can get fuel from fruit, from the sumac by the roadside, weeds, saw dust. And it remains for someone to find how this fuel can be produced commercially - better fuel at a cheaper price than we now know.

Henry Ford, 1908

The front page picture shows the EG Cel5C from *Penicillium brasilianum* with a cellulose chain in the active site and bound to the CBD. The two active site glutamic acid residues are marked in red, and the aromatic amino residues are marked in white.

The Cel5C core was modelled using *Thermoascus aurantiacus* EGI GH5 (PDB code 1GZJ) as a template. The Cel5C CBD was modelled using the CBD of CBHI from *Trichoderma reesei* (PDB code 1CBH) as a template. Both model were built with the homology model program "Nest" (Petrey *et al*., 2003). Linker and cellulase chain is an artistic impression created with PyMOL (DeLano, 2002). Esben Peter Friis, Novozymes A/S, is acknowledged for great help in the modelling.

Preface

The present work contains results of my Ph.D. study carried out at the Center for Microbial Biotechnology, BioCentrum-DTU, Technical University of Denmark in the period September 2002 to November 2005. The study was financed by a grant from the Technical University of Denmark.

Chapters two through four were updated and expanded from a book contribution entitled "Bioethanol production from lignocellulosic biomass" (Paper H).

Beyond work performed at DTU, I also carried out research at three other locations. In the end of 2003, Professor Folke Tjerneld in the Department of Biochemistry at Lund University invited me to purify cellulolytic enzymes from *Penicillium brasilianum* under the supervision of Ph.D. student Johan Börjesson. I truly enjoyed my stay at Lund from both a scientific and a personal level. During my time at Lund, I learned to apply protein purification theory to real life problems, tools I use every day as Protein chemist at Novozymes A/S

In the spring of 2004, I also had the opportunity to perform research in Davis, California with Novozymes Biotech, and in Bagsværd with the department Fungal Gene Technology, Novozymes A/S. I worked to master molecular biology tools required for my project. This opportunity was possible as a result of an invitation from Joel Cherry. I am very thankful for this training opportunity.

I have learned a lot while working as a Ph.D. student. I am particularly grateful for the guidance I received from my PhD supervisor, Lisbeth Olsson. Lisbeth gave me valuable freedom to pursue my scientific interests. For this and for many fruitful discussions I am indebted to her.

Finally I would like to express my most sincere gratitude to my wife Astrid Mørkeberg Krogh for supporting me throughout my study and my thesis writing. I look forward to continuing to support her as she finishes her thesis.

Following my PhD, I have been fortunate to get a position as research scientist at Novozymes. In my current work, I have continued to the work towards commercial production of ethanol from lignocellulosic biomass.

Kristian B. R. Mørkeberg Krogh

March 2008

Bagsværd, Denmark

List of publications

Papers included in this thesis

A: Krogh, K.B.R., Mørkeberg, A., Frisvad, J.C., and Olsson, L. (2004). Screening genus *Penicillium* for producers of cellulolytic and xylanolytic enzymes. *Applied Biochemistry and Biotechnology*, **113-16**, 389-401.

B: Jørgensen, H., Mørkeberg, A., Krogh, K.B.R., and Olsson, L. (2004) Growth and enzyme production by three *Penicillium* species on monosaccharides. *Journal of Biotechnology*, **109**, 295-299.

C: Jørgensen, H., Mørkeberg, A., Krogh, K.B.R., and Olsson, L. (2005) Production of cellulases and hemicellulases by three *Penicillium* species: effect of substrate and evaluation of cellulose adsorption by capillary electrophoresis. *Enzyme and Microbial Technology*, **36**, 42-48.

D: Krogh, K.B.R.M., Harris, P.V., Olsen, C.L., Johansen, K.S., Hojer-Pedersen, J., Borjesson, J., and Olsson, L. Cloning and characterization of a GH3 β-glucosidase from *Penicillium brasilianum* including a novel method for measurement of glucose inhibition on cellobiose hydrolysis. *Submitted*.

E: Krogh, K.B.R.M., Kastberg, H., Jørgensen, C.I., Berlin, A., Harris, P.V., and Olsson, L. A novel GH5 endoglucanase from genus *Penicillium* and its adsorption to lignin. *Submitted*.

Work not included in this thesis

Patents and Patent applications

F: Patent WO2007019442 A3

Polypeptides having beta-glucosidase activity and polynucleotides encoding same. Krogh, K., and Harris, P.

G: Patent WO2007109441 A2

Polypeptides having endoglucanase activity and polynucleotides encoding same. Harris, P., Krogh, K., Vlasenko, E., and Lassen, S. F.

Other papers and contributions

H: Olsson, L., Jørgensen, H., Krogh, K.B.R., and Roca, C.F.A. (2005) Bioethanol Production from Lignocellulosic Material. *In* Polysaccharides, Structural Diversity and Functional Versatility. 957-993, Marcel Dekker, New York.

I: Olsson, L., Sørensen, H.R., Dam, B.P., Christensen, H., Krogh, K.B.R.M., and Meyer, A.B.S. (2006) Separate and simultaneous enzymatic hydrolysis and fermentation of wheat hemicellulose with recombinant xylose utilizing *Saccharomyces cerevisiae*. *Applied Biochemistry and Biotechnology*, **129-132**, 117-129.

J: Andersen, N., Johansen, K. S., Michelsen, M., Stenby, E.H., Krogh, K.B.R.M., and Olsson, L. (2008) Hydrolysis of cellulose using mono-component enzymes shows synergy during hydrolysis of Phosphoric Acid Swollen Cellulose (PASC) but competition on Avicel. *Enzyme and Microbial Technology*, **42**, 362-370.

Table of contents

Chapter 1

Introduction

Lignocellulosic materials are abundant and renewable, and are presently of particular interest. Developments aim to use lignocellulose as the starting material in many biotechnological processes. Examples of lignocellulosic materials are energy crops, wood residues, agricultural residues and municipal waste. However, the nature of the lignocellulose is complex and not easily accessible (Chapter 3). Cellulose and hemicellulose can be hydrolysed into monosaccharides using cellulolytic and hemicellulolytic enzymes. The major monosaccharide produced by the hydrolysis of lignocellulosic material is glucose. Glucose is the most important carbon source for many microorganisms. However, it is important to use all monosaccharides produced by the hydrolysis of lignocellulose to support a move from petroleum-based to bio-based production of commodities and fine chemicals within the chemical industry. The American Department of Energy published a report in 2004 entitled "Top Value Added Chemicals from Biomass" (Werpy and Petersen, 2004). This report identified several compounds that can be produced from lignocellulosic sugars in biotechnological processes (Table 1.1).

Table 1.1 Top value added chemicals from biomass (Werpy and Petersen, 2004)

1,4 succinic, fumaric and malic acids
2,5 furan dicarboxylic acid
3 hydroxy propionic acid
aspartic acid
glucaric acid
glutamic acid
itaconic acid
levulinic acid
3-hydroxybutyrolactone
glycerol
sorbitol
xylitol/arabinitol

These compounds either have a direct chemical use or they can be used as building blocks for other chemicals (Figure 1.1 presents possible derivatives from succinic acid). In a typical process scheme for producing a given chemical using a petroleum-based process, high temperatures and a catalyst are used, whereas in a bio-based production the temperatures are significantly lower, thus leading to a large reduction in energy consumption. One chemical receiving tremendous attention these days is ethanol. Ethanol can be used as a blend in gasoline or to substitute gasoline completely (Chapter 2). Ethanol is currently being produced from sugar or starch containing crops. But in order to supply the vast amounts of ethanol needed in the transportation sector, lignocellulose must be used as raw material.

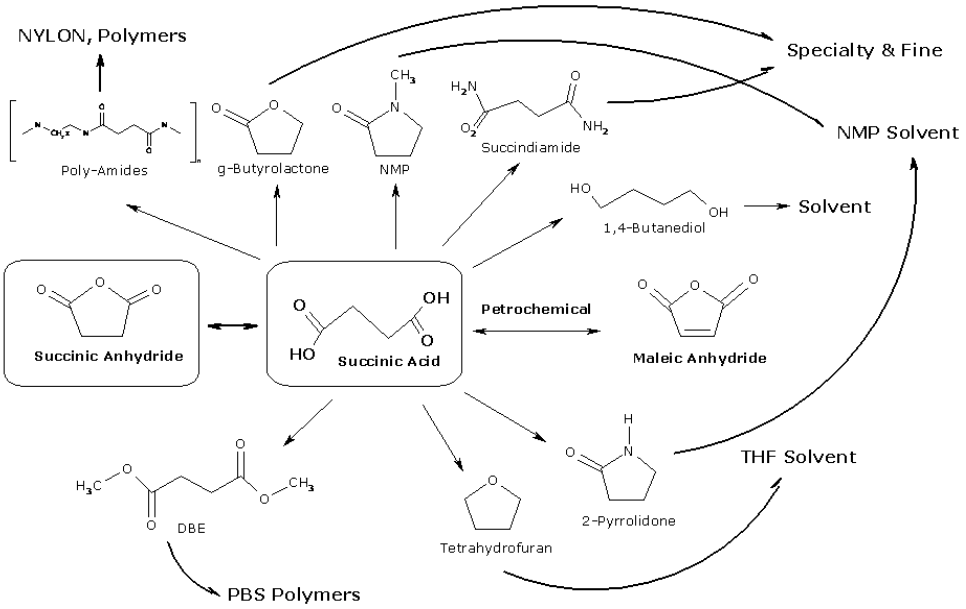


Figure 1.1 Compounds derived from succinic acid. Maleic anhydride is the building block produced in the petrochemical industry today. NMP: N-methyl-pyrrolidone, THF: tetrahydrofuran, DBS: dibasic esters (in figure represented by di-methyl succinate), PBS: polybutylene succinate. .

A driver for the bio-based production of chemicals, especially ethanol, is that oil reserves and production are expected to peak within this decade (Campbell and Laherrère, 1998). The UK Offshore Operators Association's recently published their yearly report for the production of oil and natural gas on the UK Continental Shelf. In 2006 the overall production decreased 9 %, to the lowest level since 1992 (UKOOA, 2007). The most recent numbers from The Danish Energy Agency for Danish oil production in the North Sea demonstrated that the production remained at the same level from 2000 to 2005. Meanwhile, the demand for oil for the transportation sector is rising in Asia, especially India and China, as a result of an increased number of vehicles. In China, there are fewer than 20 cars per 1,000 inhabitants whereas the US

ownership is 800 cars per 1,000 inhabitants (Harks, 2006). A second key driver for the large interest in ethanol is that domestic production of transportation fuel will decrease the dependency of foreign oil. This could lead to less political tension and a more stable economy, especially for the US (Sheehan and Himmel, 1999). President George W. Bush addressed this topic in January 2007 in his 7th State of the Union. He said, "We must continue investing in new methods of producing ethanol — using everything from wood chips, to grasses, to agricultural wastes." A third driver for a renewable transportation fuel is the awareness of global warming. The use of ethanol reduces the emission of the known greenhouse gas carbon dioxide. In February 2007, The Intergovernmental Panel on Climate Change (IPCC) adopted a major assessment of climate science made by more than 600 experts from 40 countries. The report stated that carbon dioxide most negatively impacted the greenhouse gas responsible for global warming (IPPC, 2007). In 2003, the European Union Biofuels Directive set a goal that 5.75 % of the overall transport fuel supply shall come from biofuels by the year 2010.

The production of ethanol from lignocellulosic material requires the release of monomeric sugar units constituting cellulose and hemicellulose. Lignocellulosic plant material is resistant to microbial attack and enzymatic hydrolysis due to a very complex structure (Chapter 3). Therefore, pretreatment of the lignocellulosic material is required in order to change the structure, thus making cellulose more amenable to enzymatic hydrolysis. Today, the prevalent process scheme is first a mechanical degradation to increase the surface area of the material. The hydrolysis of cellulose and hemicelluloses is carried out by a physical and/or chemical pretreatment step to increase the surface area further and followed by enzymatic hydrolysis (Figure 1.2) (Chapter 4). The enzymatic route has three different configurations (i) SHF - separate hydrolysis and fermentation, (ii) SSF - simultaneous saccharification and fermentation, and (iii) CBP - consolidated bioprocessing. A prerequisite process step for SHF and SSF is the production of cellulolytic and hemicellulolytic enzymes (produced either on-site or by industries specializing in this process).

One identified bottleneck in the production of ethanol from lignocellulosic material is the cost of cellulolytic enzymes (Galbe and Zacchi, 2002; Himmel *et al*., 2007). In 2002 the American National Renewable Energy Laboratory (NREL) initiated collaborations with the two global leaders of enzyme production, Novozymes and Genencor. The aim was to reduce enzyme cost significantly over a period of three years (Merino and Cherry, 2007). The most extensively studied microorganism producing cellulolytic enzymes is the filamentous fungus *Trichoderma reesei,* which is also the preferred microorganism for industrial production (Tolan and Foody, 1999).

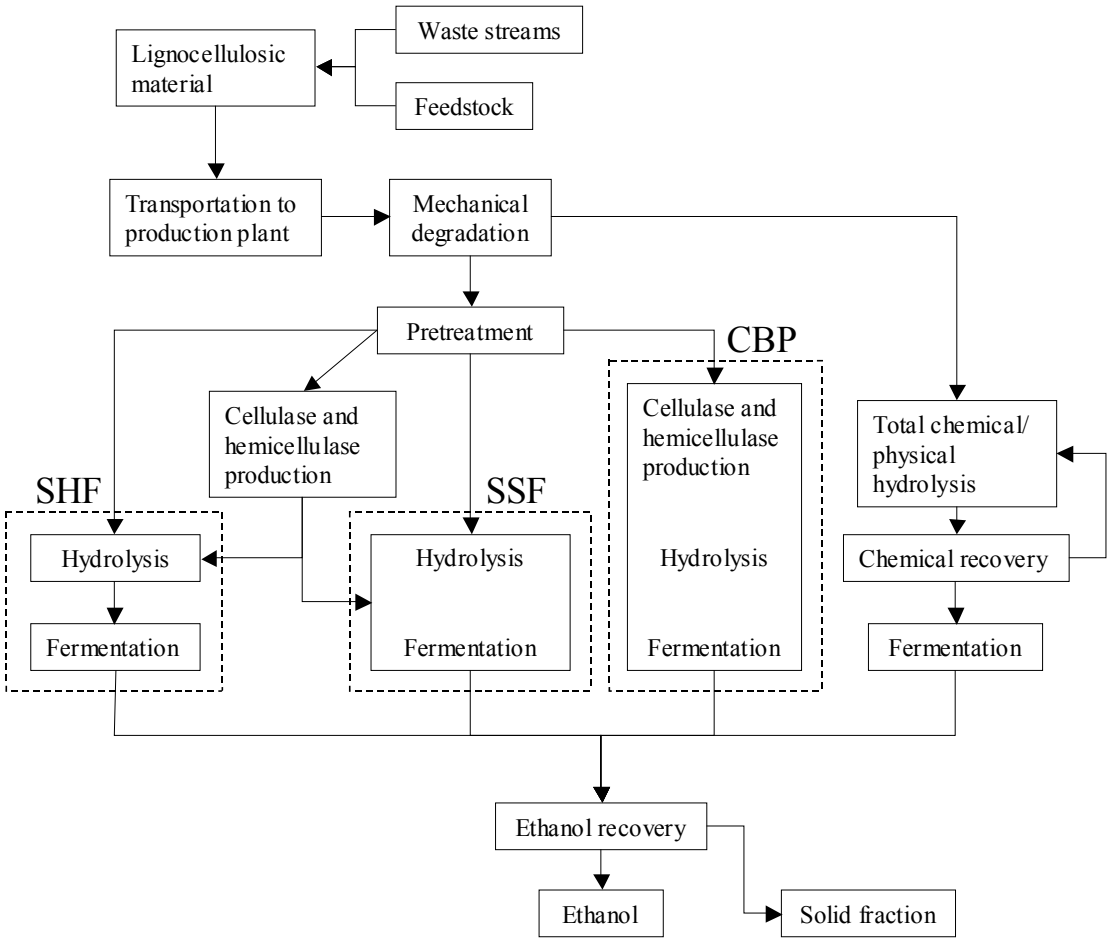


Figure 1.2 Schematic overview of the production of bioethanol from lignocellulosic material. Three possible process schemes are depicted. Separate hydrolysis and fermentation (SHF). Simultaneous saccharification and fermentation (SSF). Consolidated bioprocessing (CBP).

In this thesis, microorganisms from genus *Penicillium* were investigated for their production of cellulolytic and hemicellulolytic enzymes (Paper A) and from this investigation three strains were selected for closer investigation (Paper B). *Penicillium brasilianum* was found to produce a very interesting system of cellulolytic enzymes when cultivated on cellulose. These results led to further investigations of the individual components in the cellulolytic enzyme system. A genomic library was made and screened for genes encoding cellulolytic enzymes. This resulted in a GH3 β-glucosidase cloned and characterised in detail (Paper D). After pretreatment, the cellulose structure remained quite recalcitrant and a hydrolysis time of 48 to 72 hours was necessary. The long reaction time requires stable enzymes, and the β-glucosidase was found to have excellent thermostability (Paper D). The identification of a GH5 endoglucanase gene also resulted in expression, and the produced endoglucanase showed good thermostability (Paper E). In the enzymatic hydrolysis of biomass, another obstacle is the non-productive adsorption of cellulolytic enzymes to the

pretreated lignocellulosic material. The discovered endoglucanase was expressed in *Aspergillus oryzae*, and this expression supplied sufficient amounts of the cloned endoglucanase to study this adsorption phenomenon (Paper E).

Chapter 2

Bioethanol

Ethanol, ethyl alcohol, fuel alcohol are all names used for the product C_2H_5OH. The prefix 'bio' indicates origin from a biological process. Today 95 % of the ethanol produced is bioethanol, whereas the remaining 5 % is synthetically made from crude oil, gas or coal (F.O.Licht, 2006).

2.1 History and interests in ethanol

Around 1900, Henry Ford built his first vehicle, the quadricycle, which used ethanol as fuel. Subsequently, the Model-T was fuelled by ethanol. During the same period, gasoline emerged as the more favourable transportation fuel, and the demand for ethanol decreased. This view was dominant until the price of oil dramatically escalated in 1973, and again in 1979 creating an oil crisis. These oil crises demonstrated the world's dependence on oil, and they were the impetus for intensified research toward alternative energy sources. In recent decades, international awareness of increasing CO_2 concentration levels in the atmosphere and concern for global warming led to the formulation of the Kyoto Protocol in 1997. Under the Kyoto Protocol, many countries made a commitment to decrease their emission levels of greenhouse gases. In 2004, CO_2 concentration in the atmosphere was 377 ppm (Keeling and Whorf, 2004). This was higher than the pre-industrial average concentration of 280 ppm (Neftel *et al*., 1985), and the highest concentration during the last 420,000 years (Petit *et al*., 1999). Combustion of both fossil fuels and bioethanol results in production of CO_2, however, the predominant belief is that net CO_2 release is lower when using bioethanol as opposed to fossil fuels. A relatively small number of scientists believe that the use of bioethanol from lignocellulosic materials results in higher CO_2 release notably T. W. Patzek (Pimentel and Patzek, 2005). Numerous scientists in the field of bioethanol life cycle analysis have argued against several of the parameters and system boundaries used in the calculations of Pimentel and Patzek. The primary reason is that Patzek estimated a large amount of external energy must be supplied, as opposed to all other studies where this value is zero, as it is met by lignin from the lignocellulosic material (Lynd *et al*., 2006). In order to reach the highest possible net reduction in CO_2 release it

is crucial to use the whole plant and not only the starch fraction (Farrell *et al*., 2006; Hammerschlag, 2006).

2.2 Ethanol as transportation fuel

Bioethanol has distinct advantages compared to other alternative fuels; it is a liquid fuel, and it is compatible with current vehicles and blendable with gasoline. In addition, bioethanol can, to a large extent, use the motor fuel distribution infrastructure with only minor modifications. When looking at the mileage, the volumetric energy content of ethanol is approximately two-thirds of the volumetric energy content in gasoline; therefore mileage should be reduced by 33 % using ethanol as fuel instead of gasoline. However, ethanol combustion has a 15 % higher efficiency due to a combination of a high heat of vaporization, a high octane number and a high gas to volume change (Bailey, 1996), thus the mileage efficiency for ethanol is actually reduced by 20-25 % in comparison to gasoline (Wyman, 1996). Flexible Fuel Vehicles, currently on the market, can run on mixtures of ethanol and gasoline, with an ethanol volume content up to 85 % (E85), and all new cars with a catalyst can run on E10 (Galbe and Zacchi, 2002). The blending of ethanol and gasoline decreases the emission of carbon monoxide, volatile organic compounds and hydrocarbons. It should be noted that NO_x emission is increased in blends from pure gasoline up to E50, but with E85 the NO_x emission is decreased by 20 % when compared to pure gasoline (Bailey, 1996; Hsieh *et al*., 2002). The emission of reactive aldehydes, in particular acetaldehyde and formaldehyde, is increased when the blend ratio of ethanol to gasoline increases. As a result of the general decrease in emission of pollutants using a oxygenated additives, the US made the 1990 Clean Air Act Amendment(s) which mandated that oxygenated additives (methyl tertiary butyl ether, MTBE, Ethyl tertiary butyl ether, ETBE or ethanol) should be added to at least a level of 2 % by weight of oxygen in order to decrease urban pollution in areas with superfluous levels of carbon monoxide (Wheals *et al*., 1999). Critical amounts of MTBE found in ground waters have later led to reluctant use of MTBE in gasoline as it can be toxic to humans, and a number of states in the US have banned the use of MTBE. Ethanol has already been used as transportation fuel for quite some time, and in Brazil alcohol programs during the last three decades have resulted in that almost all gasoline sold is oxygenated as E22 (Wyman, 1996). In Denmark, Statoil introduced Bio95 in May 2006, which is E5.

2.3 Production of bioethanol from sugar and starch crops

Currently, the feedstock for bioethanol production is sugar crops (cane and beet) and starch crops (grains, primarily maize). One difference between starch crops and sugar crops is that the molasses from the sugar crops can be fermented directly. Starch is a

polysaccharide consisting of glucose units connected through α-glucosidic bonds. The structure of starch is crystalline, as found in cellulose, but starch molecules can be solubilised via heat treatment, whereas cellulose cannot. The solubilization makes the starch easily accessible for the α-amylase and amyloglucosidase that release the fermentable glucose (F.O.Licht, 2006). The two major producers in world today are Brazil and the USA. They produce more than two thirds of the total amount of ethanol produced in the world, 49.8 billion liters (table 2.1). In comparison, the American oil consumption for motor gasoline was 520 billion liters in 2005 (US Energy Information Administration, 2006). The use of maize for production of bioethanol has raised issues concerning food or fuel bioethanol. In the beginning of 2007, the "tortilla crisis" in Mexico made headlines in the NY Times on February 1st 2007 ("Thousands in Mexico City Protest Rising Food Prices"). The article claimed that rapidly growing ethanol production in the US had dramatically increased the demand for maize, therefore increasing the price on maize and maize derived products. This had significant effects for poor people in Mexico, since maize tortillas are central to the diet of the poor in Mexico. In reality, a fraction of the drastic price increase due to increased demand for the production of bioethanol. The major reason was a combination of increasing price on energy (including energy used to produce tortillas), a sub par maize harvest in South America and speculation.

Table 2.1 Top10 bioethanol producers in the world in 2006 (F.O.Licht, 2006)

Country	Bioethanol produced, billion liters
U.S.A.	19.2
Brazil	16.7
China	3.90
India	2.00
France	0.95
Russia	0.77
Germany	0.55
Spain	0.48
Thailand	0.44
South Africa	0.41

2.4 Production of bioethanol from lignocellulose

Large potential for bioethanol production lies within the possibility of including lignocellulose as a substrate, *e.g.* in wheat 35 % of the harvested plant is straw and 45 % is grain (Claasen *et al.*, 1999). However, there are several hurdles to pass. The actual knowledge of large-scale production of bioethanol is limited and based on assumption since no commercial cellulose-to-ethanol plant exists. In cost projections, calculations are done on the basis of a production plant on an order of magnitude higher capacity than the existing demonstration plants. The optimum production plant size would range from 1600 to 5000 tonnes per day (Galbe and Zacchi, 2002; Wooley *et al.*, 1999b;

Wyman, 2002). The optimum size will be dependent on several parameters including the biomass availability and quality, transportation costs, labour costs, capital costs, etc. No commercial lignocelluloses-to-ethanol commercial plants exist. Pilot and demonstration plants have been built and several are under construction. Knowledge from these demonstration and pilot plants is of crucial importance in the design of a large-scale process.

The Canadian company Iogen Corp has a demonstration plant with a capacity of 40 tonnes of biomass per day. The substrate is wheat, oat, and barley straw. The plant can produce 3 million liters of ethanol per year (Foody, 2006). In 2005, Abengoa Bioenergy started to build a demonstration plant in Babilafuente (Salamanca), Spain. By the end of 2007, the plant will process 70 tonnes of agricultural residues, such as wheat straw, each day and produce over 5 million liters of fuel grade ethanol per year. Since 2004, the Swedish company SEKAB has operated a pilot plant in Örnsköldsvik with a daily capacity of 2 tonnes (dry weight) of wood chippings. The annual production could be up to 150.000 liters of bioethanol. In Denmark, two independent pilot plants are operating. The IBUS – Integrated Biomass Utilisation System (which has a capacity of 25 tonnes of wheat straw per day) and BioGasol, a newly started pilot plant operating at The Technical University of Denmark. BioGasol has planned to build a demonstration plant with a daily capacity of 110 tonnes of biomass (dry weight) and an annual production of 10 million liters bioethanol. The Chinese company China National Cereals, Oils and Foodstuffs Corp (COFCO) has recently began to build a pilot plant converting cellulose to ethanol. In some of the pilot and demonstration plants, the cellulose-to-ethanol process is in connection with existing starch-to-ethanol plants. The general consensus is that there will be commercial lignocellulose-to-ethanol plants up and running by 2012. The US Department of Energy, DOE, took a major step in the development of commercial –scale cellulosic ethanol plants. The DOE set aside 385 Million US $ for six plants to be built with completion dates ranging from 2009 to 2011 (Service, 2007). In March 2007, the Dutch company Nedalco announced plans to build a full-scale bioethanol plant for the production of ethanol from lignocellulose with an annual capacity of 200 million litres.

Chapter 3

Lignocellulose

Lignocellulose is a generic term describing the main constituents in most plants, namely cellulose, hemicellulose and lignin. Cellulose is a glucose polysaccharide, hemicelluloses are polysaccharides with a backbone of different hexoses and pentoses, and lignin is a complex network of different phenyl propane units. In general, cellulose gives rigidity, but to confer the rigidity, *e.g.* to a stem, there is a need for other materials that can stick and glue the polysaccharides together. The stickiness is caused by hemicellulose and the glue is lignin.

Starch can easily be converted into bioethanol, and if no American corn crops were used for food purposes, the yearly bioethanol production would be 45 billion liters, approximately 10 % of the American 2005 annual gasoline consumption (Service, 2007). This highlights the necessity of an additional starting material if bioethanol shall replace gasoline. Lignocellulosic material is a cheap and abundant raw material that also can be used for bioethanol production. During photosynthesis, the sun powers the transfer of electrons to atmospheric CO_2 and it is converted into carbohydrates. The energy and carbon contained in the carbohydrates can then be used as raw material for production of alternative energy sources. The annual production of lignocellulose in the world is around 130 billion tonnes (Lutzen *et al.*, 1983). In comparison, the proved oil reserves are 143 billion tonnes (BP statistical review of world energy, June 2002). There are, however, many obstacles to the amount of lignocellulosic material available, such as accessibility, yield per area, degradability, cropping practice or local technology of harvesting (Kuhad and Singh, 1993).

3.1 Cellulose

The most prevalent component in lignocellulose is cellulose. The rigidity of cellulose is a direct consequence of its structure. The cellulose chain is a linear polymer of glucose monomers linked by β-1,4-glucosidic bonds. Every second residue is rotated 180 ° around the longitudinal direction, and the repeating unit is cellobiose. The linearity of the cellulose chain is stabilised through intramolecular hydrogen bonds (Figure 3.1) (Gardner and Blackwell, 1974). The cellulose chain consists of about 10000 D-glucose

molecules (Sjöström, 1993a), and these chains assemble into microfibrils. Each chain participates in the microfibril, just like a small thread would participate in a rope. Typically, 36 cellulose chains with the same direction, but with different starting and ending points, assemble into one microfibril with a diameter of 5-15 nm. The microfibril is greatly stabilised through intermolecular hydrogen bonds (Gardner and Blackwell, 1974). These intra- and intermolecular interactions in a microfibril make cellulose very resistant to degradation. Even though cellulose is a homopolysaccharide, the structure is heterogeneous. In areas with less order, the intermolecular bonds are weaker and the cellulose structure is more amorphous. In some microfibrils, the outer cellulose chains are more amorphous than the highly structured core of the microfibril, and the crystallinity of cellulose is a sort of macroscopic measure for the sum of the microscopic crystalline and amorphous regions (Sjöström, 1993b). Filter paper is considered to be highly crystalline cellulose and IUPAC has recommended the hydrolysis of filter paper as a standard measurement for total cellulolytic activity (Ghose, 1984).

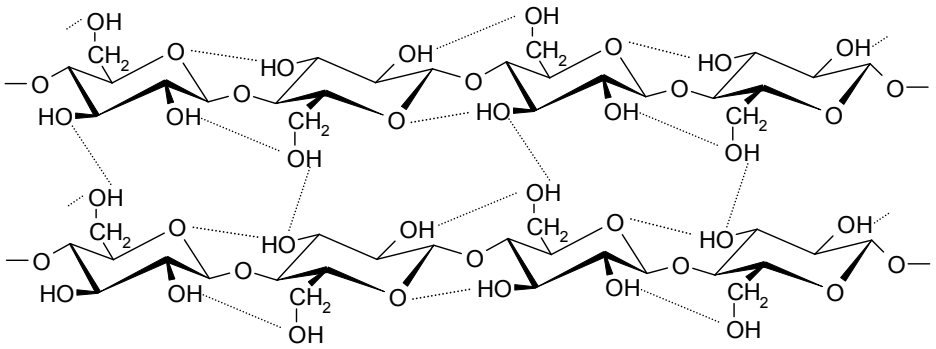


Figure 3.1 Intra- and intermolecular hydrogen bonds in cellulose.

3.2 Hemicellulose

Hemicellulose is one of the components in plant cell wall material that interlocks the cellulose microfibrils. Hemicellulose refers to a wide variety of heterogeneous short and branched polysaccharides. In comparison to cellulose, hemicellulose is a far more complex polysaccharide. Where cellulose is a single chain made of glucose monomers, hemicellulose can be a branched chain polysaccharide of both different hexoses and pentoses. The hexose monomers are mainly D-glucose, D-mannose, D-galactose, and the pentose monomers are mainly D-xylose and L-arabinose. Another monomeric component is uronic acids (Sjöström, 1993b). There are different types of hemicellulose, defined in accordance with the backbone composition and the side chains, *e.g.* arabinoxylan has a backbone of xylose residue with arabinose residues as side chains. Most often the name of the specific hemicellulose type describes both the composition and the structure; the last part of the name refers to the backbone composition and the

first part refers to the substituents. For a more detailed description of hardwood and softwood hemicelluloses (Sjöström, 1993b) and for grass hemicelluloses (Carpita, 1996; Carpita and Gibeaut, 1993). The extent of backbone substitution varies, and in regions with low substitution, there is no steric hindrance for interactions between different chains. The low substituted backbone can be bound by hydrogen bonds to either another low substituted hemicellulosic backbone or to a cellulose chain (Carpita, 1983).

In hardwood (angiosperm tree) *e.g.* birch, beech, poplar, aspen or oak, the predominant hemicellulose type has a backbone of D-xylopyranose residues, linked with β-1-4 bonds. This hemicellulose type is named xylan based on the high content of xylose in the backbone. The xylan from birch wood and beech wood is referred to as glucuronoxylan, because some of the xylose molecules have an α-(1-2) linked 4-*O*-methylglucuronic acid substituent. The average degree of polymerisation is 110, and every tenth xylose residue carries an α-(1-2) linked 4-*O*-methylglucuronic acid. The xylan is further acetylated on C-2 and/or C-3 with an average of two acetyl groups per five xylose residues (Sjöström, 1993b).

In softwood (gymnosperm tree) *e.g.* spruce, pine or fir, the dominant hemicellulose fraction is glucomannan. In glucomannan, the backbone consists of glucose and mannose in the ratio of one glucose residue per three to four mannose residues. The glucomannan backbone is acetylated with an average of one acetyl group per three to four backbone residues. Furthermore, the backbone is also substituted with single galactose units through α-1-6 bonds. In the backbone, the ratio of galactose side chains to glucose falls in two general groups, either one galactose residue per ten glucose residues or one galactose residue per glucose residue (Sjöström, 1993b). A secondary hemicellulose fraction in softwood is arabinoglucuronoxylan, which has a xylan backbone like the glucuronoxylan fraction from hardwood, but with an α-(1-2) linked 4-*O*-methylglucuronic acid substituent for every five xylose residues and two α-(1-3)-L-arabinofuranose substituents for every 15 xylose residues (Sjöström, 1993b). The arabinoglucuronoxylan fraction in softwood is not acetylated as it is in hardwood (Fengel and Wegener, 1989).

In the grass family (poaceae), *e.g.* rice, wheat, oat or switchgrass, the backbone structure of the hemicellulose is mainly glucuronoarabinoxylan as in hardwood (Carpita, 1996). The xylan backbone is substituted with glucuronic acid by an α-(1-2) bond, but in contrast to the glucuronic acid in hardwood, the glucuronic acid is not methylated. A more frequent substituent is an α-L-arabinofuranose attached through an α-1-2 or α-1-3 bond. The xylose can be either mono- or di-substituted (Ordaz-Ortiz and Saulnier, 2005). The extent of backbone substitution varies from 0.1 to almost one branching per xylose residue. Cinnamic acids are another group of substituents, and they can bind to side chain units through ester bonds (Fry, 1982). These cinnamic acids,

primarily ferulic acid, can covalently bind to each other and thereby cross-link different backbones, or they can act as attachment sites for lignin (Iiyama *et al*., 1994).

3.3 Lignin

The word lignin originates from the Latin lignum which means tree, and lignin is the chemical substance conferring rigidity and recalcitrance to microbial attack not only in trees but also in plants (for a more detailed description of lignin chemistry (Higuchi, 1997; Sjöström, 1993a)). In lignocellulosic material, lignin is the most abundant non-polysaccharide and it is a high molecular mass material composed of phenylpropene units. An interesting difference between the synthesis of cellulose and hemicellulose and that of the lignin polymer is that lignin is synthesised by radical coupling of randomly organised monomeric units. In contrast, cellulose and hemicellulose are synthesised enzymatically in a structured manner (Nimz, 1974). The three units, which comprise the lignin complex network, are guaiacyl (from the precursor coniferyl alcohol), syringyl (from the precursor sinapyl alcohol) and *p*-hydroxyphenyl (from the precursor *p*-coumaryl alcohol) (Figure 3.2).

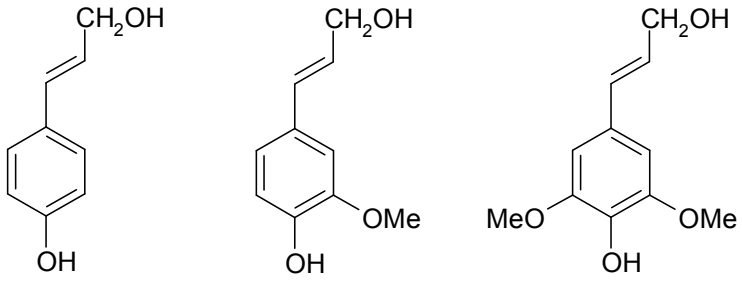


Figure 3.2 Lignin monomeric building blocks. From left to right *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol.

These monomers are joined by carbon-carbon or ether bonds (Figure 3.3). The most frequent linkage is an arylglyceryl-β-aryl ether linkage, constituting more than half of the lignin bonds in both hardwood and softwood (Adler, 1977). The relative amounts of the three precursors differ significantly in softwood, hardwood and grasses. Softwood contains mainly guaiacyl units. For example, there are 94 % guaiacyl units in spruce (Ericksson *et al*., 1973) and 86 % guaiacyl units in pine (Glasser and Glasser, 1981). In hardwood lignin, syringyl units constitute up to 45 % of the lignin (Wayman and Parekh, 1990). The syringyl unit has two methoxy groups on the phenyl ring, whereas guaiacyl has one that confers different chemical properties and reactivity. One theory is that guaiacyl lignin keeps the lignin-cellulose more densely packed than syringyl lignin. Hardwood lignin with a higher syringyl content has been found easier to extract by alkaline extraction as compared to softwood lignin (Ramos *et al*., 1992). The more densely packed guaiacyl lignin-cellulose complex in softwood restricts

cellulose hydrolysis. Vessel elements in hardwood have a higher content of guaiacyl units as compared to other cells found in hardwood (Musha and Goring, 1975), and after extensive hydrolysis these vessel elements found in the remaining unhydrolysed material (Ramos *et al.*, 1992).

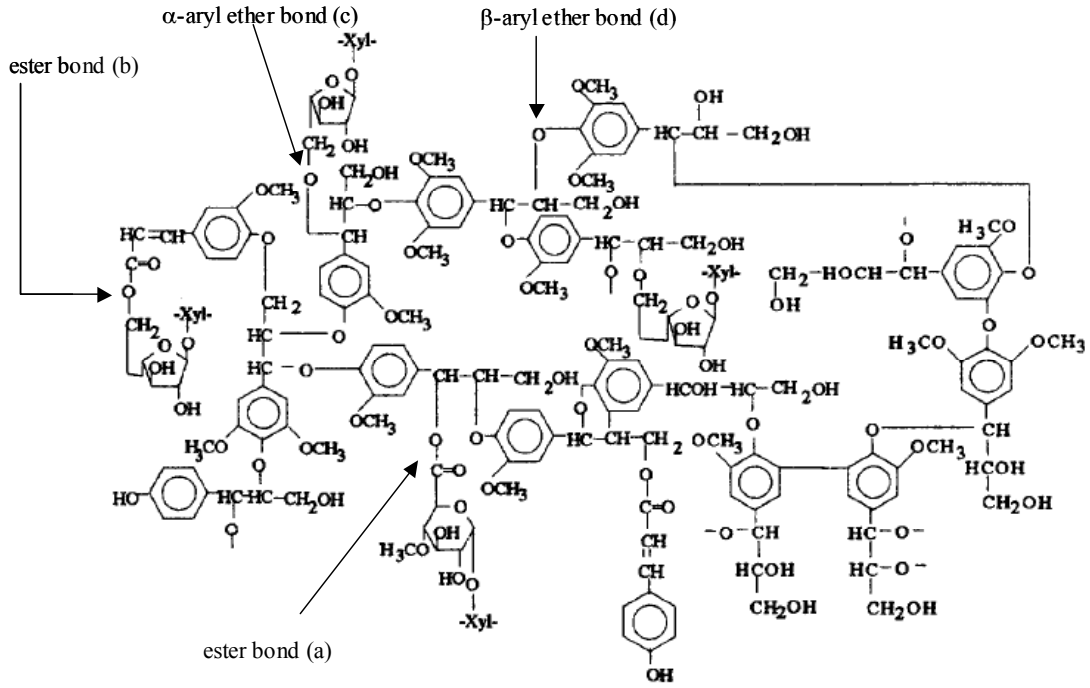


Figure 3.3 A tentative structure of wheat straw lignin with attachments points for lignin on the hemicellulose (modified (Sun *et al.*, 1997)). a) ester bond between xylan α-1 linked 4-*O*-methylglucuronic acid and a guaiacyl unit; b) ester bond between ferulic acid and 5-*O*-arabinofuranoside α-1 linked to a xylan; c) α-aryl ether bond between a guaiacyl unit and 5-*O*-arabinofuranoside α-1 linked to a xylan; and d) arylglyceryl-β-aryl ether bond between two syringyl units.

One of the reasons for structural enforcement of lignocellulosic material by lignin is the covalent bond to the hemicellulose fraction that creates a cross-linked and branched network of lignin and hemicellulose. The covalent binding between lignin and hemicellulose (Figure 3.3) can be an ester bond to a uronic acid substituent or to a hydroxycinnamic acid substituent in xylan, or a more stable ether bond to an α-L-arabinofuranose in arabinoxylan or to a galactose in glucomannan (Iiyama *et al.*, 1994; Sjöström, 1993a; Sun *et al.*, 1997). Computational calculations demonstrate reason to believe that lignin is intertwined with both single cellulose chains and microfibrils. This lignin protection gives rise to chemical resistance and attachment points for hemicellulose (Shevchenko and Bailey, 1996).

3.4 Composition of lignocellulosic material

Plants have two types of cell walls that differ in function, composition, and in primary and secondary cell walls. Primary cell walls surround growing and dividing plant cells. These walls provide mechanical strength but must also expand to allow the cell to grow and divide. In the primary cell wall the cellulose microfibrils are loosely ordered and they are interwoven with hemicellulose and pectin (Figure 3.4.A). The much thicker and stronger secondary wall (Figure 3.4.B), which accounts for most of the carbohydrate in biomass, is deposited once the cell has ceased to grow. The secondary walls are further strengthened by the incorporation of lignin. The cellulose microfibrils in the secondary cell walls are wound around the plant cell in a neatly organized matrix of hemicellulose and lignin with different orientations in each layer (Figure 3.4.C).

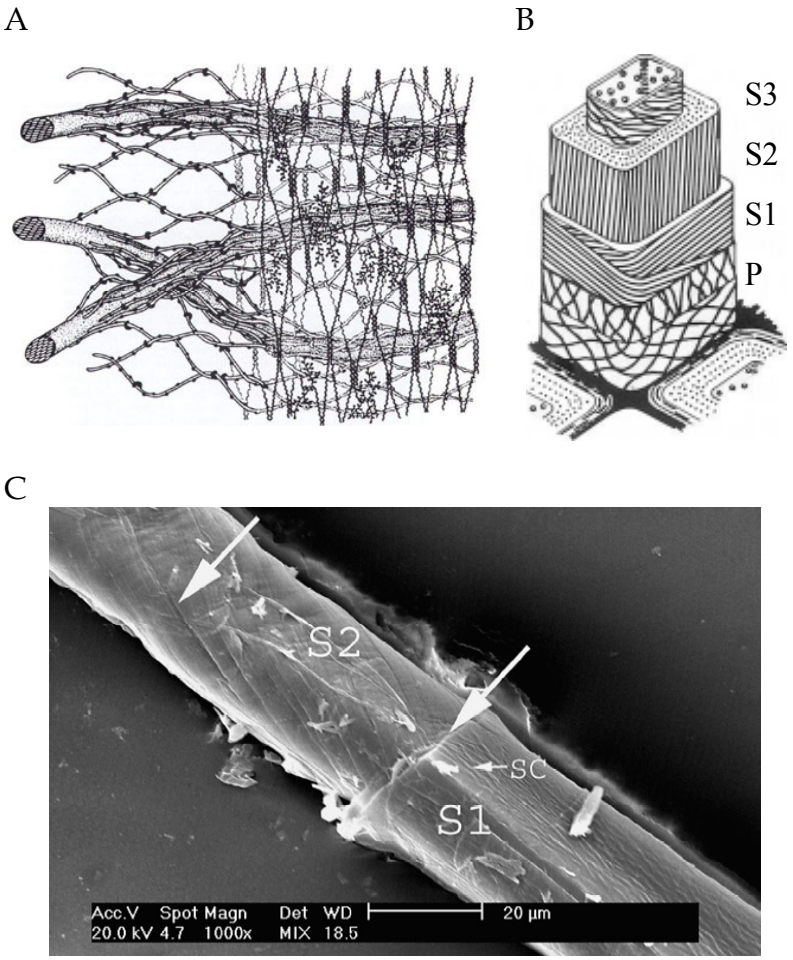


Figure 3.4 Plant cell walls. A) Primary cell wall, three horizontal cellulose microfibrils in a matrix of hemicellulose and vertical pectin (Carpita and Gibeaut, 1993). B) Wood cell structure with primary cell wall (P) and secondary cell wall (S1-S3) of a softwood tracheid (Fengel and Wegener, 1989). C) SEM micrograph of a softwood tracheid. S1 and S2 are different layers of the secondary cell wall (Upper left arrow indicates a crack in S2 layer, lower right arrow indicates a fracture between S1 and S2, SC is surface corrugations) (Brändström, 2004)

The overall composition of lignocellulose in plant material depends on the plant type and the growth conditions (Bjerre *et al*., 1996; Goel *et al*., 1996). The average composition of lignocellulose (w/w) is 40-45 % cellulose, 20-30 % hemicellulose and 15-25 % lignin (table 3.1). In general, wood has a higher content of cellulose and lignin, which favours rigidity. Straw, which is more flexible, has higher hemicellulose content. The cellulose content of softwood and hardwood is similar, but softwood contains more lignin and less hemicellulose (Szmant, 1986). The hemicellulose composition between softwood and hardwood is also different (Table 3.1).

Table 3.1 Percent dry weight (% w/dw) composition of lignocellulose from plants.

Feedstock	Cellulose	Hemicellulose	Main fraction	Lignin	Reference
Hardwood[a]	41	28	glucuronoxylan	22	(Sjöström, 1993b)
Softwood[b]	40	17	glucomannan	28	(Sjöström, 1993b)
Wheat straw	38	33	arabinoxylan	9	(Ahring *et al*., 1996)
Rice straw	32	24	arabinoxylan	13	(Linko, 2002)
Switchgrass	43	36	arabinoxylan	22	(Goel *et al*., 1996)

[a] birch, [b] spruce

The distribution between cellulose, hemicellulose and lignin influences the relative amount of the sugars present in lignocelluloses (Table 3.1). The ethanol yield and productivity in the production of bioethanol is highly dependent on the sugars for fermentation. The main sugar is glucose mainly coming from cellulose (Table 3.2). In softwood, the hemicellulose fraction in lignocellulose is mainly comprised of glucomannan, why the second most abundant sugar is mannose in this material and not xylose as in hardwood and grasses (Table 3.2). Different monomeric composition will impact pretreatment steps, enzymatic hydrolysis, and the fermentation (Figure 1.2). By knowing the specific sugar composition, it is also possible to estimate the structure of the hemicelluloses present in a given plant material. This can be used to help predict and determine the optimal pretreatment conditions. Because hemicelluloses present after pretreatment significantly reduce cellulose hydrolysis (Öhgren *et al*., 2007), this knowledge can also help determine hemicellulolytic enzymes required to break down hemicelluloses during enzymatic hydrolysis. The sugar composition of the lignocellulosic material is also relevant when the hydrolysed material has to be fermented.

Table 3.2 Monomeric composition (% w/dw) of lignocellulose samples from plants.

Feedstock	Glu	Xyl	Gal	Ara	Man	Reference
Hardwood						
Beech	42.9	20.8	-	1.5	0.9	(Wiselogel *et al*., 1996)
Birch	38.2	18.5	-	-	1.2	(Hayn *et al*., 1993)
Poplar	49.9	17.4	1.2	1.8	4.7	(Wiselogel *et al*., 1996)
Willow	36.8	12.7	-	1.2	1.9	(Eklund, 1994)
Softwood						
Pine	46.4	8.8	-	2.4	11.7	(Wiselogel *et al*., 1996)
Spruce	43.4	4.9	-	1.1	12.0	(Tengborg *et al*., 1998)
Grasses						
Corn stover	35.6	18.9	-	2.9	0.3	(Hayn *et al*., 1993)
Rice straw	34.2	24.5	-	-	-	(Wiselogel *et al*., 1996)
Switchgrass	31.0	20.4	0.9	2.8	0.3	(Wiselogel *et al*., 1996)
Wheat straw	38.2	20.4	0.9	2.8	0.3	(Wiselogel *et al*., 1996)

- below detection limit.

3.5 Changing the lignocellulose composition

During the last two decades, extensive efforts have focused on optimising the productivity and composition of different feedstocks for bioethanol production. Today, the belief that lignocellulosic material can be used as a carbon source for ethanol production has increased and there is great interest in optimising the composition of the plants to produce efficient feedstocks. *Populus* clones are one type of plant that has shown promise to become a feedstock. Many experiments conducted with different *Populus* clones have resulted in hybrids with high productivity. In addition, there is new knowledge about how growth conditions influence the final composition of the plant material (Debell *et al*., 1997; Sarath *et al*., 2008). Through these experiments, the output per field of *Populus* has been increased. Moreover, the proper growth conditions can help to make the lignocellulosic material more amendable to pretreatment and enzymatic hydrolysis for the production of bioethanol. It is anticipated that new information arising from a US Department of Energy (DOE) led full genome sequencing project of a *Populus* species (Wullschleger *et al*., 2002) will also provide stimulus for using *Populus* species as a feedstock for bioethanol fermentation. CAFI (Biomass Refining Consortium for Applied Fundamentals and Innovation) is collaboration between 5 American Universities and NREL. At present, they are conducting a large study using the leading pretreatment technologies on the same *Populus* batch.

The chemical composition of lignocellulose can be changed in order to ease the degradation and to improve the ethanol yield and productivity (Coleman *et al*., 2008).

A selection of different hybrid poplar clones has resulted in a clone with a significantly higher cellulose and lower lignin content (Wiselogel *et al*., 1996). The relative amount of each lignin monomer in a tobacco plant has been modified resulting in a significant increase in cellulose hydrolysis (Vailhé *et al*., 1996). Furthermore, transgenic aspen trees (genus *Populus*) with a substantial decrease in lignin content have produced and characterised (Hu *et al*., 1999). The lower lignin content was compensated by an increase in cellulose content so that the total cellulose-lignin mass remained the same.

Another potential feedstock for use in bioethanol production is switchgrass. In 1992, the DOE initiated a program (at Oak Ridge National Laboratory) to use switchgrass in a biofuel feedstock development program. In breeding experiments, researchers identified the possibility of combining important biofuel traits such as high yield, high lignocellulose content and low ash and mineral content into one ideal biofuel (Lemus *et al*., 2002). The choice of variety, specific location, and the cultural practice (harvest frequency, fertilization and plant spacing) have proved to be important parameters for the annual production of lignocellulosic material from switchgrass (Hopkins *et al*., 1995a; Hopkins *et al*., 1995b; Sanderson *et al*., 1996). An overview of switchgrass as a bioenergy crop has been made (McLaughlin *et al*., 1999). Almost all growth experiments with switchgrass have been conducted in the US; however, some growth experiments have demonstrated that switchgrass can be cultivated successfully in parts of Europe as well (Christian *et al*., 2002).

Chapter 4

Pretreatment of lignocellulose for enzymatic hydrolysis

Lignocellulosic plant material is very resistant to microbial attack and enzymatic hydrolysis due to a very complex and strong structure as described in chapter three. Pretreatment of the lignocellulosic material is therefore required to change the structure to make cellulose more accessible to enzymatic hydrolysis. The first step in pretreatment is a mechanical step to reduce the size and thereby increase the surface area of the lignocellulose. The second pretreatment step is a physical and/or chemical step. There have been numerous attempts to pretreat lignocellulose using various chemicals and temperatures. The objectives of the pretreatment are to increase the enzymatic accessibility of the cellulose and the hemicellulose. One must balance multiple parameters to operate at the right conditions. If the conditions are too harsh, the sugars can be degraded to by-products that inhibit subsequent fermentation steps, such as furfural and hydroxymethyl furfural (Sun and Cheng, 2002). Pretreatment is a costly step in the production of ethanol from lignocellulose and the process equipment for the pretreatment must also be simple, robust, and cheap (Wooley *et al*., 1999a). The cost of lignocellulosic biomass has been estimated to constitute 25-40 % of the total production cost of bioethanol (Gregg *et al*., 1998; Kadam *et al*., 2000; von Sivers and Zacchi, 1995). It is therefore important to recover as many sugars from cellulose and hemicellulose as possible in the pretreatment step.

Below, pretreatment methods specifically used for pretreating substrates in this Ph.D. study will be presented. Other realistic methods to be applied in large-scale operation will also be presented. The methods described are dilute acid hydrolysis, steam explosion, ammonia fiber explosion (AFEX), wet oxidation, and organosolv. For a more comprehensive review of pretreatment methods, see (Galbe and Zacchi, 2007; Sun and Cheng, 2002).

4.1 Dilute acid hydrolysis

Dilute acid with no enzymes added has been used for the complete hydrolysis of lignocellulosic material on a commercial scale since the beginning of the 1930s (the Scholler or Madison percolation process (Jones and Semrau, 1984; Parisi, 1989)). The most generally used acid is H_2SO_4 due to lower price and fewer problems with corrosion compared to *e.g.* HCl. However, the glucose yield seldom exceeds 55-60 % of the theoretical yield using this method (Kadam *et al*., 2000; Lee *et al*., 1999; Parisi, 1989). Furthermore, at elevated temperatures, pentoses from the hemicellulose, and to a lesser extent the hexoses, are rapidly degraded to by-products. Degradation by-products are unwanted because they can inhibit the subsequent fermentation steps and they lower yield. Therefore the dilute acid hydrolysis has often been divided into a two-step process. In the first step, mainly the hemicellulose is hydrolysed at less severe conditions with temperatures around 170-190 °C and acid concentrations around 0.5 to 1.2 % (w/w) (Esteghlalian *et al*., 1997; Kadam *et al*., 2000; Kim *et al*., 2002; Neureiter *et al*., 2002). Thereafter, the remaining solids, which are mainly cellulose are removed and treated in the second step at higher temperatures (200-230 °C) and higher acid concentration (up to 2.5 % (w/w)) (Galbe and Zacchi, 2002; Kadam *et al*., 2000; Kim *et al*., 2002). The harsh conditions in the second step still lead to inhibitory degradation by-products. Enzymatic hydrolysis substitutes for the second step and thereby prevents these inhibitory by-products from being formed (Duff and Murray, 1996; Esteghlalian *et al*., 1997; Vlasenko *et al*., 1997).

4.2 Steam explosion

A pretreatment process that has attracted much interest is steam explosion. It is a commonly used method to pretreat various types of lignocellulosic material (Saddler *et al*., 1993; Sun and Cheng, 2002). Steam explosion can either be performed with an acidic catalyst or without (auto hydrolysis).

4.2.1 Auto hydrolysis

In steam explosion without a catalyst (auto hydrolysis), the lignocellulosic material is heated with high-pressure steam to temperatures from 160 to 250 °C for times from several seconds up to 10 min (Saddler *et al*., 1993). Thereafter, the pressure is released and the material undergoes an explosive decompression. The high temperatures promote the formation of organic acids from acetyl groups present in the material, and these acids cause an auto hydrolysis (Mansfield *et al*., 1999). Although steam explosion has successfully been used for pretreatment of hardwood and agricultural residues like

straw, it is not very effective for pretreatment of softwood (Keller, 1996; Overend and Chornet, 1987; Saddler *et al*., 1993).

4.2.2 Acid catalysed steam explosion

The addition of an acidic catalyst in steam explosion has been recognized as a way to increase the digestibility of cellulose by improving the hydrolysis of hemicellulose and decreasing the production of degradation by-products from the sugars (Clark *et al*., 1989; Saddler *et al*., 1993; Sun and Cheng, 2002). Most commonly, the lignocellulosic material is infused with either H_2SO_4 or SO_2 before the steam explosion. During the steaming, SO_2 is converted by oxidation into H_2SO_4, which then acts as the actual catalyst (Saddler *et al*., 1993). The use of SO_2 is advantageous compared to H_2SO_4 as the gas penetrates easier and faster into the material and SO_2 does not result in as serious corrosion problems as H_2SO_4 (Galbe and Zacchi, 2002). Hydrolysis of willow resulted in higher total yield of glucose and xylose using SO_2 compared to H_2SO_4 (Eklund *et al*., 1995). The use of SO_2 for hydrolysis of softwood has also been found to be advantageous relative to H_2SO_4 as it results in the same sugar yields, but a hydrolysate with better fermentability (Tengborg *et al*., 1998). In general, SO_2 catalysed steam explosion is regarded as one of the most effective pretreatment techniques for softwood material (Hsu, 1996; Keller, 1996). The conditions reported as optimal for the pretreatment of a number of different substrates to obtain high digestibility of the cellulose and maximum recovery of the hemicellulose sugars are the use of 0.5 to 6 % (w/w) of H_2SO_4 or SO_2. The temperatures are in the range 175 to 215 °C for 2 to 10 min. Like in dilute acid hydrolysis, the combination of high temperature and acidic conditions promotes formation of degradation by-products from the released hemicellulose sugar residues. To improve the recovery of sugars and simultaneously make the cellulose fraction more accessible to enzymatic hydrolysis, two-step strategies for steam explosion have also been tested. In the first step, the steam explosion is performed using a low severity factor (temperature below 180 °C)(equation 4.1) to solubilise the hemicellulose fraction. Then, the cellulose fraction is subjected to a

$$severity\ factor \quad = \quad \log_{10}\left[t \cdot \exp\left(\frac{T - 100}{14.75} \right) \right]$$

Equation 4.1 Calculation of the severity factor with t for time (in minutes) and T for temperature (in Celsius).

second steam explosion step at higher temperature (above 210 °C) (Galbe and Zacchi, 2002; Söderström *et al*., 2002). The same severity factor can be achieved using various combinations of pretreatment time and temperature. We note, however, that temperature has larger influence on the severity factor than pretreatment time. In a given interval of temperature and time, the same severity factor will result in the same

susceptibility to enzymatic hydrolysis, and the severity factor can therefore be used as a design parameter.

4.3 Ammonia freeze explosion (AFEX)

The ammonia freeze explosion is performed in a way similar to steam explosion. The lignocellulosic material is infused with 1-2 kg of liquid ammonia per kg of biomass and heated to around 50 to 90 °C under 10 to 20 atm pressure. After 15 to 30 min, the pressure is explosively released (Holtzapple *et al*., 1991; Teymouri *et al*., 2005; Vlasenko *et al*., 1997). The dry matter recovery is close to 100 % since AFEX is basically a dry-to-dry process, since the ammonia is in gaseous phase after explosion. With the ammonia evaporated after explosion, there is no wash stream in the process (Teymouri *et al*., 2005). The pretreatment with ammonia results in a decrystallisation of the cellulose and the explosive release of the pressure causes a disruption of the fibre structure. These alterations in the structure of the cellulose and lignin increase the accessible surface area and result in enhanced enzymatic digestibility (Holtzapple *et al*., 1991). Unfortunately, the method does not significantly hydrolyse the hemicellulose fraction. Hemicellulolytic enzyme activity is therefore required during the enzymatic hydrolysis. AFEX has proven to be efficient for pretreatment of herbaceous crops and grasses with low lignin content, but it is not as efficient for material with high lignin content, *e.g.* softwood, due to the insignificant removal of lignin from the insoluble material (Holtzapple *et al*., 1991; Sun and Cheng, 2002; Vlasenko *et al*., 1997). However, the main advantage of this method is the low formation of inhibitors that can reduce the performance of the microorganisms in the fermentation stage. The costs of ammonia and environmental concerns necessitate the recovery of ammonia used in the process, which increases the capital and operating costs for this pretreatment process.

4.4 Wet oxidation

Pretreatment of agricultural residues by wet oxidation has proven to be an efficient method for solubilisation of hemicellulose and lignin, and to increase the digestibility of cellulose. The oxidation is performed at temperatures from 170 to 200 °C and at pressures from 10 to 12 bar O_2 for 10 to 15 min. The addition of oxygen combined with temperatures above 170 °C make the process exothermic, thereby reducing the total energy consumption (Ahring *et al*., 1996). Under the stated conditions, a major part (up to 60-70 %) of the lignin is removed from the insoluble fraction and simultaneously the hemicellulose is solubilised and the cellulose is made more accessible to enzymatic hydrolysis (Bjerre *et al*., 1996; McGinnis *et al*., 1983). Na_2CO_3 has been demonstrated to decrease the formation of inhibitory compounds, *e.g.* furfural and hydroxymethylfurfural (Ahring *et al*., 1996). The use of alkaline wet oxidation for the

pretreatment of wheat straw has been extensively investigated. For wheat straw at a dry matter loading of 6 %, the optimal condition has been found to be 190 °C, 12 bar O_2, 6.5 g/L Na_2CO_3 for 10 minutes. These conditions resulted in a hemicellulose yield of 70 % (xylose and arabinose) and 96 % recovery of the cellulose of which 65 % could be converted into glucose by enzymatic hydrolysis (Ahring *et al*., 1996; Klinke *et al*., 2002; Schmidt and Thomsen, 1998). The same conditions have been found to be optimal for spruce (Palonen *et al*., 2004a). Using these conditions for wheat straw, there was no formation of inhibitory degradation products like Hibbertś ketones, levulinic acid, furfural and 5-hydroxymethylfurfural. However, other inhibitors were still formed (Klinke *et al*., 2002). Wet oxidation has also been used for pretreatment of corn stover with a glucose yield after enzymatic hydrolysis of 77 % and a yield of hemicellulose sugars (xylose and arabinose) of around 60 % of the theoretical.

4.5 Organosolv

In the organosolv process, a mixture of an organic solvent and water is used as cooking liquor and an acid catalyst, for example H_2SO_4, can be added. During the organosolv process, the lignin is dissolved by acid-catalysed hydrolysis of the aryl ether bonds, especially the β-aryl ether bonds (Sarkanen, 1990). Lignin is then recovered by flashing the pulping liquor to atmospheric pressure, followed by rapid dilution with water. Other coproducts such as hemicellulose sugars and furfural are recovered from the water-soluble stream. The formation of more hydroxyl groups in lignin during the organosolv process increases the solubility and makes it more useful for manufacturing lignin-based chemicals (Camarero *et al*., 1999).

Lignol Innovations Corp., a Canadian company based in Vancouver, has built a pilot plant for the organosolv process to be applied in their lignocellulose biorefinery platform (See (Pan *et al*., 2005) for a schematic representation of the process). On a softwood mixture of spruce, pine, and Douglas fir, the operating conditions are 40–60 % ethanol (w/w) as pulping liquor with H_2SO_4 added as catalyst to pH, 2.0–3.4, 185–198 °C as reaction temperature and cooking time of 30–60 min, and a pulping liquor:wood ratio of 7–10:1. The cellulose conversion to glucose ranged from 72 % at a cellulose concentration of 100 g/L to 100 % when the cellulose concentration was 20 g/L (Pan *et al*., 2005). On a hardwood sample, 85 % of the glucose and 72 % of the xylose could be recovered (Pan *et al*., 2006). One drawback of the method is that there are several expensive process steps required to recycle the organic solvent, making this process more costly than others previously described (Sun and Cheng, 2002).

4.6 Summary of pretreatment methods

The sugar yields obtained in the different pretreatment methods are usually evaluated by enzymatic hydrolysis of the cellulose fraction. This gives a combined measure of how many sugars that have been released together with the digestibility of the cellulose fibre. An effective pretreatment method renders the cellulose more accessible and susceptible to enzymatic hydrolysis, and it releases the hemicellulosic sugars without degrading the sugars to the various inhibitory by-products. A direct comparison of the yield obtained by the different methods is often made difficult by the fact that the yield of hemicellulose by some researchers only includes xylose whereas the yield by others includes several hemicellulose sugars. An overview of the presented pretreatments methods can be seen in Table 4.1.

CAFI (Biomass Refining Consortium for Applied Fundamentals and Innovation) is collaboration between 5 American Universities and NREL. They have conducted a large study using the leading pretreatment technologies on the same substrate, corn stover. The results have been published in a special edition of *Bioresource Technology* volume 96 (2005). This is a very interesting study since identical analytical methods have been used to provide comparative performance data. Economic assessments have also been made to estimate each pretreatment cost on a consistent basis. Substantial differences have been found in sugar release patterns during different pretreatment and the following enzymatic hydrolysis. These differences have implications for the choice of process, enzyme, and fermentative microorganism. As mentioned in the special edition, it is important to stress that the data obtained pertain specifically to corn stover. Similar results can be expected for straws from other grasses, but completely different data may be obtained on hard- and softwood species.

A recent and very important advance in the process for enzymatic hydrolysis is the design of a reactor allowing for dry matter loadings up to 40 % (w/w) (Jørgensen *et al*., 2007). The higher the dry matter loading, the higher the sugar concentration is after enzymatic hydrolysis. This leads to higher concentrations of ethanol after fermentation and therefore significantly reduced distillation costs (Galbe *et al*., 2007). One major advantage of steam explosion and AFEX pretreatment is that these pretreatment methods operate at high dry matter loadings. Some pretreatment methods, such as dilute acid hydrolysis, wet oxidation and organosolv, lead to a partly depolymerised and solubilised hemicellulose fraction in a water waste stream. In this stream, the concentration of hemicellulose sugars is low and high amounts of energy are required to recover these sugars.

Table 4.1 Advantages and disadvantages for pretreatment methods for lignocellulosic material.

Method	Advantages	Disadvantages
Dilute acid	Less problems with corrosion compared to concentrated acid	Formation of degradation inhibitory by-products
	Two-step method gives good recovery of both glucose and hemicellulose sugars	Advanced reactor design needed to obtain high yields
		Low sugar concentration in exit stream
Steam explosion (with/without addition of acidic catalyst)	Effective method for softwood when acidic catalyst added	Formation of degradation products
	High yield of glucose and hemicellulose sugars – especially in the two-step method	An additional step for removal of lignin often required
Ammonia freeze explosion, AFEX	Low energy input as the pretreatment is performed at temperatures below 100 °C	High cost of both the large amount of ammonia used and of the process to recover ammonia
	Low formation of inhibitors	The hemicellulose fraction needs to be hydrolysed by enzymes
		Not suitable for softwood due to negligible removal of lignin
Wet oxidation	Minimal formation of inhibitors	Cost of oxygen and alkaline catalyst (Na_2CO_3)
	Efficient removal of lignin	
	Exothermic process minimises the energy input	
Organosolv	High value lignin produced	High cost for recovery of organic solvent
	Effective removal of lignin	
		Hemicellulose sugars in low concentration

Chapter 5

Cellulolytic enzymes from filamentous fungi

To efficiently produce ethanol from lignocellulose, pretreated lignocellulose must be broken down into simple sugars that can be used in the fermentation process. The insoluble material obtained after pretreatment consists mainly of cellulose and lignin. Most pretreatment methods effectively solubilise a considerable part of the hemicellulose fraction (See Chapter 4). The lignin complex can be degraded enzymatically to the phenylpropene monomers. Microorganisms degrading lignin have been widely studied, such as white rot fungi and the enzyme system from *Phanerochaete chrysosporium* (Shoemaker and Leisola, 1990). However, no methods exist to ferment lignin monomers to ethanol. Therefore, the ligninolytic enzymes will not be described. An overview can be found elsewhere (Saha, 2004). Because a large fraction of the hemicellulose is solubilised and partly hydrolysed during pretreatment, and because pretreatment of different plant material results in different hemicelluloses, the most investigated group of enzymes used following pretreatment are cellulolytic enzymes. It should be mentioned, however, that it is still not known how the presence of xylanases and mannanases affect the performance of the cellulolytic enzymes (Mansfield *et al*., 1999). Investigations in this area have just been initiated, and results from sulphur dioxide steam-pretreated corn stover have shown that residual xylan in the solid fraction after pretreatment reduce the hydrolysis rate and also the final yield of glucose (Öhgren *et al*., 2007).

The production of cellulolytic enzymes occurs almost exclusively in microorganisms, bacteria and fungi. Cellulolytic enzymes are also found in plants where they participate in leaf abscission, ripening of fruits, and cell wall growth (Loopstra *et al*., 1998). Few reports can also be found on cellulolytic enzymes in higher eukaryotes *e.g.* in termites (Watanabe *et al*., 1997). To get reduced carbon compounds required for energy production, microorganisms produce cellulolytic enzymes to degrade the insoluble cellulose into soluble oligomers. These oligomers can be either directly taken up and metabolised or can be degraded to glucose and then taken up.

Cellulolytic enzymes can be divided into two distinct groups, enzymes produced by aerobic microorganisms and produced by anaerobic microorganisms. Aerobic microorganisms secrete individual cellulolytic enzymes whereas anaerobic

microorganisms produce multienzyme complexes called cellulosomes (for a more in depth taxonimic review of cellulolytic microorganisms, see (Lynd *et al*., 2002)). The cellulosome from genus *Clostridium* is well characterised (Schwarz, 2001). Fungi producing enzymes with cellulotytic properties secrete the cellulolytic enzymes into the external milieu. Cellulolytic enzymes for industrial purposes most often come from filamentous fungi such as *Aspergillus niger*, *Humicola insolens*, and *Trichoderma reesei*. Enzymes from *T. reesei* are primarily used in cellulose-to-ethanol pilot plants.

5.1 History of *Trichoderma reesei*

During World War II, the American Army was concerned by the rate at which their cotton materials were deteriorating in tropical regions. Too much cargo space was being wasted merely to replenish non-functional items. Several laboratories were therefore set-up to identify the cause of the deterioration problem. One laboratory established in the Quartermaster Corps (later named the U.S. Army Natick laboratory), was led by Harvard Professor William H. Weston. They believed the source to be a microorganism. Therefore, they put forward large efforts to determine the causal microorganism, and its mechanism(s) of action. An overview of the microorganisms found and characterised by their effort has been presented (Reese *et al*., 1950). One microorganism, *Trichoderma viride* QM6a (QM for Quartermaster) was found to produce large amounts of cellullolytic enzymes (Reese *et al*., 1950) and it is the sole progenitor of the many mutants being used today. Later on phylogenetic studies using PCR-fingerprinting demonstrated that the isolated *Trichoderma* species was phylogenetically different from *T. viride* (Kuhls *et al*., 1996). The isolated species is now named *Trichoderma reesei* in honour of Edwin Reese of the U.S. Army Natick Laboratory.

One round of irradiation of QM6a resulted in a strain producing two times more cellulolytic enzyme activity (Mandels *et al*., 1971), and a second round of irradiation resulted in QM9414 producing four times more cellulolytic enzyme activity. In 1979, an important hyper producing strain (derived from QM6a) *T. reesei* Rut C30 was developed at the American Rutgers University by (Montenecourt and Eveleigh, 1979). This strain has a truncated form of the protein (Cre1) mediating glucose repression in *T. reesei* (Ilmén *et al*., 1996). Deletion of the *cre1* is lethal for the strain, whereas the truncated form allows the strain to grow, and the carbon catabolite repression is relieved enabling production of cellulolytic enzymes on easy metabolisable sugars. *T. reesei* is an asexually reproducing filamentous fungi (an anamorph or belonging to fungi imperfecti), *Hypocrea jecorina* has been identified as the sexual state (teleomorph), and this name should take priority over the anamorph-name (Kuhls *et al*., 1996).

5.2 Cellulose degradation

In the late 1950s, the International Union of Biochemistry started the International Commision on Enzymes. This Enzyme Commission (EC) classified enzymes according to their substrate specificity by assigning each enzyme a specific set of numeric identifiers. Cellulolytic enzymes belong to EC 3.2.1.x, a group of glycosidases hydrolysing *O*- and *S*-glycosyl compounds. According to the recommendations of the International Union of Biochemistry and Molecular Biology, cellulolytic enzymes are divided into three classes (Figure 5.1):

- Exo-1,4-β-D-glucanases or cellobiohydrolases (CBH) (EC 3.2.1.91) cleave off cellobiose units from the ends of cellulose chains. This class also includes the less common exo-1,4-β-D-glucanhydrolases (E.C. 3.2.1.74), which liberate D-glucose from the terminal ends of the cellulose chain.
- Endo-1,4-β-D-glucanases (EG) (EC 3.2.1.4) hydrolyse internal β-1,4-glucosidic bonds randomly in the cellulose chain.
- 1,4-β-D-glucosidase (BG) (EC 3.2.1.21) hydrolyses cellobiose to glucose and also cleaves of glucose units from cellooligosaccharides.

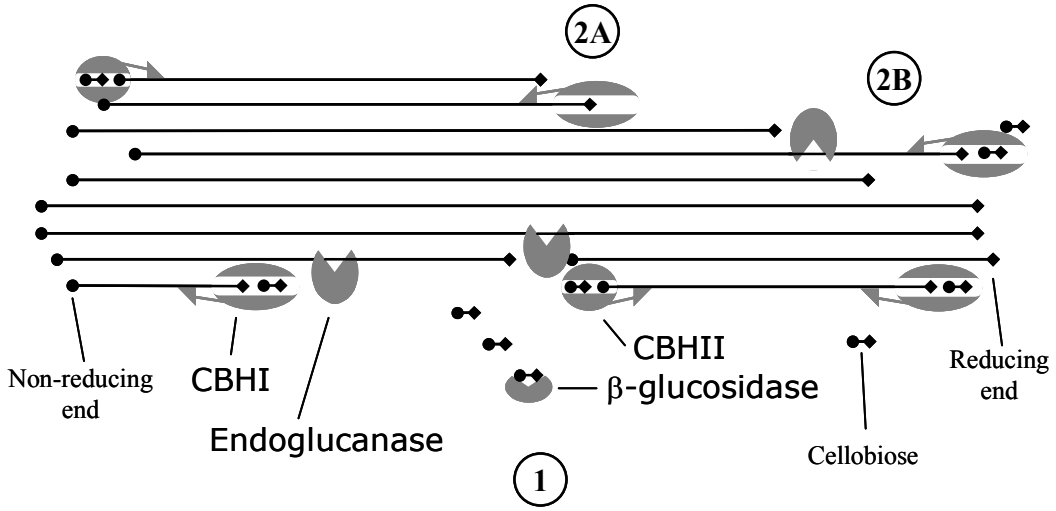


Figure 5.1 Model describing the degradation of cellulose by cellulases. **1 –** The traditional endo-exo model. Endoglucanases make random cuts in the cellulose chains. This makes new ends available for the cellobiohydrolases (CBHI and CBHII), which cleave off cellobiose units from the ends of the cellulose chains. Released cellobiose is hydrolysed into glucose by the action of the β-glucosidases. **2A and 2B –** This figure shows an extension of the endo-exo model, namely, the obstacle model. In **2A**, a CBHI has been blocked by an overlaying cellulose chain (an obstacle). Due to the strong binding of CBHI to the cellulose chain by the cellulose binding domain and the glycosyl binding sites in the tunnel, the enzyme cannot be released from the cellulose chain and becomes unproductively bound. In **2B**, an endoglucanase cuts the cellulose chain before CBHI reaches an obstacle, and thereby preventing the unproductively binding of CBHI. The CBHI trapped in 2A will not be "activated" before the upper cellulose chain is removed by the CBHII (Olsson *et al.*, 2005).

BGs are not cellulolytic enzymes because their preferred substrate is cellooligosaccharides preferably cellobiose and not cellulose. In the context of cellulose degradation, BGs are necessary to degrade cellulose into the monomeric building block, glucose. Within each enzyme class, most fungi are capable of producing multiple enzymes with similar enzymatic properties, but having distinct physical properties (molecular mass and isoelectric point) (Wood, 1985). EGs are the most diverse class of enzymes in the cellulose degrading system. Several EGs with different molecular masses and isoelectric points have been identified in *T. reesei* (Tolan and Foody, 1999) and *Humicola insolens* (Schulein, 1997). *T. reesei* produces two cellobiohydrolases, at least seven endoglucanases and two β-glucosidases (Table 5.1) (Karlsson *et al*., 2001; Saloheimo *et al*., 2002). Several other enzymes with predicted cellulolytic properties have been found using cDNA from *T. reesei* (Foreman *et al*., 2003). By using the EC system, substrate specificity can be identified. However, cellulolytic enzymes often have broad specificity towards complex lignocelluloses material. Therefore, the EC number might be misleading. EG is also active on different types of hemicelluloses. For example, EGII from *T. reesei* and EGb1 and EGb2 from *Penicillium brasilianum* have been shown to have hydrolytic activity towards mannan (Jørgensen *et al*., 2003a; Macarrón *et al*., 1996).

Table 5.1 Cellulolytic enzymes produced by *Trichoderma reesei*.

Enzyme	GH Family	Relative amount, % total protein	Reference
CBHI	Cel7A	60-75	(Nidetzky *et al*., 1994)
CBHII	Cel6A	15-20	(Teeri *et al*., 1987)
EGI	Cel7B	5-10	(Penttila *et al*., 1986)
EGII	Cel5A	1-10	(Saloheimo *et al*., 1988)
EGIII	Cel12A	1-5	(Okada *et al*., 1998) (Tolan and Foody, 1999)
EGIV	Cel61A	0-1	(Saloheimo *et al*., 1997) (Goedegebuur *et al*., 2002)
EGV	Cel45A	1-5	(Saloheimo *et al*., 1994) (Tolan and Foody, 1999)
EGVI	Cel74A	-[a]	(Coleman *et al*., 2006a)
EGVII	Cel61B	-[a]	(Coleman *et al*., 2006b)
BGI	Cel3A	1-2	(Barnett *et al*., 1991) (Tolan and Foody, 1999)
BG2	Cel1A	n.q.[b]	(Takashima *et al*., 1999)

[a] not quantified; enzyme discovered from cDNA, [b] not quantified

5.3 GH classification

In 1991, Bernard Henrissat proposed a new classification system for glycoside hydrolases, GH (Henrissat, 1991). Rather than substrate specificity, this classification system is based upon amino acid sequence similarities and hydrophobic cluster analysis. Data for GH families are available online at the CAZy (Carbohydrate Active EnZymes) website. Currently, this system comprises 111 GH families, and it is continuously updated at URL http://afmb.cnrs-mrs.fr/CAZY/. GH families share the same structural features. This property can be used to reveal evolutionary relationships between different GH families and to derive mechanistic information from protein sequence data (Henrissat *et al*., 1998). Due to these advantages, the GH system has become a powerful tool for scientists working with GHs. In families with many entries, the enzymes can be divided into subfamilies with a reliable predictive power for the substrate specificity (Stam *et al*., 2006). Broader use and increased database entries in the CAZy will continue to improve the GH classification system. It is now possible to look for GH subfamilies with specific cellulolytic activity to identify GHs that would be predicted to have a desired activity. Some of the most important families in the field of cellulolytic enzymes are families 1, 3, 5, 6, 7, and 12. Some of the most prominent cellulolytic enzymes within the different classes are found in the same family. GH families 5, 6, and 7 comprise many cellobiohydrolases, families 5, 6, 7, and 12 comprise several endoglucanases, and BGs are in families 1 and 3. When using the GH classification scheme, the name of the microorganism followed by the primary substrate for hydrolysis is also included with the specific enzyme (Henrissat *et al*., 1998). As an example, cellulolytic GHs are named Cel followed by the family number. If the microorganism produces several cellulolytic GH belonging to the same family, the name is followed by a capital letter. For example: the third GH5 EG in *Penicillium brasilianum* is named Cel5C. The origin of the enzyme can be indicated with a prefix. The aforementioned GH5 EG from *P. brasilianum* is named *Pb*Cel5C.

5.4 Molecular architecture

Fungal EGs and CBHs have a two-domain structure. Specifically, a large catalytic core domain is connected to a cellulose binding domain (CBD) with a flexible linker (Schulein, 1997).

5.4.1 Catalytic domain

The active site in the catalytic core domain is divided into three distinct structures: a tunnel (Figure 5.3.A), a cleft (Figure 5.3.B), and a pocket. The substrate specificity for a given cellulolytic enzyme is often predicted from the active site structure. The pocket

and the tunnel shape characterise exo-acting enzymes, because a chain needs to enter with one end first. In the crystal structure of the predominant cellulolytic enzyme produced by *T. reesei* CBHI, the active site is positioned in a long tunnel (Divne *et al*., 1994). The detailed structure of this long tunnel (Figure 5.2) has been identified using cellooligomers and catalytic deficient mutants of CBHI. Ten well-defined subsites for the cellulose chain have been found from positions –7 to +3 with the active site being position between –1 and +1 (Divne *et al*., 1998). The long tunnel makes CBHI a very specific exo-acting enzyme acting that processes cellulose from one end of the chain. Another exo-acting active site is the active site in BG. The pocket structure for BG that removes glucose residues from cellooligomers, preferably cellobiose, has been identified in a GH3 BG from barley (Varghese *et al*., 1999). Here, the glucose monomer fits in the pocket beyond the active site. Relative to CBHI and BG, endoglucanases have an open active site positioned in a cleft (Figure 5.3.B) The open site makes is possible for EGs to bind to the interior parts of the cellulose fibres (Figure 5.3.D) giving the EG its endo-activity. The cleft has been identified in the crystal structure of EGI (Cel7B), the EG produced in largest quantity in *T. reesei* (Kleywegt *et al*., 1997).

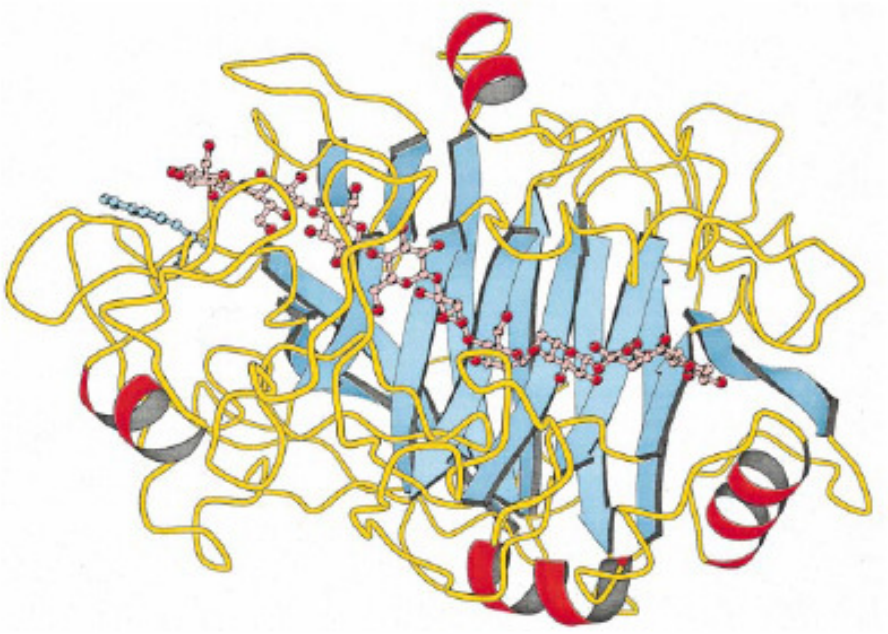


Figure 5.2 Schematic representation of the *Tr*CBHI catalytic domain with a cellooligomer bound in sites -7 to +2. Secondary-structure elements are coloured as follows: β-strands, blue arrows; α helices, red spirals; loop regions, yellow coils. The cellooligomer is shown in pink as a ball-and-stick object (Divne *et al*., 1998).

5.4.2 Linker

The catalytic core and the cellulose-binding domain are connected via a flexible linker of 22-44 kDa long. Longer linkers have also been reported (Saloheimo *et al.*, 1997). Because of the flexible nature, it is difficult to obtain structural data on the linker. Therefore, the linker and CBD are seldom depicted on 3D-models (Figure 5.3.A and B). The linker is rich in serine and threonine residues. In addition, the linker is typically glycosylated (Harrison *et al.*, 1998; Maras *et al.*, 1999). The glycosylation is believed to provide rigidity and to ensure that the catalytic domain and the CBD are correctly positioned next to each other (Figure 5.3.C) (Srisodsuk *et al.*, 1993). Glycosylation can also serve a role in protecting the very labile linker against proteolysis.

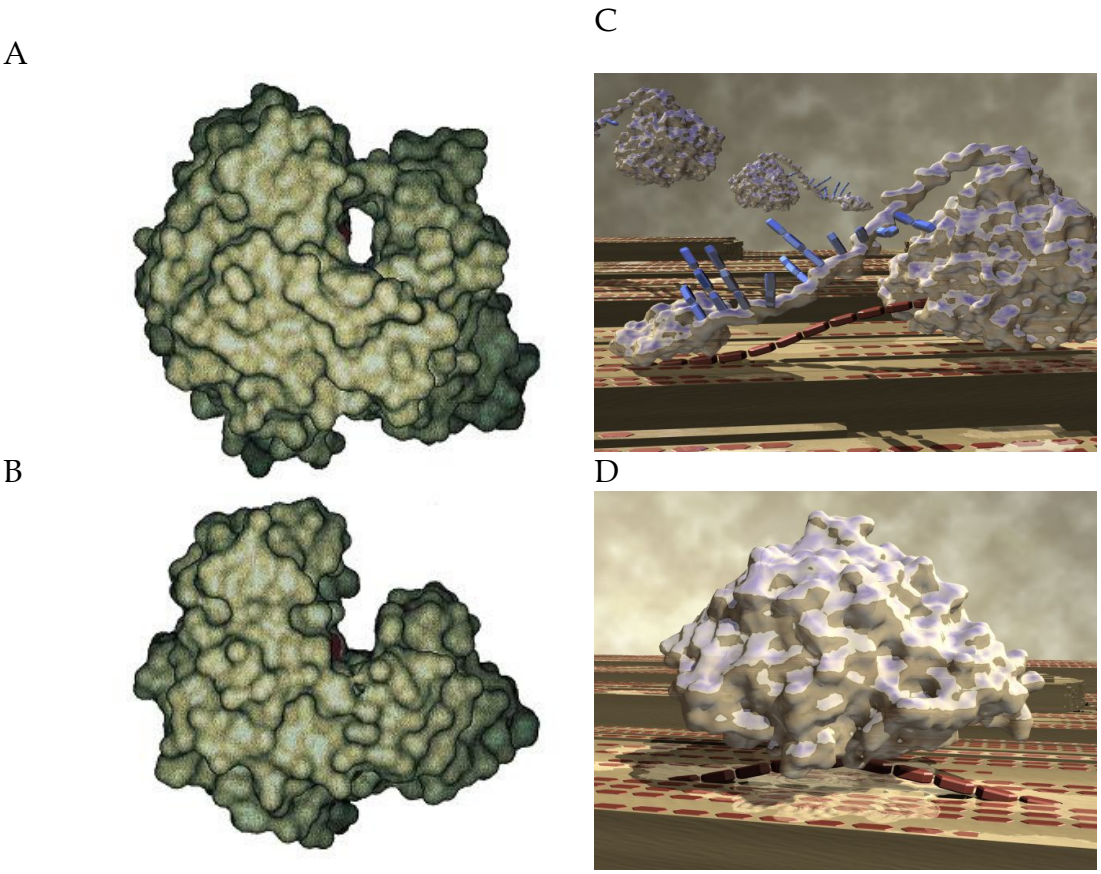


Figure 5.3 Structure of CBH and EG. **A** *Humicola insolens* CBHII (Varrot *et al.*, 1999). **B**. *Thermobifida fusca* endoglucanase E2 (Varrot *et al.*, 1999). Computer animation of *T. reesei* **C** CBHI with a cellulose chain entering the tunnel shaped active site, and **D** EG on the surface of a cellulose fiber. **C** and **D** are pictures made in corporation between NREL and Pixel Kitchen.

5.4.3 Cellulose binding domain

The cellulose-binding domain (CBD) binds to cellulose to bring the catalytic core in close contact with the cellulose chain. This action increases the hydrolysis rate.

Removal of the CBD reduces hydrolytic activity by reducing the number of interactions between cellulose and the enzyme (Tomme *et al*., 1988). Removal of the CBD from CBHI (Cel7A) and CBHII (Cel6A) from *T. reesei*, for example, reduced the adsorption of the catalytic core to bacterial microcrystalline cellulose by an order of magnitude (Palonen *et al*., 1999). Surprisingly, the CBD, which has no active site, has been shown to play another role in cellulose hydrolysis, namely, non-hydrolytic disruption of cellulose structure. The CBHI CBD from *Penicillium janthinellum* bound to and disrupted cotton fibres, and during hydrolysis the CBD showed synergy with an EG (Gao *et al*., 2001).

CBDs are frequently also named CBM, carbohydrate-binding modules. CBM is a classification system used in the CAZy database. A CBM is defined as a "contiguous amino acid sequence within a carbohydrate-active enzyme with a discrete fold having carbohydrate-binding activity". As with the GH classification scheme, CBMs are classified into families based upon amino acid sequence similarities and hydrophobic cluster analysis. Fungal CBDs are all classified into the CBM1 family, which contains proteins comprising 36 to 38 amino acids in a wedge-like structure (Kraulis *et al*., 1989). The cellulose binding function is mediated by aromatic residues on a flat surface in the CBD (Mattinen *et al*., 1998). Strikingly, the distance between these aromatic residues matches the distance between repeating units in cellobiose (Tomme *et al*., 1995). It has recently been suggested (Mulakala and Reilly, 2005) that the CBM wedges itself under a free cellulose chain on the crystalline cellulose surface which then "feeds" into the catalytic domain of the cellulolytic enzyme (Figure 5.4).

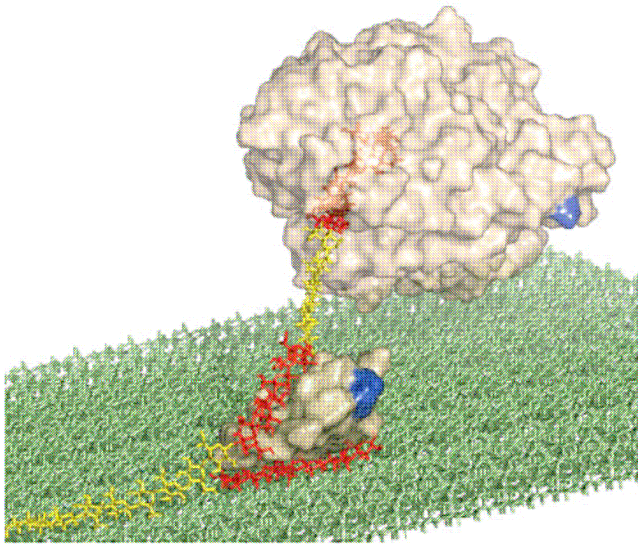


Figure 5.4 Hypothetical concerted action by *Tr*Cel7A CBD and catalytic domain to detach cellulose chain from crystal and to cleave cellobiose units from chain. Red: docked portions of cellulose chains (partly obscured by translucent catalytic domain); yellow: nondocked portions of cellulose chain; green: cellulose crystal; blue: points of attachment of O-glycosylated linker to catalytic domain and to CBD (Mulakala and Reilly, 2005).

5.5 Synergism in cellulose hydrolysis

In enzymatic reactions, synergism occurs when the combined activity of two enzymes exceeds the sum of the individual enzyme activities. In the hydrolysis of filter paper, a one to one mixture of CBHI and EGI from *T. reesei* resulted in 23 % (w/w) conversion, whereas the individual conversion was 6 % and 8 % using CBHI and EGI, respectively (Gama *et al*., 1998). This synergism is explained by the well described endo-exo model (Ståhlberg *et al*., 1993; Teeri, 1997). EGs make random cuts in the cellulose chain creating new starting points for the processive action of the CBHs (Figure 5.1 **1**). The endo-exo model has been extended to the obstacle model to account for synergism in the early phase of cellulose hydrolysis when there should be sufficient free cellulose ends for full CBH activity and therefore no synergism (Eriksson *et al*., 2002b; Karlsson *et al*., 1999). In the obstacle model, CBHs remain adsorbed to the cellulose chain due to tight binding between cellulose and the substrate-binding site in tunnel of the CBH, even though the CBH encounters an obstacle (Figure 5.1 **2A**). Here, the CBH is not active before the obstacle is removed. The obstacle can be an overlying cellulose chain (Väljamäe *et al*., 1998). Another model for synergism is the exo-exo model, in which synergism is found between a CBH acting from the reducing end of the cellulose chain and a CBH acting from the non-reducing end (Wood, 1985). The exo-exo synergism is less understood than the endo-exo model. However, one explanation can be found in the obstacle model in which an overlying cellulose chain can be removed by a CBH acting from the other end of the cellulose chain than the CBH being stuck on the underlying chain (Figure 5.1 **2A**)(Mansfield *et al*., 1999). Another explanation is that CBHII has some endo-activity. This is consistent with a report for CBHII from *Humicola insolens* (Boisset *et al*., 2000).

5.6 Measurement of cellulolytic enzyme activities

Among the cellulolytic enzymes produced by a microorganism, it is almost impossible to quantify the amount of each cellulolytic enzyme especially since several enzymes are produced within each enzyme class and on top; most enzymes have broad substrate specificities. A very good review on soluble and insoluble substrates and different set-ups for assaying the individual cellulolytic enzyme activity was recently published (Percival Zhang *et al*., 2006). In our laboratory a nice method has been established using capillary electrophoresis to quantify individual enzymes in the cultivation broths (Jørgensen *et al*., 2003b). However, it is required to have the enzymes purified before the analysis starts, since it is necessary to know the migration time and the detection response for each enzyme.

5.6.1 Total cellulolytic enzyme activity

A frequent method to measure the total cellulolytic activity for an enzyme mixture is the filter paper activity, FPA (Tolan and Foody, 1999). In this assay, the hydrolysis of filter paper (cellulose) is measured through the number of free reducing ends being produced during hydrolysis . However, the heterogeneity of the insoluble cellulose in combination with the complexity of the cellulase system influences the measurements. In spite of this, FPA is still one of the best methods for measuring total cellulolytic enzyme activity. In a screen for cellulolytic enzymes, a low filter paper activity does not necessarily rule out that a specific enzyme in the mixture may be of interest. With the molecular biology tools, an interesting enzyme can be produced in another host with high yields. Therefore, it is not a prerequisite that native microorganisms produce high amounts of the enzyme of interest. Rather, it is important that specific enzyme activities are captured to provide broad hydrolysis activity across multiple substrates. Of greatest interest is specific activity (enzymatic activity based on the amount of the enzyme, therefore it is also important to measure the amount of protein secreted. In screening Penicillia for their production of cellulolytic enzymes (see **Paper A**), the measured filter paper activity for each culture broth showed large variation from species to species. The apparent difference in filter paper activity from species to species was largely due to changes in the amount of secreted protein. A plot of measured extracellular protein and filter paper activity showed that higher protein concentrations resulted in higher filter paper activities (**Paper A**). The correlation between protein secretion and filter paper activity presents a problem familiar to scientists working with microbial screening for the discovery of new enzymes. The challenge is how to determine the amount of protein produced, since the media often are complex containing small peptides, insoluble substrate and cell mass, which makes protein concentration determination difficult.

5.6.2 Individual enzyme activities

The BG activity can be assayed on either cellobiose or *para*-nitrophenyl-β-D-glucopyranoside. Cellobiose is the natural substrate, and the formation of glucose can be measured by several methods. Activity on the chromogenic substrate *para*-nitrophenyl-β- D-glucopyranoside is measured through detection of the released *para*-nitrophenol (Wood and Bhat, 1988). An artificial substrate like *para*-nitrophenyl-β-D-glucopyranoside is often used for enzyme kinetics due to availability, price, and time saving activity measurements. One argument often brought forward is that, the use of artificial substrates does not mimic real life substrates. This is true, and one has to use caution when correlating specific activities measured on artificial substrates to activities measured on real life substrates, as I also found in this study using *para*-

nitrophenyl-β-D-glucopyranoside and cellobiose (**Paper D**). With no hesitation, however, artificial substrates can be used to measure residual activity when investigating enzyme stability.

EG activity is frequently measured using carboxymethyl cellulose or hydroxyethyl cellulose. The activity can be measured by the formation of reducing sugar by DNS (Miller, 1959). The substrates can also be azo-dyed and the activity is then quantified by the formation of small dyed soluble cellooligomers. Due to the bulky side groups on the cellulose chain, cellulose cannot enter the tunnel in the cellobiohydrolase (Divne *et al*., 1998). Little interference is observed in EG activity assays from the cellobiohydrolase present in crude enzyme mixtures.

There are a number of difficulties in measuring CBH activity in a mixture of cellulolytic enzymes. With a purified cellobiohydrolase, the CBH activity can be measured using pure cellulose with different crystallinity, either crystalline cellulose (*e.g.* bacterial microcrystalline cellulose), amorphous/crystalline cellulose (*e.g.* Avicel), or amorphous cellulose (*e.g.* phosphoric acid swollen cellulose) (Schulein, 1997). Released cellobiose can be quantified by HPLC or reducing sugar analysis. A chromogenic substrate *para*-nitrophenyl-β-D-cellobiopyranoside can be used to determine the CBH activity, but as for the cellulase substrates the major problem is that the substrates all are hydrolysed to some extent by endoglucanases, endoxylanases, β-galactosidases, and β-glucosidases (Biely *et al*., 1997; Nidetzky and Claeyssens, 1994). The BG activity in a mixture of cellulolytic enzymes can be inhibited by adding D-glucono-1,5,-δ-lactone, a BG specific inhibitor (Deshpande *et al*., 1984).

Chapter 6

Cellulolytic enzymes from *Penicillium brasilianum*

To exploit biomass as a resource for the production of sugars to be used in biofuel production, most research focused on using cellulolytic enzymes from *Trichoderma reesei* for the enzymatic hydrolysis step. Unfortunately, all *T. reesei* strains originate from one strain. Therefore the diversity of well-characterised cellulolytic enzymes is small. However, extensive work characterizing cellulolytic enzymes from *T. reesei* has led to valuable insights into enzyme structure, synergy and activity, all of which can useful in the understanding of cellulolytic enzymes from other microorganisms. Fungal cellulolytic enzymes can be produced in high amounts and many interesting enzymes have been found in *Chaetomium*, *Phanerochaete*, *Schizophyllum*, *Aspergillus*, *Fusarium*, *Myrothecium*, and *Penicillium* (Lynd *et al*., 2002).

The potential for producing large amounts of active fungal cellulolytic enzymes combined with large diversity in these enzymes has made fungal cellulolytic enzymes for hydrolysing cellulose in commercial bioethanol production very appealing. However, enzymatic hydrolysis of pretreated biomass is a complex process to understand in detail, the reasons being a large difference between biomass substrates, pretreatment methods and conditions, a heterogeneous and insoluble substrate. Revealing the intense interest in the topic, several books have been written and a pubmed search of 'cellulose hydrolysis' resulted in 3,000 hits. Enzyme hydrolysis research has addressed many issues including: synergy between enzymes, enzyme stability, productive and non-productive adsorption to different substrates, heterogeneity of the substrates (Figure 6.1), changes to substrates during hydrolysis, removal of hydrolysis end products, among others. Some of these issues will be discussed later in this chapter.

- **High specific activity on the substrate of choice**
- **A high pH and temperature stability (72h of hydrolysis)**
- **Thermostable – operation at highest possible temperature**
- **Flexibility – to allow for different process conditions**
- **Low lignin adsorption**
- **Maintain activity at high ethanol concentration**
- **Low substrate and product inhibition**
- **Cheap**

Figure 6.1 Important parameters for a cellulolytic enzymes to be applied in the production of bioethanol.

6.1 Screening genus *Penicillium* for cellulolytic enzymes

The genus *Penicillium* is predominantly found in forest soil where large amounts of plant material are degraded (Christensen *et al*., 2000). Species belonging to the genus *Penicillium* are well known producers of cellulolytic enzymes that have interesting properties in cellulose degradation (Castellanos *et al*., 1995; Karboune *et al*., 2008; Martins *et al*., 2008; van Wyk, 1999). In addition, genus *Penicillium* is in a taxonomical sense very different from genus *Trichoderma* (Figure 6.2), which may increase the probability of finding novel cellulolytic enzymes. Figure 6.2 is based on functionality/phenotype (classification), and not phylogeny (cladification) The department BioCentrum at The Technical University of Denmark has a very large and well-described culture collection of filamentous fungi curated by Professor Jens Christian Frisvad. In collaboration with Professor Frisvad, we searched the culture collection to find a *Penicillium* species producing interesting cellulolytic enzymes that might find utility for bioethanol production. The culture collection contains more than 16,000 Penicillia strains, and in collaboration with Professor J. C. Frisvad we selected 12 *Penicillium* species (**Paper A**).

Kingdom	Phylum	Class	Subclass	Order	Family	Genus	Species
Fungi	Ascomycota	Eurotiomycetes	Eurotiomycetidae	Eurotiales	Trichocomaceae	*Thermoascus*	*aurantiacus*
						Penicillium	*brasilianum*
							janthinellum
							funiculosum
							purpurogenum
						Aspergillus	*niger*
							oryzae
							nidulans
							aculeatus
							kawachii
				Onygenales	Onygenaceae	*Chrysosporium*	*lucknowense*
		Sordariomycetes	Sordariomycetidae	Sordariales	Chaetomiaceae	*Chaetomium*	*thermophilum*
						Thielavia	*terrestris*
					Sordariaceae	*Neurospora*	*crassa*
			Hypocreomycetidae	Hypocreales	Nectriaceae	*Fusarium*	*oysporum*
					Incertae sedis	*Myrothecium*	*verrucaria*
					Hypocreaceae	*Trichoderma*	*reesei*
							longibrachiatum
							koningii
							harzianum
		Saccharomycetes	Saccharomycetidae	Saccharomycetales	Dipodascaceae	*Geotrichum*	*klebahnii*
		Leotiomycetes	Leotiomycetidae	Helotiales	Bulgariaceae	*Bulgaria*	*sp*
		Dothideomycetes	Dothideomycetidae	Capnodiales	Mycosphaerellaceae	*Cladosporium*	*sp*
		Incertae sedis	Incertae sedis	Incertae sedis	Incertae sedis	*Humicola*	*insolens*
							grisea
	Basidiomycota	Agaricomycetes	Agaricomycetidae	Polyporales	Phanerochaetaceae	*Phanerochaete*	*chrysosporium*
					Polyporaceae	*Coriolus*	*versicolor*
						Poria	*placenta*
				Agaricales	Schizophyllaceae	*Schizophyllum*	*commune*
	Zygomycota						

Figure 6.2 Classification of cellulase producing microorganisms in Kingdom Fungi. based on suggested organisms (Lynd *et al.*, 2002) and organisms frequently mentioned in the literature. Incertae sedis: uncertain position – its broader relationship is unknown.

To identify suitable Penicillia strains, I screened for strains that could grow on lignocellulose containing media. The 12 screened Penicillia strains tested belonged to the two subgenera *Furcatum* and *Biverticillium*. Their phenotypic classification into subgenera was found to reflect their production of cellulolytic enzyme activities and classification can sometimes be used as a predictor as to which direction to look for a particular enzyme activity (**Paper A**). The Penicillia tested were also phylogenetically different, and as example the teleomorph form of *P. brasilianum* belongs to genus *Eupenicillium* whereas *P. pinophilum* belongs to genus *Talaromyces.*

In the screens, *T. reesei* Rut-C30 was used as reference. This is because of its use as a model organism in the production of cellulolytic enzymes. *P. brasilianum* was found to produce the highest amount of protein (560 mg/L) in the external medium compared to the other Penicillia. While the *T. reesei* reference strain secreted similar amounts of protein in to the medium, the specific[1] filter paper activity (1.21 FPU/mg) was the highest for the cellulolytic enzymes from *P. brasilianum* compared to 0.69 FPU/mg for the mixture from *T. reesei* (**Paper A**). The cultivations in the screen were done in shake flasks, but later cultivations of *P. brasilianum* in reactors under controlled conditions resulted in a specific activity of 1.18 FPU/mg, a very good result compared to 0.84 FPU/mg measured for a typically used commercial mixture for cellulose hydrolysis 3:1 (v/v) Celluclast and Novozym 188 (Jørgensen and Olsson, 2005).

Beyond identifying the best strain, *P. brasilianum,* a high specific activity for the individual enzyme classes was used to also find efficient enzymes among the Penicillia strains. The purification of a cellulolytic enzyme from a wild type cultivation broth typically results in a very low amount due to low expression and many purification steps. Therefore, a high enzyme production was required to obtain sufficient amounts of each cellulolytic enzyme for characterization and in the long run also to select a microorganism with the potential to be a commercial production strain. We identified three potentially valuable strains: *P. pinophilum*, *P. persicinum*, and *P. brasilianum* (Table 6.1).

An increase in enzyme production is a key requirement for the development of industrial microorganisms that will serve to produce enzymes for the hydrolysis step in bioethanol production. Previous experiments with random mutagenesis have demonstrated that the production of cellulolytic enzymes in *P. pinophilum* could be increased four times after only three rounds of mutagenesis (Brown *et al*., 1987). A similar increase in the production of cellulolytic enzymes has been found in the initial development of *T. reesei* as a production microorganism for cellulolytic enzymes

[1] The term "specific activity" refers to the activity relative to the total amount of protein present. This broader definition will be used for enzyme mixtures and purified enzymes.

(Mandels *et al*., 1971). Random mutagenesis could potentially be a useful strategy for enhancing the production of cellulolytic enzymes in the three strains we identified.

6.2 Growth and production of cellulolytic enzymes in selected Penicillia

The three selected Penicillia species were characterised further to measure the effect of carbon source on the specific growth rate and production of cellulolytic enzymes. Specifically, the strains were grown on glucose and xylose as carbon sources. One scenario for production of cellulolytic enzymes in the production of bioethanol is using a waste stream as carbon source for the production of cellulolytic enzymes. Both glucose and xylose could likely be present in such a waste stream. We observed that *P. brasilianum* grew 2-fold faster than the other *Penicillium* species on glucose with a maximum specific growth rate of 0.18 h^{-1} for *P. brasilianum* compared to 0.08 h^{-1} for *P. pinophilum* and 0.09 h^{-1} for *P. persicinum*. On xylose, the maximum specific growth rate for *P. brasilianum* was 0.14 h^{-1}, which was lower compared to growth on glucose. However, the maximum specific growth on xylose rate was still higher for *P. brasilianum* compared to *P. persicinum* with a maximum specific growth rate of 0.09 h^{-1} (Table 6.1) (**Paper B**).

Table 6.1 Specific growth rate in batch cultivation of selected Penicillia (**Paper B**).

Strain	Substrate	μ (h^{-1})	Replicates
P. pinophilum IBT4186	Glucose	0.08±0.00	2
P. persicinum IBT13226	Glucose	0.09	1
	Xylose	0.09	1
P. brasilianum IBT20888	Glucose	0.18±0.04	4
	Xylose	0.14±0.02	3

6.2.1 Carbon catabolite repression

Complete hydrolysis of most biomass will result in a mixture of monosaccharides, mainly glucose originating from cellulose, and a mixture of hemicellulose constituents: xylose, arabinose, mannose, and galactose. These sugars can be a cheap carbon source for the production of biomass degrading enzymes. On a mixture of these monosaccharides, the selected Penicillia metabolised glucose first. It was also found that the production of cellulolytic and hemicellulolytic enzymes was repressed during cultivation on glucose. This phenomenon is known as carbon catabolite repression, which is defined as the repression of certain sugar-metabolising genes in favour of glucose utilization genes when glucose is the predominant carbon source in the environment of the cell. In respect to evolution, it also makes sense for the fungus not to keep producing cellulolytic enzymes when the already secreted enzymes release sufficient amounts of glucose to sustain growth. Carbon catabolite repression of

cellulolytic enzymes have been reported for both species of genus *Penicillium*, *Aspergillus*, and *Trichoderma* (Chavez *et al*., 2006; de Vries *et al*., 1999; Mach and Zeilinger, 2003). Carbon catabolite repression is mediated through Cre1 in *Trichoderma* and the homologues CreA in *Aspergillus* (Ruijter and Visser, 1997). CreA/Cre1 is a DNA-binding protein able to bind to certain consensus sequences in the promoter region of a gene thereby preventing transcription (Strauss *et al*., 1995). Closer analysis of *P. brasilianum* revealed three consensus binding-sites for Cre1/CreA in the promoter region of *Cel5C* (**Paper E**). Consistent with carbon catabolite repression in our strains, I observed that the three tested Penicillia did not produce cellulolytic or hemicellulolytic enzyme activities during growth on glucose. Furthermore, CreA binding sequences have been previously observed in *Penicillia* strains. The consensus CreA binding sequence has also been found in the promoter for a GH5 EG from *P. janthinellum* (Mernitz *et al*., 1996) and for a GH10 xylanase from *Penicillium canescens* (Serebryanyi *et al*., 2002). This phenotypic analysis indicates that a CreA homologue likely exists in the genus *Penicillium*.

Before glucose was completely exhausted (2 to 4 g/L in the broth), low BG activity was detected in the culture broths of the three Penicillia (**Paper B**), the BG production is therefore less repressed than the other cellulolytic enzymes. The genome of *Trichoderma reesei* v2.0 have been made publicly available through the Joint Genome Institute as "*Trichoderma reesei* v2.0", and the promoter region of the predominant BG, Cel3A was investigated for possible CreA binding sites using the consensus sequence (5'-SYGGRG-3'). No CreA binding sites were found in the promoter of *Tr*Cel3A. This is consistent with the observed repression alleviation in the investigated Penicillia.

6.2.2 Induction

Induction of cellulolytic and hemicellulolytic enzymes is important for producing an enzyme mixture with broad specificity capable of digesting many types of bio-feedstocks. In *Aspergillus niger,* xylose has been shown to have a dual role both as repressor and as inducer (de Vries *et al*., 1999). The protein XlnR has been found to be a transcriptional activator that mediates the expression of the cellulolytic and xylanolytic system in *A. niger* (Gielkens *et al*., 1999; Hasper *et al*., 2004; van Peij *et al*., 1998) and in *A. oryzae* (Marui *et al*., 2002). During cultivation of the three Penicillia on xylose, low amounts of both cellulolytic and xylanolytic enzyme activities were measured in the culture broth. Cultivation of *P. brasilianum* on xylose resulted in one per mil of the EG activity measured after cultivation on cellulose, and five per mil of the endoxylanase activity measured after cultivation on xylan (**Paper B**). The consensus binding sequence for XlnR was found in the promoter region of GH5 Cel5C from *P. brasilianum* (**Paper E**) suggesting that XlnR can mediate the expression of cellulolytic and

hemicellulolytic enzymes in *P. brasilianum*. Putative XlnR binding sites have also been reported for other Penicillia, *e.g. P. citrinum* (Tanaka *et al*., 2005).

The production of cellulolytic and hemicellulolytic enzymes is primarily induced by cultivation on cellulose and hemicellulose containing media. Cultivation on xylose was found to induce low levels of cellulolytic and hemicellulolytic enzyme activity in the three investigated Penicillia. As expected, a greater induction of cellulolytic and hemicellulolytic enzymes was observed when the Penicillia were cultivated on cellulose and different types of hemicellulose. Irrespective of the substrate, both types of enzymes were induced (**Paper C**). However, the cellulolytic enzymes were induced to a larger extent with cellulose as carbon source compared to xylan, and the resulting EG activity was 100 times higher after cultivation on cellulose. With xylan as carbon source compared to cellulose, the resulting endoxylanase activity was 10 times higher (**Paper B**). Induction of both classes of enzymes was expected since biomass degradation, in nature, requires the whole palette of biomass degrading enzymes. After cultivation on the same type of cellulose, the ratio between the different cellulolytic enzyme activities varied for the three Penicillia. This result suggests that regulation is different among the three species.

Cultivation of the three Penicillia either on xylan from oat spelts or on xylan from birch wood resulted in different levels of hemicellulolytic enzyme activities. The specific endoxylanase activity was highest for *P. pinophilum* regardless of carbon source, and cultivation of this fungus resulted in general in the highest specific activities for the hemicellulolytic enzymes. The backbone in both hemicelluloses used as substrate was xylose units; in oat spelt xylan the main substituent is arabinose, whereas it is glucuronic acid in birch wood xylan. The change, in the profile of the biomass degrading enzymes with respect to the carbon source, is consistent with previous results where the hydrolysis efficiency of an enzyme preparation was improved when the enzymes were produced with the target material present (Baker *et al*., 1997; McMillan *et al*., 2001). In spite different carbon sources, the general trend was that cellulolytic enzymes were produced during cultivation on cellulose and hemicellulolytic enzymes were produced during cultivation on hemicelluloses (Figure 6.3) (**Paper A** and **Paper C**).

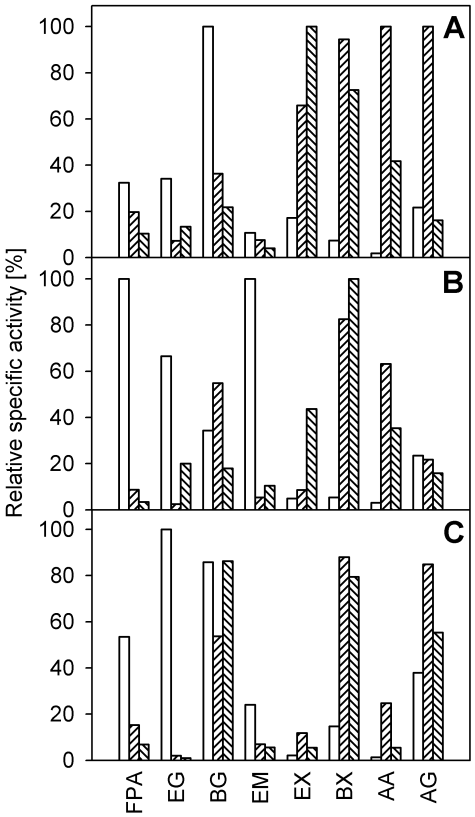


Figure 6.3 Relative specific enzyme activities after cultivation of (A) *P. pinophilum*, (B) *P. persicinum* and (C) *P. brasilianum* on either cellulose (□), oat spelts xylan (/) or birchwood xylan (\). For each enzyme activity, the specific activities are relative to the highest value obtained by any of the three fungi on one of the three carbon sources. Activities: FPA, filter paper activity; EG, endoglucanase; BG, β-glucosidase; EM, endomannase; EX, endoxylanase; BX, β-xylosidase; AA, α-arabinofuranosidase; AG, α-galactosidase. Highest values were: FPA: 1.31 FPU/mg; EG: 88 U/mg; BG: 6.2 U/mg; EM: 13 U/mg; EX: 170 U/mg; BX: 1.8 U/mg; AA: 1.3 U/mg; AG, 4.2 U/mg. (**Paper C**).

Enzymatic hydrolysis of cellulose results in the production of several degradation products that each potentially can induce the production of cellulolytic enzymes (Beguin and Aubert, 1994). Several BGs have transglycosylation activity, as well as hydrolase activity. Transglycosylation can lead to the formation of different glucose dimers such as: sophorose (β-1,2), laminaribiose (β-1,3) or gentiobiose (β-1,6) (Saloheimo *et al*., 2002), and these compounds are known to induce the production of cellulolytic enzymes in different fungi (Hrmová *et al*., 1991; Kawamori *et al*., 1986; Nogawa *et al*., 2001). During hydrolysis of cellobiose, the GH3 BG from *P. brasilianum* was found to produce at least one glucose dimer not being cellobiose that potentially can act as inducer (**Paper D**).

6.2.3 Selection of *Penicillium brasilianum*

Enzymatic hydrolysis of pretreated biomass is a complex process to understand in detail, the reasons being a large difference between biomass substrates, pretreatment methods and conditions, a heterogeneous and insoluble substrate. A number of important parameters is listed in figure 6.1. To understand and optimise the hydrolysis of cellulose, we focused on efficient carbon utilization and how to efficiently convert cellulose and hemicelluloses into usable building blocks for bioethanol fermentation.

Cellulose is a recalcitrant substrate and high enzyme loadings are required for hydrolysis. The loading is usually five to ten mg enzyme per g cellulose, and therefore it is important to have relatively large amounts of enzyme available. One challenge in working with wildtype microorganisms is that they seldom produce large quantities of enzyme mixtures used to obtain our biotechnological objectives. The actual enzyme available for characterization is often reduced further due to losses during purification. Here, for example, only 50μg of a GH3 BG from *P. brasilianum* was purified from a mixture of cellulolytic enzymes. The low yield was a combination of BGs constituting only a small fraction of the secreted proteins by cellulolytic microorganisms and no prior information on the BGs from *P. brasilianum*, why the purification protocol was not optimised (**Paper D**).

The cellulolytic enzymes from the three Penicillia were comprised of equivalent BG activity, as determined by hydrolysis of steam-pretreated spruce and when compared to a commercial product Celluclast 1.5L FG (Figure 6.4). The enzyme mixture from *P. brasilianum* yielded a saccharification of 48 % saccharification of steam-pretreated spruce, and 55 % with BG activity in surplus. The same saccharification yields were observed for the other Penicillia enzyme mixtures. Celluclast, however, performed poorly with less than 20 % saccharification without a surplus of BG activity. With surplus BG activity in the hydrolysis reaction, Celluclast reached a slightly higher saccharification of 59 % compared to the Penicillia (**Paper C**). The ratio between BG

and filter paper activity ranged from 1.9 to 7.6 for the Penicillia and it was 0.4 for Celluclast. *Penicillium ethinulatum* has recently also been reported to produce a well-balanced mixture of cellulolytic enzymes, not limited by lack of one enzyme activity (Martins *et al*., 2008). Since *P. brasilianum* produced the highest amount of cellulolytic enzymes and it has the highest specific growth rate, we decided to continue with further investigations of this fungus.

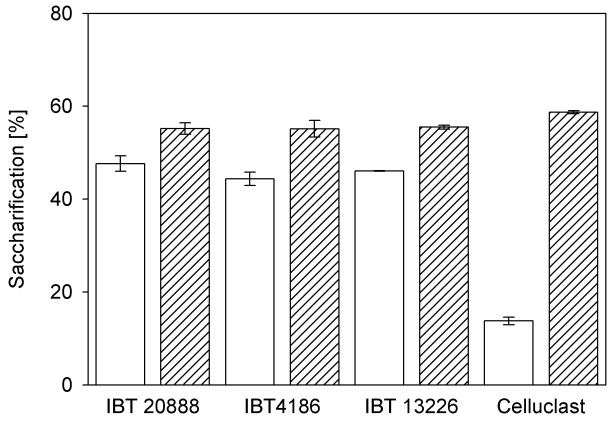


Figure 6.4 Hydrolysis of steam pretreated spruce at 40 °C for 24h by enzyme preparations from *P. brasilianum* IBT20888, *P. pinophilum* IBT4186, *P. persicinum* IBT13226, and Celluclast 1.5L FG. The enzyme loading was 25 FPU/(g cellulose), and the hydrolysis was performed without (no colour) and with (dashed) a surplus of BG activity. The saccharification is calculated relative to the theoretical amount (n=2) (**Paper C**).

6.3 Cellulolytic enzyme system from *Penicillium brasilianum*

The cellulolytic enzymes produced by *P. brasilianum* were investigated in closer detail for their ability to hydrolyse cellulose from different pretreated biomass samples. Both the time course and the effect on hydrolysis by enzyme loading were investigated. At a relatively high enzyme loading 25 FPU/ g cellulose (35 mg protein/ g cellulose), the cellulolytic enzyme system from *P. brasilianum* degraded the cellulose fraction in steam-pretreated spruce (SPS) faster and to a larger extent than a commercial mixture (3:1 Celluclast 1.5L: Novozym 188 [CN]) traditionally used for cellulose hydrolysis. However, at a lower enzyme dosage the two enzyme mixtures performed similarly (Figure 6.5 A and B). On wet-oxidised wheat straw CN reached a higher degree of saccharification than the cellulase fraction from *P. brasilianum* at high and low enzyme loadings (Figure 6.5 C and D). In contrast to acidic-steam pretreatment, which removes hemicelluloses, the wet-oxidization pretreatment removes lignin. The observed difference between the performance of individual enzyme systems on the two

substrates may therefore be a result of different pretreatment methods. The observation that not one enzyme system was optimal for two different substrates is important. This suggests that the best approach for developing an enzyme mixture for degrading biomass is to use enzymes from multiple hosts or enzymes induced differently.

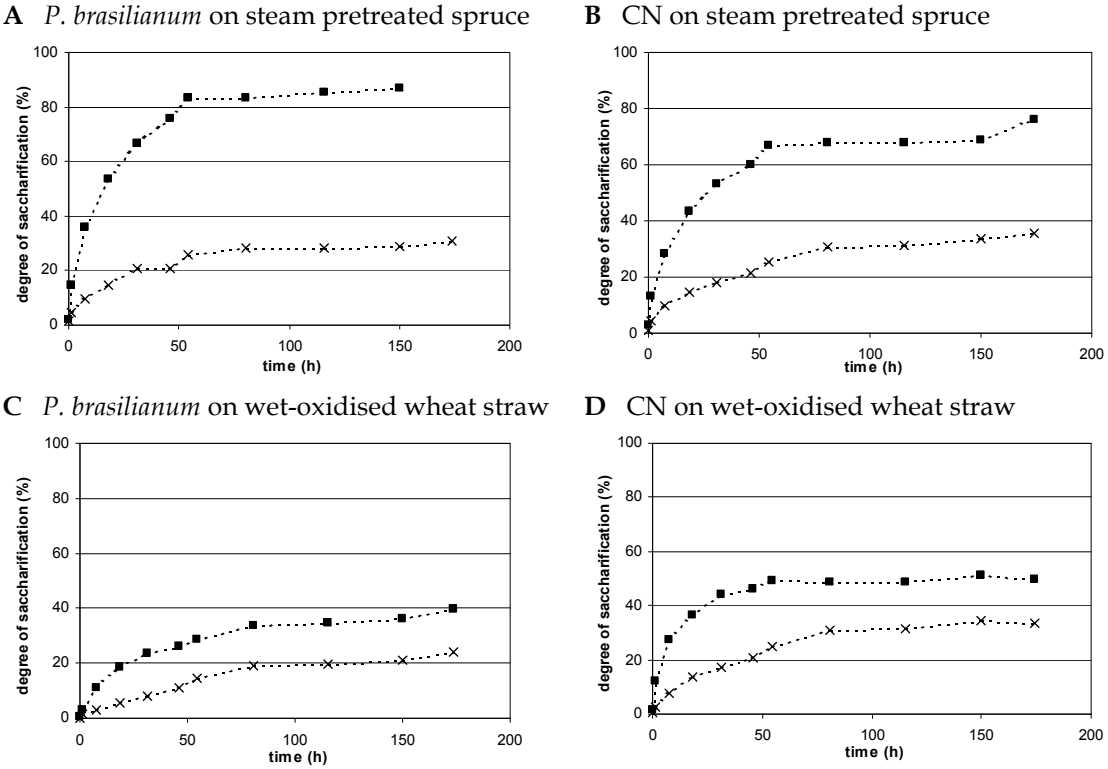


Figure 6.5 Hydrolysis of pretreated biomass samples using enzymes from *Penicillium brasilianum* and Celluclast supplied with Novozym 188 (CN). **A** Enzyme mixture from *P. brasilianum* **B** Celluclast/Novozym 188 on SO_2 impregnated spruce followed by steam pretreatment, **C** enzyme mixture from *P. brasilianum* **D** Celluclast/Novozym 188 on wet-oxidised wheat straw. The enzyme loading was 5 FPU/ g cellulose (✕) and 25 FPU/g cellulose (■).The Celluclast/Novozym 188 was a 3:1 mixture of Celluclast 1.5L and Novozym 188 and the filter paper activity was measured for this mixture. The cellulose concentration was 25 g/L in 50mM acetate pH 5.0, and the temperature was 50 °C.

To investigate differences in performance between the two enzyme systems, I investigated the hydrolytic properties of each enzyme system on different model substrates. The enzyme system from *P. brasilianum* was investigated for its ability to degrade carboxy-methyl cellulose (CMC), a substrate used to measure EG activity. The results demonstrated that the cellulolytic enzyme system from *P. brasilianum* degraded CMC faster, and to a larger extent than CN (Table 6.2). CMC hydrolysis reached a maximum of 2.6 out of 10 glucose backbone residues being released compared to 1.9

for CN (unpublished data). The level of released glucose for CN was reached after 48 hours of hydrolysis, whereas the same level was reached after 1.5 hours for the enzyme system from *P. brasilianum*. The ability to reach a higher level indicated that some of the EGs from *P. brasilianum* had other properties than the EGs originating from Celluclast.

Table 6.2 Hydrolytic performance of enzyme system from *P. brasilianum* and of commercial enzyme preparations on model substrates and pretreated biomass samples.

		Enzyme system[a]					
Substrate	Activity[b]	*P.bras*	CN	U	CNU	CU	CUP
CMC[c]	Glc	+++[d]	+	nd[e]	nd	nd	nd
Pachyman (β-1,3)	Glc	+++	+	nd	nd	nd	nd
Spruce (SO_2)	Glc	+++	++	nd	nd	nd	nd
Wheat straw (wet-ox)	Glc	+	++	nd	nd	nd	nd
Arabinoxylan (soluble)	Ara, Xyl	+++	+	++	++	++	++
Arabinoxylan (Insoluble)	Ara, Xyl	+++	nd	++	nd	nd	nd
Xyloglucan	Xyl, Glc	+	++	nd	nd	nd	nd
Vinasse	Ara, Xyl	++	+	nd	nd	nd	nd
Brewers spent grains	Ara, Xyl, Glc	++	+	++	++	++	++
Peahulls	Ara, Xyl, Glc, Gal	++	+	++	nd	nd	nd

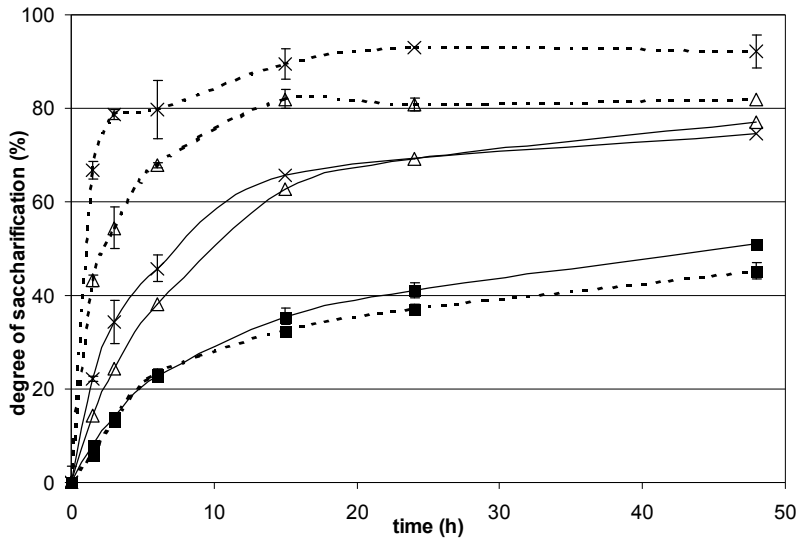
[a] Enzyme dosage is same enzyme protein/dry matter, *P.bras*: *P. brasilianum*, C: Celluclast 1.5L FG, N: Novozym 188, U: Ultraflo L, P: *P.bras* [b] Released sugars measured, [c] Carboxy methyl-cellulose, [d] +++: very good, ++: good, +: bad, [e] nd: not determined.

6.4 Hemicellulolytic enzyme system from *Penicillium brasilianum*

Due to the previously described cellulolytic properties for the enzyme mixture from *P. brasilianum*, it was decided also to investigate the hemicellulolytic properties of the enzyme mixture from *P. brasilianum*. The substrates were brewers spent grain (a commercial low value by-product from beer and alcohol production) and arabinoxylan from wheat. These two hemicellulosic substrates represent two different types of arabinoxylan. The arabinoxylan in brewers spent grain is a quite insoluble and recalcitrant hemicellulose fraction remaining after ethanol production from grain. Whereas, the arabinoxylan from wheat is soluble arabinoxylan isolated from the from wheat endosperm. On both hemicelluloses, the enzyme system from *P. brasilianum* released significantly more arabinose and xylose as compared to CN (Table 6.2). This was expected since Celluclast has been developed as a cellulolytic enzyme preparation. Ultraflo (Novozymes) is a commercial enzyme mixture with hemicellulolytic activities. The enzymes from *P. brasilianum* released more xylose and more arabinose from soluble arabinoxylan, whereas Ultraflo was slightly better on recalcitrant and heterogeneous hemicelluloses from brewers spent grain (Figure 6.6). One reason for the better performance of *P. brasilianum* can be the presence of ferulic acid esterases. These esterases improve the hydrolysis of plant cell wall material (Crepin *et al*., 2004) by opening up the lignin-hemicellulose structure (Figure 3.3). *P. brasilianum* has been

found to produce at least two ferulic acid esterases, and during hydrolysis of brewers spent grain, the release of xylose and arabinose correlated to the simultaneous release of ferulic acid (Panagiotou *et al*., 2007).

A



B

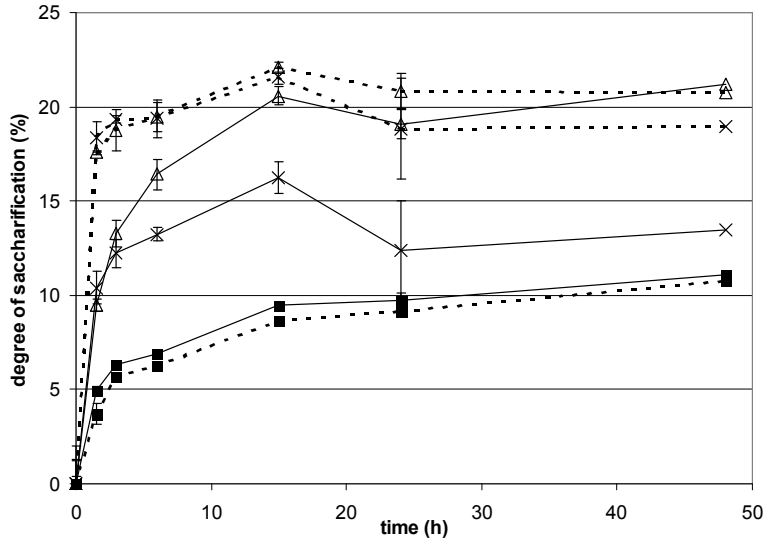


Figure 6.6 A Hydrolysis of soluble arabinoxylan from wheat (Megazyme) and **B** brewers spent grain (Carlsberg) with a substrate concentration of 100 g/L in 50mM acetate pH 5.0. Xylose release is a solid line, and arabinose release is a dotted line. The enzymes used were CN (■), *P. brasilianum* (✕), and Ultraflo (△). The enzyme loading was 25 FPU/ g substrate. Ultraflo was dosed at the same protein level as CN. The hydrolysis time was 48 hours, and the temperature was 50 °C. The hydroysis was made as duplicate experiments.

The recalcitrant nature of biomass requires long reaction times for hydrolysis, which requires enzyme stability. The stability of the individual hemicellulolytic enzymes was tested during 5 days of incubation at relevant conditions (pH 5.0 and T=50 °C). The residual α-L-arabinofuranosidase and β-xylosidase activity were higher than 60 % after 5 days of incubation for the enzyme mixtures from *P. brasilianum* and CN (Figure 6.7). After 3 days of incubation, the residual endoxylanase activity was less than 10 % for CN and 50 % for the enzyme mixture from *P. brasilianum*, and *P. brasilianum* therefore produces at least one xylanase with better thermostability than CN (Figure 6.7). The endoxylanase stability data for the enzyme mixture from *P. brasilianum* indicated that the enzyme mixture contained at least two xylanases, one with inferior stability and one with significantly superior thermostability at pH 5.0 compared to CN (Figure 6.7).

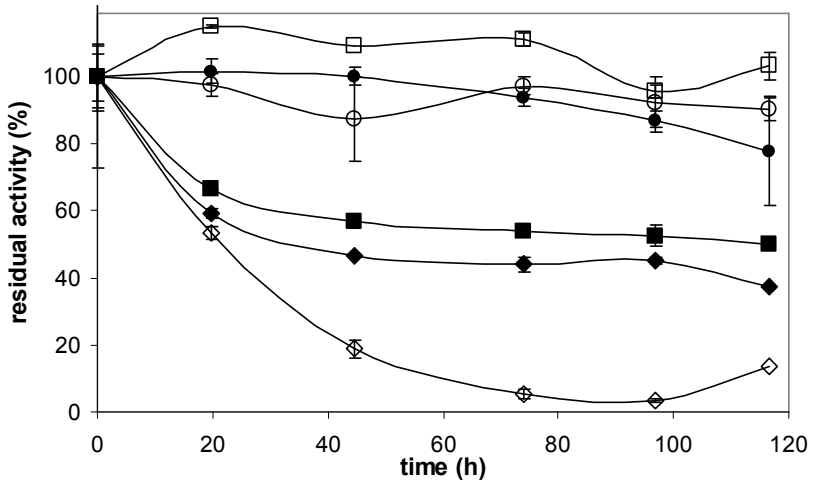


Figure 6.7 Stability of hemicellulolytic enzyme activities found in the enzyme mixture from *P. brasilianum* (filled symbols) and Celluclast (open symbols). For the activity, squares represent β-xylosidase, circles α-L-arabinofuranosidase, and diamonds endoxylanase (n=3).

6.5 Cellulolytic enzymes from *Penicillium brasilianum*

A frequent challenge in enzyme biochemistry is purifying enough enzyme to carry out characterization experiments. This was the case with cellulolytic enzymes purified from *P. brasilianum* cultivation broths. A great opportunity to pursue the investigations of the cellulolytic enzymes from *P. brasilianum* arose with an invitation from Joel Cherry to clone and express cellulolytic enzymes from *P. brasilianum* in his laboratories at Novozymes Inc. in California. The aim of the collaboration was to produce cellulolytic enzymes heterologously in order to have the enzymes as mono components and in amounts allowing both enzyme characterizations and hydrolysis studies. My goal was to clone at least one CBH, one EG, and one BG, so it would be possible to reconstitute the cellulolytic system and to investigate the synergy between the

individual enzymes. The approach taken was to create a genomic library and screen this library for genes encoding cellulolytic enzymes using probes designed on the basis of N-terminal amino acid sequences of already purified cellulolytic enzymes and conserved regions known for the specific GH families (Figure 6.8). With the genes identified, they were to be expressed in an *Aspergillus oryzae* strain known to produce high titres of secreted proteins.

Purification of cellulolytic enzymes from cultivation broth

N-terminal sequencing (10-20 AA of 200-1000 AA)

Good genomic library (size and coverage)

Probe preparation (N-terminal sequence and consensus sequence)

Screen the genomic library

Sequence all the found DNA fragment (genome walking for non full-length genes)

Prepare an expression construct

Transform into the production hosts (*A. oryzae* and *A. niger*)

Screen 20 transformants in each host to find the best producer

Spore purification of top3 producers (3 rounds)

Shake flask evaluation of production

Production in 2L scale

Purification

Figure 6.8 Flow chart of cloning and expression work in *P. brasilianum*.

6.5.1 *Penicillium brasilianum* genes encoding cellulolytic enzymes

Isolated DNA from *P. brasilianum* was fragmented to a size of 3 to 6 kb ensuring that most of the genes found in the screen for cellulolytic enzymes were full length (figure 6.8). The genomic library screened contained 35,000 clones. With a fragment size of 3 kb and an assumed genome size of 34 Mb (as reported for *Aspergillus niger* (Cullen, 2007)), the coverage of the genome was 3.0X. The likelihood of finding the cellulolytic enzymes searched for was 95 %[2]. Since several of the enzymes were purified and

[2] The propability of finding a gene, p, was calculated from a Poisson distribution as

Number of clones = ln[1-p]/ln[1-(fragment size/genome size)].

characterised beforehand, I chose to search for these specific cellulolytic enzymes (the native enzymes listed later in Table 6.4). By screening the genomic library, I found nine DNA sequences encoding cellulolytic enzymes. Four of the nine sequences were full length (Table 6.3). The full sequence for the remaining cellulolytic enzymes was determined by genome-walking approach. Having this information the genes can be cloned and expressed.

Table 6.3 Overview of DNA sequences found during genome screening for sequences encoding cellulolytic enzymes in *Penicillium brasilianum*.

Class	GH family	Probes[a]	Sequence	Expressed & purified
BG	Cel3A	N/C	Full	yes
BG	Cel3B	N/C	Partial	no
EG	Cel5A	N/C	Partial	no
EG	Cel5B	N/C	Partial	no
EG	Cel5C	N/C	Full	yes
EG	Cel12A	N/C	Partial	no
CBH	Cel6A	C/C	Partial	no
CBH	Cel7A	N/N	Full	no
CBH	Cel7B	C/C	Full	yes

[a] N: primer designed from N-terminal amino acid, C: primer designed from consensus sequence.

The approach taken to find sequences encoding cellulolytic enzymes did not lead to the finding of new cellulolytic enzyme sequences, since prior knowledge of the amino acid sequences was used to design the probes used to find the genes. If the goal is to find a completely unknown cellulolytic enzyme, the genomic library can be created such that the clones are screened for enzyme activity instead of sequence. One clear advantage of starting with the purified enzyme is that it ensures that the final heterologously produced enzyme maintains the same characteristics as the native enzyme.

6.5.2 Heterologous production

When a heterologous host is chosen, the glycosylation of the enzyme can be changed due to the different glycosylation "machinery" of the new host (Kowarik *et al*., 2006). Expression of *Tr*Cel7A in *Aspergillus niger* has been demonstrated to result in increased N-glycosylation (Jeoh *et al*., 2008). Change in growth conditions can also occur, for example, studies have demonstrated that glycosylation of native *Tr*Cel7A varies considerably with growth conditions (Stals *et al*., 2004). There are no general rules on how changes in glycosylation pattern will impact enzyme characteristics such as specific activity and stability. O-glycosylation of the linker in carbohydrases is known to affect the stability (Neustroev *et al*., 1993) and the conformation of the enzyme (Receveur *et al*., 2002). N-glycosylation has reportedly affected stability and specific activity (Neustroev *et al*., 1993), and in other cases no effect has been observed (Boer *et al*., 2000). With no general rules for the effects of changed enzyme glycosylation, it

must be tested for the specific enzyme heterologously expressed if possible. The heterologous production of Cel3A changed neither the specific activity nor the molecular weight of the enzyme (**Paper D**). However, heterologous production of Cel5C increased the degree of glycosylation while specific activity remained the same (**paper E**).

I succeeded in having three cloned and expressed cellulolytic enzymes from *P. brasilianum*: one BG, one EG and one CBH (Table 6.4). Given that *P. brasilianum* produced a well-balanced mixture of cellulolytic enzymes compared to Celluclast, and most work in the field of cellulolytic enzymes had been done on different CBHs and EGs, characterization of the BG was priority. Apart from the BG deficiency in Celluclast, the major difference between the mixture of cellulolytic enzymes from *P. brasilianum* and *T. reesei* is that the enzyme mixture from *P. brasilianum* contains more EG and less CBH without a loss in specific filter paper activity, and therefore I decided to characterise the cloned EG after the BG characterization. Thirdly, the CBH was to be characterised, but due to time limitations this enzyme still awaits characterization.

Table 6.4 Overview of available cellulolytic enzymes from *P. brasilianum*.

Class	GH family	Availability	Name[a]	Comment
BG	Cel3A	native & recombinant		
EG	Cel5C	native & recombinant	EGb1 & EGb2	EGb2 is identical to Cel5C and EGb1 is a degradation product of Cel5C without CBM.
EG	Cel12A	native	EGa	EGa N-terminal amino acid sequence is found in Cel12A DNA sequence.
CBH	Cel7A	native	CBHb	N- and internal MS-sequences confirmed identity to CBHb.
CBH	Cel7B	recombinant		
CBH	Cel6	native	CBHa	N-terminal blocked, so identity to the Cel6A gene has not been checked

[a] refers to names used in previous papers (Jørgensen *et al.*, 2003b; Jørgensen *et al.*, 2003a; Jørgensen and Olsson, 2005)

6.6 β-glucosidase, Cel3A

Two BGs were identified during purification of the cultivation broth of *P. brasilianum* after cultivation on a mixture of cellulose and hemicelluloses, Cel3A and Cel3B. From the N-terminal amino acid sequence, both sequences were classified as GH3s. Cel3A was present in significantly higher amounts than Cel3B, and Cel3A was therefore further purified to homogeneity (**Paper D**). The native and the recombinant BG had the same molecular mass of 115 kDa, which is a typical mass for a BG (Bhatia *et al.*, 2002). The BG had a level of 20 kDa glycosylation. This level of glycosylation is commonly

found for BGs from filamentous fungi (Chirico and Brown, Jr., 1987; Dan *et al*., 2000; Decker *et al*., 2000). The BG had highest (61.4 %) identity to a characterised GH3 BG from *Talaromyces emersonii* (GenBank AY072918) and 59.5 % identity to a GH3 BG from *T. reesei* (GenBank AY281374). The *Pb*BG was found to have excellent stability, and the residual activity was 100 % after 24 hours of incubation at 60 °C. In comparison, the commercial BG preparation Novozym 188 had only 50 % residual activity after 24 hours of incubation at 60 °C. This stability is higher than reported for most other fungal BGs incubated at 60 °C (**Paper D**). An increased temperature during hydrolysis will result in a higher hydrolysis rate, and therefore increased thermostability is required. The CBHI from *T. reesei* has already been engineered to work optimally at 60°C (Viikari *et al*., 2007).

With a purified BG at hand, a complete cellulase mixture could be made with different ratios of the enzymes needed for complete cellulose hydrolysis. The two types of cellulose used were phosphoric acid swollen cellulose (PASC) and Avicel. The three monocomponent enzymes used to make different mixtures of the individual enzymes were *Pb*Cel3A, *Humicola insolens* Cel6A (CBHII) and *Hi*Cel45A (EGV). Avicel has significantly higher crystallinity than the amorphous PASC and the degree of hydrolysis was too low to evaluate the BG effect on this substrate. The limiting factor for Avicel hydrolysis was reducing the hydrolyzed cellulose into smaller oligosaccharides, and the surplus of BG readily converted all released cellobiose to glucose. PASC is far more accessible for cellulolytic enzymes, and higher hydrolysis rates resulted in an accumulation of cellobiose. *Pb*Cel3A was required to reduce this accumulation, thereby decreasing the strong cellobiose inhibition of CBH. The addition of *Pb*Cel3A generated the highest conversion (Andersen *et al*., 2008a).

The developed LC-MS method for measuring glucose inhibition of cellobiose hydrolysis revealed that a glucose dimer different from cellobiose was formed during the process. This result demonstrated that *Pb*Cel3A also could do transglycosylation. The production of transglycosylation compounds would be very interesting for further investigation since non-metabolisable glucose dimers produced at a low cost could prove to be very useful as inducers in the production of cellulolytic enzymes. At present, different cellulosic substrates are used in the production of cellulolytic enzymes. An elegant solution would be the cultivation of a fungal strain relieved of carbon catabolite repression with glucose or xylose as its carbon source with a non-metabolisable inducer.

6.7 Endoglucanase, Cel5C

Following cultivation on cellulose, an important difference between the cellulolytic enzyme system produced by *P. brasilianum* as compared to *T. reesei* was a relatively

higher content of EGs in the cellulolytic enzyme system ((Table 5.1 and (Jørgensen *et al*., 2003a)). The main EG constituents of the cellulolytic system from *P. brasilianum* were Cel5C and Cel12A. Based on sequence information, *Pb*Cel5C was most closely related to the GH5 FII-CMCase from *Aspergillus aculeatus* (Takada et al., 2002), and to the EG from *Thermoascus aurantiacus* (Hong et al., 2003). Cel12A has the highest similarity to a GH12 EG from *Aspergillus aculeatus* (Ooi *et al*., 1990). A reason for working with Cel5C is that the full length DNA sequence was found in the screening for cellulolytic genes in *P. brasilianum*. More importantly, Cel5C has a CBM involved in the hydrolysis of crystalline cellulose as compared to Cel12A, which does not. The CBM is important for the hydrolysis rate (Tomme *et al*., 1988), and in the specific case of Cel5C, earlier results demonstrated that the hydrolysis of Avicel was reduced when Cel5C lacked the CBM (Jørgensen *et al*., 2003a).

Two GH5 EGs, EGb1 and EGb2 (Table 6.4) have been purified in our group (Jørgensen *et al*., 2003a). We concluded that *Pb*Cel5C and EGb2 are synonymous and that EGb1 is a degradation product of these (**paper E**). The structure of Cel5C has common traits with other cellulolytic enzymes namely a catalytic core, a linker and a CBM. The CBM belongs to CBM family 1, a family with more than 241 members (CAZy count 3Jan2008) almost exclusively of fungal origin. The CBM is positioned at the C-terminal end, a trait *Pb*Cel5C shares with its closest relatives in the GH5 family. This contrasts with the predominant N-terminal position of CBM1 in most other fungal GH5 EG family members, such as *T. reesei* (Saloheimo *et al*., 1988), *Humicola insolens* (Dalboge and Heldt-Hansen, 1994), and *P. janthinellum* (Mernitz *et al*., 1996). The *Pb*Cel5C linker was heavily O-glycosylated. This property has also been reported for several other CBHs and EGs (Hui *et al*., 2002). After 20 hours of incubation at pH 5.0 and 50 °C, *Pb*Cel5C retained full activity. If the temperature was raised to 60 °C no residual activity was detected (**paper E**).

6.8 Cellobiohydrolase

Two full-length CBH genes were obtained from the work on the genomic library encoding *Pb*Cel7A and *Pb*Cel7B. *Pb*Cel7A was found to have a CBD from CBM1 at the C-terminal extremity as for *Tr*Cel7A, *Tr*Cel7B, and *Tr*Cel5A, while *Pb*Cel7B was found to have neither a CBD nor a linker. A search among publicly available protein sequences showed that the deduced amino acid sequence of both CBHs showed highest similarity to fungal Cel7 cellobiohydrolases. I decided to start with cloning and expression of the *Pb*Cel7B, since it had a higher degree of novelty with only few other reported CBHs without a CBD .

6.8.1 Cel7A

To classify the discovered cellobiohydrolase, a similarity search was conducted with the omission of the linker and the CBD, since a N-terminal or C-terminal CBD would influence the search result. The enzymes with the highest similarity all belonged to GH family 7. The enzymes with highest similarity were CBHa from *P. janthinellum*, *Pb*Cel7B, and CBHa from *Aspergillus niger* (Table 6.5).

Table 6.5 Similarity between *Pb*Cel7A catalytic domain and selected Cel7 enzymes.

Enzyme	Organism	Identity	Reference
CBHa	*Penicillium janthinellum*	86.1 %	(Koch *et al*., 1993)
Cel7B	*Penicillium brasilianum*	80.7 %	This study
CBHa	*Aspergillus aculeatus*	70.9 %	(Takada *et al*., 2002)
CBHI	*Phanerochaete chrysosporium*	66.9 %	(Covert *et al*., 1992)
CBHI (Cel7A)	*Trichoderma reesei*	66.0 %	(Shoemaker *et al*., 1983)
EGI (Cel7B)	*Trichoderma reesei*	39.4 %[a]	(Penttila *et al*., 1986)

[a] Not ranking 5th in similarity, but selected for comparison.

The CBD was found to belong to CBM1 as all other fungal CBDs do. The *Pb*CBD showed the most identity (88.9 %) to the CBD from a cellobiohydrolase from *P. janthinellum* (Figure 6.9) (Koch *et al*., 1993).

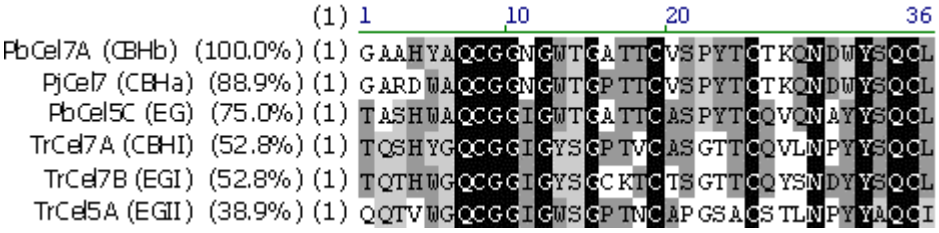


Figure 6.9 Alignment of the CBD from *P. brasilianum* with selected CBDs. Black amino acid residues are highly conserved.

6.8.2 Cel7B

The enzymes with highest identity to the deduced amino acid sequence of the cloned CBH were CBHa from *P. janthinellum* and CBHI from *Phanerochaete chrysosporium* (Table 6.6). With *Pb*Cel7A already discovered, the cloned CBH was named *Pb*Cel7B. *Pb*Cel7B was found to have only a catalytic core as earlier reported for CBHa from *Aspergillus niger* (Gielkens *et al*., 1999) and for CBHI from *Phanerochaete chrysosporium* (67.8 % identity with *Pb*Cel7B)(Covert *et al*., 1992). The nucleotide sequence of the *cel7B* gene encodes a polypeptide of 452 amino acids. Using the SignalP software program (Nielsen *et al*., 1997), a signal peptide of 17 residues was predicted. The predicted N-terminal sequence was confirmed by N-terminal sequencing. The predicted mature protein contains 435 amino acids of 45,8kDa with a pI of 3.98. The molecular mass was

found to be higher around 55kDa when measured by SDS-PAGE. The heterologously expressed *Pb*Cel7B was treated with an endoglycosidase removing N-linked glycosylations. Both treated and untreated *Pb*Cel7B was analysed by LC-MS, which determined the molecular weight very precisely. The MS-spectra revealed that the N-terminal was pyroglutamic acid, and that *Pb*Cel7B has both O- and N-linked glycosylations. Both predicted N-glycosylation sites were indeed glycosylated. Extensive O-glycosylation in the linker region has been reported for several other CBHs and EGs (Hui *et al*., 2002), but since Cel7B does not have a linker, the observed O-glycosylation must be on the surface of the catalytic core as it is for Cel6A (CBHII) from *Humicola insolens* (Varrot *et al*., 1999).

Table 6.6 Similarity between *Pb*Cel7B and selected Cel7 enzymes

Enzyme	Organism	Identity	Reference
CBHb (Cel7A)	*Penicillium brasilianum*	80.7 %	This study
CBHa	*Penicillium janthinellum*	70.9 %	(Koch *et al*., 1993)
CBHI	*Phanerochaete chrysosporium*	67.8 %	(Covert *et al*., 1992)
CBHa	*Aspergillus aculeatus*	67.5 %	(Takada *et al*., 2002)
CBHI (Cel7A)	*Trichoderma reesei*	63.9 %[a]	(Shoemaker *et al*., 1983)
EGI (Cel7B)	*Trichoderma reesei*	41.2 %[a]	(Penttila *et al*., 1986)

[a] Not # 4 and 5 in identity, but selected for comparison reasons.

The CBD is a prerequisite for the CBH hydrolysis of crystalline cellulose, but on more amorphous cellulose the CBD is of minor importance (Linder and Teeri, 1997). It has been suggested that cellulolytic enzymes with CBDs are most important in the beginning of cellulose hydrolysis when the substrate needs to be solubilised. Once a part of the substrate has been made more accessible, cellulolytic enzymes without CBDs may be more efficient. No CBHs without a CBD have been identified in *T. reesei*, but the cellulolytic enzyme mixture from *T. reesei* contains protease activity that through proteolysis produce a fraction of CBHs without a CBD during hydrolysis (Dienes *et al*., 2007).

The factors determining the hydrolysis rate of phosphoric acid swollen cellulose (PASC) have been investigated using monocomponent enzymes including the three monocomponent enzymes from this PhD study. In the hydrolysis of PASC with *Pb*Cel3A, *Humicola insolens* Cel6A (CBHII) and *Hi*Cel45A (EGV), *Pb*Cel7B increased hydrolysis most in comparison to the other CBHs tested - *Hi*Cel7A (CBHI) and *Hi*Cel6A (CBHII) (Andersen *et al*., 2008b).

6.9 Enzyme kinetic determinations

In my work characterizing BG from *P. brasilianum*, I discovered some limitations of using artificial substrates for enzyme characterization. One of these limitations was the

ability to measure glucose inhibition of a BG. This obstacle led to the development of an elegant assay to measure this inhibition.

6.9.1 Artificial substrates for enzyme characterization

Artificial substrates are often used for enzyme kinetics due to availability, price, and time saving activity measurements. This is also the case for BG, where cellobiose is the natural substrate. However, *para*-nitrophenyl-β-D-glucopyranoside (pNP-glucose) has frequently been used as substrate in the literature. Michaelis-Menten kinetic studies using these two substrates resulted in different measured maximum specific activities, and a 30-fold difference in substrate affinity. A clear difference was also observed for the inhibition constants using different inhibitors. D-glucono-1,5,-δ-lactone (GL) mimics the transition state analogue in cellobiose hydrolysis. I used GL, as well as glucose, to investigate BG inhibition. In the hydrolysis of pNP-glucose using *Pb*Cel3A, GL inhibited the hydrolysis 20 times more strongly than glucose, and in cellobiose hydrolysis GL inhibited the hydrolysis 100 times stronger than glucose (**Paper D**). None of the above mentioned ratios between inhibition constants were found when the BG from Novozym188 was characterised (**Paper D**). Therefore, it is important that both the substrate and the inhibitor are the same when comparing inhibition data from the literature. All of these observations are very important to keep in mind when setting up a new assay or when comparing results reported in different published reports.

6.9.2 Glucose inhibition of BG activity

Currently, determining glucose inhibition of cellobiose hydrolysis is almost impossible, since measuring the released glucose in the presence of excess glucose poses a large analytical challenge. For this reason, pNP-glucose has previously been used as substrate. In this PhD study, a method was developed to measure glucose inhibition of cellobiose hydrolysis.

Constant reaction rates are required in the determination of kinetic parameters, and to ensure a constant reaction rate during cellobiose hydrolysis, only a fraction of the cellobiose can be hydrolyzed. Thus, at the lowest cellobiose concentration used for kinetic measurements (0.22 mM corresponding to $0.14K_M$ as determined in **Paper D**), only very small amounts of glucose are produced. At this cellobiose concentration, 10 % conversion will result in 0.044 mM glucose (8 mg/L). When glucose is present at a concentration of $5K_i$ (5.5 mM – 0.99 g/L), high analytical precision is required to detect the relatively small changes in the amounts of glucose produced. The developed method quantified glucose inhibition accurately using glucose-^{13}C and MS. In the assay glucose-$^{13}C_6$ was used as inhibitor instead of glucose, as testing showed that the labelling did not change the inhibition properties. Not only is glucose-$^{13}C_6$ an inhibitor

that can be distinguished from the produced (naturally labelled) glucose by mass spectrometry, but it also served as an internal standard for quantification (Stokvis *et al*., 2005), thereby allowing an accurate determination of the glucose produced and the inhibition constant. The inhibition constant for BG Cel3A in cellobiose hydrolysis was found to be 1.1 mM for glucose (0.2 g/L). Using the estimated kinetic parameters, the maximum specific activity will be reduced by 80 % when the glucose and cellobiose concentration is 1 g/L (**Paper D**).

6.10 Adsorption of cellulolytic enzymes on lignocellulose

During the hydrolysis of pretreated lignocellulosic substrates, it has been reported that enzymes from the cellulolytic system adsorb to the lignin fraction as well as to the cellulose fraction. The adsorption to lignin not only limits the hydrolysis, it also reduces the possibility of recycling the enzymes, which is a way to reduce the total cost (Lee *et al*., 1995). A study has shown that after complete hydrolysis of pure cellulose (Avicel) almost all cellulolytic protein could be recovered (Boussaid and Saddler, 1999), whereas on pretreated spruce only 45 % of the cellulolytic protein could be recovered after complete hydrolysis (Palonen *et al*., 2004b). The effect of enzyme adsorption onto lignin has also been demonstrated by alkaline H_2O_2-catalysed removal of the lignin fraction in steam pretreated Douglas fir. After lignin removal, the sugar yield increased significantly from 59 % to 82 %, and the enzyme dosage could be reduced six times (Yang *et al*., 2002).

6.10.1 Effect of a surfactant, Tween20

Previous studies on the hydrolysis of filter paper, a pure cellulosic substrate, demonstrated that the hydrolysis was reduced when lignin from pine was added. The reduction in hydrolysis was diminished when different surfactants were added (Sewalt *et al*., 1997). In the hydrolysis of SO_2 pretreated spruce (SPS) I observed a significant increase in hydrolysis rate when Tween20 was added to the hydrolysis reaction (Figure 6.10). The increase in hydrolysis rate was less pronounced on wet-oxidised wheat straw. During wet-oxidization a large lignin fraction of the lignin is removed by oxidization and the remaining fraction of the lignin is most likely also oxidised to an extent causing reduced enzyme adsorption. Both the effect of Tween20 and oxidization of the lignin fraction after wet-oxidization suggested that hydrophobic interactions take place between enzyme and lignin, as also suggested by (Berlin *et al*., 2005a; Berlin *et al*., 2005b; Palonen *et al*., 2004b; Tu *et al*., 2007). BSA is known to adsorb to surfaces (Haynes and Norde, 1994) and it has been demonstrated that BSA can increase the hydrolysis rate to the same extent as Tween20 but no additional effect has been observed when both were added (Eriksson *et al*., 2002a). The mechanism of action for

both Tween20 and BSA is that they both bind to the hydrophobic parts of the lignin complex thereby preventing hydrophobic interactions between the cellulase and the lignin complex (Eriksson *et al*., 2002a; Yang and Wyman, 2006).

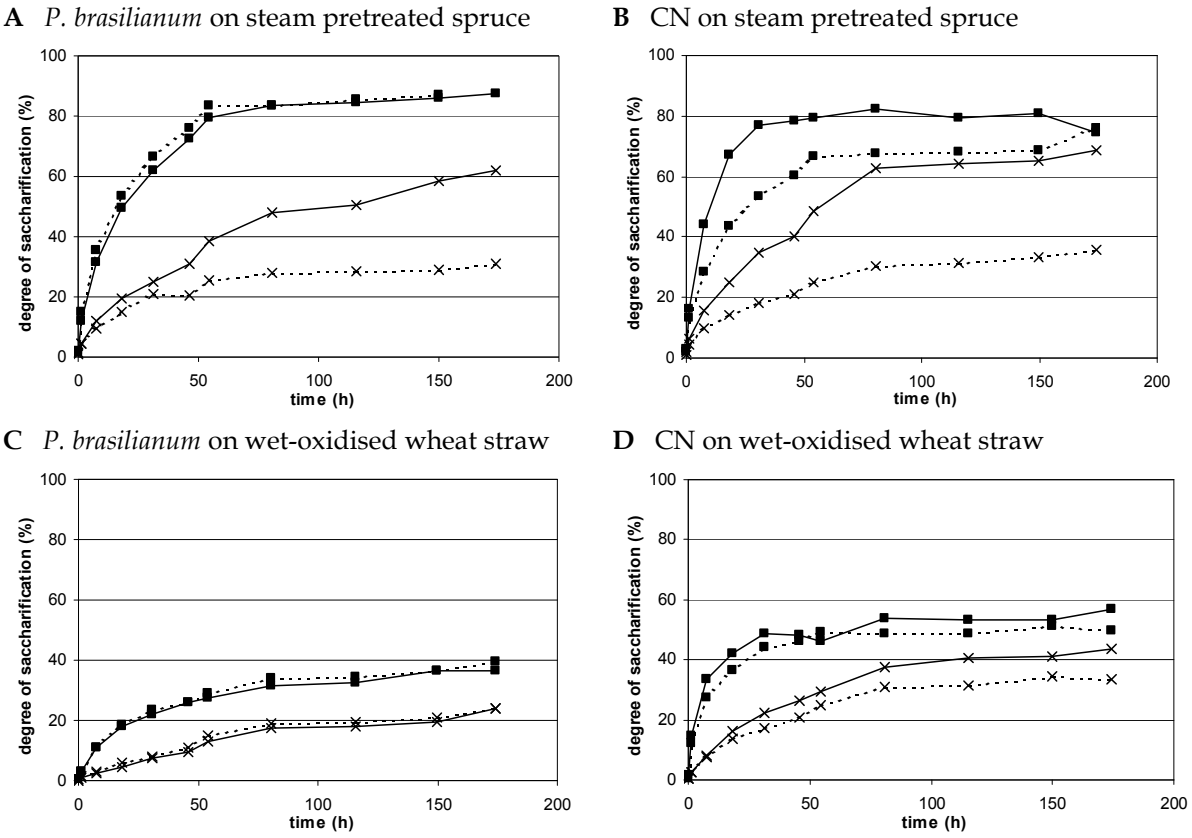


Figure 6.10 Hydrolysis of pretreated biomass samples using enzymes from *Penicillium brasilianum* and Celluclast supplied with Novozym 188 (CN). **A** Enzyme mixture from *P. brasilianum* **B** Celluclast/Novozym 188 on SO_2 impregnated spruce followed by steam pretreatment, **C** enzyme mixture from *P. brasilianum* **D** Celluclast/Novozym 188 on wet-oxidised wheat straw. The enzyme loading was 5 FPU/ g cellulose (✕) and 25 FPU/g cellulose (■). Dotted lines represent hydrolysis without Tween20 and full lines represent hydrolysis with Tween20. The Tween20 concentration was 0.05 g/g DW. The Celluclast/Novozym 188 was a 3:1 mixture of Celluclast 1.5L and Novozym 188 and the filter paper activity was measured for this mixture. The cellulose concentration was 25 g/L in 50mM acetate pH 5.0, and the temperature was 50 °C.

6.10.2 Basics of Cel5C adsorption to Avicel and SPS

In my hydrolysis studies of SPS, there appeared to be a protein loading above which, the effect of Tween 20 was reduced. To investigate the adsorption phenomenon more rigorously, I used my monocomponent Cel5C EG and a pure cellulosic substrate, Avicel, and SPS. Different temperatures were investigated for adsorption, and even though higher temperatures would result in faster equilibrium, I decided to conduct the adsorption experiments at 4 °C, because higher temperatures resulted in hydrolysis

and therefore concurrent change of substrate (**Paper E**). The adsorption parameters are assumed to be independent of the temperature, so the only effect of an elevated temperature will be that the system reaches equilibrium faster. For both substrates, equilibrium between enzyme adsorbed and enzyme in solution was reached after three hours, with 90 % of the equilibrium value reached after half an hour. The adsorption to both Avicel and SPS was found to follow The Langmuir isotherm. Adsorption has previously been described using the Langmuir isotherm for *T. reesei* CBHI (cel7A) and EGII (Cel5A) on softwood SPS (Palonen *et al*., 2004b) and on hardwood SO_2 impregnated steam pretreated willow (Karlsson *et al*., 1999) and also for the whole cellulolytic enzyme mixture from *T. reesei* (Ooshima *et al*., 1990). The maximum adsorbed amount (W_{max}) was found to be in the same range for the adsorption on Avicel and SPS, (40 and 49 mg/g substrate, respectively). In spite of the similar W_{max} for the two substrates, the Langmuir isotherms were different due to the adsorption equilibrium constants. The affinity for SPS was an order of magnitude lower than for Avicel (**Paper E**). To explain the observed effect of Tween20 at low enzyme loadings (5 FPU/g cellulose) and not at high FPA loadings (25 FPU/g cellulose) in the hydrolysis of SPS using *P. brasilianum* cellulolytic enzyme mixture (Figure 6.10A), a rough estimation was made based on several assumptions. During SPS hydrolysis the cellulose concentration was 25 g/L, with 60/40 % cellulose/lignin in SPS, and the lignin concentration was 17 g/L. The maximum adsorbed Cel5C could be 800 mg Cel5C / L. With 25 g cellulose/L and an enzyme loading of 5 FPU/g cellulose and 25 FPU/ g cellulose, respectively, the concentration of cellulolytic enzyme protein was 160 and 800 mg/L, respectively (the specific activity for the *P. brasilianum* mixture was determined to be 0.77 FPU/mg). This calculation demonstrates that the amount of cellulolytic enzyme adsorbed to the lignin fraction is an important parameter in the hydrolysis of pretreated spruce. At low enzyme loadings, the nature of the Langmuir isotherm suggests that a relatively large fraction of the added *Pb*Cel5C adsorb to lignin. This relatively large adsorption to lignin at low enzyme loadings can be seen as a threshold to pass in the amount of FPA added before significant changes in the hydrolysis rate are observed. It will be of great importance to investigate the adsorption of individual cellulolytic enzymes to lignin, since these enzymes most likely will have different Langmuir isotherms due to the number and position of surface-exposed hydrophobic residues. The effects of surface-exposed amino acid residues will be discussed in section 6.10.3.

On SPS the level of affinity for *Pb*Cel5C has been reported for *Tr*Cel5A (Palonen *et al*., 2004b). The 10-fold lower affinity for SPS compared to Avicel is somewhat surprising since 60 % of SPS is cellulose. This observation highlighted the importance of not just investigating adsorption to pure substrates. In adsorption studies on pure cellulose, one may consider using BMCC (bacterial microcrystalline cellulose) instead of Avicel, since BMCC has a higher uniformity than Avicel, which is a cellulose preparation

obtained from wood fibres by partial acid hydrolysis (Gilkes *et al*., 1992). Pretreatment can change both the cellulose surface area and crystallinity (Mansfield *et al*., 1999), and the lignin is also known to redistribute during pretreatment which can result in a changed number of available cellulose binding sites (Wong *et al*., 1988). Therefore it can be difficult to fully elucidate the reason for the 10-fold lower *Pb*Cel5C affinity on SPS compared to Avicel, since several changes occur simultaneously during pretreatment of substrates. Sulphonation could be an additional explanation for the lower affinity to spruce that has been SO_2 impregnated before steam pretreatment compared to Avicel. Softwood is particularly susceptible to sulphonation due to the high content of coniferyl alcohol in the lignin complex (Mooney *et al*., 1998) and 5 % sulphonation (mole sulphonation per mole lignin monomer) has been reported in this instance (Shin and Rowell, 2005). With the introduction of additional hydrophilic groups into the lignin, the hydrophobic interaction between the cellulose binding domain and the cellulose in the proximity of the sulphonated lignin residue may be less favoured.

6.10.3 Cel5C adsorption to cellulose and lignin

The cellulose-binding domain (CBD) is of crucial importance both for the adsorption of cellulolytic enzymes to cellulose and for efficient hydrolysis (as presented in 5.4.3). A study on the adsorption of *T. reesei* CBHI (Cel7A) and EGII (Cel5A) demonstrated that the CBD for both enzymes was the domain that mainly caused adsorption to lignin (Palonen *et al*., 2004b). A recent study on the adsorption of CBHI *Tr*Cel7A and EGI (*Tr*Cel7B) to lignin isolated from spruce showed that *Tr*Cel7B adsorption was higher than *Tr*Cel7A adsorption (Börjesson *et al*., 2007). The authors speculated that more hydrophobic interactions between lignin and the surface exposed aromatic residues on the CBD resulted in the higher observed adsorption.

To investigate the influence of the CBD from *Pb*Cel5C on the enzymes' adsorption to lignin, a comparison was made to other CBDs from known cellulolytic enzymes (Figure 6.11A). The CBD of *Pb*Cel5C and of *Tr*Cel7B had the highest number of aromatic surface residues, but not in the same positions (Figure 6.11C). On top, the CBD of *Pb*Cel5C was found to have a tryptophan on position five. This position is found on the flat face of the CBD (Figure 6.11B), which confers cellulose adsorption as presented in section 5.4.3. The substitution Y5W has also been reported to result in higher adsorption to cellulose for *Tr*Cel7A (Linder *et al*., 1995).

A

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PbCel5C (EG)     TASHWAQCGGIGWTGATTCASPYTCQVQNAYYSQCL
TrCel7A (CBHI)   TQSHYGQCGGIGYSGPTVCASGTTCQVLNPYYSQCL
TrCel7B (EGI)    TQTHWGQCGGIGYSGCKTCTSGTTCQYSNDYYSQCL
TrCel5A (EGII)   QQTVWGQCGGIGWSGPTNCAPGSACSTLNPYYAQCI
PbCel7A (CBHb)   GAAHYAQCGGNGWTGATTCVSPYTCTKQNDWYSQCL
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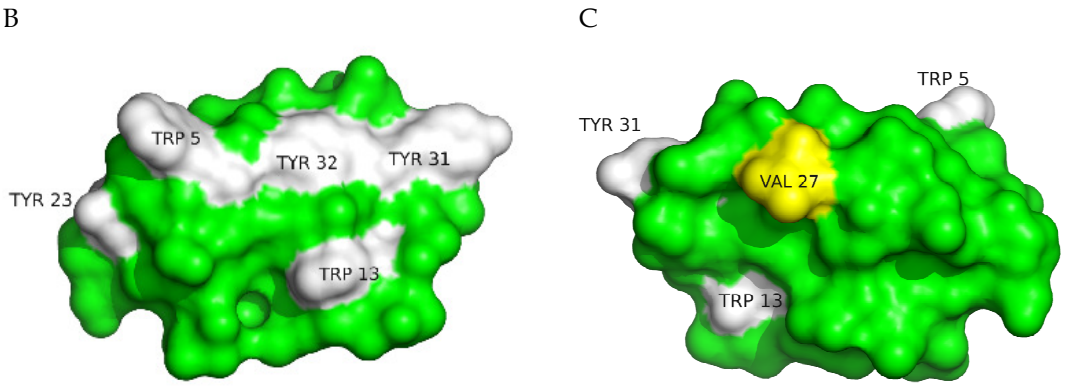

Figure 6.11 A Alignment of CBD from *P. brasilianum* Cel5C and *T. reesei* Cel5A, Cel7A, and Cel7B. Aromatic residues are in bold and residues responsible for cellulose adsorption are underlined. The conserved cysteines involved in disulfide bands are shaded grey. The Prosite consensus pattern is C-G(2)-x(4,7)-G-x(3)-C-x(4,5)-C-x(3,5)-[NHGS]-x-[FYWMI]-x(2)-Q-C. **B** Model structure of the CBD from *Pb*Cel5C. **C** The same structure rotated 180°; the yellow amino acid (valine) is a tyrosine in *T. reesei* Cel7B. Aromatic residues exposed on the surface are marked with white. Numbering starts at first residue in Figure 6.11A. The structure was calculated using the structure 1CBH from *Trichoderma reesei* Cel7A (Kraulis *et al*., 1989).

Adsorption to lignin is not solely caused by the CBD and the catalytic domain of *Tr*Cel5A has been found to have higher affinity for lignin compared to the *Tr*Cel7A catalytic domain (Palonen *et al*., 2004b). It has been suggested that the higher affinity of the *Tr*Cel5A catalytic domain is due to the more open active site in Cel5A compared to the "tunnel" active site in *Tr*Cel7A. In the active site there are aromatic residues exposed to the surface of the enzyme with the purpose of positioning the cellulose fibre correctly for hydrolysis (Palonen *et al*., 2004b). The position of the fiber is stabilised through hydrophobic interactions between aromatic residues and the glucopyranoside rings. The protein structure of *Pb*Cel5C shows several aromatic residues positioned in the open active site (Figure 6.12). The structure of *Pb*Cel5C also revealed that there are several aromatic residues outside of the binding site for cellulose exposed on the surface. One possibility for reducing the adsorption to lignin without altering the hydrolytic activity on cellulose would be to change these residues. To prevent structural changes, it is suggested to substitute these amino acids with smaller residues, it could either be to a less hydrophobic amino acid such as alanine or to a polar amino acid like serine.

A B

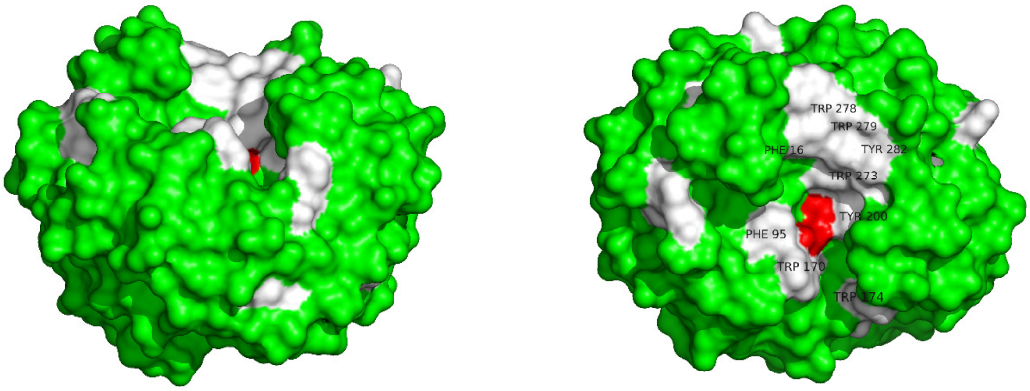

Figure 6.12 A model structure of *Pb*Cel5C. Numbering starts at N-terminal. The catalytic residues are Glu133 and Glu240. A Active site seen from the side. **B** Active site seen from above. Aromatic residues exposed on the surface are marked with white, and the active site is marked with red. The structure was calculated using the crystal structure 1H1N of EGI from *Thermoascus aurantiacus* (van Petegem *et al*., 2002). This EG had 71 % identity to Cel5C.

6.10.4 Adsorption to different biomass substrates

In different parts of the world, there are a large variety of substrates available in sufficient quantities for commercial hydrolysis to monomeric sugars. Among the grasses, rice straw is a potential substrate in Asia and local regions of the USA. Corn stover is a potential substrate in the USA and China, and wheat straw is available in Europe. The softwoods spruce and pine are a potential substrate in the Northern hemisphere. My results demonstrated that there is a significant difference in adsorption to different types of biomass, and that even the pretreatment method is of importance for the adsorption of cellulolytic enzymes to lignin (**Paper E**). These results add to the statement that the commercial process of lignocellulose-to-ethanol is a very complex process and experimentation is necessary to optimise the production of ethanol at individual production sites.

Chapter 7

Concluding remarks and future perspectives

Lignocellulosic materials are abundant and renewable. Therefore, they can be valuable resources to use as raw materials in many biotechnological processes. One identified bottleneck in the enzymatic release of glucose from the cellulose fraction in lignocellulose is the enzyme cost. Therefore, I have in this study investigated the possibility of finding new and better cellulolytic enzymes, which can overcome the obstacles of lack of thermostability and non-productive adsorption to lignin.

In this PhD study, 12 species from genus *Penicillium* were pre-selected for their ability to degrade cellulose when screened for their production of cellulolytic enzymes. These filamentous fungi were found to be very diverse with respect to the biomass degrading enzymes and growth (**Paper A**). My results demonstrated that novel cellulolytic enzymes that will find utility in bioethanol production are yet to be found. A screening for biomass degrading enzymes mainly focus on the ability of the microorganisms to produce the wanted enzymes. However, very long reaction times, due to the recalcitrant nature of cellulose, are to be expected and therefore stability measurements of the cellulolytic systems are important to study after the initial screening.

Among the *Penicillium* species tested, *P. brasilianum* was found most importantly to produce a well-balanced mixture of the individual cellulolytic enzymes, to secrete relatively high concentrations of protein (560 mg/L in shake flasks and twice as much in well controlled reactors), and to be fast growing with a maximum specific growth rate of 0.18 h^{-1} (**Paper A** and **Paper B**). The production of cellulolytic enzymes during cultivation of *P. brasilianum* was regulated in a way similar to that of other fungi with carbon catabolite repression mediated by CreA/Cre1related protein and induction by growth on cellulose and hemicellulose (**Paper B** and **Paper C**). In a commercial process for converting lignocellulose into bioethanol, the substrate and pretreatment method will be dependent on many factors such as local substrate availability, possible excess energy from for example a nearby power generation plant, price on process equipment, microorganism for fermentation,. Therefore, no substrate for enzymatic hydrolysis will be alike. For this reason, knowledge of individual cellulolytic enzymes can, in the long run, be used to produce a mixture of cellulolytic enzymes working optimally for each specific substrate. In *P. brasilianum,* analysis of the promoter regions for the genes *cel3A*

and *cel5C* (**Paper E**) revealed different binding sites for the carbon catabolite repressor protein, differences that was reflected in the actual production of these cellulolytic enzymes (**Paper B** and **Paper C**). Changing the promoter region for the individual genes encoding the cellulolytic enzymes will be a solution to optimise the mixture of cellulolytic enzymes for the hydrolysis of a specific substrate.

In the genomic library, I created from *P. brasilianum*, I found nine genes encoding cellulolytic enzymes. Four of these were full-length sequences. Classification of the genes showed that *P. brasilianum* was found to have sequences encoding cellulolytic enzymes belonging to the typical glycosyl hydrolase families: two GH3 BGs, three GH5 EGs, one GH12 EG, one GH6 CBH and two GH7 CBH. Successfully, one enzyme from each class of cellulolytic enzymes was heterologously expressed. The three expressed enzymes were a BG Cel3A, an EG Cel5C, and a CBH Cel7B. The heterologous expression allowed substantial characterization of these three enzymes, and yet demands future investigation. Heterologous production of the three cellulolytic enzymes made it possible to do extensive studies on the hydrolysis of different cellulosic materials leading to a mathematical model describing the hydrolysis (Andersen, 2007).

A common choice of operation in cellulose hydrolysis is three days at 50 °C. In a process with separate hydrolysis and fermentation, the hydrolysis can run at a higher temperature to increase the hydrolysis rate. In light of this, it is especially worthwhile mentioning that I found Cel3A to have excellent thermostability as compared to other Cel3 BGs in the literature (**Paper D**). Cel3A was compared to Novozym 188, a commercial BG enzyme preparation, and after 24h of incubation at 60 °C, Cel3A had 100 % residual activity. The commercial BG preparation from *Aspergillus niger* only had 50 % residual activity (**Paper D**). In order to better understand the structural reasons for this thermostability, I investigated the possibility of a structural model based on the only reported crystal structure for a GH3 BG. Unfortunately, this GH3 BG from barley (Hrmova *et al*., 2005), has such little structural similarity to *Pb*Cel3A that no useful information could be obtained using the barley GH3 BG as a scaffold for structure modelling. Comparing the thermostability of the *P. brasilianum* enzymes, there was found a significant difference in thermostability for Cel3A and Cel5C. Cel3A had full residual activity after 24h incubation at 60 °C whereas Cel5C had no residual activity (**Paper E**). The difference in the stability between the two *P. brasilianum* enzymes demonstrated that no general prediction on thermostability could be made from characterization of just one enzyme.

The characterization of the different cellulolytic enzymes conducted in this study demonstrated that it is of crucial importance to work with the real substrates when the kinetic parameters have to be determined. A comparison of the kinetic parameters for two different BGs on an artificial substrate did not to correlate to the kinetic parameters

found when using the real substrate being cellobiose (**Paper D**). During hydrolysis of cellulose, glucose will accumulate. Large amounts of glucose will inhibit BG, resulting in a build up of cellobiose, which again is a strong CBH inhibitor. So far, it has been very difficult to quantify glucose inhibition of cellobiose hydrolysis because of the analytical challenges associated with detecting a very small change in the glucose produced relative to the high inhibition concentration of glucose present. I succeeded in establishing a method to circumvent this obstacle by using fully labelled glucose-13C as inhibitor and differentiating it from the produced glucose using LC-MS analysis (**Paper D**).

One of the major limitations in the enzymatic hydrolysis of pretreated biomass is that the cellulolytic enzymes adsorb to the lignin fraction in the biomass samples. This results in either prolonged hydrolysis or a higher enzyme loading to reach the same degree of hydrolysis. Adsorption studies showed that the amount of enzyme adsorbing to lignin is widely dependent on the substrate and of the pretreatment conditions. For Cel5C, the enzyme adsorption to lignin was found in the range of 6 to 33 % for softwood, hardwood and grasses. The exception was rice straw lignin that adsorbed 82 % Cel5C (**Paper E**). A reduction in the adsorption to lignin would therefore significantly reduce the cost of cellulolytic enzymes. Future investigations of Cel5C would be of interest since it has both a catalytic core and a CBM that can adsorp to lignin. Through molecular biology, hydrophobic aromatic amino acid residues identified in this project on the surface of the catalytic domain and on the CBM could be substituted with less hydrophobic residues. Perhaps the removal of certain aromatic residues could reduce lignin adsorption without loss of activity on cellulose.

In conclusion, the work performed during this PhD study has focused on the enzymatic hydrolysis of cellulose in the process of producing glucose from biomass. Many interesting cellulolytic enzymes were found in *P. brasilianum* and scientific work lies ahead for further investigation of these enzymes and to test them in the specific choice of operation (simultaneous or separate hydrolysis and fermentation), pretreatment method and operating parameters, and type of lignocellulose. Important aspects in the choice of assays used for characterization of these different enzymes have been highlighted. Furthermore, I have learned that it is an extremely complex process to make ethanol from biomass. For example: the choice of pretreatment method will affect energy consumption, materials used for pretreatment reactor, cellulose degradability, enzyme adsorption, release of hemicellulosic sugars, and inhibitor formation for hydrolysis and fermentation. The large complexity of a full commercial scale production of bioethanol from a given substrate will require a holistic point of view in order to be successful.

Chapter 8

Dansk populærvidenskabelig sammenfatning

Halmstrå, majsstængler, elefantgræs, bark, træflis og stivelse er eksempler på energikilder, der findes i store mængder i naturen, og til forskel fra fossile energikilder så er disse materialer fornybare. Alt plantemateriale indeholder lignocellulose i større eller mindre grad. I hvede for eksempel er halvdelen stivelse og den anden halvdel lignocellulose. Lignocellulose er en fælles betegnelse for plantecellevægsmateriale opbygget af cellulose, hemicellulose og lignin. Cellulose og hemicellulose er polysakkarider, opbygget af forskellige sukre. Til forskel fra stivelse, hvor de enkelte sukkermolekyler kan frigives forholdsvist nemt, så er lignocellulose et svært nedbrydeligt materiale. Dette skyldes i sagens natur, at lignocellulose giver planter struktur. En anvendelse af plantemateriale er afbrænding for at producere energi. Idag er en anden meget interessant mulighed at frigive de utrolige mængder af sukkermolekyler, som plantematerialet består af. Mange kemikalier, der på nuværende tidspunkt fremstilles udfra olie, vil kunne fremstilles udfra sukker. Ethanol kan fremstilles udfra disse sukre og betegnes bioethanol for at markere, at den er produceret fra biologiske udgangsmaterialer. Denne bioethanol er en fuldgod erstatning for benzin som transportbrændstof, og der er adskillige grunde til interessen i bioethanol: Olieknaphed, verdens olieressourcer er endelige, national produktion af transportbrændstof skaber større uafhængighed af oliestater, øget velstand i verden medfører en større bilpark og endelig frygt for global opvarmning grundet drivhuseffekten.

Lignocellulosens sukre kan frigøres ved en fysisk/kemisk forbehandling af plantematerialet efterfulgt af en enzymatisk hydrolyse, hvor der anvendes cellulose og hemicellulose nedbrydende enzymer. Til forskel fra hydrolyse af stivelse, som er en yderst velkendt metode idag, så er hydrolysen af forbehandlet lignocellulose fortsat på den begyndende del af indlæringskurven med adskillige pilotanlæg under opførelse. En identificeret udfordring ved produktionen af bioethnaol er udgiften til enzymer. En utrolig stor forskningsindsats er blevet lagt i forståelsen af det cellulose nedbrydende enzymsystem fra svampen *Trichoderma reesei*. Det er nødvendigt med et samspil mellem tre enzymklasser for at nedbryde cellulose: endoglukanaser, cellobiohydrolaser og β-glukosidaser. Hver svamp producerer flere enzymer indenfor hver enzymklasse.

Genus *Penicillium* findes fortrinsvist i skovens muldlag, hvor store mængder plantemateriale bliver omsat, og svampe fra *Penicillium* er nogle af de hyppigst forekommende i disse omgivelser. I dette ph.d. studie er produktionen af cellulosenedbrydende enzymer undersøgt i adskillige *Penicillium* arter med det formål at finde nye cellulytiske enzymer, der kan reducere enzymudgiften ved produktion af bioethanol. Blandt de undersøgte *Penicillium* arter viste det sig, at *Penicillium brasilianum* producerer en velbalanceret blanding af cellulosenedbrydende enzymer, der ikke skal tilføres ekstra enzymaktiviteter for at kunne nedbryde cellulose effektivt, som det i dag er tilfældet for kommercielle enzymprodukter til cellulosenedbrydning.

Nærmere studier af det cellulosenedbrydende enzymsytem fra *P. brasilianum* førte til oprensning af enkelte væsentlige enzymer nødvendige for cellulosenedbrydning. De oprensede enzymer blev brugt som udgangspunkt for en nærmere undersøgelse af de cellulosenedbrydende enzymer i *P. brasilianum*, og ni gener kodende for disse blev fundet. En GH3 β-glukosidase, en GH5 endoglukanase og en GH7 cellobiohydrolase blev produceret i større mængder ved heterolog produktion i en anden værtsstamme med høj proteinproduktion. De producerede mængder enzym tillod tilbundsgående karakterisering af dem i dette projekt, og samtidigt tillod det også anvendelse af samme enzymer i andre projekter. Den fundne GH3 β-glukosidase var særdeles termostabil ved sammenligning både med en kommerciel preparation og med andre rapporterede β-glukosidaser. Samme ekstraordinære termostabilitet blev ikke fundet for GH5 endoglukanasen, og det er derfor ikke muligt at slutte, at alle cellulosenedbrydende enzymer fra en organisme har samme termostabilitet.

Under cellulosenedbrydning dannes der i visse processkonfigurationer høje koncentrationer af glukose. Hidtil har det været meget svært at måle, hvorledes glukosekoncentration indvirker på β-glukosidase aktiviteten, men i dette projekt blev et nyt assay udviklet. Dette assay anvender kulstof-13 mærket glukose, som inhibitor for den enzymatiske nedbrydning af cellobiose til glukose. Den under hydrolysen frigivne glukose kan i det nye assay ved massespektrometrisk analyse separeres og kvantificeres i forhold til den anvendte kulstof-13 mærkede glukose.

Under den enzymatiske hydrolyse af cellulose og hemicellulose er det stort problem, at de anvendte enzymer binder til lignindelen i det forbehandlede plantemateriale, og så længe disse enzymer sidder på lignin, så er de ikke aktive. Strukturstudier af den producerede GH5 endoglukanase og sammenligning med andre cellulosenedbrydende enzymer identificerede forskelle, som kan være af betydning for bindingens størrelse. Binding mellem GH5 endoglukanasen og lignin fra forskellige typer af plantemateriale forbehandlet på forskellig vis viste, at både typen af plantemateriale og forbehandling er af væsentlig betydning for graden af binding. I en kommende bioethanol fabrik skal der således udføres detaljerede adsortionsstudier for at optimere den enzymatiske hydrolyse.

Chapter 9

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Chapter 10

Publications

Paper A

Krogh, K.B.R., Mørkeberg, A., Frisvad, J.C., and Olsson, L. (2004). Screening genus *Penicillium* for producers of cellulolytic and xylanolytic enzymes. *Applied Biochemistry and Biotechnology*, **113-16**, 389-401.

Paper B

Jørgensen, H., Mørkeberg, A., Krogh, K.B.R., and Olsson, L. (2004) Growth and enzyme production by three *Penicillium* species on monosaccharides. *Journal of Biotechnology*, **109**, 295-299.

Paper C

Jørgensen, H., Mørkeberg, A., Krogh, K.B.R., and Olsson, L. (2005) Production of cellulases and hemicellulases by three *Penicillium* species: effect of substrate and evaluation of cellulose adsorption by capillary electrophoresis. *Enzyme and Microbial Technology*, **36**, 42-48.

Paper D

Krogh, K.B.R.M., Harris, P.V., Olsen, C.L., Johansen, K.S., Hojer-Pedersen, J., Borjesson, J, and Olsson, L. Cloning and characterization of a GH3 β-glucosidase from *Penicillium brasilianum* including a novel method for measurement of glucose inhibition on cellobiose hydrolysis. *Submitted*.

Paper E

Krogh, K.B.R.M., Kastberg, H., Jørgensen, C.I., Berlin, A., Harris, P.V., and Olsson, L. A novel GH5 endoglucanase from genus *Penicillium* and its adsorption to lignin. *Submitted.*

Paper F

Patent application WO2007019442 A3

Krogh, K., and Harris, P. (2005) Polypeptides having beta-glucosidase activity and polynucleotides encoding same.

Paper G

Patent application WO2007109441 A2

Harris, P., Krogh, K., Vlasenko, E., and Lassen, S. F. (2006) Polypeptides having endoglucanase activity and polynucleotides encoding same.

Paper A

Screening genus *Penicillium* for producers of cellulolytic and xylanolytic enzymes

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Screening Genus *Penicillium* for Producers of Cellulolytic and Xylanolytic Enzymes

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Abstract

For enzymatic hydrolysis of lignocellulosic material, cellulolytic enzymes from *Trichoderma reesei* are most commenly used, but, there is a need for more efficient enzyme cocktails. In this study, the production of cellulolytic and xylanolytic enzymes was investigated in 12 filamentous fungi from genus *Penicillium* and compared with that of *T. reesei*. Either Solka-Floc cellulose or oat spelt xylan was used as carbon source in shake flask cultivations. All the fungi investigated showed coinduction of cellulolytic and xylanolytic enzymes during growth on cellulose as well as on xylan. The highest filter paper activity was measured after cultivation of *Penicillium brasilianum* IBT 20888 on cellulose.

Index Entries: Cellulolytic enzymes; hemicellulolytic enzymes; enzymatic hydrolysis; coinduction.

Introduction

Today, an international awareness of the increasing CO_2 concentration in the atmosphere has resulted in the formation of the Kyoto Protocol, which has led many countries to make the commitment to decrease the emission of CO_2. One way of decreasing CO_2 emissions could be substitution of fossil fuels with renewable energy sources. The net production of CO_2 is significantly lower when bioethanol produced from plant materials is used as transportation fuel instead of fossil fuels, since CO_2 is assimilated

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during photosynthesis *(1)*. To meet the future demand for bioethanol, not only starch but also lignocellulosic materials need to be used as substrate.

The polysaccharides in the raw materials need to be hydrolyzed before the sugar monomers can be fermented to ethanol. Today, enzymatic hydrolysis is regarded as a method with great potential. One major obstacle to overcome is the high cost of cellulolytic enzymes. In 2001, the United States Department of Energy formed a contract with two commercial producers of cellulolytic enzymes in an attempt to achieve a 10-fold decrease in the cost of the cellulolytic enzymes (www.ott.doe.gov/biofuels/research_partnerships.html).

The decay of plant material in nature is partly owing to the production of cellulolytic and hemicellulolytic enzymes in microorganisms. Filamentous fungi, such as *Trichoderma reesei*, *Penicillium pinophilum*, and *Humicola insolens* have demonstrated the capability of secreting large amounts of cellulolytic enzymes *(2)*. The most extensively studied microorganism producing cellulolytic enzymes is the filamentous fungus *T. reesei* which is the preferred microorganism for industrial production of cellulolytic enzymes. One of the main limitations of the cellulolytic system from *T. reesei* is the low amount of β-glucosidase (BG) *(3)*. Low BG activity leads to a buildup of cellobiose during hydrolysis, which inhibits the activity of the cellobiohydrolases (CBHs) to a larger extent than glucose does *(4)*. Therefore extra BG needs to be added for an efficient hydrolysis of cellulosic materials *(5)*.

Microorganisms grow in various habitats in nature, and they have therefore adapted to various physical and chemical conditions. In the search for microorganisms that efficiently can degrade lignocellulose, several species from genus *Penicillium* were tested. In forest soil, where large amounts of plant materials are degraded, an abundance of *Penicillium* species is present *(6)*. Because of this fact and that enzyme mixtures from various *Penicillium* species have been shown to perform well in the hydrolysis of different kinds of lignocellulosic material *(7–9)*, we screened 12 different *Penicillium* species for their production of cellulolytic and xylanolytic enzymes.

Materials and Methods

Strains

The filamentous fungi screened were all from the genus *Penicillium* (Table 1) and were selected from the culture collection at BioCentrum-DTU, Technical University of Denmark. The filamentous fungi *T. reesei* Rut C30 was used as reference strain.

Preparation of Inoculum

Each strain was received on a Czapek yeast autolysate agar plate from the culture collection. Spores were transferred to a potato dextrose agar (PDA) (Difco, Detroit, MI) plate and the PDA plates were kept at the optimal temperature for growth (Table 1). After 2 wk the strains on the PDA

Table 1
Subgenus, Optimal Growth Temperature, and Origin for 12 *Penicillium* Strains Investigated

Strain	Subgenus	Temperature (°C)	Isolated from
P. allii IBT 3803	*Penicillium*	25	Garlic, Denmark
P. persicinum IBT 13226	*Furcatum*	25	Soil, USA
P. simplicissimum IBT 13237	*Furcatum*	30	Flannel bag, South Africa
P. simplicissimum IBT 15303	*Furcatum*	30	Feed, Norway
P. brasilianum IBT 20888	*Furcatum*	30	Seaweed, Denmark
P. pinophilum IBT 4186	*Biverticillium*	30	Maize, India
P. funiculosum IBT 5816	*Biverticillium*	30	Citric acid (10 %), Australia
P. pinophilum IBT 10872	*Biverticillium*	30	Maize, India
P. rubicundum IBT 10943	*Biverticillium*	30	Cultivated soil, United States
P. aculeatum IBT 18363	*Biverticillium*	30	Rhizosphere of bamboo, Taiwan
P. verruculosum IBT 18366	*Biverticillium*	30	Soybeen seed, Taiwan
P. minioluteum IBT 21486	*Biverticillium*	25	Fruit, Denmark

plates had produced spores, which were suspended in 0.1% (v/v) Tween-80 (P-1754; Sigma, St. Louis, MO).

Shake Flask Cultivations

An amount of each spore suspension was transferred to a 500-mL shake flask in order to obtain a spore concentration of 10^6 spores/mL. The medium was a modified Mandels and Weber medium *(10)*, in which the concentration of KH_2PO_4 was increased by 50% to improve buffer capacity. The initial volume in the shake flask was 150 mL. The carbon sources were either 2% (w/v) Solka-Floc cellulose (FCC200; Fiber Sales & Development) or 2% (w/v) oat spelt xylan (X-0627; Sigma). Cultivations were carried out aerobically at 150 rpm and at the optimal growth temperature for each strain (Table 1) and *T. reesei* Rut C30 was cultivated at 30°C. Samples were taken at regular time intervals during the cultivations, filtered through a 0.22-µm low-protein-binding filter (Cameo 25 GSS; Osmonics), and the filtrates were stored at –20°C.

Enzymatic Assays

Filter Paper Activity

Total cellulolytic activity was measured using the filter paper assay (FPA) according to Ghose *(11)* based on an estimation of the released reducing sugars by dinitrosalicylic (DNS) acid *(12)*.

Xylanase Activity

Xylanase (XA) activity was measured through the degradation of birch-wood xylan (7500.1; Roth, Karlsruhe, Germany) according to Bailey et al. *(13)* based on an estimation of the released reducing sugars by DNS acid *(12)*.

Endoglucanase and Endoxylanase Activities

Endoglucanase (EG) and endoxylanase (EX) activities were measured using azo-carboxymethyl cellulose (Megazyme, Bray, Ireland) and azo-xylan (Megazyme), respectively, as substrate as described by Jørgensen et al. *(14)*.

BG, β-Xylosidase, and α-L -Arabinofuranosidase Activities

BG, β-xylosidase (BX), α-L -arabinofuranosidase (AF), and β-galactosidase activities were measured using *p*-nitrophenyl-β-D-glucopyranoside (73676; Fluka), *p*-nitrophenyl-β-D-xylopyranoside (Sigma N-2132), and *p*-nitrophenyl-α-L-arabinofuranoside (N-3641; Sigma), respectively, as substrate as described by Jørgensen et al. *(14)*.

CBH Activity

CBH activity was measured in a 1 m*M* *p*-nitrophenyl-β-D-cellobioside (N-5759; Sigma) substrate solution at pH 4.8 with 50 m*M* sodium citrate. The substrate solution also contained 1.3 m*M* D-glucono-1,5-δ-lactone in

order to inhibit BG from hydrolyzing the substrate and thereby overestimating the CBH activity *(15)*. Otherwise, the procedure was as described for BG, BX, and AF activity.

Determination of Protein

Intracellular Protein

Mycelium was washed twice with 0.9% (w/v) NaCl followed by three extraction steps. In each extraction step the mycelium was boiled for 10 min with 1 *M* NaOH and the supernatant was collected after centrifugation *(16)*. The amount of protein in the pooled supernatant was measured based on the biuret method *(17)*.

Extracellular Protein

The concentration of extracellular protein was quantified using the "Bio-rad total protein" assay based on the Bradford *(18)* method with γ-globulin (G-7516; Sigma) as standard. The assay was performed using an analytical robot (Cobas Mira; Roche, Rotkreutz, Switzerland).

Results and Discussion

Production of cellulolytic and xylanolytic enzymes was investigated in 12 *Penicillium* species isolated from different habitats (Table 1), as well as in the well-characterized fungus *T. reesei* Rut C30. These filamentous fungi were cultivated aerobically for 220–240 h in shake flasks with 20 g/L of cellulose (Solka-Floc) or 20 g/L of xylan from oat spelts as carbon source. The cell mass concentration was estimated through measurements of intracellular protein. Cellulolytic enzyme production was characterized through measurements of BG, EG, and CBH activity, and total cellulolytic activity was determined as FPA. Xylanolytic enzyme production was investigated by measuring BX, EX, and AF activity, and the total XA activity was determined through the degradation of birchwood xylan.

Growth

To keep the production time as short as possible, it is important that the microorganism grows relatively fast. In samples containing insoluble substrates, the amount of cell mass cannot be determined by measurement of dry matter. The general trend in cell mass concentration, estimated indirectly from the amount of intracellular protein, was a faster initial growth on xylan compared to cellulose, but a higher final cell mass concentration when cellulose was used as carbon source compared to xylan (data not shown). Xylan is less ordered and has fewer hydrogen bonds than cellulose, and the higher initial growth rate on xylan may be owing to the structure of xylan, which is more accessible for the enzymes than cellulose. *P. verruculosum* IBT 18366, *P. brasilianum* IBT 20888, and *T. reesei* RUT C30 reached the highest cell mass concentration after cultivation on cellulose, and after cultivation on xylan, *P. persicinum* IBT 13226 and *P. verruculosum* IBT 18366 reached the highest cell mass concentration.

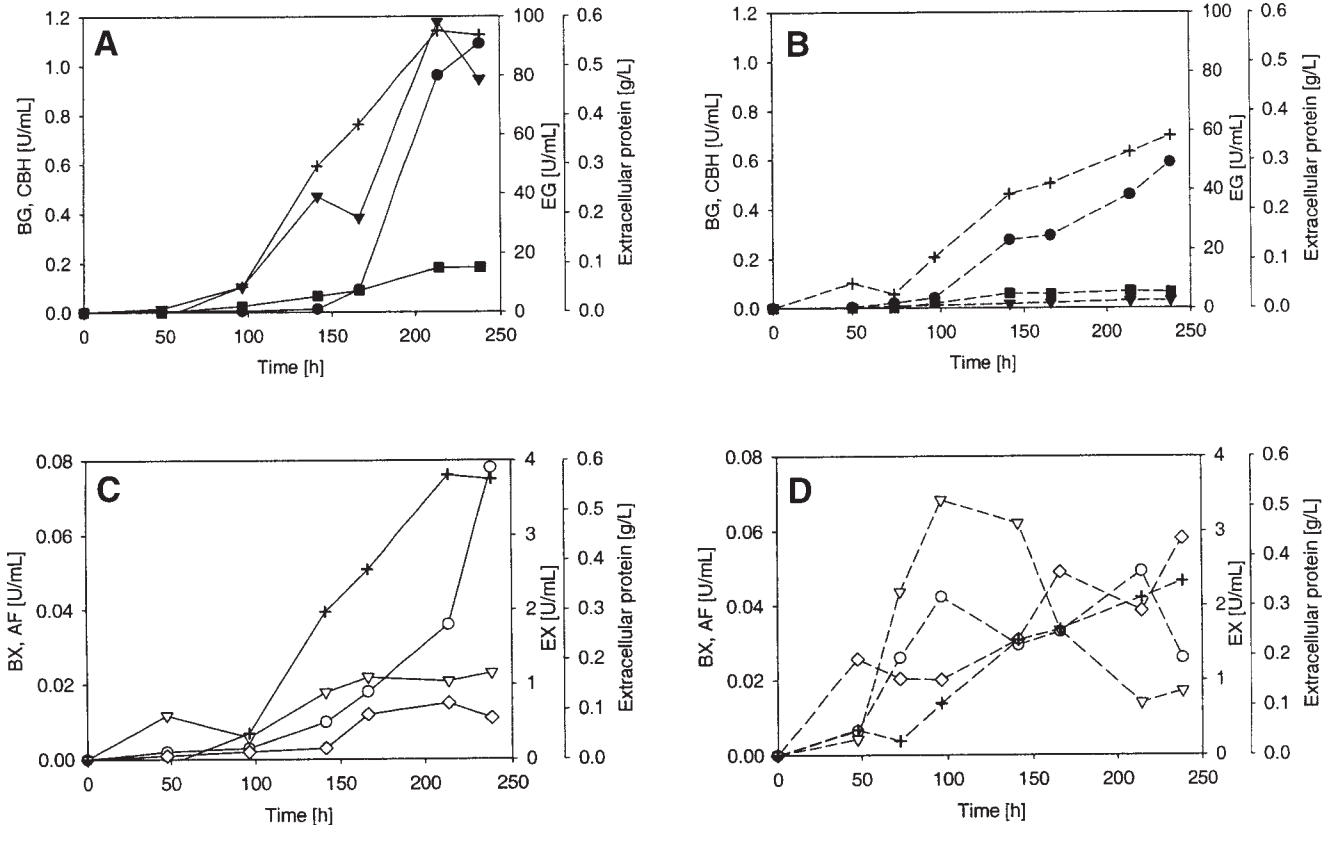

Fig. 1. Cellulolytic and xylanolytic activities and extracellular protein during time course of the cultivation of *P. brasilianum* IBT 20888. **(A)** Cellulolytic activities on cellulose; **(B)** cellulolytic activities on xylan; **(C)** xylanase activities on cellulose; and **(D)** xylanolytic activities on xylan. (●) BG; (■) CBH; (▼) EG; (○) BX; (◇) AF; (▽) EX; (+) extracellular protein; (—) cultivation on cellulose; and (- - -) cultivation on xylan.

Enzyme Production

In all cultivations, the general trend was a steady increase in cellulolytic and xylanolytic enzyme activities in the cultivation broth during the time course, as previously demonstrated by Schulz and Hirte *(19)* in a screening experiment of different *Penicillium* species cultivated on a plant hydrolysate. The same trend was observed during both cultivation on cellulose and on xylan (as exemplified in Fig. 1 A–D). Even though each measured enzyme activity increased during the time course of the cultivation, the specific activity of all enzymes was not constant in all cultivations. During cultivation of *P. brasilianum* IBT 20888 on cellulose, BG activity increased 10 times from 170 to 240 h, but CBH and EG activity only doubled (Fig. 1A). Increasing xylanolytic activity throughout cultivation on cellulose has been observed in *T. reesei (20)*, and increasing cellulolytic activity throughout cultivation on xylan has been demonstrated for an *Aspergillus* species *(21)*. Activities of cellulolytic enzymes in the supernatant were generally higher during growth on cellulose than during growth on xylan (Fig. 1A, 1B), whereas the activities of xylanolytic enzymes were higher during growth on xylan than during growth on cellulose (Fig. 1C, 1D). Our study demonstrated that there was a coinduction between cellulolytic and xylanolytic activities whether the substrate for cultivation of the fungus was cellulose or xylan. Furthermore, EX activity decreased in some cultivations when the substrate was xylan (data not shown).

Cellulolytic Enzymes

Cultivation of the different filamentous fungi for 230 h on cellulose resulted in different concentrations of extracellular protein and FPA, ranging from 0.01 to 0.78 g/L and from 0.02 up to 0.68 filter paper units (FPU)/mL, respectively. A relatively high protein concentration was shown to be correlated with a high FPA during growth on cellulose (Fig. 2). The specific FPA was found to be 0.77 FPU/mg of protein through linear regression with a regression coefficient of 0.77. A similar specific activity has been shown to result from a cultivation of *P. pinophilum (22)* on a substrate containing both cellulose and hemicellulose. Comparison of the specific FPA resulting from the growth of *P. occitanis* and *T. reesei* QM9414 on cellulose showed that the specific FPA was higher for the *Penicillium* species than for *T. reesei (23)*, as observed for *P. brasilianum* IBT 20888 and *T. reesei* Rut C30 in the present study. The *Penicillium* species with the highest FPA after growth on cellulose were *P. brasilianum* IBT 20888, *P. verruculosum* IBT 18366, *P. pinophilum* IBT 10872, and *P. minioluteum* IBT 21486. The highest FPA (0.68 FPU/mL) was measured after cultivation of *P. brasilianum* IBT 20888; this FPA was even higher than the 0.54 FPU/mL resulting from growth of *T. reesei* Rut C30 (Table 2). Fungi with low FPA might contain specific enzymes with interesting properties, such as higher specific activity for single enzymes or a lower product inhibition; thus, it might be interesting to also investigate these strains in further detail. If a fungus produces

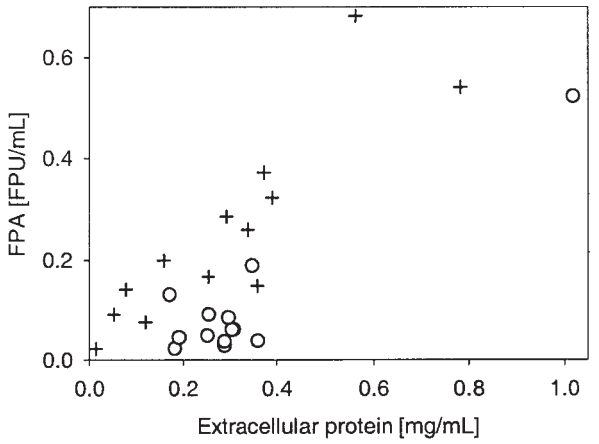

Fig. 2. FPA vs protein concentration in cultivation broth after cultivation of fungi on cellulose (+) and xylan (○). Each point in the plot represents the final protein concentration and the corresponding measured FPA in the cultivation broth for each fungus on each substrate.

Table 2
Cellulolytic Activities for *T. reesei* Rut C30 and for Four *Penicillium* Species with Highest FPA After Cultivation in Shake Flasks[a]

	BG (U/mL)	EG (U/mL)	CBH (U/mL)	FPA (FPU/mL)
T. reesei Rut C30	0.03 (0.31)	87 (44)	0.16 (0.07)	0.54 (0.52)
P. brasilianum IBT 20888	1.09 (0.59)	98 (2.6)	0.18 (0.07)	0.68 (0.19)
P. verruculosum IBT 18366	0.97 (0.33)	12 (0.5)	0.08 (0.02)	0.37 (0.13)
P. pinophilum IBT 10872	2.45 (0.80)	6 (1.3)	0.07 (0.07)	0.32 (0.06)
P. minioluteum IBT 21486	1.70 (0.78)	9 (1.2)	0.11 (0.06)	0.29 (0.05)

[a]Numbers not in parenthesis are activities after cultivation on cellulose, and numbers in parenthesis are activities after cultivation on xylan. Owing to assay limitations, the measured CBH activity should be seen as a quantitative indication.

a single enzyme with a desirable property, it is possible to increase the secretion of this enzyme through mutagenesis. It has been demonstrated that FPA in a *P. pinophilum* strain can be increased four times through three rounds of mutation and selection of the best-producing strain *(24)*.

BG activity was more than one order of magnitude higher for the four *Penicillium* species than BG activity resulting from cultivating *T. reesei* Rut C30. EG activity was one order of magnitude higher for *T. reesei* Rut C30 and *P. brasilianum* IBT 20888 than for the other three *Penicillium* species, which may provide an explanation for the lower FPA obtained from *P. verruculosum* IBT 18366, *P. pinophilum* IBT 10872, and *P. minioluteum* IBT 21486.

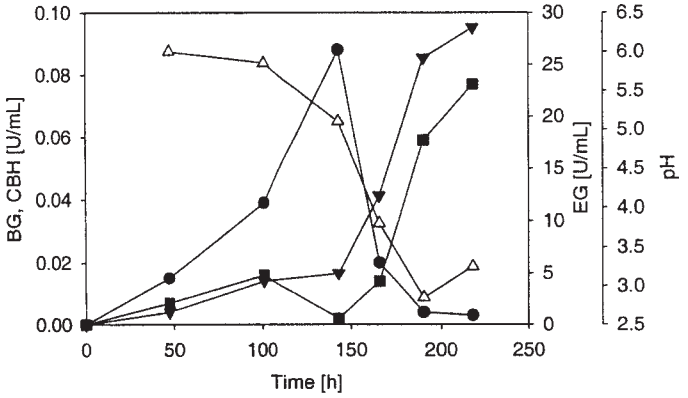

Fig. 3. Cellulolytic activities and pH during cultivation of *P. persicinum* IBT 13226 on cellulose (●) BG; (■) CBH; (▼) EG; and (△) pH.

When considering the results for FPA after cultivation of the fungi on xylan, the filter paper activity showed less correlation to the concentration of extracellular protein than when the fungi were grown on cellulose (Fig. 2). *T. reesei* Rut C30 had a much higher FPA, 0.52 FPU/mL, than any of the other fungi when grown on xylan (Table 2). The FPA for *T. reesei* Rut C30 was one order of magnitude higher than the FPA for *P. pinophilum* IBT 10872, although the only activity that was higher for *T. reesei* Rut C30 was the EG activity. The FPA could not be fully explained by the measured cellulolytic activities, for instance after growth on xylan, the cellulolytic activities were all lower for *P. verruculosum* IBT 18366 than for *P. pinophilum* IBT 10872, and yet *P. verruculosum* IBT 18366 yielded higher FPA (Table 2).

BG activity seemed to deviate from the general trend in that the activity of the individual cellulolytic enzymes increased throughout the cultivation (Fig. 1). In some cultivations, the measured BG activity decreased in the middle or toward the end of the cultivation, as exemplified in Fig. 3. After growing *P. persicinum* IBT 13226 on cellulose for 140 h, BG activity started to decrease, and after 191 h no BG activity could be measured in the cultivation broth. In this time period, the pH dropped concurrently to a value of 2.85. For *T. reesei* Rut C30 and *P. minioluteum* IBT 21486, BG activity also decreased as the pH dropped to a value of 3.0. The pH instability of BG has been reported for other microorganisms: several species of *Aspergillus (25)*, *Thermomyces lanuginosus (26)*, and also *Trichoderma harzianum (27)*. Other experiments (data not shown) have demonstrated that during cultivation of *P. persicinum* IBT 13226 on cellulose in well-controlled bioreactors with pH control, BG activity reached a significantly higher value, 1.3 U/mL, compared to the present experiments, in which no activity was detected.

Table 3
Xylanase Activities for *T. reesei* Rut C30 and Four *Penicillium* Species with Highest Xylanase Activity After Cultivation in Shake Flasks[a]

	BX (U/mL)	EX (U/mL)	AF (U/mL)	XA (U/mL)
T. reesei Rut C30	1.67 (0.07)	3.9 (59)	0.17 (0.42)	16 (176)
P. persicinum IBT13226	ND (0.19)	3.8 (30)	ND (0.15)	2 (105)
P. funiculosum IBT 5816	0.08 (0.33)	0.39 (10)	0.02 (0.20)	2 (42)
P. simplicissimum IBT 13237	ND (ND)	0.30 (1.6)	0.01 (0.21)	7 (31)
P. simplicissimum IBT 15303	ND (ND)	3.4 (2.7)	ND (0.21)	19 (23)

[a]ND, not detected. Numbers not in parenthesis are activities after cultivation on cellulose, and numbers in parenthesis are activities after cultivation on xylan.

Xylanolytic Enzymes

Cultivation of filamentous fungi on xylan resulted in extracellular protein concentrations for the *Penicillium* species in the range of 0.17–0.36 mg/mL and 1.02 mg/mL for *T. reesei* Rut C30. The XA activity was measured to be between 1.5 and 105 U/mL for the *Penicillium* species and 176 U/mL for *T. reesei* Rut C30. No linear correlation was observed between the XA activity and the amount of secreted protein, although the highest XA activities were measured in cultivation broths with relatively high protein concentrations (data not shown). Among the *Penicillium* species examined, *P. persicinum* IBT 13226 had the highest XA activity (105 U/mL) after growth on xylan. *P. funiculosum* IBT 5816 had the second highest XA activity of 42 U/mL (Table 3). XA activity was in the range of 1.5–19 U/mL after the fungi were grown on cellulose, and like on xylan, no linear correlation between XA activity and the amount of secreted protein was observed (data not shown). A possible explanation for the missing linear correlation could be the heterogeneity of hemicellulose. Owing to adaptation to the habitat, each fungus may have changed the regulation of individual hemicellulolytic enzymes to the given conditions, but during growth on xylan from oat spelts in the present experiment, the fungus might still produce enzymes needed to hydrolyze the "original" substrate. Therefore, the best producers found in a screening experiment heavily depend on the chosen substrate.

The highest EX and AF activities were measured after cultivation of *T. reesei* Rut C30 on xylan, (59 and 0.42 U/mL, respectively) (Table 3). These activities were with a few exceptions almost twice as high as any of the activities obtained after cultivation of the *Penicillium* species. BX activity reached by far the highest activity after cultivation of *T. reesei* Rut C30 on cellulose. This was not the case for the *Penicillium* species that had the highest BX activity when they were cultivated on xylan. XA activity could not, like FPA, be fully explained by the individual xylanolytic enzymes, as demonstrated in a comparison of *T. reesei* Rut C30 and *P. simplicissimum* IBT 15303 after growth on cellulose.

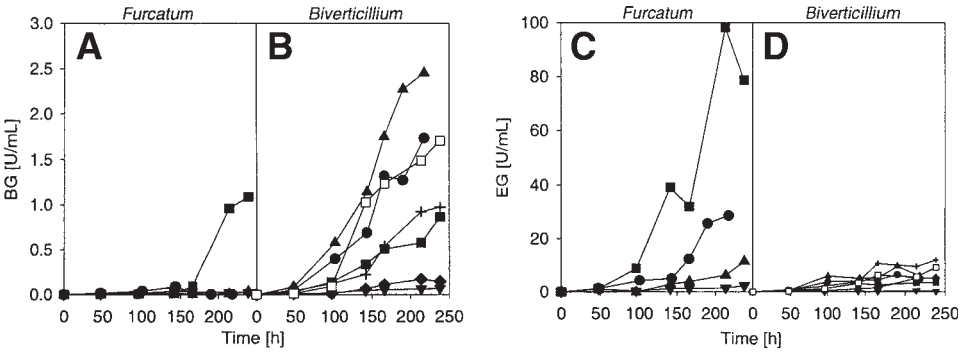

Fig. 4 BG activity during cultivation on cellulose for **(A)** subgenus *Furcatum* and **(B)** subgenus *Biverticillium*; and EG activity during cultivation on cellulose for **(C)** subgenus *Furcatum* and **(D)** subgenus *Biverticillium*. Subgenus *Furcatum*: (●) *P. persicinum* IBT 13226; (▼) *P. simplicissimum* IBT 13237; (▲) *P. simplicissimum* IBT 15303; (■) *P. brasilianum* IBT 20888. Subgenus *Biverticillium*: (●) *P. pinophilum* IBT 4186; (■) *P. funiculosum* IBT 5816; (▲) *P. pinophilum* IBT 10872; (▼) *P. rubicundum* IBT 10943; (◆) *P. aculeatum* IBT 18363; (+) *P. verruculosum* IBT 18366; (□) *P. minioluteum* IBT 21486.

Comparison of Enzyme Profiles in Subgenera Within Genus Penicillium

The genus *Penicillium* can be classified into different subgenera according to differences in morphology, physiology, and secondary metabolite production (Table 1). The *Penicillium* subgenus *Biverticillium* often occurs on wood, paper, and textile-related plant products *(28)*. By contrast, *P. brasilianum*, *P. simplicissimum*, and related species belong to the subgenus *Furcatum*, which often occurs in grassland soils *(6)*.

Comparisons of the cellulolytic activities resulting from growth on cellulose and xylanolytic activities resulting from growth on xylan for each subgenus showed that BG activity was higher for the subgenus *Biverticillium* than for the subgenus *Furcatum* during cultivation on cellulose (Fig. 4A, B). EG activity tended to be highest for the subgenus *Furcatum* during growth on cellulose (Fig. 4C, D). An investigation of the xylanolytic activities showed that six of seven fungi from the subgenus *Biverticillium* reached a higher BX activity than the maximum BX activity for the subgenus *Furcatum*. EX activity did not reveal any difference between the two subgenera (data not shown).

In the future, when a larger number of filamentous fungi in each subgenus have been screened for their production of cellulolytic and xylanolytic enzymes, a powerful tool to search for characteristics in the subgenera will be multivariate data analysis. In the search for an enzyme mixture with desirable properties for a given application, knowledge of enzyme activities characterizing each subgenus during growth on a given substrate can be valuable.

Other Screening Experiments

In the present study, the fungi were cultivated in submerged cultures, which can be habitats that are far from the conditions that each fungus has adapted to during evolution. Solid-state fermentation could very well be another interesting way to cultivate the microorganisms of interest for cellulase and hemicellulase production. It has earlier been shown that when *P. citrinum* was grown on rice husks, cellulolytic activity was three times higher in the solid-state fermentation than in the submerged culture *(29)*.

Conclusion

The filamentous fungi investigated showed coinduction of cellulolytic and xylanolytic enzymes. During growth on cellulose, products from the hydrolysis of cellulose also induced production of xylanolytic enzymes, and during growth on xylan, products from the hydrolysis of xylan also induced the production of cellulolytic enzymes.

FPA was used to evaluate how well suited filamentous fungi could be as a producer of cellulolytic enzymes. *P. brasilianum* IBT 20888 cultivated on cellulose resulted in the highest FPA, an activity that was even higher than the FPA resulting from growth of *T. reesei* Rut C30. *P. brasilianum* IBT 20888 was different from the other *Penicillium* species in the way that it produced almost an order of magnitude higher EG activity than any of the other species. Even though differences in single cellulolytic activities among the fungi were found, a linear correlation was observed between the amount of extracellular protein and the FPA measured after cultivation of each fungus on cellulose. The screening among the different filamentous fungi belonging to *Penicillium* showed that many species are interesting as producers of cellulolytic and xylanolytic enzymes, but further cultivation experiments need to be performed under more controlled conditions. Cultivations in shake flasks can be used as a relatively fast approach when screening many microorganisms for their production of cellulolytic and xylanolytic enzymes, but certain characteristics may not be apparent owing to the nature of shake flask cultivations.

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Paper B

Growth and enzyme production by three *Penicillium* species on monosaccharides

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Short communication

Growth and enzyme production by three *Penicillium* species on monosaccharides

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Abstract

The growth and preference for utilisation of various sugar by the *Penicillium* species *Penicillium pinophilum* IBT 4186, *Penicillium persicinum* IBT 13226 and *Penicillium brasilianum* IBT 20888 was studied in batch cultivations using various monosaccharides as carbon source, either alone or in mixtures. *P. pinophilum* IBT 4186 and *P. persicinum* IBT 13226 had a μ_{max} around 0.08–0.09 h^{-1} using either glucose or xylose as carbon source. The μ_{max} of *P. brasilianum* IBT 20888 was 0.16 and 0.14 h^{-1} on glucose and xylose, respectively. Glucose was found to exert repression on the catabolism of mannose, galactose, xylose and arabinose. The three species were able to utilise all the tested monosaccharides, but arabinose was only slowly metabolised. Glucose was also found to repress the production of endoglucanases, endoxylanases and β-xylosidases. After glucose depletion, the fungi started producing β-glucosidase and endoglucanases. Xylose did not repress the enzyme production and it induced the production of endoxylanases and β-xylosidases.

Keywords: *Penicillium pinophilum*; *Penicillium persicinum*; *Penicillium brasilianum*; Glucose repression; Cellulases; Xylanases

1. Introduction

Cellulases and hemicellulases are produced by a wide range of microorganisms during growth on lignocellulosic material. These enzymes degrade the polysaccharides constituting the major fractions of lignocellulosic material into sugars, from which the growth of the microorganism can be sustained. The regulation of the enzyme production has been extensively investigated in *Trichoderma* and *Aspergillus* (de Vries and Visser, 2001; Mach and Zeilinger, 2003). In *Trichoderma* and *Aspergillus*, glucose represses the metabolism of other monosaccharides like xylose and galactose—known as the carbon catabolite repression (Ilmén et al., 1996; Scazzocchio et al., 1995). Furthermore, glucose is a strong repressor of the production of cellulases and hemicellulases. The protein CreA in *Aspergillus* and the homologous Cre1 in *Trichoderma* has been found to mediate the transcriptional regulation of the genes encoding cellulases, hemicellulases and enzymes for metabolism of other sugars (de Vries and Visser, 2001; Mach and Zeilinger, 2003). In *Aspergillus*, xylose has also been demonstrated to

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mediate catabolite repression, although not as strong as glucose, but simultaneously xylose has been found to be involved in the induction of the production of some xylanases (de Vries et al., 1999).

The knowledge about the regulation of the enzyme production in *Penicillium* species is more limited than for *Aspergillus* and *Trichoderma*. Nevertheless, a number of *Penicillium* species have proven to be efficient producers of cellulases and hemicellulases (Brown et al., 1987; Chaabouni et al., 1994). In this study, the effect of glucose and xylose on the metabolism of other sugars and the production of cellulases and xylanases was studied in batch cultivations using three different *Penicillium* species.

2. Materials and methods

2.1. Cultivation conditions

Penicillium pinophilum IBT 4186, *Penicillium persicinum* IBT 13326 and *Penicillium brasilianum* IBT 20888 were from the culture collection at BioCentrum-DTU. The bioreactors were inoculated with spores propagated on rice according to Jørgensen et al. (2003), but *P. pinophilum* IBT 4186 was first grown in a shake flask with 100 ml of medium (see below) with 10 g l^{-1} glucose and 12 ml of this culture was transferred to rice to propagate spores.

The batch cultivations were carried out aerobically at 30 °C (*P. pinophilum* IBT 4186 and *P. brasilianum* IBT 20888) or at 25 °C (*P. persicinum* IBT 13226) according to Jørgensen et al. (2003). The medium composition was based on the medium of Mandels and Weber (1969). For each 10 g l^{-1} of carbon source the medium contained 0.75 g l^{-1} Bacto peptone (Difco) and 0.25 g l^{-1} yeast extract (Difco). The concentration of Tween 80 was 0.2 ml l^{-1} and Struktol SB2121 antifoam (Qemi Int., USA) 1 ml l^{-1}. The carbon source was autoclaved separately from the mineral medium. Samples were filtered through a 0.22 μm low protein-binding filter (Cameo 25 GSS, Osmonics, USA). Biomass was measured by drying biomass samples filtered on Whatman no. 1 filter paper for 24–48 h at 105 °C. The CO_2 concentration in the off gas was measured using a Brüel and Kjær acoustic gas analyser (Christensen et al., 1995).

2.2. Enzymatic assays

Endoxylanase and endoglucanase activity was measured using azo-arabinoxylan and azo-carboxymethyl cellulose (Megazyme, Ireland), respectively, as substrate. For both assays, 500 μl of a solution of 5.4 g l^{-1} of substrate in 50 mM sodium acetate buffer pH 4.8 was mixed with 50 μl of sample and incubated at 50 °C for 15 min. The reaction was terminated by the addition of 750 μl of stop solution. Stop solutions were prepared according to the instruction from the supplier of the substrates. After 20 min at room temperature, the samples were centrifuged at 2000 × *g* for 10 min and the absorbance of the supernatant was read at 620 nm. Standard curves prepared using purified endoxylanase (Megazyme endo-β-xylanase 880 U ml^{-1}) or endoglucanase (Megazyme EGII 1000 U ml^{-1}) from *Trichoderma* sp. were used to calculate the enzyme activities.

The activity of β-glucosidase and β-xylosidase was measured as described previously (Jørgensen et al., 2003).

2.3. Quantification of sugars and extracellular metabolites

Glucose, xylose, glycerol, gluconic acid, pyruvate, acetate and ethanol were separated on HPLC using an Aminex HPX-87H column (Bio-rad, Hercules, CA) according to Zaldivar et al. (2002). Glucose, arabinose, galactose, mannose and xylose were separated on a Dionex HPLC system in a CarboPac PA1 column according to Zaldivar et al. (2002).

3. Results and discussion

The three fungal species *P. pinophilum* IBT 4186, *P. persicinum* IBT 13226 and *P. brasilianum* IBT 20888 has been selected as potential good producers of cellulases and/or hemicellulases in a screening of a number of fungi belonging to the genus *Penicillium* (Krogh et al., 2003). The three species were investigated in batch cultures using various monosaccharides as carbon source to characterise their growth and to study the effect of the carbon source on the production of cellulases and hemicellulases.

3.1. Growth and sugar uptake

Either glucose or xylose was used as carbon source at a concentration of 40 g l^{-1}. The cultivations with *P. brasilianum* IBT 20888 without the addition of the complex nutrients peptone and yeast extract were performed using 30 g l^{-1} of carbon source. *P. pinophilum* IBT 4186 and *P. persicinum* IBT 13226 had approximately the same maximum specific growth rate (μ_{max}) on both xylose and glucose, 0.08–0.09 h^{-1} (Table 1). During growth of *P. brasilianum* IBT 20888 on glucose, μ_{max} was $0.18 \pm 0.04\,h^{-1}$ ($n = 2$) with the addition of peptone and yeast extract to the medium and $0.15 \pm 0.02\,h^{-1}$ ($n = 2$) without, or on average $0.16 \pm 0.03\,h^{-1}$ (Table 1). On xylose, μ_{max} were 0.15 and $0.14 \pm 0.03\,h^{-1}$ ($n = 2$) with and without peptone and yeast extract, respectively. The only significant effect of omitting peptone and yeast extract was a 10 h longer lag phase compared to the lag phase on the complex medium. The μ_{max} obtained in this study were within the range reported for other Penicillia grown on glucose or sucrose (Goudar and Strevett, 1998; Petruccioli et al., 1995).

The biomass yield (Y_{sx}) was found to be 0.5 g g^{-1} (dry-weight per sugar) for both *P. persicinum* IBT 13226 and *P. brasilianum* IBT 20888 on both glucose and xylose (Table 1), which has also been reported for other fungi (Goudar and Strevett, 1998). Omission of peptone and yeast extract had no significant effect on Y_{sx} for *P. brasilianum* IBT 20888. *P. pinophilum* IBT 4186 had significantly lower Y_{sx} compared to the two other fungi and a carbon balance revealed that 30–40% of the carbon in the consumed sugar could not be accounted for in biomass, CO_2 and glycerol. Some *Penicillium* species produce glucose oxidase, which converts glucose into gluconic acid, and Y_{sx} down to 0.1 g g^{-1} have been reported (Petruccioli et al., 1995). The production of gluconic acid by *P. pinophilum* IBT 4186 was verified by HPLC analysis, thereby explaining the low biomass yields.

The preference for sugar utilisation was investigated by cultivating *P. pinophilum* IBT 4186 and *P. persicinum* IBT 13226 on a mixture of sugars. The sugar concentrations were chosen to mimic the typical composition in the hydrolysate of lignocellulosic material (glucose 15 g l^{-1}, xylose 10 g l^{-1}, mannose, galactose, arabinose 5 g l^{-1}). *P. brasilianum* IBT 20888 was studied on a mixture containing glucose (10 g l^{-1}), arabinose (5 g l^{-1}) and xylose (5 g l^{-1}). As also observed for other microorganisms (Scazzocchio et al., 1995), glucose was the first sugar to be metabolised and no uptake of the other sugars was observed before glucose depletion (Fig. 1). After glucose depletion, mannose, xylose and galactose started to be metabolised. All three sugars were utilised simultaneously by *P. pinophilum* IBT 4186 and *P. persicinum* IBT 13226, but the uptake rate of xylose was higher than the uptake rate of galactose (Fig. 1). Arabinose was the last sugar to be utilised and the uptake rate was considerably lower than for the other sugars by all three species

Table 1
Growth characteristics during batch growth on either glucose or xylose

Strain	Substrate	μ (h^{-1})	Y_{sx} (g g^{-1})
P. pinophilum IBT 4186	Glucose	0.08 ± 0.00[a]	0.33 ± 0.07
P. persicinum IBT 13226	Glucose	0.09	0.56
	Xylose	0.09	0.51
P. brasilianum IBT 20888	Glucose	0.16 ± 0.03[b]	0.47 ± 0.04
	Xylose	0.14 ± 0.02[c]	0.48 ± 0.02

[a] Average of two cultivations.
[b] Average of four cultivations—two with and two without peptone and yeast extract.
[c] Average of three cultivations—one with and two without peptone and yeast extract.

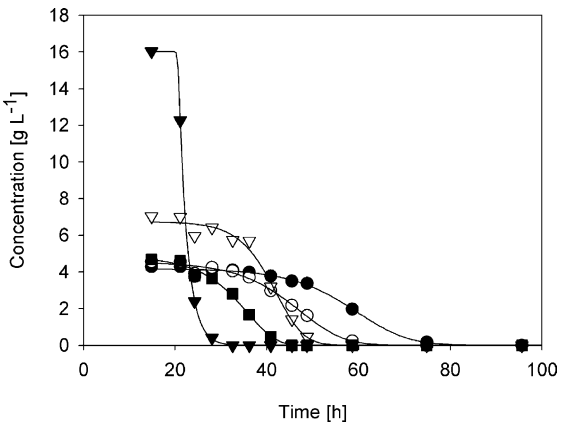

Fig. 1. Consumption of sugars by *P. pinophilum* IBT 4186 during batch growth on a mixture of (▼) glucose, (▽) xylose, (●) arabinose, (○) galactose and (■) mannose.

(Fig. 1). *P. persicinum* IBT 13226 and *P. brasilianum* IBT 20888 did not begin to metabolise arabinose before all other sugars were depleted. Xylose and arabinose have part of their pathway in common and during growth of *Aspergillus* on xylose some of the enzymes involved in the arabinose catabolism are expressed (Witteveen et al., 1989). However, results from this study indicate that xylose is either repressing the expression or inhibiting some of the enzymes involved in the catabolism of arabinose as no arabinose consumption started before xylose depletion.

3.2. Enzyme production on glucose and xylose

Glucose was also found to repress the production of cellulases and xylanases. During exponential growth on glucose none of the three species produced enzymes with endoglucanase, β-glucosidase, endoxylanase or β-xylosidase activity (Fig. 2A). Similar results have been reported for other *Penicillium* and *Aspergillus* strains (Chavez et al., 2002; van Peij et al., 1998; Wang and Gao, 1999). In *Aspergillus*, the protein CreA, which is also responsible for the carbon catabolite repression, represses the expression of genes encoding cellulases and xylanases in the presence of glucose (de Vries and Visser, 2001). A consensus sequence for binding of CreA has also been found in the promoter of cellulase and xylanase genes from Penicillia (Chavez et al., 2002; Mernitz et al., 1996). Low β-glucosidase activity was measured in the medium at glucose concentrations below 2–4 g l^{-1}. This indicates that the regulation of the β-glucosidase genes differs from the regulation of the endoglucanase genes or the repression of these genes by glucose is less strong. The endoxylanase and β-xylosidase activity remained very low also after complete glucose utilisation, indicating that the transcription of these enzymes needs to be induced (Fig. 2A).

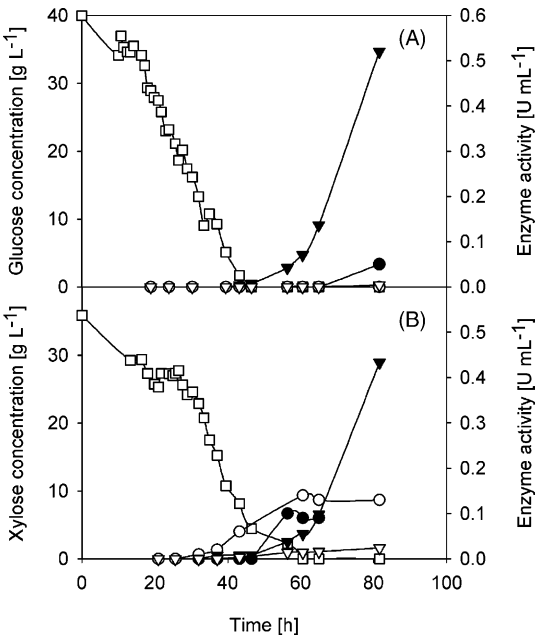

Fig. 2. Sugar and enzyme activities in the medium during growth of *P. brasilianum* IBT 20888 on glucose (A) or xylose (B). (●) Endoglucanase activity, (○) endoxylanase activity, (▼) β-glucosidase activity, (▽) β-xylosidase activity, (□) sugar concentration.

The cultivations on xylose revealed detectable activities of all four measured enzyme activities in the medium already before xylose depletion for all three species (Fig. 2B). The most pronounced difference compared to the cultivation on glucose was the production of endoxylanases and β-xylosidases in the presence of xylose (Fig. 2). As has also been reported for *Aspergillus niger*, although xylose can be regarded as an repressing sugar, it has a dual role as xylose is also a prerequisite for the expression of xylanases (de Vries et al., 1999; Gielkens et al., 1999). This effect of xylose was also found to hold for fungi from genus *Penicillium* in this study.

In conclusion, this study showed that glucose is repressing the utilisation of a number of other sugars as well as the production of cellulases and xylanases in the studied *Penicillium* species. Xylose is a less repressing sugar as other sugars, with the exception of arabinose, can be utilised simultaneously and the enzyme production is also less repressed. The production of xylanases even seems to require the presence of xylose.

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Professor Jens Christian Frisvad (BioCentrum-DTU, Technical University of Denmark) is thanked for providing the fungi. The work was financially supported by the Danish Technical Research Council (STVF).

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Paper C

Production of cellulases and hemicellulases by three *Penicillium* species: effect of substrate and evaluation of cellulose adsorption by capillary electrophoresis

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Production of cellulases and hemicellulases by three *Penicillium* species: effect of substrate and evaluation of cellulase adsorption by capillary electrophoresis

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Abstract

The production of cellulases and hemicellulases by *Penicillium pinophilum* IBT 4186, *P. persicinum* IBT 13226 and *P. brasilianum* IBT 20888 was studied during well-controlled batch cultivations using various polysaccharides as carbon source. Generally, the use of cellulose as carbon source resulted in production of cellulases whereas xylan resulted in production of xylanases, but the enzyme production by *P. pinophilum* IBT 4186 was less strictly regulated by the carbon source compared to the two other species. Capillary electrophoresis (CE) was used to quantify the main five cellulases produced by *P. brasilianum* IBT 20888 during cultivations on cellulose. It was found that the concentration of cellulases possessing a cellulose-binding module (CBM) was unaltered by an increased cellulose concentration in the cultivations, whereas the concentration of some cellulases without the cellulose-binding module was increased. The lack of correlation between initial cellulose concentration and final filter paper activity in the medium could therefore be assigned to adsorption of a fraction of the cellulases produced by *P. brasilianum* IBT 20888 onto remaining cellulose.

Keywords: *Penicillium pinophilum*; *Penicillium persicinum*; *Penicillium brasilianum*; Capillary electrophoresis; Hydrolysis; Steam pretreated spruce

1. Introduction

A complete cellulase system consists of cellobiohydrolases (exoglucanases), endoglucanases and β-glucosidases. As the nature of hemicellulose is more heterogeneous than cellulose a complex mixture of enzymes is required for its degradation: endoxylanases, β-xylosidases, endomannanases, β-mannosidases, α-L-arabinofuranosidases and α-galactosidases [1]. Efficient enzymatic hydrolysis of lignocellulosic material, e.g. for use in bioethanol production, therefore requires a complex, but balanced mixture of enzymes.

The regulation of cellulase and hemicellulase production in both *Trichoderma* and *Aspergillus* has been thoroughly investigated. A number of different proteins involved in the transcriptional regulation of the genes encoding cellulases and hemicellulases have been identified [2,3]. The expression of the enzymes has been found to be positively regulated via inducer molecules originating from the degradation of lignocellulosic material, e.g. cellobiose, D-xylose and L-arabinose [1]. In *Aspergillus*, the transcription activator XlnR has been identified to be involved in the induction of both cellulases and hemicellulases [1]. The protein ACEII with similar function has been found in *Trichoderma* [4]. Despite of the similarities in the regulatory proteins discovered in these two fungi, essential differences in the regulation do exist. Sophorose is an effective inducer of cellulase genes and some xylanase genes in *Trichoderma* [2]. This disaccharide does, however, not function as an inducer of an endoglucanase gene in *Penicillium janthinellum* [5] and

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has no effect on the expression of several cellulolytic genes in *A. niger* [6]. The fungi respond differently to inducers in medium and the choice of carbon source might therefore strongly effect the composition of the produced enzyme preparation.

An inherited problem associated with cellulase production is the presence of a cellulose-binding module (CBM) on most cellulases, which results in strong adsorption of the enzymes onto cellulose [7]. The enzyme preparations produced in cultivations using cellulose as carbon source might therefore be biased by adsorption of a part of the produced cellulases onto remaining cellulose in the bioreactor. However, this is difficult to evaluate as most fungi produce more enzymes with similar activity that cannot be distinguished with traditional enzymatic assays used to measure the cellulase production in most studies.

Although *Trichoderma* strains are efficient producers of cellulases, a frequently reported problem with the cellulase preparations produced by hyperproducing *T. reesei* strains is the low β-glucosidase activity that reduces the performance during hydrolysis of lignocellulosic material [8,9]. *Penicillium* species with the ability to produce high cellulase and hemicellulase titres have been described [10], but much less is known about the regulation and production of these enzymes by *Penicillium* species compared to *Trichoderma* and *Aspergillus*.

In the present study, three fungal species belonging to genus *Penicillium*, selected after a previous screening of a larger number Penicillia [11], were investigated thoroughly in well-controlled batch cultivations. The effect of the carbon source on the production of cellulases and some hemicellulases was investigated in cultivations using various polysaccharides as carbon source. We have earlier developed a quick method for separation and quantification of five different cellulases produced by *P. brasilianum* IBT 20888 [12]. In this study, the issue relating adsorption of cellulases onto cellulose in the medium was investigated by using CE to measure the production of individual enzymes as function of the substrate concentration during the cultivation. Finally, the performance of the produced enzyme preparations was evaluated by hydrolysis of steam pretreated spruce and compared to a commercial enzyme preparation.

2. Material and methods

2.1. Strains and preparation of inoculum

The three fungal species *P. pinophilum* IBT 4186, *P. persicinum* IBT 13226 and *P. brasilianum* IBT 20888 from the culture collection at BioCentrum-DTU were investigated. Fungal spores were stored in a 17% (v/v) glycerol suspension at −80 °C. *P. persicinum* IBT 13226 and *P. brasilianum* IBT 20888 spores for inoculation of the bioreactors were propagated on rice according to [13] by inoculation of the rice with spores from the glycerol solution. After 1–2 weeks at 25–30 °C, the spores covering the rice were harvested by addition of 100 mL of 0.1% (v/v) Tween 80 in sterile water. *P. pinophilum* IBT 4186 was first grown in a shake flask with 100 mL of medium (see below) with 10 g L^{-1} glucose. After 3–6 days at 30 °C, 12 mL of this culture was transferred to rice to propagate spores. The bioreactors were inoculated to give a spore concentration of 10^6 spores mL^{-1}.

2.2. Medium

The medium composition was based on the medium of Mandels and Weber [14] containing per L for each 10 g L^{-1} of carbon source: 2.0 g KH_2PO_4, 1.4 g $(NH_4)_2SO_4$, 0.3 g Urea, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.3 g $CaCl_2$, 5 mg $FeSO_4 \cdot 7H_2O$, 1.56 mg $MnSO_4 \cdot H_2O$, 1.4 mg $ZnSO_4 \cdot 7H_2O$, 2.0 mg $CoCl_2$, 0.75 g bacto peptone (Difco), 0.25 g yeast extract (Difco). Tween 80 was added to a concentration of 0.2 mL L^{-1}. Foaming was controlled by the addition of 1 mL L^{-1} of Struktol SB2121 antifoam (Qemi Int., USA). The carbon source was autoclaved separately from the mineral medium and subsequently added to the bioreactor. The carbon sources were Solka-Floc 200 FCC cellulose (Fiber-sales & Development Corp., USA), oat spelts xylan (Sigma X 0627) and birchwood xylan (Sigma X 4252). The substrate concentration was, if otherwise not stated, 40 g L^{-1}. The cultivation on the mixture of Solka-Floc, oat spelts xylan and birchwood xylan was conducted using 30 g L^{-1} of cellulose and 5 g L^{-1} of each type of xylan.

2.3. Cultivation conditions

The batch cultivations were carried out aerobically at 30 °C (*P. pinophilum* IBT 4186 and *P. brasilianum* IBT 20888) or 25 °C (*P. persicinum* IBT 13226) in well-controlled four-baffled 5 L in-house-manufactured bioreactors with a working volume of 4 L. The pH was maintained at 5.0 by the addition of either 2 M NaOH or 2 M HCl. The agitation was 300–500 rpm and the aeration rate was 4 L min^{-1} (1 vvm). Samples were withdrawn at regular time intervals during the cultivations, filtered through a 0.22 μm low protein-binding filter (Cameo 25 GSS, Osmonics, USA) and stored at −20 °C.

2.4. Enzymatic assays

Filter paper activity was determined according to [15] and reducing sugars were measured by the DNS method [16], using glucose as standard. The xylanase activity was measured by the method of [17] using birchwood xylan (Roth 7500, Karlsruhe, Germany). Reducing sugar was measured by the DNS method using xylose as standard. Mannanase activity was measured using locust bean gum (Sigma G-0753) in 50 mM Na-citrate buffer, pH 5.3, as substrate [18]. Reducing sugar was measured by the DNS method using mannose as standard.

Endoxylanase activity was measured using azo-arabinoxylan (Megazyme, Bray, Ireland) as substrate and endoglucanase activity was measured using azo-carboxymethyl cellulose (Megazyme, Bray, Ireland) as substrate according to [19]. Standard curves prepared by using purified endoxylanase (Megazyme endo-β-xylanase 880 U mL^{-1}) or endoglucanase (Megazyme EGII 1000 U mL^{-1}) from *Trichoderma* sp. were used to calculate the enzyme activities.

The activity of β-glucosidase, β-xylosidase, α-galactosidase and α-L-arabinofuranosidase was measured at pH 4.8 using 1 mM *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, *p*-nitrophenyl-α-D-galactopyranoside and *p*-nitrophenyl-α-L-arabinofuranoside, respectively, as substrate as described previously [20].

2.5. Measurement of extracellular protein

Extracellular protein was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA), which is based on the Bradford method. The analysis was performed using a Cobas-Mira analyzer (Roche, Switzerland), and γ-globulin (Sigma G-7516) was used as standard.

Separation and quantification of enzymes by CE was performed according to [12]. Purified enzymes; two cellobiohydrolases (CBHa and CBHb), three endoglucanases (EGa, EGb1 and EGb2) and one endoxylanase (XYL) from *P. brasilianum* IBT 20888 [20] were used for identification and quantification of enzymes in the CE method.

2.6. Hydrolysis of steam pretreated spruce

The steam pretreatment of the spruce has been performed according to [21]. Steam pretreated spruce (SPS) was washed with distilled water before use to remove soluble sugars. The cultivation samples were concentrated and buffer exchanged to 50 mM sodium acetate pH 4.8 before the hydrolysis study using an Ultrafree 15 ultrafiltration unit with a 5 kDa cut-off (Millipore).

The hydrolysis was performed in 2.0 mL plastic tubes (CM-LAB, Vordingborg, Denmark) using an enzyme loading of 25 FPU (g cellulose)$^{-1}$ and a cellulose concentration of 21.7 g L^{-1}, corresponding to 38.5 g SPS L^{-1}. The commercial cellulase preparation Celluclast 1.5 L FG (Novozymes, Denmark) was used as reference. The hydrolysis was performed with and without the addition of 32.5 U β-glucosidase (g cellulose)$^{-1}$ from the commercial β-glucosidase preparation Novozym 188 (Novozymes, Denmark). The total volume was adjusted to 1 mL with 50 mM sodium acetate buffer pH 4.8. The hydrolysis was carried out at 40 °C with continuous mixing by inversion of the tubes at a speed of 10 rpm. After 24 h, the hydrolysis was terminated by filtering the samples through a 0.22 μm filter. The amount of glucose and xylose released was quantified using an HPAEC-PAD system according to [22].

3. Results

To get more knowledge about how various carbon sources constituting lignocellulosic material (*e.g.* cellulose and xylan) effect the enzyme production and thereby the composition of the final enzyme preparation, the three species were cultivated on three different polymeric carbon sources. Furthermore, it was investigated if all three species respond similarly to the applied carbon sources. Finally, the capability of the produced enzyme preparations to hydrolyse lignocellulosic material was tested on steam pretreated spruce and compared to a commercial enzyme preparation.

3.1. Enzyme production profile

The enzyme production by the three fungi during growth on three polymeric substrates (Solka-Floc cellulose (SF), oat spelts xylan (OX) and birchwood xylan (BX)) was determined by measuring the protein concentration and a number of enzyme activities throughout the cultivations. During growth on SF, the protein concentration increased steadily throughout the cultivation of the three species. The same trend was observed for the endoglucanase and β-glucosidase activities whereas the other measured enzyme activities (mannanase, xylanase, endoxylanase, β-xylosidase, α-galactosidase and α-L-arabinofuranosidase) increased until 120–160 h and then remained constant (data not shown).

The enzyme production profiles in cultivations using either BX or OX as carbon source were more complex as represented by the enzyme production in *P. brasilianum* IBT 20888 (Fig. 1). The endoxylanase and xylanase activity in the

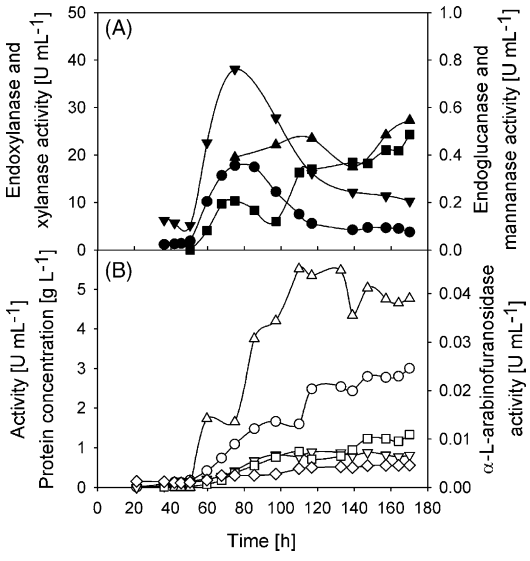

Fig. 1. Enzyme activities and protein concentration in the cultivation broth during growth of *P. brasilianum* IBT 20888 on 40 g L^{-1} of birchwood xylan. In (A) (■) endoglucanase activity, (▲) mannanase activity, (●) endoxylanase activity, (▼) xylanase activity. In (B) (△) α-L-arabinofuranosidase activity, (○) β-glucosidase activity, (▽) β-xylosidase activity, (□) α-galactosidase activity, (◊) protein concentration.

Table 1
Final protein concentrations and filter paper activities after cultivation of *P. pinophilum* IBT 4186, *P. persicinum* IBT 13226 and *P. brasilianum* IBT 20888 on various carbon sources

Carbon source[a]	*P. pinophilum* IBT 4186			*P. persicinum* IBT 13226			*P. brasilianum* IBT 20888		
	Time (h)	Protein ($g\,L^{-1}$)	FPA[b] ($FPU\,mL^{-1}$)	Time (h)	Protein ($g\,L^{-1}$)	FPA[b] ($FPU\,mL^{-1}$)	Time (h)	Protein ($g\,L^{-1}$)	FPA[b] ($FPU\,mL^{-1}$)
Solka-floc	221	0.66	0.28	236	0.61	0.80	229	1.07	0.75
Oat xylan	212	0.31	0.08	196	0.44	0.05	170	0.46	0.09
Birchwood xylan	224	0.59	0.08	224	0.45	0.02	170	0.56	0.05
Mixture	131	1.08	0.81	131	2.22	1.7	141	1.07	0.63

[a] Substrate concentration $40\,g\,L^{-1}$, except mixture: $30\,g\,L^{-1}$ Solka-floc, $5\,g\,L^{-1}$ oat xylan and $5\,g\,L^{-1}$ birchwood xylan.
[b] Filter paper activity.

medium started to increase rapidly after 50 h of the cultivation reaching a maximum at 80 h, after which the enzyme activities declined throughout the rest of the cultivation (Fig. 1A). This characteristic profile was observed on both BX and OX. In the cultivations with *P. pinophilum* IBT 4186, the maximum endoxylanase and xylanase activity was at 180–190 h and with *P. persicinum* IBT 13226 the maximum was at 90 h (OX) and 150 h (BX). The decline in endoxylanase activity in the last part of the cultivations indicated that the production of endoxylanases either stopped or was significantly reduced. Furthermore, the results showed that the stability of at least some of the endoxylanases under the given conditions was low. For all three species, the other enzyme activities measured started to increase simultaneously with the increase in endoxylanase activity, but their activity either continued to increase throughout the cultivations (endoglucanase, β-glucosidase and α-galactosidase) or reached a maximum and then remained constant throughout the rest of the cultivations (Fig. 1).

3.2. Effect of carbon source on enzyme production

High filter paper activities were as expected obtained in the cultivations containing cellulose (Table 1). However, the highest protein concentrations and also filter paper activities were in general obtained in the cultivations on a mixture of SF, OX and BX (Table 1). The cultivation of the fungi on only xylan (OX or BX) resulted in low filter paper activities, but also the total enzyme production (measured as the protein concentration) was significantly lower in the cultivations on any of the two types of xylan compared to on SF or the mixture of SF, OX and BX.

Due to the variation in the total protein production depending on the carbon source used (Table 1), the effect of the carbon source on the enzyme production is reported using specific activities ($U\,(g\,protein)^{-1}$). The specific filter paper and endoglucanase activities were significantly higher in samples from the cultivation on SF compared to those on OX or BX for all three species (Fig. 2). However, the enzyme mixtures produced by *P. persicinum* IBT 13226 and *P. brasilianum* IBT 20888 on SF had higher specific endoglucanase and filter paper activities compared to *P. pinophilum* IBT 4186. The specific mannanase activity also seemed to be highest in the cultivation on SF compared to on xylan. For *P. pinophilum* IBT 4186, the specific β-glucosidase activity was higher after the cultivation on SF compared to OX and BX (Fig. 2A), but for *P. persicinum* IBT 13226 and *P. brasilianum* IBT 20888 there was no clear correlation between carbon source and the specific β-glucosidase activity (Fig. 2B and C). The cultivation of the fungi on xylan (either OX or BX) resulted in between two- and eight-fold higher specific endoxylanase activities and between 5- and 20-fold higher β-xylosidase activities compared to the cultivations on SF (Fig. 2). However, the specific endoxylanase activities obtained were in general low in the cultivations

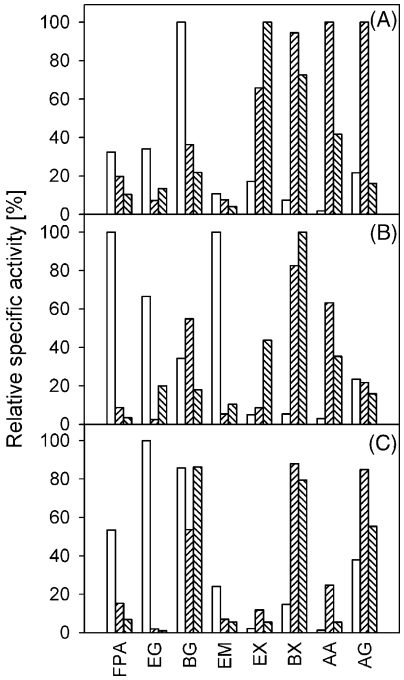

Fig. 2. Relative specific enzyme activities obtained in the cultivation of (A) *P. pinophilum* IBT 4186, (B) *P. persicinum* IBT 13226 and (C) *P. brasilianum* IBT 20888 on either cellulose (□), oat spelts xylan (▨) or birchwood xylan (▧). For each enzyme activity, the specific activities are relative to the highest value obtained by any of the three fungi on one of the three carbon sources. FPA, filter paper activity; EG, endoglucanase activity; BG, β-glucosidase activity; EX, endoxylanase activity; BX, β-xylosidase activity; AA, α-L-arabinofuranosidase activity; AG, α-galactosidase activity; EM, mannanase activity.

Table 2
Protein concentration and concentration of endoglucanases and cellobiohydrolases produced in cultivation of *P. brasilianum* IBT 20888 on Solka-Floc cellulose

Substrate concentration	Protein ($g\,L^{-1}$)	EGa ($mg\,L^{-1}$)	EGb1 ($mg\,L^{-1}$)	EGb2 ($mg\,L^{-1}$)	CBHa ($mg\,L^{-1}$)	CBHb ($mg\,L^{-1}$)
20	0.89	120	20	20	120	150
40	1.07	290	40	20	130	170

with *P. brasilianum* IBT 20888 compared to especially *P. pinophilum* IBT 4186, which produced the enzyme mixtures with the highest specific endoxylanase activities. The specific α-L-arabinofuranosidase activity produced was highest in the cultivation using xylan as carbon source, but the specific activity was more than two-fold higher after the cultivation on OX compared to BX for all three species. OX is an arabinoxylan and BX is a glucuronoxylan [23] and this diversity in the nature of the substrate consequently resulted in significant differences in the enzyme production, which is in accordance with previous results from *P. purpurogenum* [24]. The specific α-galactosidase activity did not clearly correlate with the carbon source (Fig. 2).

Using a mixture of SF, OX and BX as carbon source resulted for all three species in enzyme mixtures with specific activities in between those obtained on either SF or the two types of xylan (data not shown). That is, the specific filter paper and endoglucanase activities were lower using the mixed carbon source than using SF as carbon source, but higher than using OX or BX as carbon source. Likewise, the specific endoxylanase, β-xylosidase and α-L-arabinofuranosidase activities were higher using the mixed carbon source than using SF as carbon source, but lower than using OX or BX as carbon source.

The cultivations with *P. brasilianum* IBT 20888 were performed using a substrate concentration of both 20 and $40\,g\,L^{-1}$ of SF to study the effect of substrate concentration on the yield of enzymes produced. Doubling the SF concentration resulted only in a minor increase in the final protein concentration (Table 2). The final filter paper activities and the endoglucanase activities obtained were not significantly different (around $0.8\,FPU\,mL^{-1}$ and $90\,U\,mL^{-1}$, respectively). However, the final β-glucosidase activity was increased from 1.8 to $5.7\,U\,mL^{-1}$. CE was used to measure the concentration of some of the cellulases—the three endoglucanases EGa, EGb1 and EGb2 and the two cellobiohydrolases CBHa and CBHb (Table 2). The analysis revealed that only the concentration of EGa and EGb1 were significantly higher in the cultivation on $40\,g\,L^{-1}$ compared to $20\,g\,L^{-1}$.

3.3. Hydrolysis of steam pretreated spruce

The hydrolytic performance of the enzyme preparations obtained after the cultivation of the fungi on the mixture of SF, OX and BX was evaluated on steam pretreated spruce using the same amount of filter paper activity. The enzyme solutions were concentrated and the highest filter paper activity was obtained in the preparation from *P. persicinum* IBT 13226 (Table 3). However, the ratio between the filter paper activity and the β-glucosidase activity was highest in the preparation from *P. brasilianum* IBT 20888. A commercial cellulase preparation (Celluclast) was included in the study for comparison. Celluclast had a very low β-glucosidase activity compared to the filter paper activity (Table 3). To investigate whether the enzyme preparations were deficient in β-glucosidase activity, the hydrolysis was also performed with the addition of extra β-glucosidase activity (Novozym 188).

Table 3
Enzyme activities in concentrated enzyme preparations from *P. pinophilum* IBT 4186, *P. persicinum* IBT 13226 and *P. brasilianum* IBT 20888 and in commercial enzyme preparations

Enzyme preparation	Filter paper activity ($FPU\,mL^{-1}$)	β-glucosidase ($U\,mL^{-1}$)	Ratio[a] ($U\,FPU^{-1}$)
P. pinophilum IBT 4186	4.5	18.6	4.1
P. persicinum IBT 13226	11.5	21.7	1.9
P. brasilianum IBT 20888	3.3	25.0	7.6
Celluclast	55.7	21.1	0.4

[a] Ratio between β-glucosidase and filter paper activity.

After 24 h, the use of the enzyme preparation from *P. brasilianum* IBT 20888 resulted in 48% saccharification (calculated as glucose released relative to total theoretical amount) (Fig. 3). Despite the significant difference in the ratio between filter paper activity and β-glucosidase activity (Table 3), the enzyme preparations produced by the two other fungi yielded not significantly lower saccharification compared to the enzyme preparation produced by *P. brasilianum* IBT 20888. After addition of extra β-glucosidase activity, all three enzyme preparations yielded a saccharification of 55%

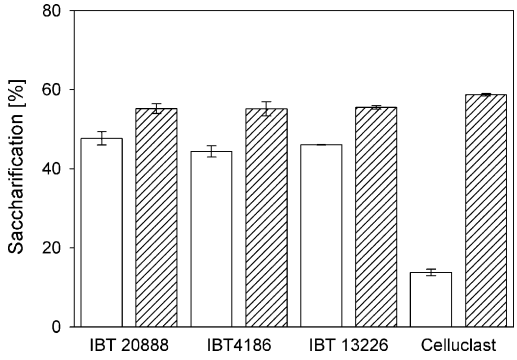

Fig. 3. Hydrolysis of steam pretreated spruce at 40 °C for 24 h by enzyme preparations from *P. pinophilum* IBT 4186, *P. persicinum* IBT 13226 and *P. brasilianum* IBT 20888 and a commercial enzyme preparation (Celluclast). The enzyme loading was 25 FPU $(g\,cellulose)^{-1}$ and the hydrolysis was performed without (□) and with addition of surplus of β-glucosidase activity (▨). The saccharification is calculated from the amount of released glucose relative to the total theoretical amount ($n = 2$).

(Fig. 3). The increased saccharification for the *Penicillium* enzyme preparations was probably due to the presence of endoglucanase activity in Novozym 188 [25]. Celluclast was clearly deficient in β-glucosidase activity (14% saccharification), but the addition of additional β-glucosidase activity (Novozym 188) up to 42.5 U β-glucosidase $(g\,cellulose)^{-1}$ resulted in a significantly improved saccharification to 59%.

4. Discussion

The three fungal species *P. pinophilum* IBT 4186, *P. persicinum* IBT 13226 and *P. brasilianum* IBT 20888 have been selected from a screening of a number of fungi belonging to the genus *Penicillium* [11]. In this investigation, the potential of these three *Penicillium* species as produces of cellulases and hemicellulases was investigated and the effect of the carbon source on the enzyme production was studied in well-controlled cultivations.

All three species produced mainly cellulases during growth on cellulose (SF) as a carbon source, whereas mainly hemicellulases were produced during growth on xylan (OX and BX). However, the three species showed some differences in the magnitude of the response to the various carbon sources. In *P. brasilianum* IBT 20888 and *P. persicinum* IBT 13226, the specific endoglucanase activities were 50- to 100-fold higher in the cultivation on SF compared to on xylan, whereas for *P. pinophilum* IBT 4186, the specific endoglucanase activities were only three- to six-fold higher on SF compared to xylan (Fig. 2). Also, the ratio between the endoglucanase and endoxylanase activity in the cultivation of *P. pinophilum* IBT 4186 on SF was around 1, whereas the ratios were 9 and 30 for *P. persicinum* IBT 13226 and *P. brasilianum* IBT 20888, respectively. The results indicate differences within the regulation of the enzyme production in the three species in this study. The enzyme production seemed to be less strictly controlled by the carbon source in *P. pinophilum* IBT 4186 compared to *P. persicinum* IBT 13226 and *P. brasilianum* IBT 20888.

A high specific mannanase activity was in general observed in the cultivations on SF (Fig. 2). This could be due to co-induction of mannanase genes, but it could also be the result of the problems associated with enzyme activity determinations. The substrates used in enzymatic assays are not always specific for just one class of enzyme and simultaneously the specificity of the enzymes might be broad. Two endoglucanases (EGb1 and EGb2) produced by *P. brasilianum* IBT 20888 have been shown to additionally have activity towards locust bean gum [20], which was used as substrate for measuring mannanase activity in this study. The observed higher specific mannanase activity after the cultivation of the fungi on SF might therefore have been due to the production of more endoglucanases having mannanase side activity and not a direct production of mannanases.

The production of five cellulases produced by *P. brasilianum* IBT 20888 was studied in more detail using CE, thereby allowing the levels of individual enzymes to be quantified, which can not be done with traditional enzymatic assays [12]. Earlier studies with *T. reesei* have shown a linear correlation between cellulase concentration and cellulase production up to 60 $g\,L^{-1}$ [26]. However, in the present investigation an increase in the cellulose concentration from 20 to 40 $g\,L^{-1}$ did neither yield a higher filter paper activity nor endoglucanase activity in the cultivation with *P. brasilianum* IBT 20888, only the β-glucosidase activity was increased three-fold. The CE measurements revealed that the final concentration of the cellulases possessing a CBM (CBHa, CBHb and EGb2) was similar using either 20 or 40 $g\,L^{-1}$ of cellulose (Table 2). Only the concentration of two endoglucanases not possessing a CBM (EGa and EGb1 [20]) were found to be noticeably higher in the cultivation on 40 $g\,L^{-1}$ of cellulose compared to the cultivation on 20 $g\,L^{-1}$. The specific activities of EGa and EGb1 on Avicel cellulose have been found to be three- to four-fold lower compared to CBHa, CBHb and EGb2 [20], and this can explain that the measured activities were not significantly increased although more EGa and EGb1 were produced. The fact that only enzymes without a CBM (EGa, EGb1 and β-glucosidases) were actually present in larger quantities in the cultivation with the high cellulose concentration could indicate that more enzyme had been produced, but a larger fraction remained adsorbed onto the cellulose in the cultivation broth after the cultivation had ended. This points out two important conclusions: (1) complete hydrolysis of all the substrate is consequently essential for maximum recover of the produced enzyme and (2) although the same substrate has been used and similar filter paper activities were obtained, the actual composition of the enzyme preparation might be significantly different due to differences in the adsorption of individual cellulases.

In hydrolysis studies, the presence of sufficient β-glucosidase activity is often critical as the accumulation of cellobiose can strongly inhibit the activity of the cellulases [27]. Celluclast and other cellulase preparations produced by *T. reesei* normally have a low β-glucosidase activity and extra β-glucosidase activity is frequently added to avoid temporary product inhibition caused by cellobiose [8,28]. The enzyme loading used in the hydrolysis of steam pretreated spruce corresponded to 10, 48, 103 and 190 U β-glucosidase $(g\,cellulose)^{-1}$ using the enzyme preparations from Celluclast, *P. persicinum* IBT 13226, *P. pinophilum* IBT 4186 and *P. brasilianum* IBT 20888, respectively. The results indicated that β-glucosidase activities above 50 U $(g\,cellulose)^{-1}$ or a ratio of two between β-glucosidase activity and filter paper activity do not result in increased saccharification at the substrate concentration used in this study (3.9% (w/v)). In commercial scale hydrolysis of lignocellulosic materials, the substrate concentration will be much higher (10–15% (w/v) [29]). Under these conditions, the much higher β-glucosidase activities in the enzyme preparations produced by the three *Penicillium* species, especially *P. brasilianum* IBT 20888, might be beneficial for the hydrolysis performance and final yields. The results verified that all three tested fungi produced

enzyme preparations that could potentially be used for hydrolysis of lignocellulosic materials and with the advantage of containing high β-glucosidase activity.

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Paper D

Cloning and characterization of a GH3 β-glucosidase from *Penicillium brasilianum* including a novel method for measurement of glucose inhibition on cellobiose hydrolysis

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Submitted

Characterization of a thermostable GH3 β-glucosidase from *Penicillium brasilianum* including a novel method for measurement of glucose inhibition on cellobiose hydrolysis

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Abstract

The extracellular β-glucosidase (BGL) from *Penicillium brasilianum* IBT 20888 was purified to homogeneity in a seven step procedure from a culture filtrate, and it was partly sequenced. Using this information the gene was cloned from a genomic library and it was successfully expressed in *Aspergillus oryzae*. Sequence analysis predicted a gene encoding a 97 kDa enzyme belonging to the family 3 glycoside hydrolases. A region in the protein (GFVMSDW) was found identical to a corresponding region, which includes the catalytic nucleophile in a GH3 BGL from *Aspergillus niger*, *Aspergillus wentii* and *Aspergillus aculeatus*. The BGL was thermostable with 100 % residual activity after 24h incubation at 60 °C at pH 4 - 6. Enzyme kinetics on *para*-nitrophenyl-β-D-glucopyranoside (*p*NP-Glc) and cellobiose revealed pronounced substrate inhibition using *p*NP-Glc as substrate and significantly lower affinity for cellobiose compared to *p*NP-Glc. Inhibition studies with *p*NP-Glc and cellobiose as substrate were performed with both glucose and D-glucono-δ-lactone as inhibitors. To characterize glucose inhibition on cellobiose hydrolysis, a new assay for determining such inhibition was developed using labeled glucose-$^{13}C_6$ as inhibitor and subsequent mass spectrometry analysis to quantify the hydrolysis rates.

Introduction

The present trend in the chemical industry is to move from a petroleum-based to a more bio-based production of commodity and fine chemicals. Cheap and abundant raw materials are required to support such a development. Currently, the predominant carbon sources are starch and sugar containing crops. Plant biomass is in general composed of approximately 40 % cellulose, which makes

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cellulose an abundant carbon source, and unlike many starch and sugar containing crops cellulose is not used for food production [1]. With the increasing demand for glucose for bio-based production processes, cellulose, a homopolymer of D-glucose, is a promising carbon source [2].

An efficient release of glucose from cellulose can be obtained by enzymatic hydrolysis, and it requires synergistic action of three classes of glycoside hydrolases: endoglucanases, cellobiohydrolases, and β-glucosidases (BGLs). Cellobiohydrolases (EC 3.2.1.91) release cellobiose (β-D-glucose-1,4-D-glucose) from the ends of the cellulose chain. Endoglucanases (EC 3.2.1.4) hydrolyze glucosidic linkages within the interior part of the cellulose chain, opening more sites for the action of cellobiohydrolases. Finally, β-glucosidases (EC 3.2.1.21) hydrolyze cellobiose to glucose [3]. In the CAZy database (http://www.cazy.org/CAZY/) enzymes hydrolyzing glycosidic bonds are classified according to structural similarities, and BGLs are predominantly classified as members of glycosyl hydrolase family GH1 or GH3 [4]. Primarily, BGLs are considered to be of great value in the hydrolysis of cellulose, since their presence results in a higher hydrolysis rate (due to decreased end product inhibition from cellobiose) and a more complete degradation, i.e. to a higher glucose yield. Other interesting applications of BGLs are release of flavors in fruits and synthesis of diverse oligosaccharides [5].

Currently, the filamentous fungus *Trichoderma reesei* is the predominant microorganism used by industry for production of cellulose degrading enzymes. During cellulose hydrolysis, the cellobiohydrolase and endoglucanase release shorter cellooligomers, primarily cellobiose, and as this fungus produce insufficient BGL activity, cellobiose accumulates. Cellobiose inhibits the action of cellobiohydrolases and endoglucanase (reviewed by [6]). Numerous studies have shown that cellulose hydrolysis is improved using cellulases from *T. reesei* supplemented with extra β-glucosidase activity [7-11]. For example, Novozym 188 is a commercial β-glucosidase enzyme preparation commonly used to supply sufficient β-glucosidase activity. Other filamentous fungi have been shown to be good producers of cellulose degrading enzymes [12;13], and in a recent study *P. brasilianum* was found to produce a more balanced mixture of cellulolytic enzymes than *T. reesei* due to a relatively high β-glucosidase activity, which led to a more efficient cellulose hydrolysis [11].

In the collaboration between the American National Renewable Energy Laboratory (NREL) and Novozymes to reduce the cellulase cost in cellulose hydrolysis, a significant contributor to the cost reduction in cellulases reported by Novozymes was the introduction of heterologously expressed BGL to a strain of *T. reesei*. There is currently a desire to further increase the temperature of cellulose saccharification in order to increase reaction rates and minimize microbial contamination. One limitation to that increase is the relatively low long-term temperature stability of available commercial BGLs.

Cellobiose is the natural substrate for BGLs, but several synthetic substrates have been used in the characterization of these enzymes, *e.g.*, methylumbelliferyl-β-D-glucopyranoside, and *para*-nitrophenyl-β-D-glucopyranoside (*p*NP-Glc), where the latter is the most frequently used. In addition to being commercially available, the major advantage of using *p*NP-Glc as a substrate for activity measurements is that BGL liberates *p*-nitrophenol that can be measured spectrophotometrically by absorbance at 405 nm. In most applications of BGLs, cellobiose is the natural substrate and thus it should be used preferentially for determination of kinetic parameters. In studies for temperature and pH stability *p*NP-Glc is a convenient substrate since it allows fast activity measurements.

Substrate inhibition has been observed for many BGLs, and several fungal BGLs have been shown to have transglycosylation activity as well as BGL activity [14-18]. During the process of hydrolysis a glycosyl-enzyme intermediate is formed through a nucleophilic attack, typically from the carboxylate of an aspartate residue [19;20]. In the glycoside hydrolase reaction, a water molecule reacts with this intermediate to release the sugar moiety, whereas in transglycoslation a hydroxyl group from either a sugar or an alcohol reacts with the sugar moiety in the glycosyl-enzyme intermediate (reviewed by [19;21]). In hydrolysis of cellobiose at high glucose concentration, the sugar can be favored over a water molecule in the release of the intermediate. This can lead to the production of different glucose dimers through transglycosylation such as: sophorose (β-1,2), laminaribiose (β-1,3) or gentiobiose (β-1,6) [22]. During the hydrolysis of cellobiose, it has been shown that a GH3 BGL from *A. niger* produced gentiobiose when the glucose concentration was greater than 5 mM [23].

BGL can be inhibited not only by high substrate concentrations but also by the products formed or by different transition state analogues. One transition state in the formation of a glycosyl-enzyme intermediate is an oxocarbonium ion-like intermediate. The planarity of this intermediate is mimicked by D-glucono-δ-lactone [19]. This transition state analogue has most frequently been investigated as an inhibitor for BGLs. Glucose at high concentration can either block the active site for the substrate or prevent the hydrolyzed substrate from leaving. The determination of glucose inhibition of cellobiose hydrolysis is presently quite difficult. To ensure constant reaction rates, only a small fraction of the cellobiose can be degraded, and at low cellobiose concentrations only very small amounts of glucose are produced. With an inhibitor (glucose) present at high concentration it requires high analytical precision to detect the relatively small changes in the amounts of glucose produced.

Here, we report the first sequence of a BGL from the genus *Penicillium*. From *P. brasilianum* a GH3 BGL was purified, a genomic library was constructed and a gene encoding a BGL was identified and heterologously expressed in *Aspergillus*

oryzae. The stability was investigated and compared to Novozym 188, a commercial product with β-glucosidase activity. The large quantities of heterologously expressed β-glucosidase made it possible to study and determine the kinetic parameters of the enzyme. The Michaelis-Menten constants K_M and V_{max} were determined using *p*NP-Glc and cellobiose as substrates. Inhibition of the hydrolysis of *p*NP-Glc and cellobiose by glucose and D-glucono-δ-lactone was investigated. A novel method to determine glucose inhibition on hydrolysis of cellobiose was developed. The kinetic parameters of the heterologously expressed BGL were compared to results obtained for the native *A. niger* BGL from Novozym 188 in order to benchmark the new *P. brasilianum* BGL.

Materials and methods

Culture conditions

Penicillium brasilianum strain IBT 20888 was grown on Mandels and Weber medium [24] supplemented per liter with 1 g of yeast extract, 3 g of bactopeptone, 30 g of Sigmacell cellulose type 20 (Sigma Chemical Co., St. Louis, MO), and 10 g of oat spelt xylan (Sigma Chemical Co., St. Louis, MO). Spores were propagated on rice according to [25] by inoculating with spores in a glycerol solution stored at -80°C . After 1 week at 30 °C, the spores were harvested using 0.1 % (v/v) Tween 80. A 5 L bioreactor, with a working volume of 4 L, was inoculated to a concentration of 1×10^6 spores per mL. The pH was maintained at 5.0 by addition of either 2 M NH_4OH or 2 M HCl. The temperature was kept at 30 °C. The aeration was 4 liters per minute and the agitation was 300-500 rpm. After 111 hours, the cultivation was terminated and the broth was filtered through a glass fiber filter (GD 120, Advantec, Japan).

Purification

The culture filtrate was concentrated and buffer exchanged to 20 mM triethanolamine/HCl pH 7.5 (TEA/HCl) using an Amicon ultrafiltration unit equipped with a PM10 membrane with 10 kDa cut-off (Millipore, Bedford, MA, USA). The enzyme purification was performed at 22 °C using a FPLC system (Amersham Bioscience, Uppsala, Sweden). All columns were from Amersham Bioscience. Between each purification step, the buffer was exchanged in the pooled fractions to the sample buffer using either an Amicon ultrafiltration unit or a 3.5 mL Microsep ultrafiltration unit with a 10 kDa cut-off (Pall Life Sciences, Ann Arbor, MI, USA). Elution of the β-glucosidase was monitored at 280 nm. After each purification step the fractions were assayed for β-glucosidase activity using *p*NP-Glc, and pooled based on specific activity and purity (SDS-PAGE).

(i) Anion-exchange chromatography on Q Sepharose HP. The column was washed with 180 mL of sample buffer (20 mM TEA/HCl pH 7.5). The enzyme was eluted with a gradient up to 50 % (over 800 mL) of 20 mM TEA/HCl pH 7.5 with 1 M NaCl. Fractions of 10 mL were collected, and fractions 81 to 85 were pooled.

(ii) Gel filtration chromatography on Superdex 75 10/300 GL. The sample buffer was 100 mM $NaCH_3CO_2$ pH 4.8 with 200 mM NaCl. The enzyme was eluted with 60 mL of the same buffer. Fractions of 2 mL were collected, and fractions 6 to 9 were pooled.
(iii) Anion-exchange chromatography on RESOURCE Q. The column was washed with 30 mL of sample buffer (10 mM sodium acetate, pH 4.8). The enzyme was eluted with a gradient up to 50 % (over 180 mL) of 500 mM $NaCH_3CO_2$ pH 4.8. Fractions of 2 mL were collected, and fractions 49 to 61 were pooled.
(iv) Anion-exchange chromatography on RESOURCE Q. The column was washed with 30 mL of sample buffer (10 mM $NaCH_3CO_2$ pH 4.8). The enzyme was eluted with a gradient up to 50 % (over 300 mL) of 500 mM $NaCH_3CO_2$ pH 4.8. Fractions of 2 mL were collected, fractions 63 to 67 were pooled.
(v) Cation-exchange chromatography on Source S. The column was washed with 31.5 mL of sample buffer (10 mM $NaCH_3CO_2$ pH 4.0). The enzyme was eluted with a gradient up to 15 % (over 120 mL) of 1 M $NaCH_3CO_2$ pH 4.0 and then with a gradient from 15 % to 100 % (over 90 mL) of 1 M $NaCH_3CO_2$ pH 4.0. Fractions of 2 mL were collected, and fractions 93 to 107 were pooled.
(vi) Gel filtration chromatography on Superdex 200 H10/300 GL. The sample buffer was 100 mM $NaCH_3CO_2$ pH 4.8 with 200 mM NaCl. The enzyme was eluted with 50 mL of the same buffer. Fractions of 0.5 mL were collected, and fractions 28 to 31 were pooled.
(vii) Hydrophobic interaction chromatography on Phenyl Sepharose HP. The column was washed with 17.0 mL of the sample buffer (1 M $(NH_4)_2SO_4$, 50 mM $NaCH_3CO_2$ pH 4.8). The enzyme was eluted with a gradient up to 100 % (over 70 mL) of 50 mM $NaCH_3CO_2$ pH 4.8. Fractions of 0.5 mL were collected, and fractions 73 to 78 were pooled.

Isoelectric focusing
Isoelectric focusing (IEF) was performed with a Pharmacia PhastSystem using IEF gels, pH 3–9 and Protein mixture (Broad pI 3.5–9.3) was used as pI marker. The gel was stained by the silver method for PhastGel IEF media.

Protein quantification
Protein concentration was determined using "Biorad total protein" assay based on the Bradford method [26] with γ-globulin (Sigma G-7516) as standard. For purified enzymes the protein concentration was determined using the protein specific extinction coefficient calculated from the algorithm of [27] used in GPMAW (Lighthouse data, Denmark).

N-terminal sequencing
A sample of the purified β-glucosidase was applied to SDS-PAGE followed by electroblotting to a ProBlott membrane (Applied Biosystems, Foster City, CA, USA). After staining, the piece of the ProBlott membrane with the β-glucosidase was placed in the blotting cartridge of Procise Protein Sequencer (Applied

Biosystems, Foster City, CA, USA). The N-terminal amino acid sequence of the β-glucosidase was determined directly using a Procise 494 HT Sequencing System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

DNA-library

After isolation of genomic DNA from *P. brasilianum*, a genomic library was constructed using a TOPO Shotgun Subcloning Kit (Invitrogen, Carlsbad, CA, USA). Briefly, total cellular DNA was sheared by nebulization under 10 psi nitrogen for 15 seconds and size-fractionated on 1 % agarose gels. DNA fragments migrating in the size range 3-6 kb were excised and eluted using a MiniElute™ Gel Extraction Kit (QIAGEN Inc., Valencia, CA, USA). The eluted fragments were size-fractionated again, excised and eluted to ensure sizes of 3-6 kb. The eluted DNA fragments were blunt end repaired using a mixture of a T4 DNA polymerase and a Klenow DNA polymerase and thereafter dephosphorylated using shrimp alkaline phosphatase (Roche Applied Science). The blunt end DNA fragments were cloned into the pCR4Blunt-TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, transformed into electrocompetent *Escherichia coli* TOP10 cells by electroporation, and plated on LB plates supplemented with 100 µg of ampicillin per ml. The electroporation resulted in 15,300 clones. Colony lifts were performed as described [28] and the DNA was cross-linked onto Hybond N+ membranes (Amersham, Arlington Heights, IL, USA) for 2 hours at 80 °C.

Probes

Based on the N-terminal amino acid sequence of the purified β-glucosidase, a forward primer was designed using the CODEHOP strategy [29]. Regions with a high degree of similarity in known β-glucosidases were used as scaffold to design a reverse primer using the CODEHOP strategy. The primers used were: forward primer: 5'-GCGCTATCGAGTCTTTCTCTGARCCNTTYTA-3' and reverse primer: 5'-GTCGGTCATGACGAAGCCNKGRAANCC-3', where R=A or G, Y=C or T, K=G or T and N=A, C, G or T, all with equal weight. A PCR reaction product of 840 bp using genomic DNA as template was excised from a 2 % agarose gel. The purified PCR product was subsequently cloned into a pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Forty ng of DNA was prepared using primers homologous to the vector, and the DNA was random-primer labeled using a Stratagene Prime-It II Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The radiolabeled gene fragment was separated from unincorporated nucleotide using a MinElute PCR Purification Kit (QIAGEN Inc., Valencia, CA, USA). The radioactive probe was denatured by adding 5.0 M NaOH to a final concentration of 0.5 M, and added to the hybridization solution at an activity of approximately 0.5 x 106 cpm per mL of hybridization solution. The mixture was incubated for 10 hours at 68 °C in a shaking water bath. Following incubation, the membranes were washed three times in 0.2X SSC (1X is 0.15 M NaCl and 0.015

M NaCitrate pH 7.0), 0.2 % SDS at 68 °C. The membranes were then dried on blotting paper for 15 minutes, wrapped in SaranWrap™, and exposed to X-ray film overnight at -80 °C with intensifying screens (Kodak, Rochester, NY, USA).

Colonies producing hybridization signals with the probe were inoculated into 1 mL of LB medium supplemented with 100 µg of ampicillin per mL and cultivated overnight at 37 °C. Dilutions of each overnight culture were made and 100 µL were plated onto LB agar plates supplemented with 100 µg of ampicillin per mL. A plate for each positive with about 40 colonies was chosen for secondary lifts. The lifts were prepared, hybridized, and probed as above. Two colonies from each positive plate were inoculated into 3 mL of LB medium supplemented with 100 µg of ampicillin per mL and cultivated overnight at 37 °C. Miniprep DNA was prepared from each colony using a Bio Robot 9600 (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. The size of each insert was determined by *Eco*R I restriction and agarose gel electrophoresis. Two clones contained a 4.5 kb insert. Sequencing revealed that the clones were identical and the vector was named pKKAB.

Vector for cloning

The β-glucosidase gene on pKKAB was amplified by PCR using the following two oligonucleotide primers: Forward 5'-AATT*TGATCA*CACCATGCAGGGTTCTACAATCTTTCTGCC-3' and reverse 5'-TTAA*CTCGAG*TTACTCCAATTGTGAGCTCAGCGG-3'. A restriction enzyme site was inserted into the 5' end of each primer, the forward primer contained a *Bcl* I site and the reverse primer contained an *Xho* I site (sites shown in italics). The PCR reaction produced a single DNA fragment of approximately 2.700 kb in length. The fragment was digested with *Bcl* I and *Xho* I and isolated by agarose gel electrophoresis, purified, and cloned into pJaL721 (WO 03/008575) digested with *Bam*H I and *Xho* I, resulting in a plasmid designated pKBK01. The sequence of the β-glucosidase gene in pKBK01 was verified by sequencing with a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Heterologous production of β-glucosidase

Aspergillus oryzae BECh2 (WO 00/30322) was transformed with 5 µg of pKBK01 as described [30]. Transformants were cultivated in 50 mL tubes for 4 days at 30 °C in 10 mL of a medium with 10 g/L yeast extract, 20 g/L peptone, and 2 % maltose. The whole broths were centrifuged at 12,100 x g and the supernatants collected. The supernatants were analyzed by SDS-PAGE using a Criterion XT Precast Gel, 10 % Bis-Tris gel in a XT MES buffer (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The transformant producing the highest titer of β-glucosidase estimated by SDS-PAGE and activity measurements was chosen for production in a bioreactor. After 5 days of cultivation on a maltose rich media, the β-glucosidase was purified. The biomass was removed from 2.5 L of cultivation broth by centrifugation and filtration. The resulting supernatant was brought to

5 liters with deionized water and ultrafiltrated on a Filtron with an 10 kDa membrane (Filtron, Northborough, MA, USA). The resulting volume of 1.2 L was adjusted to pH 8.5. The β-glucosidase solution was loaded onto a Q-Sepharose Fast Flow column (Amersham Biosciences, Uppsala, Sweden) pre-equilibrated with sample buffer (25 mM Tris pH 8.5). The β-glucosidase was eluted with a gradient up to 100 % (over 5 column volumes) 1 M NaCl in sample buffer. Fractions containing the β-glucosidase were pooled. A portion of the pool (40 ml) from the Q-Sepharose step was further purified on a Sephacryl S-200 HR (Pharmacia, Uppsala, Sweden) column pre-equilibrated in 0.1 M $NaCH_3CO_2$ pH 6.0. The β-glucosidase was eluted with the same buffer.

Enzymatic assays

Activity on *p*-nitrophenyl-β-D-glucopyranoside

β-glucosidase activity was measured on 1 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NP-Glc; Fluka 73676) in 50 mM NaCitrate buffer pH 4.80. The assays were performed in a microtiter plate at 22°C by incubation of 10 μL sample with 80 μL substrate, and 10 μL of buffer for 10 minutes (in kinetic experiments substituted with inhibitor dissolved in buffer). The reaction was terminated by the addition of 100 μL 0.5 M glycine/NaOH pH 10.0 with 2 mM EDTA. The absorbance was read at 405 nm in a microtiter plate ELISA reader. 1 unit (U) of activity was defined as the amount of enzyme hydrolyzing 1 μmol of *p*NP-Glc per minute.

Activity on cellobiose

β-glucosidase activity was also measured using 10 mM cellobiose (Fluka 22150) as substrate in 50 mM sodium acetate buffer, pH 4.80. The assays were performed at 22 °C by incubation of 10 μL sample with 80 μL substrate, and 10 μL of buffer for 10 minutes. The reaction was terminated by the addition of 25 μL 0.1 M NaOH and incubated at 65 °C for 15 minutes. Following neutralization by 25 μL 0.05 M HCl, the glucose concentration was determined using a kit: Ecoline S+ for glucose (1 2531 99 90 335 DiaSYS Diagnostics Systems GmbH). In the kit, glucose dehydrogenase catalyzes the oxidation of glucose and the amount of NADH produced is proportional to the glucose concentration. 1 unit (U) of activity was defined as the amount of enzyme hydrolyzing 1 μmol of cellobiose per minute.

pH and temperature optimum

The specific activity was investigated using 1 mM *p*NP-Glc in 50 mM NaCitrate. The pH in the substrate solution was adjusted to a pH of either 3.18, 4.16, 4.80, 6.17, 7.07, or 8.13. The temperatures investigated were 20 to 90 °C (20, 30, 40, 50, 60, 65, 70, 75, 80, 85, 90 °C). The reaction time was 10 minutes.

Stability
After 24 hours of incubation at different pH values in the range 2.0 to 10.0 and at different temperatures in the range of 20 to 80 °C, the residual activity was measured using the *p*NP-Glc activity assay.

Kinetic studies
All kinetic studies were carried out at 22 °C using various concentrations of *p*NP-Glc and cellobiose as substrates, respectivily. The β-glucosidase activity was measured using different concentrations of inhibitor. In the assay setup the 10 μL of buffer was substituted with the inhibitor dissolved in buffer. The inhibitors were glucose and D-glucono-δ-lactone in the range of 0.2 to 5K_i. For glucose inhibition on the hydrolysis of cellobiose, uniformly labeled glucose-$^{13}C_6$ (Sigma 389374) was used.

For the glucose inhibition assay, the hydrolysis rate of cellobiose was estimated by determining the release of glucose. The amount of released glucose was quantified by LC-MS using an Agilent 1100 LC coupled to an Agilent ion-trap MS. The LC separation was performed on a Luna 3 μm NH_2 column (100 mm x 2.0 mm) from Phenomenex (Torrance, CA, USA) with an isocratic flow of acetonitrile:water (90:10) at 0.5 mL/min for 10 min. The column temperature was kept a 40 °C and the injection volume was 1.0 μL. The MS was equipped with an electrospray source operated in negative mode and scanned from m/z 30-500. The capillary voltage was set to 3000 V and the remaining instrument parameters were optimized for detection of naturally and fully ^{13}C-labeled glucose. For quantification, the extracted ion chromatograms for the $[M-H]^-$ and $[M+Cl]^-$ were used for naturally and fully ^{13}C-labeled glucose, respectively. Apart from being the inhibitor in the assay, fully ^{13}C-labeled glucose was used as internal standard for quantification of glucose released during hydrolysis of cellobiose. All samples were analyzed in triplicate.

Results

After cultivation of *P. brasilianum* on a medium containing cellulose and xylan, the mycelia were removed by filtration. The filtrate was put through a series of chromatographic separation steps including ion exchange at different pH values, gel filtration, hydrophobic interaction and cation exchange step. The purification resulted in an enzyme with few, barely visible, impurities when silver stained. Based on highest sequence identity to two already characterized BGLs and a high specific activity on *p*NP-Glc and cellobiose, the enzyme was determined to be a BGL. The amount of BGL was 65 μg estimated to be more than 99 % pure. A second BGL was identified during purification; the N-terminal sequence of this BGL was found to have highest identity to another GH3 BGL, GH3 BGL from *A. niger* (Q30BH9).

A genomic library was made for *P. brasilianum* strain IBT 20888 and it was screened using nucleotide probes designed from the N-terminal sequence of the purified enzyme and of generally conserved sequences in other BGLs. It was found that the genomic coding sequence of 2751 bp (including stop codon) encodes a predicted polypeptide of 878 amino acids (GenBank accession no. EF527403). The sequence is interrupted by 2 introns of 57 bp (85-141 bp) and 57 bp (312-368 bp). Using the SignalP software program [31], a signal peptide of 19 residues was predicted. Based on the N-terminal sequence of the BGL, residues 20 through 36 appear to constitute a pro-region that is proteolytically cleaved during maturation. The N-terminal sequence of the mature BGL heterologously expressed was identical to the sequence found in the mature BGL from *P. brasilianum*. The mature protein is expected to consist of 842 amino acids with a molecular mass of 92.9 kDa. The amino acid sequence of the mature polypeptide shared 61.4 % identity to a characterized GH3 BGL from *Talaromyces emersonii* (GenBank AY072918) and 59.5 % identity to a GH3 BGL from *Trichoderma reesei* (GenBank AY281374). An alignment of the region with the proposed active site for a range of other GH3 BGLs (mentioned in [32]) revealed that the amino acid sequence Gly-Phe-Val-Met-Ser-Asp-Trp (aa 293-299) in *P. brasilianum* was identical to the sequence found in *Aspergillus niger* (CAB75696), *Aspergillus wentii* (P29090), and *Aspergillus aculeatus* (P48825). The sequence includes Asp-298, which in the active site has been demonstrated to perform the nucleophilic attack of the anomeric C1 carbon of the glucose moiety [33].

Molecular weight and pI

The molecular masses of the *P. brasilianum* BGL (BGL_{Pb}) and of the recombinant *P. brasilianum* BGL ($rBGL_{Pb}$) were both estimated to be 115 kDa by SDS-PAGE (data not shown). The pI value of BGL_{Pb} was determined to be 3.9.

Effect of temperature and pH on stability and specific activity

Due to the recalcitrance of cellulose, enzymatic hydrolysis typically lasts for several days before satisfactory conversion is achieved. In the present study, the pH and temperature stability were tested after a 24 h incubation period. After incubation at 60 °C, the $rBGL_{Pb}$ retained full residual activity in the range of pH 4.0 to 6.0, and outside this pH range no residual activity was measured. At 65 °C, the residual activity was 50 % at pH 5 and 6 and 20 % at pH 4 (data not shown). The commercial enzyme mixture Novozym 188 had full residual activity when incubated at pH 4 and 5 at temperatures up to 50f°C, and outside these pH-values less than 20 % residual activity was measured. After incubation at 60 °C, the residual activity for Novozym 188 was reduced to 50 % at pH 4 and 5.

The specific activity of the $rBGL_{Pb}$ was measured over a period of 10 minutes at different temperatures and pH values. The pH value resulting in the highest specific activity for the $rBGL_{Pb}$ was pH 4.80 at all temperatures tested (20 to 90

°C). At pH values 4.16, 4.80 and 6.17 the temperature optimum was 70 °C (Fig. 1). At pH values outside this range the temperature optimum was found at lower temperatures – an apparent consequence of the lower stability outside this pH range.

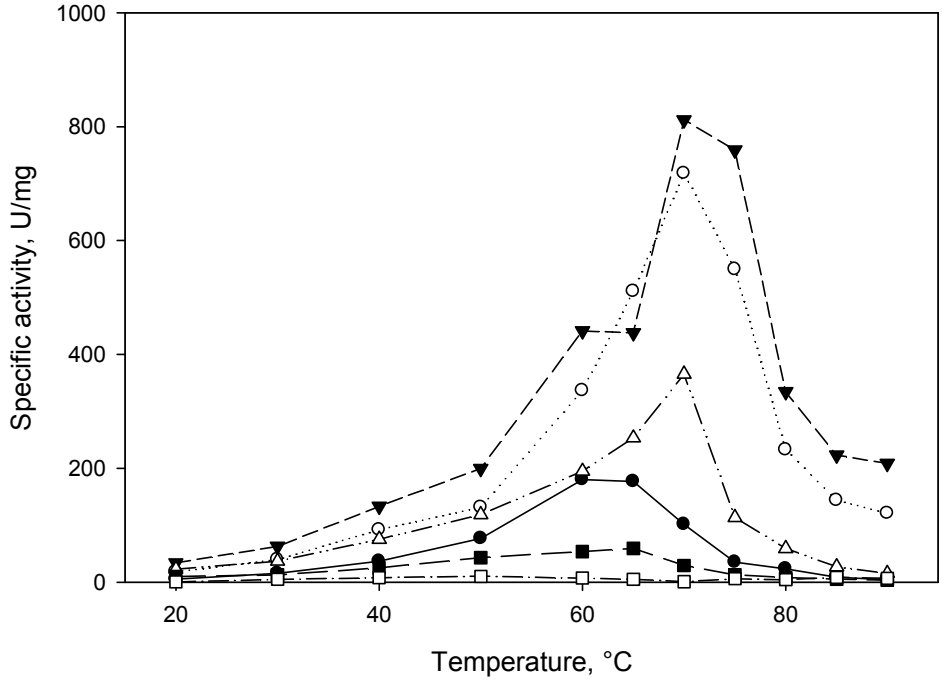

Fig. 1. Specific activity related to temperature and pH for recombinant BGL from *P. brasilianum* IBT 20888 measured by the degradation of 1 mM *p*NP-Glc at the pH values: ● 3.18; ○ 4.16; ▼ 4.80;△ 6.17; ■ 7.07, and □ 8.13. Reaction time 10 minutes. Two independent samples were prepared for each set of temperature and pH value (the average value is shown).

Substrate specificity and kinetic analysis

All kinetic experiments were conducted at pH 4.8, to ensure that the BGLs were stable and having the highest specific activity. To make sure that the substrate concentration was in excess during the hydrolysis experiments, it was carefully monitored that the substrate degradation did not exceed 10 % of the substrate. In kinetic characterization, the parameters determined for *P. brasilianum* were compared with those of a BGL purified from Novozym 188 (produced in *Aspergillus niger* and referred to as *A. niger* BGL_{An}).

The specific activity for purified BGL_{Pb} and the heterologously produced $rBGL_{Pb}$ on 1 mM *p*NP-Glc was found to be the same (data not shown). With the same N-teminal amino acid sequence and very similar molecular weights, the $rBGL_{Pb}$ was used in the kinetic analysis instead of the native BGL purified only in very low amount. The hydrolysis of *p*NP-Glc followed Michaelis-Menten (MM) kinetics when the substrate concentration was lower than 0.3 mM (Fig. 2), however, evidence of substate inhibition was seen at higher concentrations, and at 25 mM *p*NP-Glc the specific activity was reduced to 20 % of the maximum specific activity measured in the range of 0.25 mM *p*NP-Glc. In constrast, no substrate inhibition was observed in the hydrolysis of up to 10 mM cellobiose (data not shown). For BGL_{An} the hydrolysis of *p*NP-Glc followed MM kinetics with no substrate inhibition when the concentration was lower than 1.4 mM, and as with $rBGL_{Pb}$, no substrate inhibition was observed up to 10 mM cellobiose (data not shown).

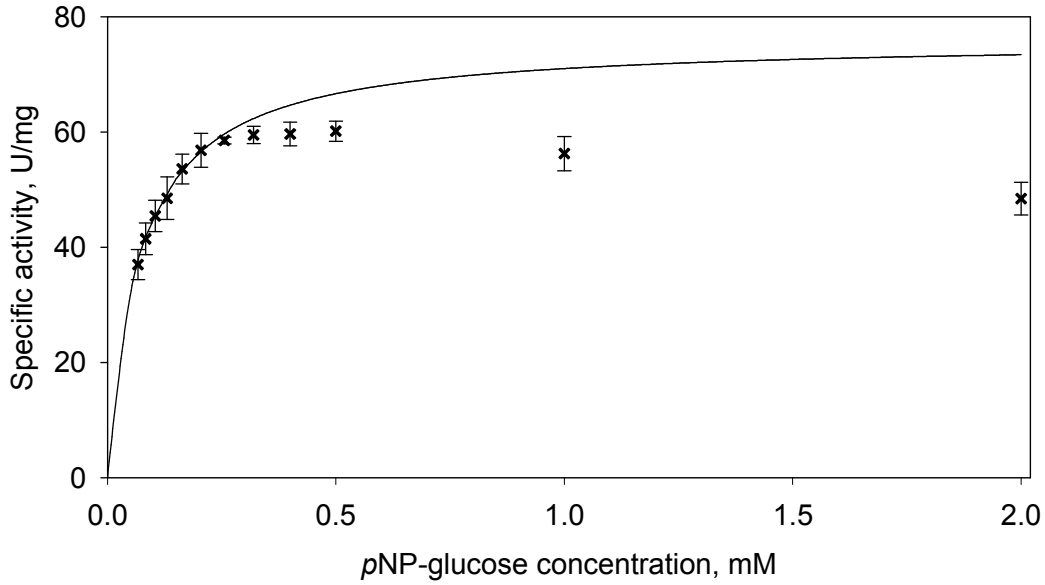

Fig. 2. Specific activity at different substrate concentrations for the recombinant BGL from *P. brasilianum* at 22 °C and pH 4.80 at different concentrations of *p*NP-Glc (n=4). (×) experimental results, (——) calculated results using the V_{max} and K_m values from table 1 using MM kinetics and no substrate inhibition component. (Note: At 25 mM *p*NP-Glc the specific activity was 15.7 U/mg).

The V_{max} and K_M values were estimated using six to nine substrate concentrations in the substrate ranges where the BGLs followed simple MM kinetics. Lineweaver-Burk (1/s, 1/v) and Dixon (s, s/v) plots were used to visually determine if MM could be applied. The MM parameters V_{max} and K_M (and K_i when glucose and D-glucono-δ-lactone were present, respectively) were calculated by least square fitting of the kinetic model. An overview of the determined kinetic parameters is given in Table 1.

Table 1. V_{max} and K_M for the hydrolysis of *p*NP-Glc and cellobiose, respectively, and inhibition constants for glucose and D-glucono-δ-lactone, respectively.

	rBGL$_{Pb}$[a]				BGL$_{An}$[b]			
	V_{max} U/mg	K_M mM	K_i (Glc[c]) mM	K_i (GL[d]) mM	V_{max} U/mg	K_M mM	K_i (Glc[c]) mM	K_i (GL[d]) mM
*p*NP-Glc	76	0.09	2.3	0.10	n.d.[e]	0.45	1.1	0.12
Cellobiose	28	1.58	1.1	0.012	n.d.[e]	0.35	1.6	0.11

[a] recombinant BGL from *P. brasilianum*, [b] BGL from *A. niger*, [c] glucose, [d] D-glucono-δ-lactone, [e] not determined.

The maximum specific activity V_{max} for the recombinant BGL from *P. brasilianum* was more than twice as high on *p*NP-Glc compared to cellobiose, 76 U/mg compared to 28 U/mg (k_{cat} of 118 s^{-1} compared to 43 s^{-1}). The affinity constant K_M was higher for cellobiose compared to *p*NP-Glc *i.e.* the enzyme has higher affinity to *p*NP-Glc, 0.09 mM compared to 1.58 mM. The BGL from *A. niger* had similar affinity for *p*NP-Glc and cellobiose.

Inhibition

A new assay for glucose inhibition on cellobiose hydrolysis

Product inhibition of BGL by glucose has in most studies been estimated using *p*NP-Glc as substrate. An advantage of using *p*NP-Glc is that the amount of glucose does not influence the absorbance measurement of released *p*NP. However, in application of BGL in cellulose hydrolysis it is important to know to which extent glucose inhibits the enzymatic hydrolysis of cellobiose, especially since cellobiose is a very potent inhibitor of CBH activity [34]. The determination of glucose inhibition on cellobiose hydrolysis requires high analytical precision to measure the small changes in glucose released during hydrolysis relative to the inhibitor concentration [35]. A way to get more glucose released is to use a higher cellobiose concentration and more BGL, but at high cellobiose concentrations, substrate inhibition increases as described by [36]. We have designed a new method including LC-MS to precisely determine glucose inhibition of cellobiose hydrolysis and Fig. 3 shows the results for inhibition of BGL$_{An}$. The inhibitor used in this newly developed assay was glucose-$^{13}C_6$. A cellobiose hydrolysis experiment to test the inhibition properties

of glucose and glucose-$^{13}C_6$ revealed that glucose and glucose-$^{13}C_6$ at the same concentration resulted in the same amount of glucose released (data not shown), so glucose and glucose-$^{13}C_6$ inhibited cellobiose hydrolysis to the same extent. In the new assay, we used mass spectrometry to distinguish the labeled inhibitor (glucose-$^{13}C_6$) from the hydrolysis product, which was naturally labeled glucose.

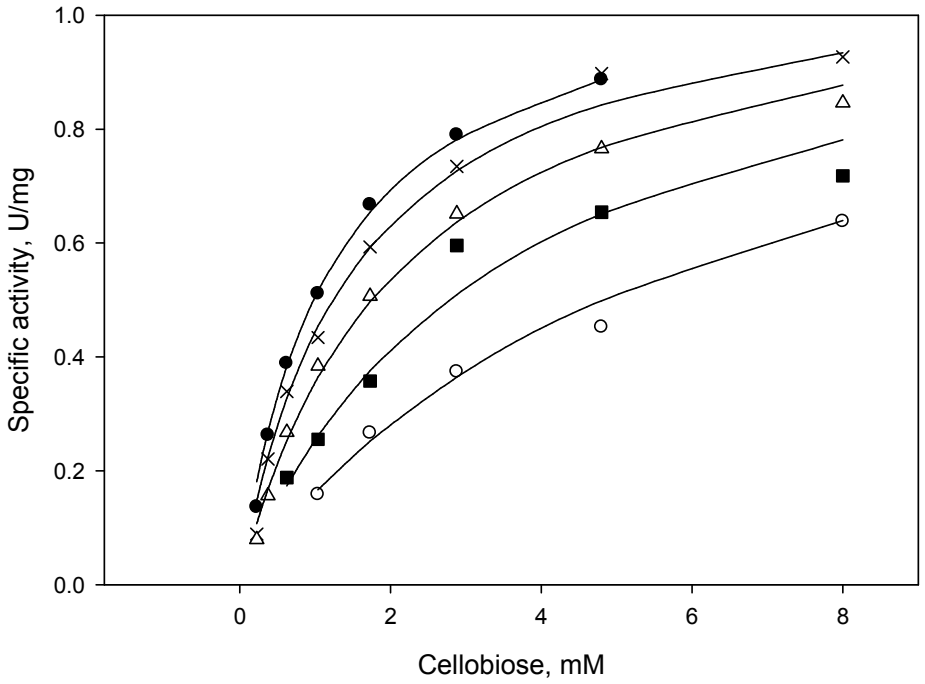

Fig. 3. Glucose inhibition of cellobiose hydrolysis for BGL_{An} with glucose-$^{13}C_6$ as inhibitor: ● 0.625 mM; × 1.25 mM; △ 2.5 mM; ■ 5.0 mM; o 10 mM. Lines represent the calculated specific activity at each inhibitor concentration using Michaelis-Menten kinetics for competitive inhibition.

After hydrolysis, the hydrolysis mixture contained cellobiose, released glucose and glucose-$^{13}C_6$. These compounds were separated in a LC step in which the glucose and the glucose-$^{13}C_6$ co-eluted. The following MS step in the analysis was ESI-MS in negative mode. The glucose and glucose-$^{13}C_6$ signal detected in the same period of time was dependent on the eluent and sample to sample variation, so the amount of (naturally labeled) glucose produced could not be determined directly. However, since the glucose-$^{13}C_6$ concentration was known, glucose-$^{13}C_6$ served as an internal standard correcting for sample and matrix variation. The amount of glucose produced was then found from the inhibitor concentration and from the ratio of glucose to glucose-$^{13}C_6$. It was possible to detect ratios of 0.01 to 100 with a standard deviation below five percent for triplicate measurements.

Inhibitors: glucose and D-glucono-δ-lactone

To describe the inhibition kinetics, the MM expression was extended with an additional term for competitive inhibition

$$\mathrm{v} = V_{\max} \cdot \frac{\mathrm{s}}{\mathrm{s} + K_M(1 + \mathrm{i}/K_\mathrm{i})}$$

v, s, and i are the variables for specific activity, substrate concentration and inhibitor concentration, respectively. The kinetic parameters V_{max}, K_M and K_i are the maximum reaction rate, the affinity constant and inhibition constant, respectively. At least five different inhibitor concentration and six substrate concentrations were tested. The inhibitor concentration was chosen to span a range of $0.2K_i$ to $5K_i$. All experiments were plotted in Lineweaver-Burk and in Dixon plots to visualize the type of inhibition. D-glucono-δ-lactone was found solely to change K_M for each BGL *i.e.* the inhibition was competitive irrespective of substrate. The effect of competitive inhibition decreases when the substrate concentration increases, since the substrate will displace the inhibitor and V_{max} is therefore not affected. With glucose as inhibitor a minor decrease was observed in V_{max}, so the inhibition was not strictly competitive. A Dixon plot for the hydrolysis of cellobiose using the rBGL*Pb* (Fig. 4) demonstrated that the slope ($1/V_{max}$) increased slightly when the inhibitor (glucose) concentration increased. The intercept with the ordinate increased as a result of an apparent higher K_M caused by competitive inhibition. However, since the V_{max} was reduced less than 20 % and the actual specific activity was well predicted using the MM expression for competitive inhibition, this expression was applied to determine the kinetic parameters (an example is shown in Fig. 3). In the experiments with cellobiose as substrate and glucose as inhibitor, BGL activity released additional amounts of glucose, thereby changing the actual inhibitor concentration. Only in the assays with the lowest inhibitor and highest cellobiose concentration did the released glucose during the assay change the

glucose inhibitor concentration significantly. In each single hydrolysis experiment, the inhibitor concentration is assumed to be constant, so in order to account for the glucose produced in the assay half of the glucose produced was added to the inhibitor concentration to estimate the average inhibitor concentration during the 10 minutes hydrolysis.

Inhibition of both *p*NP-Glc and cellobiose hydrolysis by glucose was found to be in the same range for both BGLs. Glucose was found to inhibit $rBGL_{Pb}$ hydrolysis of cellobiose more strongly (K_i 1.1 mM) than hydrolysis of *p*NP-Glc (K_i 2.3 mM), whereas glucose was found to inhibit BGL_{An} hydrolysis of *p*NP-Glc more strongly (K_i 1.1 mM) than of hydrolysis of cellobiose (K_i 1.6 mM)(Table 1). The inhibition constant for GL compared to glucose was an order of magnitude lower for both BGLs on *p*NP-Glc. In hydrolysis of cellobiose, BGL_{An} was also an order of magnitude more inhibited by GL (K_i 0.11 mM) than by glucose (K_i 1.6 mM), whereas the $rBGL_{Pb}$ was two orders of magnitude more inhibited during hydrolysis of cellobiose by GL (K_i 0.012 mM) than by glucose (K_i 1.1 mM) (Table 1).

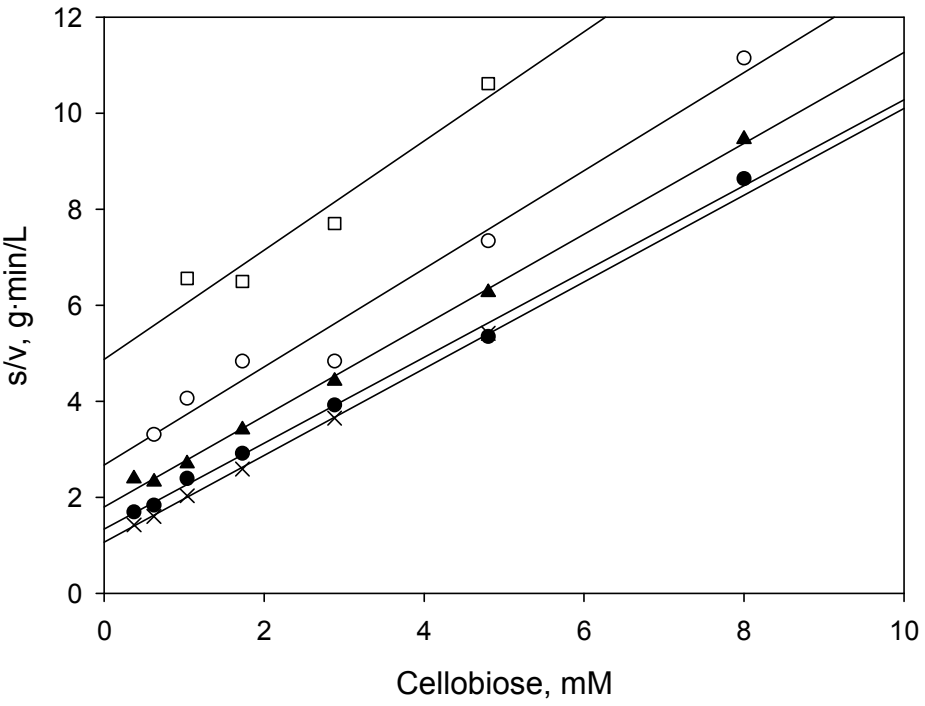

Fig. 4. Dixon plot of the $rBGL_{Pb}$ hydrolysis of cellobiose with glucose as inhibitor. Glucose at 0.625 mM (x), 1.25 mM (●), 2.5 mM (▲), 5.0 mM (○) and 10 mM (□).

Discussion

In this study, the first *bgl* gene sequence from the genus *Penicillium* is presented, *bgl1*. *Bgl1* showed highest identity to two BGLs that belong to the GH3 family, which at present, encompasses 962 GenBank/GenPept entries in CAZy [4]. A second GH3 BGL was also identified during purification. The apparent specific activity and amount in was similar, and it was decided to pursue the final purification of the BGL later named Cel3A encoded by *bgl1*.

Among BGLs in GH3, only one three-dimensional structure has been determined, a BGL from barley [37]. This structure could not be used to make a structural model of the *P. brasilianum* BGL due to too little identity. In contrast, several structures have been elucidated for cellobiohydrolases and endoglucanases. One reason for the poor knowledge on BGLs compared to the "true" cellulases is that the protein typically constitutes a very low portion of the extracellular protein secreted. In *T. reesei* only 0.5 to 1 % of the secreted protein is BGL [38]. After seven purification steps for the BGL from *P. brasilianum* the result was 65 μg of BGL in 5 mL from 2 L of culture broth supernatant, an amount of protein typically not sufficient for x-ray crystallographic studies. A larger amount of pure BGL was achieved after heterologous production in *A. oryzae*.

SDS-PAGE showed that the size of both the native and the recombinant BGL was 115 kDa, a typical size for a BGL as reviewed by [5]. The same size of native and heterologously expressed BGL indicated a similar glycosylation pattern. From the obtained sequence of the *P. brasilianum* BGL the mass was predicted to be 96.7 kDa (without glycosylation). The level of 20 kDa glycosylation is commonly found for BGLs from filamentous fungi [39-41].

The recombinant BGL from *P. brasilianum* was found to have excellent stability. The specific activity was highest for the $rBGL_{Pb}$ at pH 4.8, and the residual activity was 100 % after 24 hours of incubation at 60 °C. The commercial BGL preparation Novozym 188 had in comparison only 50 % residual activity after 24 hours of incubation at 60 °C. The stability is higher than reported for most other fungal BGLs incubated at 60 °C (see table 2). *Thermoascus aurantiacus* has been found to be equally stable, however the incubation time was half an hour compared to 24 hours in our experiments [42].

Table 2. Residual for purified BGL from different organisms. The temperature was 60 °C.

Organism	Residual activity	Incubation time and pH		reference
Aspergillus oryzae	10 %	4 h at	pH 5.0	[43]
Trichoderma viride	40 %	3 h at	pH 4.8	[44]
Candida peltata	15 %	0.5 h at	pH 5.0	[45]
Penicillium funiculosum	70 %	0.16 h at	pH 5.0	[35]
Thermomyces lanuginosus	60 %	0.5 h at	pH 6.0	[46]
Thermoascus aurantiacus	100 %	0.5 h at	pH 5.2	[47]
Penicillium brasilianum	100 %	24 h at	pH 4.8	

The rBGL$_{Pb}$ had activity on both *p*NP-Glc (an aryl-glucoside) and on cellobiose, as most characterized BGLs do [5]. rBGL$_{Pb}$ followed MM kinetics to 0.3 mM *p*NP-Glc, and above this concentration substrate inhibition was observed, as also reported for other BGLs [48]. This phenomenon of substrate inhibition is important to bear in mind when comparing specific activities for different BGLs, since substrate concentrations reported in the literature most often vary from 1 to 5 mM *p*NP-Glc.

In hydrolysis of cellobiose up to a concentration of 10 mM, no cellobiose inhibition was observed for neither of the BGLs. At this relatively high cellobiose concentration, substrate inhibition has been reported for other BGLs, a BGL from *Trichoderma viride* demonstrated substrate inhibition [44]. A BGL from *Fusarium oxysporium* has been found to produce trioses during hydrolysis of cellobiose at concentrations higher than 2.5 mM [49]. Other transglycosylation reactions reaction will result in the formation of other glucose dimers than cellobiose. When the reaction rate is measured through glucose quantification by either formation of reducing ends or by HPLC analysis, an outcome of these transglycosylation reactions will appear as substrate inhibition. However, we conclude that for the investigated BGLs transglycosylation did not supersede hydrolysis under the tested conditions.

Several kinetic studies of fungal GH3 BGLs have been performed in which the affinity constant K_M and the maximal specific activity V_{max} have been measured using *p*NP-Glc and cellobiose as substrate [43;44;50-56]. The ratio between V_{max} on *p*NP-Glc and on cellobiose ranges from approximately 1 for a GH3 BGL from *Penicillium funiculosum* [53] to 9 for a BGL from *Aspergillus oryzae* [43]. The high V_{max} for the *A. oryzae* BGL (3040 U/mg at 50 °C) on *p*NP-glc was accompanied by a relatively high affinity (0.55 mM). However, for a BGL from *Penicillium verruculosum* [57] a five fold higher V_{max} on *p*NP-Glc was accompanied by four fold higher K_M (*i.e.* lower affinity) for *p*NP-Glc compared to cellobiose. The kinetic parameters from these studies demonstrate significant kinetic differences between BGLs within the same GH family. It is going to be very interesting to investigate these differences, once the first crystal structure for a fungal GH3 BGL becomes available.

In the study of BGLs for cellulose hydrolysis, the most relevant substrate is cellobiose. Unless glucose is removed during cellulose hydrolysis, the glucose concentration will increase proportionally to the cellulose degraded. In investigations of glucose inhibition of cellobiose hydrolysis, it has been difficult to measure the small change in glucose released due to a high glucose concentration present as inhibitor. Therefore, the transition state analogue D-glucono-δ-lactone (GL) has been used to investigate the inhibition of several BGLs. GL was found to be at least an order of magnitude stronger inhibitor for both the investigated BGLs in the hydrolysis of *p*NP-Glc and cellobiose, when compared to glucose. This is in accordance with similar investigations of both inhibitors, where it is often found that GL is a more powerful inhibitor than glucose [43;58]. In spite of the fact that the GL inhibition for the $rBGL_{Pb}$ was ten times stronger compared to the BGL_{An}, the BGLs were inhibited to the same extent by glucose.

A new method using LC-MS developed in this work can quantify glucose inhibition accurately during cellobiose hydrolysis. In the assay glucose-$^{13}C_6$ was used as inhibitor instead of glucose, since the labelling did not change the inhibition properties. Not only is glucose-$^{13}C_6$ an inhibitor that can be distinguished from the produced (naturally labelled) glucose by mass spectrometry, but is also serves as internal standard for quantification [59] allowing an accurate determination of the inhibition constant. However, significant levels of transglycosylation will inevitable interfere with this method since the internal standard will be consumed.

During BGL_{An} hydrolysis of 8 mM cellobiose with 10 mM $^{13}C_6$-glucose concentration, a compound with a molecular mass corresponding to a glucose dimer made of one glucose molecule and one $^{13}C_6$-glucose molecule was detected using ESI-MS. The HPLC step did separate this dimer from cellobiose, so a possible transglycosylation product could be sophorose (β-1,2), laminaribiose (β-1,3), or gentiobiose (β-1,6). With none of these compounds used as a standard, it was not possible to quantify the amount of transglycosylation. However, no cellobiose containing one $^{13}C_6$-glucose molecule was detected. A GH1 BGL from *T. reesei* produced sophorose in the presence of glucose [60]. Thus, the developed LC-MS method can potentially be used to detect and quantify transglycosylation products.

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Abbreviations

BGL_{An}; β-glucosidase from *Aspergillus niger*; ESI-MS: electrospray ionization-mass spectrometry; GH: glycoside hydrolase; GL: D-glucono-δ-lactone; Glc: glucose;
LC: liquid chromatography, *p*NP: *para*-nitrophenol; $rBGL_{Pb}$: recombinant β-glucosidase from *Penicillium brasilianum*; TEA: triethanolamine.

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Paper E

A novel GH5 endoglucanase from genus *Penicillium* and its adsorption to lignin

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Submitted

A novel GH5 endoglucanase from genus *Penicillium* and its adsorption on lignin

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Abstract

The *cel5C* gene, coding for an endoglucanase (Cel5C) of *Penicillium brasilianum* was cloned and heterologously expressed in *Aspergillus oryzae*. This is only the second GH5 EG from the genus *Penicillium* reported in the CAZy database. Cel5C consists of a C-terminal CBM1 cellulose binding domain linked to the catalytic core via a heavily glycosylated serine and a threonine-rich linker. The promoter region of the gene has putative binding sites for both the carbon catabolite repressor CreA and the activator XlnR. The pH optimum of Cel5C was found to be 4.0 and the temperature optimum was 70 °C. At 50 °C Cel5C retained full residual activity after 20 hours of incubation at pH 5.0 and pH 6.0. The adsorption of the enzyme, investigated on Avicel and steam pretreated spruce, was found to follow the Langmuir isotherm. The maximum adsorption was similar for both substrates, 40 mg/g and 49 mg/g respectively. The affinity for Avicel was ten times higher than for steam pretreated spruce, 0.040 L/mg and 0.0035 L/mg, respectively. Non-productive binding of cellulolytic enzymes to lignin can reduce the cellulose hydrolysis rate of lignocellulosic substrates. Therefore, the adsorption on residual lignin produced from various biomass samples was investigated. Steam-exploded rice straw adsorbed significantly more Cel5C compared to steam-exploded wheat straw. Two samples of spruce SO_2-pretreated at varying conditions, resulted in different adsorption of Cel5C to the residual lignin.

Keywords: Lignocellulose; Lignin; Endoglucanase; Adsorption; *Penicillium brasilianum*; Cloning

1. Introduction

Many microbial cellulolytic systems have in the past decades attracted considerable interest from both academia and industry. Cellulolytic enzymes have been applied in several biotechnological and industrial applications and a large potential exists for future applications. Currently, cellulolytic enzymes are used in the textile industry for polishing cotton fibers and for increasing their brightness (Cavaco-Paulo, 1998), for biostoning of denims (Belghith *et. al*., 2001), in the detergent industry (Ito, 1997), and in the paper and pulp industry (Oksanen *et. al*., 2000). At present, considerable attention is devoted to developing efficient cellulolytic enzyme systems for degradation of the cellulose fraction in lignocellulose. Lignocellulosic biomass is the most abundant organic matter on earth, and its hydrolysis can supply vast amounts of sugar, primarily glucose. This glucose can be fermented into ethanol, which can be used as transportation fuel. The US Energy Information Administration estimated for 2007 that the American yearly oil consumption for motor gasoline was 3.4 billion barrels (approx. 540 billion litres) constituting 45 % of the total oil consumption (US Energy Information Administration, 2008). If a significant fraction of this requirement is to be replenished with lignocellulosic ethanol, large amounts of low-cost feedstock lignocellulose need to be converted into fermentable sugars.

Lignocellulose is comprised mainly of cellulose, hemicellulose, and lignin. Cellulose is a linear polymer constituted of β-1,4-D-glucopyranose units linearly aligned into microfibrils. These microfibrils form dense fibers stabilized by hydrogen bonds. Hemicellulose and lignin constitute the matrix holding the cellulose fibers together. Hemicelluloses are heterogeneous polysaccharides (Sjöström, 1993), often esterified with ferulic acid which acts as an anchor point for lignin (Sun *et. al*., 1997). Lignin is not a polysaccharide, but rather a high molecular weight material composed of phenylpropane monomers covalently bound to each other through either ether or carbon bonds (Nimz, 1974). The three precursor monomers of lignin are *p*-coumaryl-, coniferyl-, and sinapyl alcohol. The relative amounts of the three precursors differ significantly in softwood, hardwood, and annual plants. Softwoods such as spruce and pine contain mainly polymerized coniferyl alcohol (guaiacyl) units (Ericksson *et. al*., 1973; Glasser and Glasser, 1981). In hardwood lignin, syringyl units constitute up to 45 % of the lignin with the rest being mainly guaiacyl lignin (Wayman and Parekh, 1990). In grasses and herblike dicotyledons (such as wheat, rice, and corn), the monomeric lignin precursor is *p*-coumaryl alcohol (Monties, 1998).

The recalcitrant nature of biomass is a major barrier to effective enzymatic degradation of constituent polymers into fermentable pentoses and hexoses. The recalcitrance of lignocellulosic biomass contrasts with the easiness of enzymatic degradation of starchy substrates, such as corn or wheat grains, which can be directly hydrolyzed into fermentable carbohydrates after simple mechanical milling. A severe pretreatment step

is needed to open up the lignocellulose structure in order to speed up the hydrolysis rate (Wyman *et. al*., 2005). Acid-catalyzed steam pretreatment using either sulfur dioxide or sulfuric acid is one of the most studied pretreatment methods (Galbe and Zacchi, 2002). Generally, most biomass pretreatments result in solubilization and hydrolysis of hemicelluloses, leaving the cellulose and lignin as an insoluble fraction (Tengborg *et. al*., 2001), and in a redistribution of the remaining solids (Viikari *et. al*., 1994). An organosolv pretreatment, in which an organic solvent and a dilute acid (or a dilute base in certain cases) are used as chemical pretreating agents, has been found to remove most of the hemicelluloses and a large fraction of lignin (Kurabi *et. al*., 2005). For a review of pretreatment methods see (Olsson *et. al*., 2005). In general, the accessibility of cellulose increases as the severity of the pretreatment is intensified (Thompson *et. al*., 1992).

An efficient hydrolysis of the cellulose fraction requires cooperative action of three enzyme classes: endoglucanases (EGs), cellobiohydrolases (CBHs) and β-glucosidases. EGs are the most diverse class of enzymes in the cellulose degrading system, and several EGs with varying size and isoelectric point have been identified in enzyme complexes of most common industrial cellulolytic fungal strains such as *Trichoderma reesei* (Tolan and Foody, 1999) and *Humicola insolens* (Schulein, 1997). Fungal EGs and CBHs often have a two-domain structure with a catalytic core domain connected to a cellulose binding domain (CBD) with a flexible linker (Schulein, 1997). A fungal CBD is typically 36-38 amino acid residues long and it has a wedge-like form (Kraulis *et. al*., 1989). One of the functions of the CBD is to increase the enzyme concentration on the cellulose surface leading to higher *in situ* specific cellulolytic activity as it has been demonstrated for *T. reesei* CBHI (Cel7A) CBD on crystalline and amorphous cellulose (Tomme *et. al*., 1988). The absence of a CBD in *T. reesei* CBHI (Cel7A) and EGII (Cel5A) has been shown to reduce their adsorption to steam pretreated spruce (Palonen *et. al*., 2004) and steam-pretreated willow (Kotiranta *et. al*., 1999) and their hydrolysis of steam-pretreated willow (Kotiranta *et. al*., 1999).

In pretreated substrates, lignin has repeatedly been reported to adsorb cellulolytic enzymes resulting in decreased cellulolytic activity. After complete hydrolysis of pure cellulose (Avicel) almost all cellulolytic protein could be recovered (Boussaid and Saddler, 1999), whereas on pretreated spruce only 45 % of the cellulolytic protein was recovered after complete hydrolysis (Palonen *et. al*., 2004). Lignin obtained after complete hydrolysis of a hardwood mixture pretreated under different conditions adsorbed 30 to 45 % of the cellulolytic proteins in solution (Ooshima *et. al*., 1990). The enzyme adsorption to lignin is an important obstacle to be overcome since the cost of cellulolytic enzymes contributes significantly to the total production cost of glucose from cellulose. Several funding programs have been released to reduce the enzyme costs such as the US National Renewable Energy Laboratory (NREL)/DOE program

initiated in 2001 in collaboration with Novozymes (ZCO-1-30017-02) and Genencor (ZCO-0-30017-01) aiming a 10-fold enzyme cost reduction. Recent press releases by these major enzyme companies have claimed reduction of enzyme costs of up to 30-fold compared to the costs of commercial biomass degrading enzymes available in 2001.

The cellulolytic enzyme system from *P. brasilianum* was found to have a relatively higher content of EGs and to have a higher specific filter paper activity compared to *T. reesei* (Jørgensen *et. al*., 2003a). In particular, large quantities of GH5 EGs were secreted by *P. brasilianum*. In the present study, we identified the first gene encoding a Cel5 EG in *P. brasilianum*. So far only one GH5 EG from genus *Penicillium* has been sequenced, from *Penicillium janthinellum* (Mernitz *et. al*., 1996), but there are no published reports on characterization of this GH5 EG. Heterologous production of Cel5 from *P. brasilianum* made it possible to do basic characterization and adsorption experiments to cellulose and several pretreated lignocellulosic samples.

2. Materials and methods

2.1. Cultivation and purification of endoglucanases from P. brasilianum

The cultivation and enzyme purification procedures have been reported (purification A) (Jørgensen *et. al*., 2003a). Briefly, *P. brasilianum* strain IBT 20888 was cultivated in a Mandels and Weber medium containing cellulose and oat spelt xylan. The cultivation was performed under aerobic conditions at 30 °C with pH controlled at pH 5.0. EGb1 and EGb2 were purified using anion exchange chromatography at starting pH 7.5 or 4.6 in combination with hydrophobic interaction chromatography. Eluted fractions were pooled after measurement of CMCase activity.

2.2 Biomass samples

Residual lignins were prepared from three grasses, three softwoods, and two hardwoods. A list of the residual lignins used for the study is presented in Table 1.

Table 1. Residual lignins used for the enzyme adsorption experiments.

Substrate	Pretreatment	Abbreviation	Cellulose, % DM
Wheat straw	acid-free steam explosion[c]	SEWS	<10
Rice straw	acid-free steam explosion[c]	SERS	<10
Corn stover	dilute-acid	DACS	<10
Spruce[a]	SO_2 steam explosion	SSES1	15
Spruce[b]	SO_2 steam explosion	SSES2	<10
Lodgepole pine	Organosolv	OLP	<2
Poplar	SO_2 impregnated	SP	<10
Maple	Organosolv	OM	<2

[a]Lund University (LTH), Sweden, [b]The University of British Columbia, Canada, [c] Exogenous acid-free, released acetyl groups from the hemicellulose acidified the pretreatment.

Spruce (SSES1/LTH) pretreatment and enzymatic isolation of its residual lignin

Spruce (*Picea abies*) was steam pretreated according to Tengborg *et al*. (2001) using sulfur dioxide as acid catalyst. The sulfur dioxide concentration was 2.4 % (w/dw). The freshly chipped wood chips (2.2 to 10 mm) were impregnated with sulfur dioxide for 20 minutes at room temperature followed by steam pretreatment at 215 °C for three minutes. The steam pretreated spruce (SPS) was washed with distilled water to remove residual acid and soluble sugars. Residual lignin was prepared using extensive enzymatic hydrolysis with a 3:1 mixture of Celluclast 1.5 L FG and Novozym 188 (Novozymes, Bagsvaerd, Denmark). The activity of this mixture was 60.5±0.4 FPU/mL. SPS (50 g/L) in 50 mM Na-acetate pH 4.8 was hydrolyzed using 50 FPU/(g DW SPS) for 5 days at 50 °C and 200 rpm. Every 24 hours, the hydrolysis mixture was centrifuged for 5 min at 7,000g. The pelletized hydrolysis residues were resuspended in new buffer and a fresh cellulolytic enzyme mixture was added. After five days, the hydrolysis was terminated and the adsorbed protein was removed. The hydrolysis residues were centrifuged, washed twice with hydrolysis buffer, twice with water, twice with 70 % ethanol, twice with water and three times with 50 mM borax at pH 10 (Palonen *et. al*., 2004). Finally, the hydrolysis residues were washed thoroughly with 50 mM sodium acetate pH 4.8 to bring the pH down to the conditions to be used for adsorption studies. The lignin content was determined using the NREL procedure for determination of structural carbohydrates and lignin in biomass (Sluiter *et. al*., 2006).

Pretreatment of other lignocellulosic substrates and isolation of residual lignins

Residual lignins from 7 different substrates apart from SSES1 (Table 1) were prepared by extensive enzymatic digestion of the pretreated substrates as follows. The enzymatic hydrolysis was continuously run for 7 days, 40 FPU Celluclast 1.5L/g dry substrate, 80 CBU Novozym 188/g dry substrate (5 % w/w substrate consistency), pH 4.80, Na-acetate buffer in 1L flasks with a total reaction volume 250 mL. Tetracycline (40 µg/mL) and Cyclohexamide (30 µg/mL) were added to prevent microbial contamination. After extensive enzymatic hydrolysis proteolysis of the residual lignins was performed overnight (~16 hrs) at 37°C, 0.2 M phosphate buffer, pH 7.5, 200 rpm using a non-specific protease Pronase (Sigma, USA) as described elsewhere (Berlin *et. al*., 2006). After protease treatment, the solution was incubated at 70°C for 2 hours in order to deactivate the protease. After the protease treatment and the wash of the centrifuged residue with nanopure water, the obtained lignins were centrifuged and the precipitates were dried for three days at 40°C under vacuum. After drying the residual lignins were manually milled and screened through 80-mesh and stored in sealed vials at room temperature.

2.3. Protein determination

Protein concentration was determined using a "Biorad total protein" assay based on the Bradford method (Bradford, 1976) with γ-globulin (Sigma G-7516) as standard. The concentration of Cel5C after purification was determined using the extinction molar coefficient calculated from the amino acid sequence using the algorithm of (Skoog and Wichman, 1986) in GPMAW (Lighthouse data, Denmark).

2.4. N-terminal sequencing

The N-terminal sequence was determined as described using Edman degradation (Krogh *et. al*., 2008).

2.5. Peptide map

Gel digests by Trypsin (sequencing grade, Roche) were prepared according to the manufacturer's instructions. MALDI-TOF MS analysis of the digests was performed by the dried-droplet method on a Voyager DE-PRO workstation from Applied Biosystems using alpha-cyano-4-hydroxycinnamic acid as matrix. Analyses were run in reflector mode with positive ionization with CalMix1+2 (Applied Biosystems) as external calibration.

2.6. Molecular weight determination by MALDI-TOF MS and LC-ESI-MS

The MALDI-TOF MS procedure is described above but in this case with sinapinic acid as matrix. Analyses were run in linear mode with positive ionization with BSA (Sigma) as external calibration. The LC-ESI-MS analysis was performed on a Bruker electrospray (ESI) MicroTOF Focus (Bremen, Germany) coupled to an Agilent 1100 HPLC system where samples were desalted on a MassPREP™ On-Line Desalting Cartridge (Waters). ES Tuning mix from Agilent was used for "quasi" internal calibration. Spectra deconvolution was accomplished by the maximum entropy method using Maximum Entropy (MaxEnt) Deconvolution software from Spectrum Square Associates, Inc.

2.7. Protein deglycosylation

An aliquot of purified Cel5C was diluted to a concentration of 1 mg/mL with 50 mM Tris with 50 mM sodium maleate pH 6.2. Two microliters endoglycosidase H (5 U/mL) (cat. no. 11 644 053 001, Roche) was added to a 100 µL Cel5C solution. The mixture was incubated for 1 hour at 25 °C followed by 72 hours at 4 °C.

2.8. DNA library and screening

A DNA library was made using a TOPO Shotgun Subcloning Kit from Invitrogen as described in (Krogh *et al*. in press). Based on the N-terminal amino acid sequence of the purified EGs, a forward primer was designed using the CODEHOP strategy (Rose *et.*

al., 2003). Regions with a high degree of similarity to known EGs were used as a scaffold to design two reverse primers.

Forward primer, fwd: 5'- TTCGGTACCTCTGAGTCTGGNGCNGARTT -3'.

Reverse primer, rev1: 5'- TGATCCATATCGTGGTACTCGTTRTTNGTRTCRAA -3'.

Reverse primer, rev2: 5'- CCGTTGTAGCGACCGTARTTRTGNGGRTC -3'.

Where R=A or G, Y=C or T, K=G or T and N=A, C, G or T.

Two PCR reactions using genomic DNA as template resulted in two products of about 570 bp (fwd and rev1) and 350 bp (fwd and rev2). These products were excised from on a 2 % agarose gel. The library screen using radioactive probes and the subsequent plasmid isolation and sequencing was done as described in (Krogh *et al*. in press). The vector containing the 5.5 kb fragment of genomic DNA was named pKKAH1.

2.9. Heterologous production

The *cel5c* gene was amplified from genomic DNA by PCR using the following two oligonucleotide primers:

Forward PCR: 5'-AATT*GGATCC*ACCATGAAATACCCTCTACTCCTGGCAAC-3'.

Reverse PCR: 5'- TTAA*CTCGAG*TTACAGACACTGCGAATAATACGCATTC-3'

To facilitate cloning a restriction enzyme site was inserted into the 5' end of each primer where the forward primer contained a *Bam* HI site and the reverse primer contained an *Xho* I site (sites shown in italics). Genomic DNA was used as template in the PCR reaction. The PCR fragment was sequenced and cloned into pJAL721 (WO 03/008575). *Aspergillus oryzae* was transformed with the constructed plasmid as described elsewhere (Krogh *et. al*., 2008). The transformants were evaluated using SDS-PAGE analysis and activity measurements. Transformant KBK03 produced the highest titer of EG and it was chosen for production. After removal of the biomass, the EG was purified at 4 °C using a XK 50 column (Amersham Biosciences, Uppsala, Sweden) containing 110 g Avicel Ph 101 (Merck, Merck KGaA, Darmstadt, Germany) pre-equilibrated with 25 mM Tris (pH 7.5) prior to loading. The bound enzyme was eluted with 25 mM Tris, 1 % TEA at pH 11.0. After elution, the pH was immidiately adjusted to 7.5 in the eluted fractions, and the fractions containing the EG were pooled.

2.10. Determination of endoglucanase activity

The EG activity was assayed as described elsewhere (Jørgensen *et. al*., 2003a). The substrate was azo-carboxymethyl cellulose (Megazyme International Ireland Ltd., Bray, Ireland) and the incubation time was 15 minutes.

2.11. Determination of pH and temperature optimum and stability

The pH dependence was studied at 50 °C using the assay described above. The buffer used to dissolve the substrate was 50 mM Britton-Robinson buffer (50 mM boric acid, 50 mM acetic acid, 50 mM phosphoric acid) ranging from pH 3.0 to 10.0. The temperature dependence of the specific activity was assayed at pH 4.8 and at temperatures ranging from 20 to 80 °C. The EG stability was tested at temperatures from 20 °C to 80 °C at pH 4.8, and at pH 2 to 10 at 25 and 50 °C over a period of time of 20 hours. Incubation buffers were 10 mM Britton-Robinson buffer adjusted to the desired pH. The pH was confirmed after adding the enzyme to the incubation buffer and during the enzyme activity assay. The residual activity was measured as described above.

2.12. Adsorption experiments

The protein adsorption on a specific substrate is influenced by the concentration of the protein relative to the substrate. This adsorption can often be described by the Langmuir isotherm model under the following assumptions: 1) adsorption can not proceed beyond monolayer substrate surface coverage, 2) all sites are equivalent and the surface is uniform, 3) the ability of a molecule to absorb at a given site is independent of the occupation of neighbouring sites (Atkins, 1994). The Langmuir isotherm (equation 1) describes that

$W = W_{max}KE/(1 + KE)$ (equation 1),

where W is the amount of adsorbed enzyme, W_{max} is the maximum adsorption amount, K is the adsorption equilibrium constant, and E is the enzyme concentration in the liquid phase at equilibrium.

In the adsorption experiments the enzyme concentration was 0.01 mg/mg substrate, and the substrate concentration was 25 mg/mL in 50 mM Na-acetate buffer pH 4.8. The reaction volume was 1 mL. The microcentrifuge tubes were placed on a tilting table Desaga SM1 Universal mixer (Sarstedt, AG & Co, Germany) at a speed of 60 tilts per minute. After incubation, the microcentrifuge tubes were centrifuged at 10,000g for 3 min at 4 °C. The supernatant was filtered through a 0.2 μm nylon filter (17 mm Titan syringe filter, Sun-Sri, TN, US). The dependence of the protein adsorption on temperature was investigated at 4, 25, and 50 °C, respectively on Avicel, SPS, SSES1 and organosolv hardwood lignin (Aldrich 371017), respectively. The kinetics of the protein adsorption was studied with sampling of the supernatant for protein analysis at 0, 1, 30, 60 min, 3, 5, 8, 24 h. In the Langmuir isotherms, the enzyme concentration was as high as 1 mg/mL *ie.* 0.04 mg/mg substrate. The amount of free enzyme was determined using capillary zone electrophoresis as described elsewhere (Jørgensen *et. al*., 2003b) using triethanolamine as internal standard. Adsorbed Cel5C was calculated as the difference between the added Cel5C and the free Cel5C in the supernatant.

3. Results

The interesting properties found in previous studies for the cellulolytic enzyme system secreted by *Penicillium brasilianum* were the motivation for creating a genomic library to search for these enzyme encoding genes.

3.1. Endoglucanases from P. brasilianum

The major endoglucanases secreted by *P. brasilianum* has previously been investigated in our group (Jørgensen *et. al*., 2003b). Two of these enzymes, namely EGb1 and EGb2, were subsequently found to have the same N-terminal sequence (ASSFVWFGTSESGAEFGNQN) and thus were probably derived from the same gene. As suggested (Jørgensen *et. al*., 2003a), EGb1 is very likely a proteolytic degradation product of EGb2 in which a part or the whole cellulose binding domain has been cleaved off. The N-terminal sequence has similarity to certain members of glycoside hydrolase family 5 and it was used to design a sense-strand primer to amplify the corresponding gene from *P. brasilianum* genomic DNA. Two antisense strand primers were designed based on conserved sequences in known GH5 family members. The PCR amplification yielded two different sequences with homology to GH5, and the corresponding genes were named *cel5a* and *cel5b*. Both of these gene fragments were used to probe the genomic library of *P. brasilianum* in order to recover full-length gene sequences. Unexpectedly, the only GH5 gene recovered from this probing was a third GH5 gene sequence that we named *cel5c*. The recovery of this third sequence is in retrospect not surprising given the presence of three 25-bp regions of greater than 84 % identity between *cel5c* and the PCR-amplified region of *cel5a*. The Cel5C protein encoded by the full-length gene has a predicted N-terminal sequence that perfectly matches the EGb1 and EGb2 proteins. It also almost perfectly matched internal peptide sequences of EGb2 inferred from tandem mass spectrometry (Jørgensen *et. al*., 2003a). The minor differences in sequence are probably the result of incorrect interpretation of the tandem mass spectra. We concluded that Cel5C and EGb2 are synonymous and that EGb1 is a degradation product of these.

3.2. Promoter analysis

The sequenced 5′ noncoding region includes promoter elements of a putative TATA box at position – 80 relative to the start codon, and four putative CCAAT motifs at -45, -307, -322, and –334. Three consensus binding sites for the catabolite repressor CREA (5′-SYGGRG-3′) identified in *Aspergillus nidulans* (Cubero and Scazzocchio, 1994) were present at −125 (GTGGAG), -484 (CTGGGG), and at –530 (CCGGGG). This indicates that the *cel5C* gene could be regulated by catabolite repression.

3.3. Cel5C enzyme

The nucleotide sequence of the *cel5C* coding region is 1471 bp (including stop codon), interrupted by 4 introns (GenBank accession no. **EU379561**), and it encodes a polypeptide of 421 amino acids. Using the SignalP software program (Nielsen *et. al*., 1997), a signal peptide of 16 residues was predicted. Based on the experimentally determined N-terminal sequence of the secreted endoglucanase, residues 17 through 24 appear to constitute a pro-region that is proteolytically cleaved during maturation, probably by a kexin family protease that recognizes the dibasic Lys-Arg adjacent to the mature N-terminus. The predicted mature protein contains 397 amino acids of 42,559 Da. The molecular mass was determined to 65 kDa when measured by SDS-PAGE (data not shown) and 59.6 kDa determined by MALDI-TOF mass spectrometry. Treatment with endoglycosidase H to remove *N*-linked oligosaccharides reduced the measured MALDI-TOF mass to 57 kDa, indicating that the potential *N*-glycosylation site at residue 104 was very likely glycosylated. More detailed analysis of endoglycosidase H-treated Cel5C by LC-ESI-MS revealed a pattern of peaks spaced apart by the mass of 1 hexose unit and centered on the most intense peak at 57,040 Da. The mass difference between the measured mass and the theoretical mass presumably represents typical heavy *O*-linked glycosylation of the serine- and threonine-rich linker region between the cellulose binding domain and the catalytic domain (Maras et al., 1999). The mass difference corresponds to a modal value of 88 hexose units with an observed spread from 71 to 102.

3.4. Cel5C similarity to other GH5 family members

Among publicly available GH5 sequences, *P. brasilianum* Cel5C is most closely related to a number of known and predicted endo-1,4-ß-D-glucanases from various *Aspergillus* species such as the FII-CMCase from *Aspergillus aculeatus* (Takada *et. al*., 2002), and to the characterized endo-1,4-ß-D-glucanase from *Thermoascus aurantiacus* (Hong *et. al*., 2003). It is much more distantly related to GH5 family members with other biochemical activities such as glucan 1,3-ß-glucosidase, endo-1,4-ß-xylanase and mannan endo-1,4-ß-mannosidase (Fig. 1). The domain structure of Cel5C is identical to its closest *Aspergillus* relatives, namely the fact that it contains a C-terminal cellulose binding domain (fungal CBM1) connected to the catalytic core domain by a serine and threonine-rich linker. The catalytic core domain has conserved residues corresponding to the catalytic glutamates and to residues known to form an ion network with the catalytic glutamates (Fig. 1)

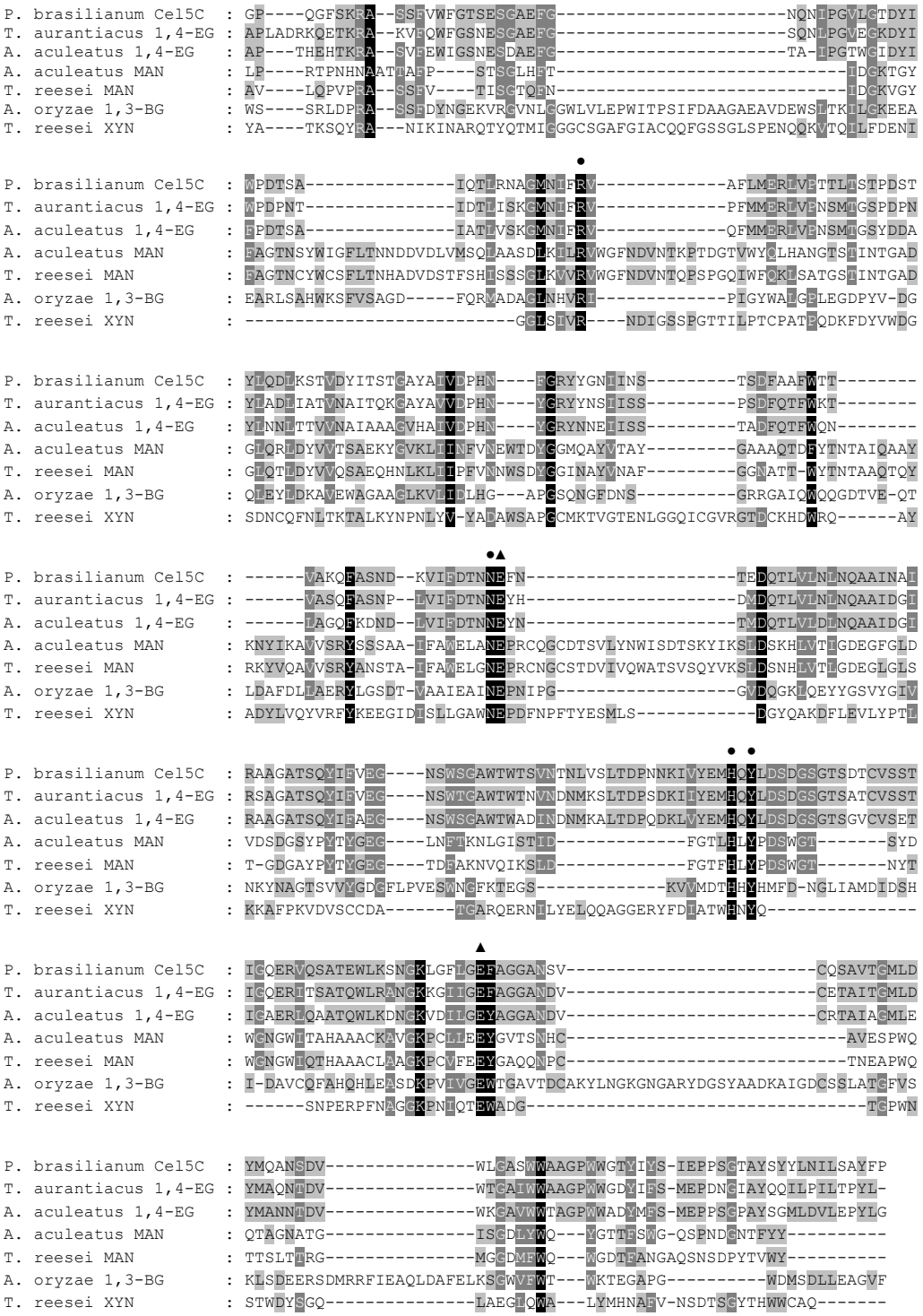

Fig. 1. Alignment of the core domain of *P. brasilianum* Cel5C with known endo-1,4-ß-D-glucanases of *Thermoascus aurantiacus* and *Aspergillus aculeatus* and with other diverse members of glycoside hydrolase family 5 including two mannosidases (MAN), a glucan 1,3-ß-glucosidase (1,3-BG) and a xylanase (XYN). The likely catalytic glutamates are marked with triangles and residues that in other GH5 proteins form an ion network with the catalytic glutamates (Hilge *et. al*., 1998; Sakon *et. al*., 1996) are marked with circles. Black background white characters represent 100 % conservation, dark gray background with white characters greater than or equal to 80 % conservation, and lighter gray with black letters greater than or equal to 60 % conservation.

3.5. Enzyme characterization

After heterologous expression, Cel5C was purified and the specific activity was measured and compared to a commercial EG from *Trichoderma longibrachiatum* (Megazyme International Ireland Ltd., Bray, Ireland). The specific activity was 51 U/mg for Cel5C and 61 U/mg for the commercial EG measured using azo-CMC as a substrate. To characterize Cel5C, pH and temperature optima were determined. The pH and temperature stability was also investigated since it is often desirable to operate at the highest possible temperature without loosing activity.

The optimum pH for EG activity was pH 4.0 with a sharp decrease in activity at pH 3.0. At pH 6.0, the EG activity was 60 % of the maximum level (Fig. 2A). The temperature optimum was 70 °C with more than 75 % of the maximum activity in the interval from 60 to 80 °C (Fig. 2B).

A **B**

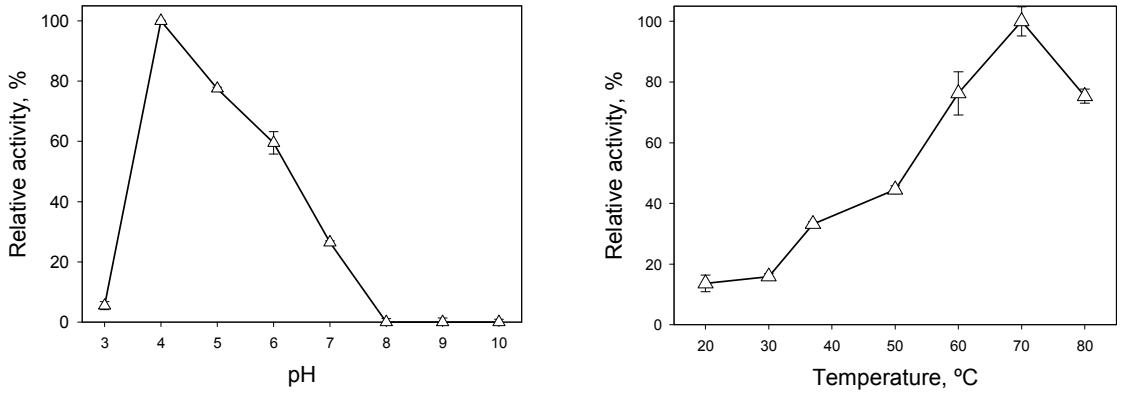

Fig. 2. Specific activity of *P. brasilianum* EG Cel5C at **A** different pH values at 50 °C (n=2) and **B** different temperatures at pH 4.8 (n=2).

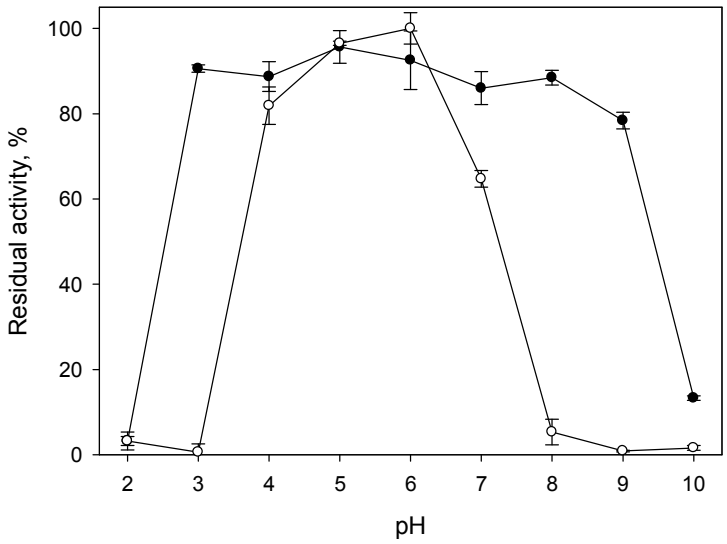

Fig. 3. Residual activity of *P. brasilianum* EG Cel5C after 20 hours of incubation at different pH values and at 25 °C (●), and 50 °C (○) (n=2). All values were normalized to 50 °C at pH 6.0.

Temperature and pH are known factors influencing the stability of enzymes. Stability of Cel5C was investigated after 20 hours of incubation at pH values ranging from 2.0 to 10.0 and at 25 °C and 50 °C . After incubation, the residual activity was measured under standard conditions. At 25 °C , Cel5C retained more than 75 % of its starting activity in the interval from pH 3.0 to 9.0. At 50 °C this interval was narrowed to pH 4.0 to 6.0 (Fig. 3). The influence of temperature was more closely investigated at the optimal pH. These results (data not shown) demonstrated that the residual activity after incubation at pH 4.0 for 20 hours started to decrease slightly at 50 °C as it is shown in Fig. 3, and at 60 °C no residual activity was detected.

3.6. Adsorption studies

Spruce is a large potential resource for production of bioethanol in Northern Scandinavia, and we studied the binding of Cel5C to the lignin fraction in steam pretreated spruce (SPS). Spruce was steam pretreated according to (Tengborg *et. al*., 2001) using sulfur dioxide as acid catalyst, largely removing the hemicellulose fraction. To study the adsorption to residual lignin, cellulose was removed from SPS by hydrolyzing the cellulose for five days with an excess of cellulolytic enzyme activity. A compositional analysis of SPS and enzyme-hydrolyzed SPS found a cellulose content of 41% and 15 %, respectively. The amount of residual cellulose we found in enzyme-

hydrolyzed SPS was significantly higher than the 4.5 % reported by Palonen *et al.* (2004) even though our enzymatic hydrolysis included two additional rounds of 24 hours hydrolysis. The 15 % cellulose content in our study may be slightly overestimated, since our wash procedure after hydrolysis included 70 % ethanol that most likely removed some of the low-molecular weight lignin. Cellulose content is important to bear in mind when discussing adsorption on the lignin fraction in enzymatically hydrolyzed SPS. However, since the cellulose was not degraded even after very extensive cellulolytic enzyme treatment, we assume that it was not accessible to the enzymes at all, and therefore only of minor importance for studying enzyme adsorption on lignin.

Influence of incubation time and temperature on adsorption

The EG adsorption was investigated at 4, 25 and 50 °C to Avicel, SPS, enzymatically hydrolyzed SPS (SSES1) and organosolv extracted hardwood lignin. Adsorption on Avicel decreased from 80 % at 4 °C to 71 % at 25 °C and 64 % at 50 °C . A temperature dependent decrease was also observed for SSES1. The adsorption on SPS did not change with temperature, whereas the adsorption on lignin increased with increasing temperature, which is consistent with a hydropobic component in the interactions rather the an ionic component (Table 2). The action of the cellulolytic enzyme system continuously changes the structure of the substrate as the hydrolysis proceeds (Himmel *et. al*., 2007). Capillary electrophoresis of the broths after incubation showed that the amount of neutral compounds increased with increasing temperature. A neutral compound will neither be attracted nor repelled to the electrodes, and it will therefore move with the electroosmotic flow in the capillary. At the pH-value used for measuring protein adsorption most types of sugar will be uncharged. This strongly indicated that the incubation with EG hydrolyzed the substrate. To minimize the change in substrate during adsorption, the temperature was set to 4 °C in the following adsorption studies.

Table 2. Adsorption of *P. brasilianum* Cel5C at varying temperatures and on different substrates at a loading of 0.01 g/g substrate (n=2). Adsorption time was 1h.

	% Cel5C adsorbed		
	4 °C	25 °C	50 °C
Avicel	79.6±0.0	71.1±1.9	64.4±0.8
SPS[a]	32.6±1.2	34.6±0.7	35.9±1.0
SSES1[b]	28.8±1.8	24.7±0.3	19.9±0.1
Hardwood lignin[c]	5.3±0.1	14.5±0.9	16.9±0.3

[a]Steam Pretreated Spruce, [b]enzyme hydrolyzed SPS – SSES1 in Table 1 and [c]organosolv lignin from a mixture of hardwood.

Adsorption is not an instantaneous process, and it takes time before equilibrium is reached. Therefore we investigated the time course for Cel5C adsorption on Avicel cellulose and on steam pretreated spruce. For both substrates, a constant level of adsorption was observed after three hours (with the same level observed after 5, 8 and

24 hours), and 90 % of the equilibrium value was reached after half an hour (data not shown). Since the adsorption reached equilibrium after three hours, this time was chosen for further studies of adsorption.

Langmuir isotherms

The EG adsorption on SPS and Avicel was investigated at the conditions found in previous experiments - three hours of adsorption at 4 °C. The parameters in the Langmuir isotherm were optimized using non-linear regression, and the smoothed curves in Fig. 4 were calculated using these estimated parameters (Table 3). The predicted amount of bound EG at equilibrium fitted the experimental values well.

Table 3. Langmuir parameters for the adsorption of *P. brasilianum* EG Cel5C to Avicel and steam pretreated spruce

	W_{max} mg/g cellulose[b]	K L/mg[b]
Avicel	40	0.040
SPS[a]	49	0.0035

[a]Steam Pretreated Spruce. [b] 1 mg enzyme = 0.023 μmol using the predicted mature Mw.

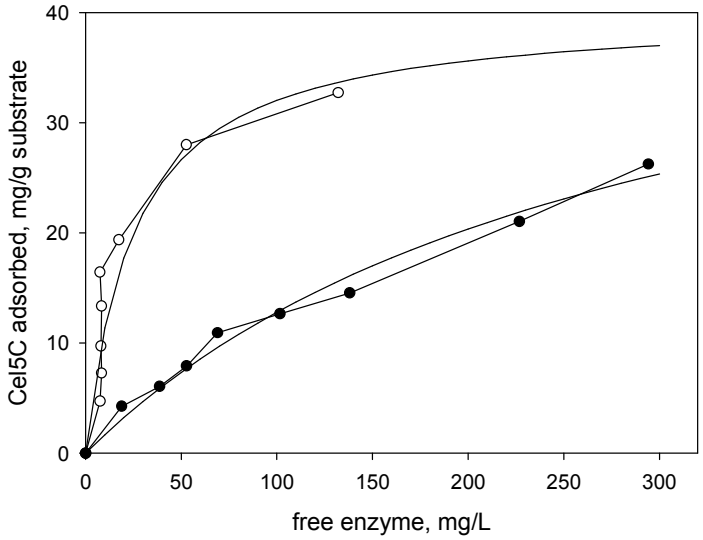

Fig. 4. Langmuir adsorption isotherm for *P. brasilianum* EG Cel5C adsorption on Avicel (○) and SPS (●). The substrate concentration was 25 g/L. The curves were calculated from W_{max} and K.

The maximum adsorbed amount (W_{max}) was in the same range for the adsorption on Avicel and SPS, 40 and 49 mg/g substrate, respectively. In spite of the similar W_{max} for the two substrates, the Langmuir isotherms appeared differently, due to the different adsorption equilibrium constants. The affinity for SPS was an order of magnitude lower than for Avicel (Table 3). At the point where the adsorbed amount of Cel5C was half of the maximum amount, the equilibrium concentration ($1/K$) was 25 and 290 mg/L for Avicel and SPS, respectively.

Adsorption to biomass samples

The adsorption to lignin was investigated for various types of biomass, and to solely evaluate the adsorption on lignin, residual lignins were prepared by extensive enzymatic digestion using commercial cellulolytic enzyme preparations. The residual lignins were prepared from three grasses, three softwoods and two hardwoods. In the adsorption experiments Avicel was used as a reference. An industrial enzyme loading for cellulose hydrolysis is widely considered to be around 1 % w/w (Decker *et. al*., 2003) and a comparable enzyme loading was chosen for studying Cel5C adsorption on the residual lignin.

The adsorption on rice straw residual lignin was significantly higher than the adsorption on any of the other residuals lignins (Fig. 5). Rice straw and wheat straw were pretreated in the same way, but the ash content was high as 15 % in rice straw (Alex Berlin, unpublished results) and this could be a reason for the higher enzyme adsorption observed on rice straw. The dilute-acid pretreatment is considered by NREL to be a promising process step in the use of corn stover for bioethanol production (Aden *et. al*., 2002) and DACS adsorbed the smallest amount of Cel5C among the grasses tested. The residual lignin from wheat straw adsorbed more Cel5C than the residual lignin from corn stover. The softwood residual lignin, with a higher guaiacyl content compared to hardwood lignin, adsorbed a larger amount of Cel5C compared to the hardwood residual lignin irrespective of pretreatment. The SO_2-catalyzed steam-exploded spruce from two batches differed in adsorption, 26 % and 33 % respectively. Among hardwoods, the overall lowest adsorption was found on organosolv maple, which only adsorbed 6 % Cel5C.

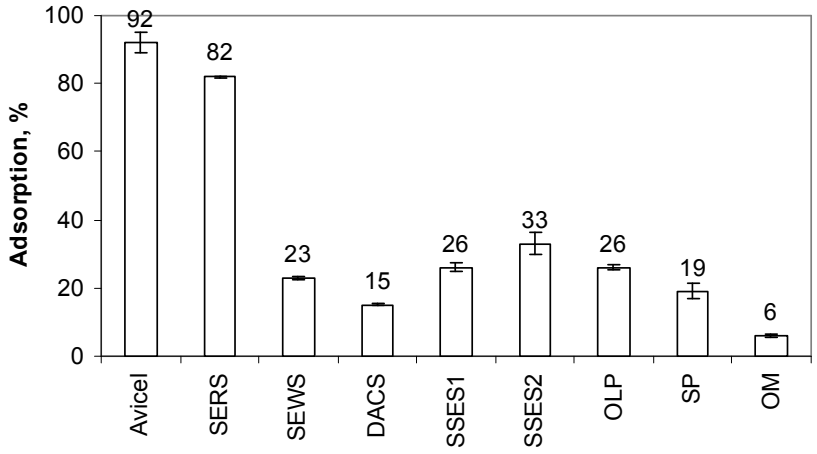

Fig. 5. Adsorption on residual lignin from lignocellulosic samples, which were pretreated and then hydrolyzed extensively by enzymes to remove cellulose. The substrate concentration was 25 g/L. The adsorption was 3 hours at 4 °C (n=2). SERS: acid-free steam-exploded rice straw, SEWS: acid-free steam-exploded wheat straw, DACS: dilute-acid pretreated corn stover. SSES1: SO_2-catalyzed steam-exploded spruce from Lund University, Sweden, SSES2: SO_2-catalyzed steam-exploded spruce from The University of British Columbia, Canada, OLP: organosolv lodgepole pine, SP: SO_2-catalyzed poplar, and OM: organosolv maple.

4. Discussion

Endoglucanases (EGs) are the most diverse class of enzymes amongst cellulolytic enzymes. In the carbohydrate active enzymes database (CAZy) EGs (EC 3.2.1.4) are found in families GH5, 6, 7, 8, 9, 12, 44, 45, 48, 51, 61, and 74 (Coutinho and Henrissat, 1999), but so far GH8, 48, and 51 only contain EGs of bacterial origin. The database had ultimo February 2008 208 bacterial and 163 eukaryotic EG entries, and it is continuously updated at URL http://afmb.cnrs-mrs.fr/CAZY/.

GH5 is a large family of EGs and so far GH5 comprises 50 eukaryotic and 95 bacterial EGs. The identified cellulolytic enzyme from *P. brasilianum* was based on sequence similarity classified as GH5. Substrate specificity demonstrated that the *P. brasilianum* GH5 enzyme was a cellulase, and it was named Cel5C. The enzyme cloned in this study is only the second EG from genus *Penicillium* identified by CAZy. The first EG also a GH5 has been identified from *P. janthinellum* (Mernitz *et. al*., 1996). So far no reports have been published on the biochemical characterization of a *Penicillium* EG. Two additional GH5 putative EGs of *P. brasilianum* were also partially cloned during this study, but since full-length clones were never obtained their study was not pursued further.

The structure of Cel5C has common traits with other cellulolytic enzymes; a catalytic core, a linker and a CBM. The CBM belongs to CBM family 1, a family with more than 200 members, almost exclusively of fungal origin. The CBM is positioned at the C-terminal end, a trait Cel5C shares with its closest relatives in the GH5 family but in contrast to the predominant N-terminal position of CBM1 in most other fungal GH5 EG-family members. Heavy glycosylation has been observed for other fungal EGs, *eg.* EG "F-II CMCase" from *Aspergillus aculeatus* (Takada *et. al*., 2002). One *N*-linked glycan attachment site was predicted and experimentally shown in the amino acid sequence of Cel5C. However the bulk of the glycosylation is *O*-linked and occurs in the serine- and threonine-rich linker region where our analysis demonstrated a range of 71 to 102 hexose residues. Extensive *O*-glycosylation in the linker region with mainly di-and trisaccharides has been reported for several other cellobiohydrolases and EGs having a linker region (Hui *et. al*., 2002). In the two domain structure, glycosylation can play several important roles both to protect the very labile linker against proteolysis and to give needed spatial separation between the catalytic core and the CBD (Srisodsuk *et. al*., 1993).

The promoter region of *cel5c* was found during the library screening, and promoter analysis was performed to get a better understanding of the cellulolytic gene regulation. The upstream sequence of the *cel5c* gene contains a TATA box that is necessary for strong promoter activity. An additional element for strong promoter activity is the CCAAT box, one of the most common elements found in eukaryotic

promoters. The upstream sequence contains four putative CCAAT box sites. The CCAAT box at nt position –45 is too close to the start site (Mantovani, 1998) and is unlikely to function as a transcriptional regulator. In TATA-containing promoters the CCAAT box is preferentially located in the –80/–100 region relative to the transcriptional start site. However, the three remaining boxes in the *cel5c* gene are found somewhat further upstream. A similar far-upstream positioning is found in the promoter of the eglA endoglucanase gene of *Aspergillus nidulans* {Chikamatsu, 1999 6110 /id}. The fact that *P. brasilianum* has been shown to be a good producer of EG (Jørgensen *et. al*., 2003a) suggests that the CCAAT boxes ensure strong promoter activity in spite their distance from the translational start site. The upstream sequence also contains three putative binding sites for the transcriptional glucose-responsive repressor protein CreA, as also found in the promoter for the GH5 EG from *P. janthinellum* (Mernitz *et. al*., 1996). This repressor has been found to exert carbon catabolite repression in *Aspergillus* (de Vries and Visser, 2001) and its homologue Cre1 in *T. reesei* (Mach and Zeilinger, 2003). Earlier findings by (Jørgensen *et. al*., 2005) demonstrated that enzyme production in *P. brasilianum* is repressed by glucose, and the upstream CreA binding sites found in this study gives strong indications that a CreA like protein mediates glucose repression. In *A. niger,* the protein XlnR has been found to be a transcriptional activator that mediates the expression of the xylanolytic system (van Peij *et. al*., 1998). When *A. oryzae* was cultivated on either xylose or xylan, not only the hemicellulolytic enzyme system but also the cellulolytic enzyme system were induced (Marui *et. al*., 2002). The presence of the XlnR binding site in the promoter region of *cel5c* suggests that the expression of the cellulolytic system in *P. brasilianum* can be mediated by an XlnR-like activator as found in *Aspergillus*. Putative XlnR binding sites have been reported for xylanolytic enzymes in *Penicillium citrinum* (Tanaka *et. al*., 2005).

Cellulose is by nature relatively recalcitrant to enzymatic hydrolysis even after pretreatment, and the hydrolysis to glucose takes several days. For efficient hydrolysis, the stability of the cellulolytic enzymes employed at the given conditions is a very important parameter. The most commonly reported optimal conditions for cellulose hydrolysis using a fungal cellulolytic enzyme system is pH 5.0 and 50 °C (Castellanos *et. al*., 1995). At these conditions, Cel5C was shown to be a promising candidate with 78 % of the maximum specific activity, and 100 % residual activity after 20 hours incubation. The specific activity was highest at 70 °C, comparable to a thermostable GH5 EG from *A. niger* (Akiba *et. al*., 1995). However, few EGs have been reported to be optimally active at higher temperatures, one example being EG from *Thermoascus aurantiacus* which was optimally active at 75 °C (Wojtczak *et. al*., 1987).

Avicel and SPS were used for studying the adsorption kinetics. Avicel is predominantly crystalline cellulose and SPS is a heterogenous mixture of cellulose,

residual hemicellulose, ash, and lignin. Two assumptions for the Langmuir isotherm are that the substrate binding sites are equivalent and the surface was uniform. In spite of the invalidity of these assumptions for Avicel and SPS, the Langmuir isotherm was still found to describe the adsorption well. The maximum amount of Cel5C that could adsorb to Avicel was at similar level as found for an EG from *T. reesei* (Ooshima *et. al*., 1983). Interestingly, the same level of maximum adsorption was found for SPS, however, the affinity was a magnitude lower. The same level of affinity for SPS has been reported for EG Cel5 from *T. reesei* (Palonen *et. al*., 2004). Given the fact that only half the SPS is cellulose and the maximum adsorption was the same, Cel5C may adsorb to the residual lignin. Another explanation can be that the removal of hemicellulose during pretreatment opens up the cellulose structure resulting in more binding sites compared to crystalline cellulose (Wong *et. al*., 1988). It is known that lignin redistributes during pretreatment (Wong *et. al*., 1988), which also can result in a changed number of available cellulose binding sites.

The lower affinity after sulfur dioxide steam pretreatment can also be explained by sulphonation. Softwood is particularly susceptible to sulphonation due to the high content of coniferyl alcohol in the lignin complex (Mooney *et. al*., 1998). When treated at 70 °C for 1 day at pH 3 with sodium sulfite, lignocellulose from juniper (softwood) has been sulphonated to an extent of 5 % mole sulphonation per mole lignin monomer (Shin and Rowell, 2005). For cellulolytic enzymes with a CBD, the adsorption to cellulose is primarily due to hydrophobic interactions between aromatic residues on the flat surface of the CBD and the cellulose surface (Mattinen *et. al*., 1998). With introduction of more hydrophilic groups into the lignin, the hydrophobic interaction between the cellulose binding domain and the cellulose in the proximity of the sulphonated lignin residue may be hindered.

Adsorption of enzymes to lignin does not only limit the hydrolysis efficiency, it also reduces the potential of developing effective enzyme recycling schemes, which is considered a possible way of reducing the enzyme cost share in bioethanol production (Lee *et. al*., 1995). The biomass samples were all chosen to be potential and relevant substrates for bioethanol production in different parts of the world. Within the grasses, rice straw is a potential substrate for bioethanol in China other Asian countries and localized regions of the US, corn stover is a potential substrate in the US and China, and wheat straw in Europe, Canada, US, and South America. The softwoods spruce and pine are substrates with potential for bioethanol production in the Northern hemisphere. Hardwoods such as maple and polar have potential in particular in Eastern Canada and the North East of the USA. Adsorption of Cel5C to rice straw was significantly higher than to any of the other biomass samples including two other grasses. For hardwood, the adsorption on sulfur dioxide treated poplar (19 %) was lower than found for a GH5 EG from *T. reesei* on sulfur dioxide treated willow (55 %)

(Kotiranta *et. al.*, 1999). This highlights the possible differences contributed by substrate type, enzyme type and pretreatment conditions. In order to make a very accurate comparison between adsorption to residual lignin from two biomass samples, the pretreatment will have to use the same equipment under the same conditions.

From the adsorption studies to residual lignin from pretreated softwood samples, significant differences in Cel5C adsorption on two different sulfur dioxide treated spruce samples were found. This clearly demonstrated that in hydrolysis of pretreated lignocellulose for sugar production it is of utmost importance to make pilot scale experiments under the same conditions as expected to be used in large scale.

We have identified a thermostable endoglucanase, Cel5C from *P. brasilianum* belonging to GH5, and under commonly used conditions for cellulose hydrolysis, Cel5C demonstrated good stability. The binding properties of cellulolytic enzymes to cellulose and lignin influence the hydrolysis rate, and knowledge on binding is therefore of great importance when designing a process for enzymatic hydrolysis of lignocellulose. The adsorption results of our study demonstrated that not only is the pretreatment of importance, but also the specific conditions. Information on microscopic composition and structure of the pretreated material will be valuable for understanding the mechanisms of enzymatic hydrolysis.

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Paper F

Polypeptides having beta-glucosidase activity and polynucleotides encoding same

Kristian Krogh, and Paul Harris

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(54) Title: POLYPEPTIDES HAVING BETA-GLUCOSIDASE ACTIVITY AND POLYNUCLEOTIDES ENCODING SAME

(57) Abstract: The present invention relates to isolated polypeptides having beta-glucosidase activity and isolated polynucleotides encoding the polypeptides. The invention also relates to nucleic acid constructs, vectors, and host cells comprising the polynucleotides as well as methods for producing and using the polypeptides.

Paper G

Polypeptides having endoglucanase activity and polynucleotides encoding same

Paul Harris, Kristian Krogh, Elena Vlasenko, and Soren Flensted Lassen

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(74) Agent: **STARNES, Robert**; Novozymes, Inc., 1445 Drew Avenue, Davis, CA 95618 (US).

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(54) **Title:** POLYPEPTIDES HAVING ENDOGLUCANASE ACTIVITY AND POLYNUCLEOTIDES ENCODING SAME

(57) **Abstract:** The present invention relates to isolated polypeptides having endoglucanase activity and isolated polynucleotides encoding the polypeptides. The invention also relates to nucleic acid constructs, vectors, and host cells comprising the polynucleotides as well as methods for producing and using the polypeptides.

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92*bis*.1 and Administrative Instructions, Section 422)

To:

STARNES, Robert
Novozymes, Inc.
1445 Drew Avenue
Davis, CA 95618
ETATS-UNIS D'AMERIQUE

Date of mailing *(day/month/year)*
18 October 2007 (18.10.2007)

Applicant's or agent's file reference
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IMPORTANT NOTIFICATION

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1. The following indications appeared on record concerning:

☒ the applicant ☒ the inventor ☐ the agent ☐ the common representative

Name and Address	State of Nationality	State of Residence
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☐ the person ☐ the name ☐ the address ☐ the nationality ☐ the residence

Name and Address	State of Nationality	State of Residence
KROGH, Kristian, Bertel, Romer, Morkeberg Klirevaenget 35 2880 Bagsvaerd Denmark	DK	DK
	Telephone No.	
	Facsimile No.	
	Teleprinter No.	

3. Further observations, if necessary:
The person identified in Box 2 should be added to the record as applicant for the United States of America only and inventor for all designated States.

4. A copy of this notification has been sent to:

☒ the receiving Office
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☐ the International Preliminary Examining Authority
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The International Bureau of WIPO
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