Technical University of Denmark

DTU

Enzymatic Hydrolysis of Cellulose
Experimental and Modeling Studies

Andersen, Natalija; Stenby, Erling Halfdan; Michelsen, Michael Locht

Publication date:
2007

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

DTU Library
Technical Information Center of Denmark

Enzymatic Hydrolysis of Cellulose

Experimental and Modeling Studies

Natalija Andersen

Ph.D. Thesis
October 2007

BioCentrum-DTU
TECHNICAL UNIVERSITY OF DENMARK

PREFACE

The present work represents the result of my PhD study carried out as a part of the research school Novozymes Bioprocess Academy, which is collaboration between the industrial partner, Novozymes A/S, and two departments at the Technical University of Denmark (DTU), BioCentrum and Department of Chemical Engineering. Experimental part of this study was conducted at Center for Microbial Biotechnology (CMB), BioCentrum - DTU, while modeling study was conducted at IVC-SEP group, Chemical Engineering department. The study was carried out in the period January 2004 to December 2006. During my PhD study I received grants from Otto Mønsteds Fond to cover the expenses for participation at conferences in the USA and Chile.

I would very much like to thank my four supervisors, Professor Lisbeth Olsson (CMB), Docent Michael L. Michelsen (IVC-SEP), Professor Erling H. Stenby (IVC-SEP) and Senior Scientist Katja S. Johansen (Novozymes) for their valuable guidance, inspiration, and support throughout the whole period. Lisbeth, thank you for your enormous enthusiasm, always positive thinking, and encouragement when the things got rough. Michael, you helped me a lot with modeling and all my small and big questions about it, and I am very grateful for that. Erling, you contributed a lot to our discussions by your holistic view and by ability to see things from a different angle, and Katja, I thank you for valuable discussions on enzymes and for providing the industrial point of view.

During my PhD study I co-supervised Cleo C.W. Chang who conducted her master project on enzymatic hydrolysis of cellulose. She did a great work and I wish her all the best in her PhD and future career.

I would also like to thank all my former and present colleagues at CMB and Chemical Engineering department, for three very good and pleasant years. It has been a pleasure to be a part of this open-minded, international environment. However, a special thank to Kristian Krogh for interesting and inspiring brain-stormings and lots of help in the lab, and to Kianoush Hansen, Tina Johansen and Jette Mortensen for technical support. Susan, Maya and Gianni, it was great shearing an office with you.

Finally, I would like to thank my family and friends for their enormous support during the last three years. I would especially like to thank my parents Milica and Zvonimir Popovic for both moral and economical support during my studies, and to my wonderful daughter, Sara for being so loving, nice and easy.

Natalija Andersen

August, 2007

Copenhagen, Denmark

CONTENTS

LIST OF ABBREVIATIONS

ANTS	8-aminonaphthalene-1,3,6-trisulfonic acid
BET	Bennet-Emmit-Teller
BG	β-glucosidase
BMCC	Bacterial micro-crystalline cellulose
BSA	Bovine serum albumin
C_1	Glucose
C_2	Cellobiose
C_3	Cellotriose
C_4	Cellotetraose
C_5	Cellopentaose
C_6	Cellohexaose
CBH	Cellobiohydrolase
CBM	Carbohydrate binding module
CD	Catalytic domain
CMC	Carboxymethyl cellulose
CrI	Crystallinity index
DMSO	Dimethyl sulfoxide
DNS	Dinitrosalicylic acid
DP	Degree of polymerization
EG	Endoglucanase
FPA	Filter paper activity
HPAEC	High performance anion-exchange chromatography
HPLC	High pressure liquid chromatography
IUPAC	International Union of Pure and Applied Chemistry
PACE	Polysaccharide analysis using carbohydrate gel electrophoresis
PAD	Pulsed amperometric detection
PAHBAH	4-Hydroxybenzoic acid hydrazide
PASC	Phosphoric Acid Swollen Cellulose
Rxn	Reaction
SSA	Specific surface area
TEMED	N,N,N',N'-Tetramethylethylenediamine

CHAPTER 1

Introduction

Cellulose is the major polymeric component of plant material and is the most abundant polysaccharide on Earth. In nature, a variety of microorganisms are known for producing a set of enzymes capable of degrading this insoluble polymer to soluble sugars, primarily cellobiose and glucose. Enzymes involved in these processes are called *cellulases* and are consisting of at least three classes of enzymes, namely, endogluganases (EG), cellobiohydrolases (CBH) and β-glucosidases (BG). Cellulases can be used in the variety of applications within food, vine, animal feed, textile and pulp and paper industry (Bhat, 2000). The application and interest in cellulases has particularly increased in recent years with the utilization of the enzymes in the production of bioethanol from lignocellulose (Sun and Cheng, 2002). Bioethanol can be blended at low concentrations with petrol (gasoline) or diesel for use in today's vehicles, and is considered to be a sustainable transportation fuel. Alternatively, if bioethanol is used in higher, or 100 % concentrations, adopted vehicles are typically needed. The main motivation for investments in research and process development concerning bioethanol production is environmental concern related to global warming. The focus is, in particular, turned towards the reduction of CO_2 emissions and other so-called green house gases. Moreover, sustainable bioethanol production would decrease the dependency on the traditional, natural oil, reserves, which can due to their restricted geographical localization cause political tension and economical instability. Under EU proposal 0547 from November 7, 2001, a series of goals were set for member states to introduce biofuels for diesel and gasoline. By 2005, 2 % of transport fuel should be accounted for by biofuels; by 2020, the goal is 20 %.

First-generation biofuels are made from food crops, such as sugar cane and corn. This can offer some CO_2 benefits and can help to improve domestic energy security. Nevertheless, concerns exist about the sourcing of feedstocks, including the impact it may have on biodiversity and land use, and competition with food crops. Second-

generation biofuels are made from non-food feedstocks, such as waste from agriculture and forestry. Second-generation biofuels could significantly reduce CO_2 production, do not compete with food crops, and, some types can offer better engine performance (www.shell.com, August 2007). Third-generation biofuels technology is directed towards, so called, synthetic biology, e.g. discovery, development and commercialization of engineered cellulase enzymes that are incorporated into the corn plants themselves, or development of crops whose lignin-content (the hard, "woody" part of plants' cell walls) has been artificially weakened and reduced, and disintegrates easy under dedicated processing techniques (www.biopact.com, August 2007).

In this thesis the focus is on the second-generation biofuels technology, and more precisely on the process of enzymatic hydrolysis of cellulose. In general terms, the production of bioethanol from lignocellulose involves a degradation of the polymeric compounds, primarily cellulose and hemicellulose, to sugars, which are then fermented by microorganisms to ethanol. The process can be performed in a number of different ways (Olsson et al., 2004). Figure 1.1 shows an example of the process steps used for the conversion of lignocellulosic-based waste material to bioethanol.
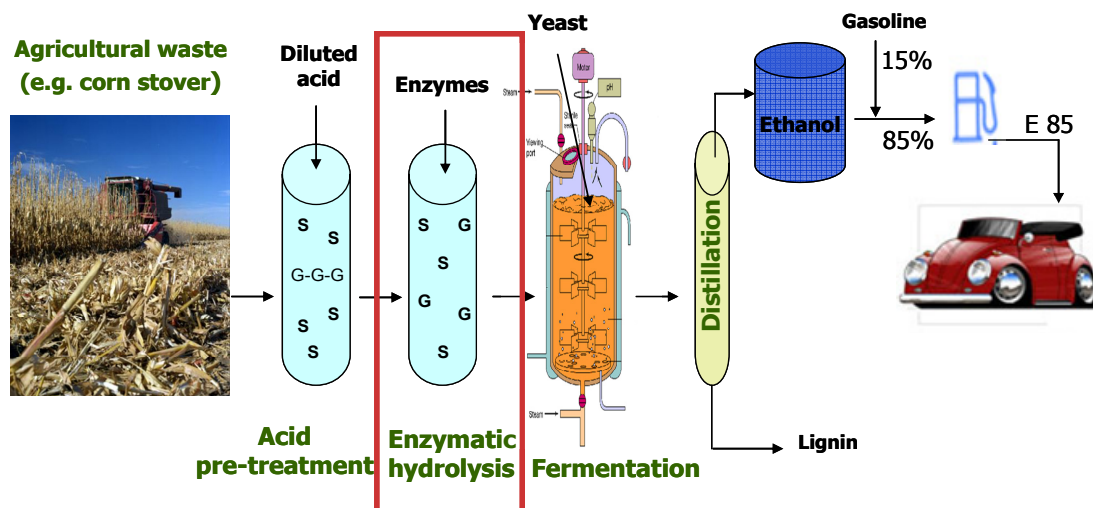
Figure 1.1: Production of bioethanol from lignocellulosic-based waste material, an example. Agricultural waste consists of approximately 38 % cellulose, 32 % hemicellulose, 17 % lignin and 13 % other compounds. Acid pre-treatment, most often with diluted acids, will degrade hemicelluloses to other sugars (S), while the degradation of cellulose to glucose (G) is accomplished in the next step, enzymatic hydrolysis. This step is framed as the focus of this thesis will be on the enzymatic hydrolysis of cellulose. Microorganisms (e.g. yeast) can ferment sugars to ethanol, which is further on distilled, and mixed with gasoline to obtain blends such as E 85 (a mixture of 15 % gasoline and 85 % ethanol).

In Chapter 2, the composition of lignocellulose (plant cell wall polysaccharides) is introduced, and moreover, the characteristics of cellulose from plant materials (used in the industrial processes), and cellulose from model substrates, readily used in research, are discussed and compared. Cellulolytic enzymes and their main characteristics are introduced in Chapter 4. One of the main characteristics of the cellulose hydrolysis process is the cooperative action, often designated synergy, of the different enzyme classes involved in the degradation process. Synergy is dependent on the variety of factors (see Chapter 5), of which substrate characteristics play an important role. The cooperative action between the three enzyme classes (EG, CBH and BG) was investigated on two model cellulose substrates (Avicel and phosphoric acid swollen cellulose, PASC) and presented in Chapter 5, Article A. Moreover, enzyme and substrate related factors affecting the hydrolysis, often represented by decrease of the hydrolysis rate in the later stage of the process, are discussed in Chapter 6 and Article B.

To better understand the fundamentals of this process, data obtained in the experimental studies were compared and evaluated against a mechanistic mathematical model describing the hydrolysis process (Chapter 7). Additionally, a novel approach, based on application of the metabolic control analysis theory, was investigated as an alternative way of modeling, and, consequently, determining the most rate controlling step(s)/enzymes of the process (Chapter 8). In general, mathematical modeling of enzymatic hydrolysis process can offer increased understanding of fundamentals of this process, and consequently lead to better choice of enzyme mixtures for hydrolysis.

1.1 **References**

Bhat MK. 2000. Cellulases and related enzymes in biotechnology. Biotechnol. Adv. 18: 355-383.

Olsson L, Jørgensen H, Krogh KBR, Roca C. 2004. Bioethanol production from lignocellulosic material. In: Polysaccharides: structural diversity and functional versatility. New York: Marcel Dekker Inc. p 957-993.

Sun Y, Cheng J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technnol. 83: 1-11.

CHAPTER 2

Cellulosic material

The aim of this PhD study was to investigate enzymatic degradation of *cellulose*. For that purpose, I chose to work with relatively well defined, cellulosic model substrate – Avicel. Nevertheless, in the broader perspective, detailed plant chemistry knowledge is important as it will improve understanding of the processes going on, as well as, help the rational experimental design, for example in choosing the appropriate enzymes for efficient degradation. In other words, plant cell wall polysaccharide architecture and the molecular makeup determine the resistance to enzymatic degradation. Therefore, I will in this chapter introduce the main components of the "real", lignocellulosic material, as well as the model cellulosic material used in the experiments.

2.1 Lignocellulose (plant cell wall polysaccharides)

Lignocellulosic biomass is the most abundant material on Earth. Its sources, including raw materials like, agricultural residues (e.g. corn stover and wheat straw), forestry residues (e.g. sawdust and mill wastes), portions of municipal solid waste (e.g. waste paper) and various industrial wastes have a great potential to be used in the industrial processes.

Lignocellulose is the collective name for the three main components of plant material, namely cellulose, hemicellulose and lignin. In plants, linear cellulose chains contribute to tensile strength, while hydrophobic amorphous lignin is responsible for chemical resistance, in particular protection against water. Hemicellulose provides bonding between cellulose and lignin (Figure 2.1). Thus, two major obstacles hinder the hydrolysis of cellulose in lingocellulosic material. They are, the recalcitrance of crystalline cellulose itself (emerging from the linear cellulose chain structure tightly bound in microfibrils), and the highly protective lignin surrounding it, and acting as a

physical barrier for microorganisms (i.e. enzymatic attack). For detailed structural models of various lignocellulosic materials see major review by Carpita and Gibeaut, 1993.
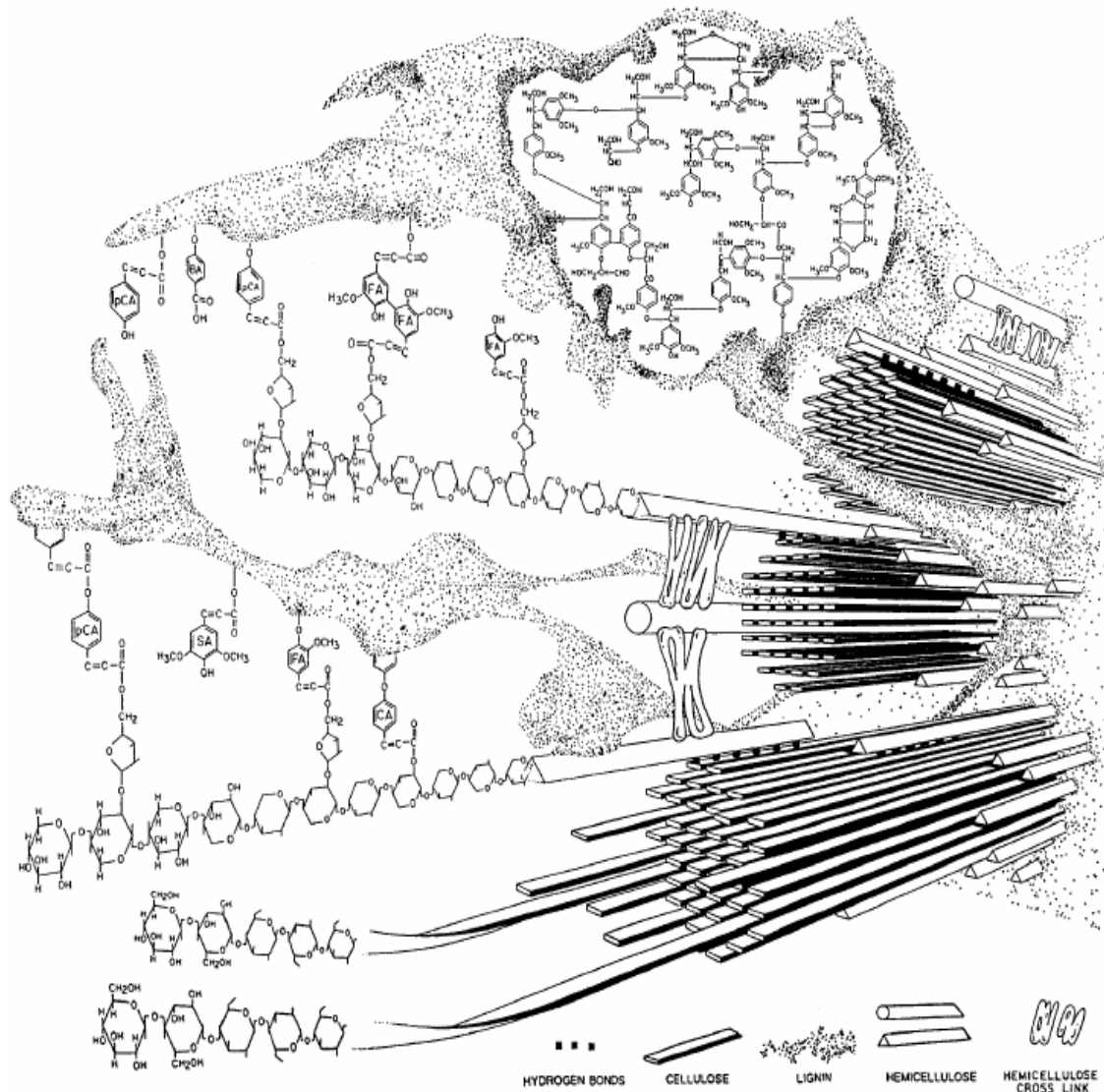
Figure 2.1: A model of the molecular structure of the main constituents of lignocellulosic material (secondary plant cell wall). Components are arranged so that the cellulose and hemicellulosic chains are embedded in lignin. Parallel cellulose chains bound tightly together by hydrogen bonds provide rigidity to the plant material, while surrounding hydrophobic lignin "glues" the components together and acts as a physical barrier for microorganisms and water. Hemicellulose connects cellulose and lignin through a network of cross-linked fibres. From Bidlack et al., 1992.

6

2.1.1 Cellulose

Cellulose is a major component of lignocellulose. Chemically, it is a simple molecule composed of linear β-1,4-linked D-glucopyranose chains (also called glucose or glucan chains). While β-1,4-linked glucose is the chemical repeating unit, the structural repeat is β-cellobiose (Varrot et al., 2003), Figure 2.2. In cellulose, glucose chains are tightly bound to each other by van der Waals forces and hydrogen bonds into crystalline structures called elementary fibril (consisting of around 40 glucan chains), about 40 Å wide, 30 Å tick and 100 Å long (Bidlack et al., 1992). Aggregates of elementary fibrils, of essentially an infinite length, and a width of approximately 250 Å, are called microfibrils (Fan, et al., 1982).

Structural repeating unit
is the disaccharide β-cellobiose
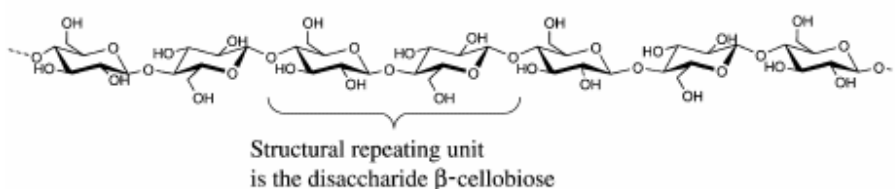
Figure 2.2: Chemical structure of cellulose. Linear β-1,4-linked glucose is the chemical repeating unit, while the structural repeat is β-cellobiose, and consequently each glucoside is oriented at 180° in respect to its neighbors. From Varrot et al., 2003.

Regions within the microfibrils with high order are termed crystalline, and less ordered regions are termed amorphous. The term "amorphous" cellulose is widely accepted even though it can be contradictory. Amorphous material is defined as material which is formless or lacks definite shape, however, amorphous cellulose probably still possesses some degree of order (O'Sullivan, 1997). Larsson, et al. (1997), investigated molecular ordering of cellulose and reported that most of the amorphous regions correspond to the chains that are located at the surface, whereas crystalline components occupy the core of the microfibril, Figure 2.3.A. A different molecular architecture of crystalline and amorphous cellulose is suggested by Moiser et al. (1999) and Tenkanen et al. (2003). They describe cellulose as being semi-crystalline, with regions of high crystallinity averaging approximately 200 glucose residues in length separated by amorphous regions, Figure 2.3.B.
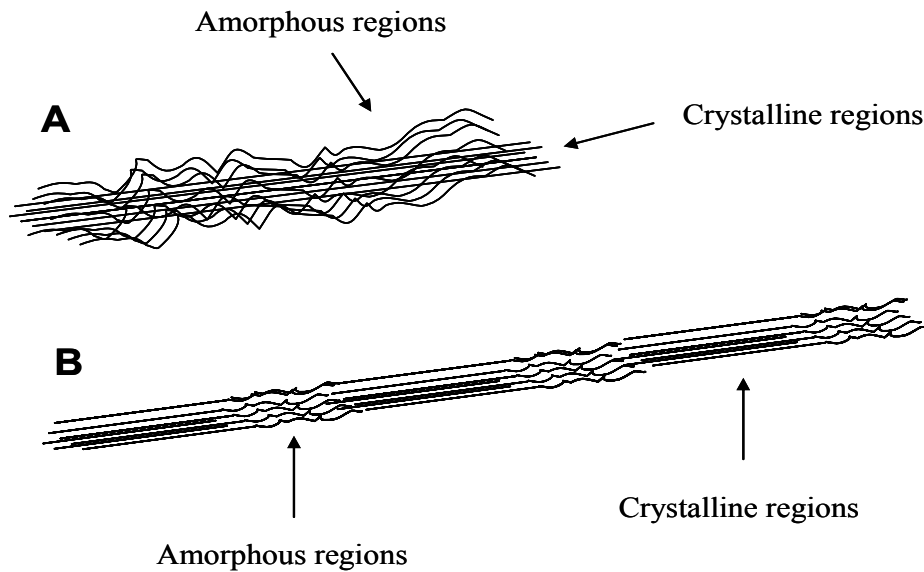
Figure 2.3: Two different views on how crystalline and amorphous cellulose is distributed within the microfibril. **A:** Crystalline cellulose is in the core of the microfibril, and it is surrounded by amorphous substrate. **B:** Crystalline and amorphous regions are being repeated in horizontal dimension.

Native cellulose has the degree of polymerization (DP) up to 10.000 β-anhydroglucose residues (Hon and Shiraishi, 1991). This means that the molecular weight is above 1.5 million [g/mol]. As the length of the anhydroglucose unit is 0.515 nm (5.15 Å), the total length of the native cellulose molecule can reach 5 μm.

Cellulose exists in seven crystal structures (polymorphs) designated as celluloses Iα, Iβ, II, III$_I$, III$_{II}$, IV$_I$, and IV$_{II}$ (O'Sullivan, 1997). In nature, cellulose Iα and Iβ are the most abundant crystal forms. Iα polymorph is meta-stabile, and thus, more reactive than Iβ. No pure sample of Iα have been found in nature. The percentage of Iα polymorph in crystalline cellulose varies from 70 % in bacterial cellulose (O'Sullivan, 1997), 64 % in cellulose isolated from algae *Valonia ventricosa*, to 20 % in ramie and cotton cellulose (Yamamoto and Horii, 1994). The co-existence of two polymorphs of native cellulose, which have different stabilities, may imply that the part of the Iα polymorph within the microfibril is most prone to the enzymatic attack.

2.1.2 Hemicellulose

Hemicellulose is the second most abundant renewable organic material, next to cellulose, on the Earth. In the conversion of lignocellulose to fuels and chemicals, utilization of hemicellulose as a byproduct is essential to make overall economics of processing wood into chemicals feasible.

Hemicelluloses are branched heterogeneous polymers consisting of many different sugar monomers like: D-xylose, L-arabinose (pentoses), D-mannose, D-glucose, D-galactose, L-rhamnose (hexoses), 4-O-methyl-D-glucuronic acid, D-glucuronic acid and D-galacturonic acid (uronic acids) (Hon and Shiraishi, 1991). The type of hemicellulose varies depending on monomer composition. The hemicelluloses from different types of wood, e.g. hardwood and softwood, differ both in structure and amount (Table 2.1). Hardwood hemicelluloses are composed mainly of xylan and make up from 20 to 37 % of the woody material, while softwood hemicelluloses consist primarily of galactoglucomannans and make up from 16 to 27 % of softwood (Hon and Shiraishi, 1991). The average DP varies between 70 and 200 depending on the wood species (Fengel and Wegener, 1983).

Table 2.1: The major hemicellulose components (from Eriksson, et al. 1990).

Hemicellulose type	Ocurrance	Amount [% of wood]	Composition			DP
			Units	Molar ratios	Linkage	
Galactoglucomannan	Softwood	5.0-8.0	β-D-Manp	3	1,4	~ 200
			β-D-Glcp	1	1,4	
			α-D-Galp	1	1,6	
			Acetyl	1		
Galactoglucomannan	Softwood	10.0-15.0	β-D-Manp	4	1,4	~ 200
			β-D-Glcp	1	1,4	
			α-D-Galp	0,1	1,6	
			Acetyl	1		
Arabinoglucuronoxylan	Softwood	7.0-10.0	β-D-Xylp	10	1,4	~ 200
			4-O-Me-α-D-GlcpA	2	1,2	
			α-L-Araf	1,3	1,3	
Arabinogalactan	Larchwood	5.0-35.0	β-D-Galp	6	1,3	350
			α-L-Araf	~2/3	1,6	
			β-L-Arap	~1/3	1,3	
			β-D-GlcpA	Little	1,6	
Glucuronoxylan	Hardwood	15.0-30.0	β-D-Xylp	10	1,4	200
			4-O-Me-α-D-GlcpA	1	1,2	
			Acetyl	7		
Glucomannan	Hardwood	2.0-5.0	β-D-Manp	~1-2	1,4	200
			β-D-Glcp	1	1,4	

DP: Degree of polymerization; β-D-Manp: β-D-Mannopyranose; β-D-Glcp: β-D-Glucopyranose; α-D-Galp: α-D-Galactopyranose; β-D-Xylp: β-D-Xylopyranose; 4-O-Me-α-D-GlcpA: 4-O-Mehyl-α-D-Glucuronic acid; α-L-Araf: α-L-Arabinopyranose; β-D-Galp: β-D-Galactopyranose; β-L-Arap: β-L-Arabinofuranose; β-D-GlcpA: β-D-Glycopyranosyl-uronic acid.

Due to their branched structure, hemicelluloses are more soluble then cellulose and they can be isolated from wood by extraction. Hemicelluloses are easily hydrolyzed by strong acid leaving cellulose and lignin intact (Liu and Wyman 2005; Lloyd and Wyman, 2005), or by strong base (Fan et al., 1982). In many cases diluted acid (0.5-1.0 % H_2SO_4) pretreatment under elevated temperatures (140-190 °C) will degrade most of the hemicellulose to soluble pentose and hexose sugars (Lloyd and Wyman, 2005). Even though this treatment is not particularly targeted towards solubilization of lignin, the lignin structure is disturbed and redistributed leading to much more favorable (pretreated) substrate for enzymatic hydrolysis (Yang and Wyman, 2004).

2.1.3 Lignin

Lignin is probably the most complex and the least characterized molecular group among the wood components. It's primary purpose is to give strength and water permeability to plants, but also to protect plants from pathogen infections. Lignin is composed of *p*-hydroxyphenoyl, guaiacyl and/or syringyl monomers linked in three dimensions. These three monomers differ in the methoxylation pattern of the aromatic ring (Douglas, 1996). As it is the case for hemicellulose, the composition and amount of lignin present in the woody material varies according to species, cell type and stage of tissue development. Lignin accounts for approximately 20-35 % of wood structure (Fan et al., 1982).

In the process of enzymatic hydrolysis of lignocellulose (Vinzant et al., 1997; Mooney et al., 1998) and in the pulp and paper industry, lignin is an undesirable component and it is generally necessary to remove it by chemical treatments. Besides being the physical barrier for the enzymes (Mansfield et al., 1999), cellulases can be irreversibly bound to lignin, consequently influencing the amount of enzyme needed for the hydrolysis, but also hindering the recovery of the enzymes after the hydrolysis (Lu et al., 2002). Two commonly used chemical pre-treatment methods (summarized by Sun and Cheng, 2002) targeted towards degradation of lignin are: 1) Oxidative delignification, where lignin biodegradation is catalyzed by the peroxidase enzyme in the presence of H_2O_2 (Azzam, 1989); and 2) Organosolv process, where an organic or aqueous organic solvent mixture with inorganic acid catalyst (HCl or H_2SO_4) is used to

break the internal lignin and hemicellulose bonds (Aziz and Sarkanen, 1989). Other treatments such as wet oxidation performed at temperatures from 170 °C to 200 °C and at pressures from 10 to 12 bar O_2, for 10 to 15 minutes, are recognized as an efficient method for partial solubilization of lignin (McGinnis et al., 1983).

2.1.4 Pretreatment of lignocellulose

In economically feasible industrial processes for conversion of lignocellulosic materials into energy carriers such as ethanol and butanol, or various other products, such as organic acids, acetone or glycerol, both cellulose and hemicellulose needs to be hydrolyzed to sugars that in proceeding steps can be further converted (Wyman, 2002). The ideal pretreatment method, thus, needs to integrate several processes; e.g. hydrolysis of hemicellulose to predominantly pentoses (5-carbon sugars), reduction, modification and/or redistribution of lignin, and reduction in crystallinity and increase of surface area of cellulose. Physical, physico-chemical, chemical and biological processes have been used for pretreatment of lignocellulosic material (Fan, et al., 1982; Sun and Cheng, 2002), Table 2.2. The drawback of the pretreatment processes, besides the obvious economical impact, is the generation of inhibitory compounds that can negatively influence the action of enzymes and microorganisms. Inhibitors are produced as a consequence of extreme pH and high temperature treatment of lignocellulosic materials (Olsson et al., 2004). Each pretreatment process should, therefore, be carefully chosen and critically justified.

Physical treatments can be classified into two general categories, mechanical and non-mechanical pretreatments (irradiation, high pressure steaming and pyrolysis), Table 2.2. A common purpose of both categories is to subdivide lignocellulosic materials into fine particles which are substantially susceptible to acid or enzymatic hydrolysis. The smaller particles have a large surface-to-volume ratio thus making cellulose more accessible to hydrolysis (Fan, et al., 1982).

Chemical pretreatment methods have been traditionally used in paper and pulp industry for delignification of cellulose and for destroying its crystalline structure. The main advantages of these methods are high effectiveness (high glucose yield) and

minimal formation of inhibitors (in particular with the concentrated acid treatment). On the other side, the need of specialized corrosion resistant equipment, and necessity of subsequent extensive washing, together with the disposal of chemical waist should be stated as the main disadvantages of these processes.

Biological pretreatment utilizes wood attacking microorganisms that can degrade lignin (Table 2.2). White rot fungi have been identified as the most promising group for the lignocellulose pretreatment (Ander and Eriksson, 1979).

Table 2.2: Summary of methods used for the pretreatment of lignocellulosic materials

Physical[a]	Chemical[a]	Biological[b]
Ball-milling	Alkali	Fungi
Two-roll milling	Sodium hydroxide	Brown rots (attack cellulose)
Hammer milling	Ammonia	*Piptoprus betulinus*
Colloid milling	Ammonium sulfite	*Laetiporus sulphureus*
Vibro energy milling	Acid	*Trametes quercina*
High pressure steaming	Sulfuric acid	*Fomitopsis pinicola*
Extrusion	Hydrochloric acid	*Gloephyllum saepiarium*
Expansion	Phosphoric acid	White rots (attack both lignin and cellulose)
Pyrolysis	Gas	*Formes fomentarius*
High energy radiation	Chlorine dioxide	*Phellinus igniarius*
	Nitrogen dioxide	*Ganoderma appalanatum*
	Sulfur dioxide	*Amillaria mellea*
	Oxidizing agents	*Pleurotus ostreatus*
	Hydrogen peroxide	Red rot (attack both lignin and cellulose)
	Ozone	*Formitopsis annos*
	Cellulose solvents	Bacteria
	Cadoxen (ethylene diamine and water)	
	CMCS	
	Solvent extraction of lignin	
	Ethanol-water extraction	
	Benzene-ethanol extraction	
	Ethylene-glycol extraction	
	Butanol-water extraction	
	Swelling agents	

[a]: From Fan et al., 1982; [b]: From Schurz, 1978; CMCS: is composed of sodium tartarate, ferric chloride, sodium sulfite, and sodium hydroxide solution.

2.2   Cellulose in model substrates

Natural lignocellulosic substrates have complicated structure and are therefore not well suited for fundamental and detailed characterization of enzymes. A wide range of different model substrates have, thus, been developed and used to demonstrate key functional differences in enzyme properties. The advantage of being able to perform experiments with model substrates is that the purity of the substrate is constant and

assured. However, those substrates do not have exact same characteristics as the cellulose have in the plant cell wall material, and thus sometimes poorly represent some of, for enzymatic hydrolysis of cellulose, crucial characteristics. The often occurring dilemma is therefore, to which degree results obtained with the model substrate can be transferred to the condition where cellulose is integrated with other chemical components (hemicellulose, and in particular lignin). Nevertheless, model substrates are extensively used in the academia for basic research and are important for increasing our fundamental understanding of the process.

In my studies, I used Avicel and phosphoric acid swollen cellulose/Avicel (PASC) as substrates. Avicel is microcrystalline cellulose derived by acid hydrolysis of wood, and is commonly considered to be a crystalline substrate. Avicel® PH-101 is a white powder with the particle size of about 50 μm. Besides Avicel, bacterial micro-crystalline cellulose (BMCC), *Valonia* cellulose, Solka Floc (a mixture of crystalline and amorphous cellulose produced by hammer-milling sulfite wood pulp), Cotton, and Whatman No. 1 filter paper are frequently used as model substrates. Summary of their main characteristics is presented in Table 2.3.

Table 2.3: Summary of main characteristics of cellulosic model substrates.

Substrate	CrI	SSA [m2/g]	DP	Reference
Valonia cellulose	~ 1	---	---	a
BMCC	0.8 - 0.95	200	600 - 2000	b,c
Cotton	0.8 - 0.95	---	1000 - 3000	b,c
Avicel	0.5 - 0.6	20	150 - 500	b,c
Wahtman No.1 filter paper	~ 0.45	---	750 - 2800	b,c
Solca Floc	0.4 - 0.7	55 - 61	750 - 1500	b,c
PASC	0	240	100 - 1000	b,c

CrI: Crystallinity index; SSA: Specific surface area; DP: Degree of polymerization; BMCC: Bacterial microcrystalline cellulose; PASC: Phosphoric acid swollen cellulose; a: Verlhac et al., (1990); b: Zhang et al., (2004); c: Zhang et al., (2006).

PASC was prepared from Avicel by phosphoric acid treatment (as described by Schulein, 1997) and it is considered to be a representative of amorphous cellulose.

Opposed to the dry, solid, powder-like composition of Avicel, PASC is relatively viscous, unclear (cloudy) liquid. It has been shown that in the cellulose swelling process, the macromolecular structure of cellulose as a moiety of fibers is maintained, while the physical properties of the sample are changed (Zhang, et al., 2006). In particular, specific surface area and sample volume have been shown to increase, due to swelling, while DP of PASC have not changed radically compared to the untreated Avicel (Zhang and Lynd, 2005; Zhang, et al., 2006). Thus, the two model cellulosic substrates are profoundly different in their physical features. In order to better define their differences, during the experimental study (Article A), the number-average-degree of polymerization of Avicel and PASC were determined, and furthermore, the molecular weights were estimated. Those results, including summary of the most important physical characteristics of Avicel and PASC are presented in Table I of Article A, presented in Chapter 5.

The accessibility of cellulose fibers/chains to the cellulolytic enzymes is essential for efficient hydrolysis process. Without a physical contact, i.e. adsorption of enzymes to the surface of the insoluble substrate, the reaction will not proceed. Thus, accessibility of cellulose is related to, in particular, specific surface area and crystallinity of the substrate, but also particle size, porosity and presence of associated compounds such as hemicelluloses and lignin. Zhang et al, 2006 investigated influence of phosphoric acid treatment of Avicel on hydrolysis and observed dramatic differences in the hydrolysis rates of the cellulosic samples before (Avicel) and after phosphoric acid treatment (PASC). Those differences have been related to the differences in structure, and thus accessibility, of the two investigated substrates (Figure 2.4). As shown in Figure 2.4, while the crystal and fiber structure of Avicel is maintained intact, the structure of PASC is evidently disordered, so that the larger surface area for binding is provided to the enzymes.
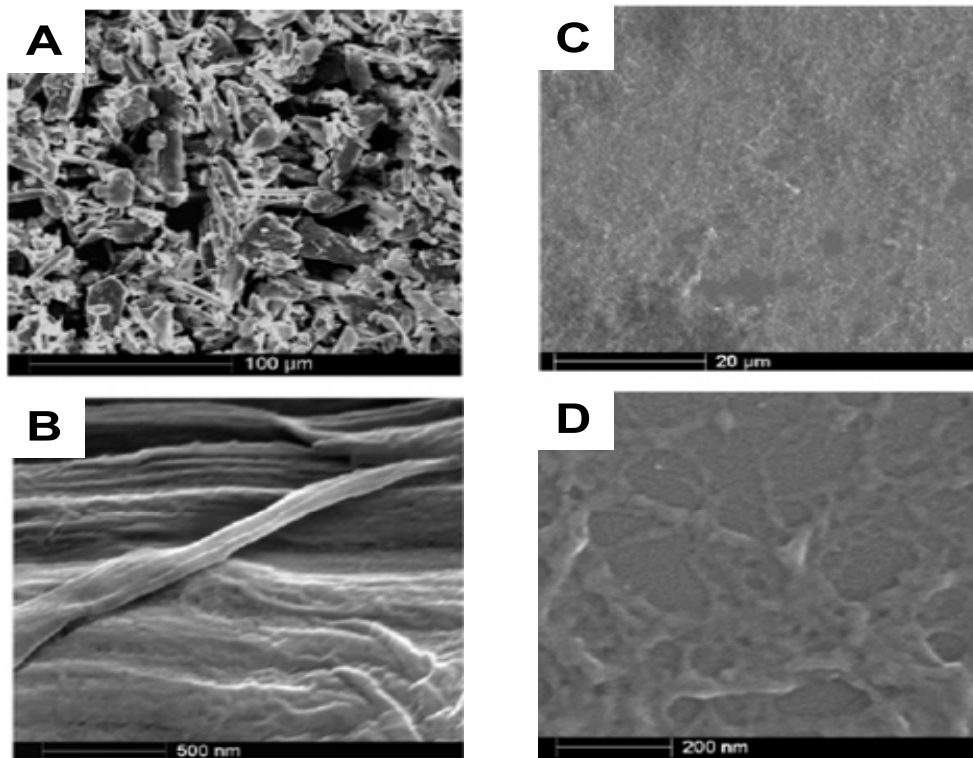
Figure 2.4: The scanning electron microscopy image of Avicel (A,B) and PASC (C,D), with two different magnifications as shown in the pictures. The crystal and fiber structure is intact in Avicel, opposed to the evident amorphous (disordered) structure of PASC samples. From Zhang, et al., 2006.

2.3   References

Ander P, Eriksson KE. 1978. Lignin degradation and utilization by microorganisms. In: Progress in industrial microbiology 14. New York: Elsevier. p 1-58.

Aziz S, Sarkanen K. 1989. Organosolv pulping – a review. Tappi. J. 72: 169-175.

Azzam AM. 1989. Pretreatment of cane bagasse with alkaline hydrogen peroxide for enzymatic hydrolysis of cellulose and ethanol fermentation. J. Environ. Sci. Health. B. 24: 421-433.

Bidlack J, Malone M, Benson R. 1992. Molecular structure and component integration of secondary cell wall in plants. Proc. Okla. Acad. Sci. 72: 51-56.

Carpita NC, Gibeaut DM.1993. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J. 3: 1-30.

Douglas CJ. 1996. Phenylpropannoid metabolism and lignin biosynthesis: from weeds to trees. Trends Plant Sci. 1: 171-178.

Eriksson K-E L, Blanchette R.A, Ander P. 1990. Biodegradation of hemicelluloses. In: Microbial and enzymatic degradation of wood and wood components, New York: Springer-Verlag. p 181-223.

Fan LT, Lee Y-H, Gharpuray MM. 1982. The nature of lignocellulosics and their pretreatments for enzymatic hydrolysis. Adv. Bichem. Eng/Biotechnol 23: 158-187.

Fengel D, Wegener G. 1983. Wood: chemistry, ultrastructure and reactions. Berlin: Walter de Gruyter & Co. 613 p.

Hon D N-S, Shiraishi N. 1991. Wood and cellulosic chemistry. New York: Marcel Dekker, inc. 1032 p.

Larsson PT, Wickholm K, Iversen T. 1997. A CP/MAS ^{13}C NMR investigation of molecular ordering in celluloses. Carbohydr. Res. 302: 19-25.

Lloyd TA, Wyman CE. 2005. Combined sugar yields for dilute sulfuric acid pretreatment of corn stover followed by enzymatic hydrolysis of the remaining solids. Bioresour. Tech. 96: 1967-1977.

Lu Y, Yang B, Gregg D, Saddler JN, Mansfield SD. 2002. Cellulase adsorption and an evaluation of enzyme recycle during hydrolysis of steam-exploded softwood residues. Appl. Biochem. Biotechnol. 98-100: 641-654.

Mansfield SD, Mooney C, Saddler JN. 1999. Substrate and enzyme charackteristics that limit cellulose hydrolysis. Biotechnol. Proc. 15:804-816.

McGinnis GD, Wilson WW, Mullen CE. 1983. Biomass pretreatment with water and high-pressure oxygen. The wet oxidation process. Ind. Eng. Chem. Prod. Res. Dev. 22: 352-357.

Moiser NS, Hall P, Ladisch CM, Ladisch MR. 1999, Reaction kinetics, molecular action and mechanisms of cellulosic proteins. Adv. Biochem. Eng. Biotehnol. 65: 23-40.

Mooney CA, Mansfield SH, Touhy MG, Saddler JN. 1998. The effect of initial pore size and lignin content of the enzymatic hydrolysis of softwood. Biores. Technol. 64: 113-119.

O'Sullivan. 1997. Cellulose: the structure slowly unravels. Cellulose 4: 173-207.

Olsson L, Jørgensen H, Krogh KBR, Roca C. 2004. Bioethanol production from lignocellulosic material. In: Polysaccharides: structural diversity and functional versatility. New York: Marcel Dekker Inc. p 957-993.

Schulein M.1997. Enzymatic properties of cellulases from *Humicola insolens*. J Biotechnol 57: 71-81.

Schurz J. 1978. Bioconversion of cellulosic substrates into energy chemicals and microbial protein. New Delhi: Symp. Proc. TK Ghose. p 37.

Sun Y, Cheng J. 2002. Hydrolysis of lignocellulosic material for ethanol production: a review. Bioresour. Tech. 83: 1-11.

Tenkanen M, Niku-Paavola M-L, Linder M, Viikari L. 2003. Cellulase in food processing Iin Handbook of food enzymology. New York: Marcel Dekker Inc. p 879-915.

Varrot A, Frandsen TP, von Ossowski I, Boyer V, Cottaz S, Driguez H, Sculein M, Davies GJ. 2003. Structural basis for ligand binding and processivity in cellobiohydrolase Cel6A from *Humicola insolens*. Structure. 11: 855-864.

Vinzant TB, Ehrman CI, Himmel ME. 1997. SSF of pretreated hardwoods: effect of native lignin content. Appl. Biochem. Biotechnol. 62: 97-102.

Wyman CE. 2003. Potential synergies and challenges in refining cellulosic biomass to fuels, chemicals and power. Biotechnol. Prog. 19: 254-262.

Yamamoto H, Horii F. 1994. *In situ* crystallization of bacterial cellulose I. Influences of polymeric additives, stirring and temperature on the formation of celluloses Iα and Iβ as revealed by cross polarization/magic angle spinning (CP/MAS) ^{13}C NMR spectrometry. Cellulose 1: 57-66.

Yang B, Wyman CE. 2004. Effect of xylan and lignin removal by batch and flow-through pretreatment on enzymatic digestibility of corn stover cellulose. Biotechnol. Bioeng. 86: 88-95.

Zhang Y-HP, Cui J, Lynd LR, Kuang LR. 2006 A. A transition from cellulose swelling to cellulose dissolution by *o*-phosphoric acid: evidence from enzymatic hydrolysis and supramolecular structure. Biomacromolecules. 7: 644-648.

Zhang Y-HP, Himmel ME, Mielenz JR. 2006. Outlook for cellulose improvement: screening and selection strategies. Biotechnol. Adv. 24: 452-481.

Zhang Y-HP, Lynd LR. 2005. Determination of the number-average degree of polymerization of cellodextrins and cellulose with application to enzymatic hydrolysis. Biomacromolecules. 6: 1510-1515.

CHAPTER 3

Analytical methods for quantification of enzymatic hydrolysis

The extent of hydrolysis is most commonly evaluated based on the products of the enzymatic reaction. Simultaneously, cellulase activity is also evaluated based on the products of the reaction. Term *cellulase* includes not only one, but several enzymes, with similar and sometimes overlapping activities, working in synergy with each other. Defining only one of the enzyme activities in the presence of other enzymes is therefore often challenging. Thus, a combination of several enzymatic assays and measuring techniques is preferred.

Methods for quantification of enzymatic hydrolysis can be divided in following groups:

1. Traditional enzyme assays
2. Chromatographic techniques
3. Other (novel) techniques

3.1   Traditional enzyme assays

Most commonly used enzyme assays are based on measuring "reducing ends" or "reducing sugars/saccharide" upon the hydrolysis reaction. Moreover, this analysis enables quantification of the degree of polymerization of cellulosic substrates.

So called "reducing ends" are free aldehyde groups in a sugar, that will be oxidized to carboxylic group, in the presence of oxidizing agents like: dinitrosalicylic acid, copper sulphate or ferricyanide. This reaction is typically followed spectrophotometrically due to the color change of the oxidizing agent (Figure 3.1).
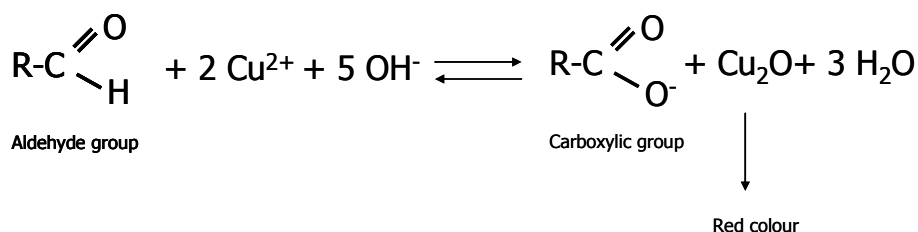
Figure 3.1: The principal of "reducing end" reaction. In this example free aldehyde group in a sugar is oxidized to carboxylic group in the presence of oxidizing agent (cupper sulphate, dinitrosalicylic acid or ferricyanide) producing red colored copper oxide that can be measured on spectrophotometer.

When measuring a mixture of carbohydrates this relatively fast and inexpensive measurement can give inexact results due to poor stoichiometric relationship between cellodextrins and glucose standard, i.e., a spectrophotometric response of glucose (commonly used as standard) and reducing agent is stronger than the response of cellobiose or the cellulose chain (Ghose, 1987; Wood and Bhat, 1988; Coward-Kelly et al., 2003). Moreover, presence of protein (cellulases) can cause interference and influence the quantification. Nevertheless, this analysis is an often used tool for the comparison of enzyme efficiency and substrate degradability.

International Union of Pure and Applied Chemistry (IUPAC) has recommended filter paper assay (FPA) as a standard measurement of total cellulase activity. The assay is based on the reducing sugar measurements (Wood and Bhat, 1988; Tolan and Foody, 1999). Although the method requires simple reagents and equipments, it is hampered by long assay times and many manual manipulations (Ghose, 1987). Filter paper is considered to be highly crystalline cellulose and thus, its degradation is depending on the combination of, primarily, endoglucanase (EG) and cellobiohydrolase (CBH) activities, but also β-glucosidase (BG) activity, an enzyme that will degrade higher soluble cellooligosaccharides and cellobiose to glucose. This method can, thus, provide means of measurement of total cellulase activity, but due to earlier mentioned poor stoichiometric relationship between glucose and other cellooligosaccharides, it is to a high extent dependent on the presence of β-glucosidase activity (Chan et al., 1989).

Kabel et al., (2005) compared 14 commercially used cellulase preparations for their efficiency, by measuring three standard activities, among which the FPA, cellobiose and xylanase activity [U/ml] and furthermore, by using more natural and therefore more

complex substrates, wheat bran and grass. They observed no relationship between measured amount of protein [mg/ml] present in the commercial cellulase samples and total enzyme activity (measured by FPA). Moreover, substantial disagreement between, the measured xylanase activity (using a standard assay), and the percentage of degraded xylan to xylose, by a commercial enzyme mixture, has been observed. They, thus, concluded that standard assays do not predict the efficiency of commercial cellulase preparations towards plant material and that the choice of an enzyme preparation is more dependent on the characteristics of the substrate rather that on the standard enzyme activities measured.

During my PhD studies I investigated a number of assays for determination of reducing ends, evaluating their detection range and stoichiometric relationship between cellobiose and glucose standard. After short introduction of the assays in sections 3.1.1 – 3.1.5, the results will be summarized in section 3.1.6.

3.1.1 Nelson-Somogyi assay

Nelson-Somogyi assay is based on the alkaline copper reagent of Somogyi (1952) and the color reagent of Nelson (1944). The assay involves the reduction of Cu^{2+} to Cu^+ by the reducing saccharides and the formation of colored compound – arsenomolybdate. The exact protocol is described in Appendix 10.1. Relatively high sugar detection range and low interference from the cellulase protein have been reported as major advantages (Zhang et al., 2006). However, Nelson's reagent contains arsenic of which high toxicity is a serious environmental problem.

In this study four different wavelengths (500 nm, 520 nm, 660 nm and 750 nm) were tested on seven glucose and cellobiose standards in the range from 0.001 mM to 0.1 mM (Appendix 10.1). In all cases linear response was observed, with the best results obtained at 750 nm as the divergence between glucose and cellobiose measurement at this wavelength was the smallest (Table 3.1).

Interference of the externally added protein, bovine serum albumin (BSA), in the concentration range of 2.5 µg/ml to 250 µg/ml was tested, and absorbance reading showed clear, though not exactly linear, increase of the absorbance in response to the

protein addition (data not shown). Reproducibility of the experiment was tested on five independent experiments with average standard deviation of 0.025 absorbance units and average error of 10 %.

Table 3.1: Comparison of the reducing saccharide assays and their performance

| | Wavelenghts [nm] | Linear range mM | Response of glucose | | Response of cellobiose | | |
			Equation of linear trendline	R^2	Equation of linear trendline	R^2	Relative response [%]
N-S	500	0.001 - 1.0	y = 0.3804x + 0.0016	0.9936	y = 0.4575x + 0.0181	0.9968	138
	520	0.001 - 1.0	y = 0.4699x - 0.0048	0.9942	y = 0.5615x - 0.0123	0.9963	138
	660	0.001 - 1.0	y = 1.5282x - 0.0267	0.9971	y = 1.71762x - 0.0011	0.9972	122
	750	0.001 - 1.0	y = 2.4826x - 0.0435	0.9973	y = 2.5708x - 0.0032	0.9988	111
PAHBAH	410	0.001 - 1.0	y = 1.3674x	0.9982	y = 0.8080x	0.9910	59
	410	1.0 - 70.0	y = 0.0152x	0.9921	y = 0.0105x	0.9908	69
DNS	575	1.0 - 20.0	y = 0.0187x - 0.0090	0.9971	not linear	n.a.	< 70
2-C	274	0.001 - 1.0	y = 1.3581x + 0.0379	0.9998	y = 0.9746x + 0.0324	0.9993	72

N-S: Nelson-Somogyi assay; PAHBAH: 4-Hydroxybenzoic acid hydrazide assay; DNS: Dinitrosalicylic acid assay; 2-C: 2-Cyanoacetamide assay; n.a.: not applicable; Relative response of cellobiose was calculated relative to the response of glucose (which was set to be 100 %).

3.1.2 4-Hydroxybenzoic acid hydrazide (PAHBAH) assay

Under alkaline conditions 4-hydroxybenzoic acid hydrazide reacts with reducing saccharide to give intensively yellow anion which adsorbs strongly at 410 nm (Lever, 1972). The assay has been improved by including bismuth as a catalyst so that the reaction can be carried out more rapidly and at lower temperatures (Lever, 1977).

The assay was tested on two ranges of glucose and cellobiose concentrations, e.g. macro-assay from 1 mM to 70 mM, and, micro-assay from 0.001 mM to 1 mM, having ten and five points, respectively (Appendix 10.2). The error (calculated as standard deviation divided by mean) was found to be 14.1 % for the macro-assay and 18.3 % for the micro-assay. Furthermore, considerable difference in absorbance response of glucose and cellobiose was observed (Table 3.1). On average, equimolar concentration of cellobiose gave absorbance response that was only 69 % of that of glucose in the macro-assay, and 59 % in the micro-assay, rather unacceptable for a quantification assay.

3.1.3 Dinitrosalicylic (DNS) assay

The stochiometry of the reaction is based on the oxidation of one mole of aldehyde functional group and simultaneous reduction of one mole of 3,5-dinitrosalycilic acid (Miller, 1959). Nevertheless, many side reactions are known to exist depending on the type of the reducing saccharide used (Wang, 2005). As a result equal amounts of glucose, cellobiose and xylose would yield different color intensity and need to be calibrated individually.

The procedure of the assay is described in the Appendix 10.3. In the concentration range from 5 mM to 10 mM of glucose and cellobiose, linearity and similar absorbance response of both sugars was observed. At concentrations higher then 10 mM, however, cellobiose absorbed less then 70 % compared to glucose (Table 3.1). At low saccharide concentrations (0.001 mM to 0.1 mM) a non-linear absorbance was observed, and thus this assay was not further investigated.

3.1.4 2-Cyanoacetamide assay

In this assay, the aldehyde group of the reducing saccharide undergoes Knoevenagel condensation with the active methylene group in 2-cyanoacetamide. The following dehydration results in formation of dienol, which absorbs ultraviolet light at 274 nm (Honda et al., 1982; Bach and Schollmeyer, 1992). This assay is reported to be very sensitive with high linearity (Bach and Schollmeyer, 1992).

The procedure of the assay is described in the Appendix 10.4. The linear range of the glucose and cellobiose standards was established in the concentration interval of 0.001 mM to 1.0 mM, with the absorbance response of cellobiose being on average 72 % of that of glucose (Table 3.1).

3.1.5 Ferricyanide assay

This assay was proposed by Park and Johnson (1949) and it involves reduction of ferricyanide ions by the aldehyde groups on the reducing saccharide under alkaline conditions to form Prussian blue (ferric ferrocyanide) measurable on spectrophotometer.

Although high sensitivity has been reported (Park and Johnson, 1949), in this study I failed to reproduce the assay despite a number of careful attempts. The protocol used is described in Appendix 10.5.

3.1.6 Summary of the results - reducing saccharide assays

The results obtained during investigation of five reducing saccharide assays are summarized in Table 3.1. In conclusion, Nielson-Somogyi assay at 750 nm was found to be the best assay for quantification of saccharides of varying sizes. The results showed that this assay had the least discrimination between glucose and cellobiose (relative response of cellobiose was 111 %), and, furthermore, it was highly reproducible.

3.2 Chromatographic techniques

Previously described traditional enzyme assays are predominantly based on detection of reducing ends after the hydrolysis reaction, and can thus give us the information on the number of glycosidic bonds that have been broken, but provide no information on the actual pattern of the hydrolysis products. Chromatographic techniques, in particular high performance anion-exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD) provides high detection sensitivity of mono-, di- and oligosaccharides, but also alditols and closely related compounds, including methylated aldoses, deoxysugars, amino sugars (Cataldi et al., 2000), provided the availability of standards for quantification. The separation mechanism is based on the weakly acidic properties of sugar molecules in basic solutions (pH above 12), while the detection employs the ability of gold electrode surface to catalyze the oxidation of polar compounds in alkaline media.

Chromatographic techniques have been applied to carbohydrate analysis by numerous authors (summarized by Cataldi et al., 2000). One of the most appreciated advantages is that the HPAEC-PAD method is not affected by the BG deficiency,

provided that the accumulated cellobiose does not inhibit the cellulase enzymes. Furthermore, detection is sensitive and consumes only a small amount of sample.

During the PhD work soluble hydrolysis products: glucose (C_1), cellobiose (C_2), cellotriose (C_3), cellotetraose (C_4), cellopentaose (C_5), and celloheksaose (C_6) were determined by HPAEC-PAD using CarboPac PA 1 column (Dionex). More details on the analytical procedure can be found in the Materials and Methods section of Article A, Chapter 5. Typical chromatogram of standard sugars, C_1 to C_6 is presented in Figure 3.2.
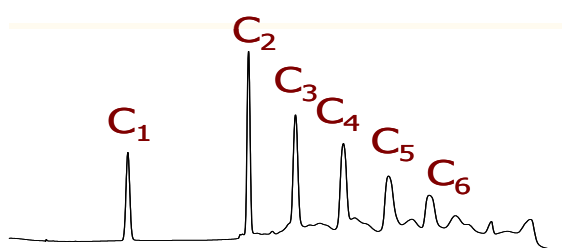
Figure 3.2: Typical chromatogram of standard sugars (200 µM). Based on the retention time and peak area of standard sugars, peaks in the sample can be identified and quantified. Note that, even though, each compound is present at the same concentration (200 µM), the height and the area of the corresponding peaks are not the same.

3.3   Other (novel) techniques

One of the often mentioned obstacles in the identification of cellulose hydrolysis products is their solubility. Shorter cellooligosaccharides with DP up to 6 are soluble in water, products of DP 7-12 are partially soluble, but higher DPs are insoluble (Zhang and Lynd, 2005). This has no impact on the measurement of the reducing sugars, but in the chromatographic techniques, only soluble hydrolysis products can be analyzed. I have therefore in the further study attempted to identify higher, insoluble cellooligosaccharides by the method presented by Goubet and coworkers (2002), primarily developed for determination of plant cell walls polysaccharides. Polysaccharide analysis using carbohydrate gel electrophoresis (PACE) relies on derivatization of reducing ends of sugars and cellooligosaccharides with a fluorophore, followed by separation of different

oligosaccharide sizes accomplished by electrophoresis under optimized conditions in polyacrylamide gels.

Most saccharides are naturally neutral in charge and can therefore not be separated by electrophoresis. The most significant breakthrough of PACE lies in derivatization step where electrically neutral saccharides are labeled with negatively charged fluorophores, resulting in saccharide derivatives that can migrate under the electric field and are visible under UV light (Jackson, 1990).

The purpose of gel electrophoresis is to separate water soluble particles through the application of electric force and the assistance of gel matrix. Electric force is the primary cause of the particle movement, while the gel matrix brings in the sieving action so that particles can be separated based on the molecular size. The principal of saccharide electrophoresis is virtually the same as the principle of protein and nucleic acid electrophoresis described earlier (Starr et al., 1996). One fundamental difference however exists, and that is the size of the sample molecule. While proteins and DNA have typical molecular masses in the range of 10.000's [g/mol], oligosaccharides have molecular mass of a few thousands, and monosaccharides are in the range of 180-300 [g/mol] (Starr et al., 1996). Thus, high percentage polyacrylamide gels must be used.

In this study, I have attempted to optimize PACE method, and apply it to identification of higher (insoluble) cellulose hydrolysis products. A summary of the study will be presented in the following section.

3.3.1 Optimization of PACE for cellulose hydrolysis studies

Since PACE have previously not been used to study large β-1,4-linked glucose oligosaccharides, following modifications and optimization of the published method (Goubet et al., 2002) were required:

- Optimization of the electrophoresis procedure, including:
  - Comparison of 4-12 % Bis-Tris gel and 20 % polyacrylamide gel,
  - Comparison of different electrophoresis buffers/buffer systems,
  - Development of standard quantification algorithms, and,
  - Testing the linear response range and reproducibility.

26

- Optimization of derivatization conditions, including testing different amounts of derivatization compound and reproducibility of the procedure.

For successful identification of the cellooligosaccharides produced after enzymatic hydrolysis, oligosaccharide standards should be used. Unfortunately, there are only a few commercially available. Thus, in this study efforts were made to create β-1,4-linked glucose oligomer ladder that could potentially be used as a standard.

Description of the method and results

The general procedure of PACE is described in Goubet, et al. (2002). The changes of the method that were introduced will be shortly discussed below. ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid) - a charged fluorophore, was used as a derivatization compound to study uncharged oligosaccharides and monosaccharides. The derivatization procedure is described in more details in Appendix 10.6.

Evaluation of 4-12 % Bis-Tris mini-gel (Invitrogen)

Though most literature has recommended using in-house made polyacrylamide gel, pre-casted 4-12 % Bis-Tris mini-gel (Invitrogen) was tested initially because it is convenient (commercially available) and it does not require working with the extremely toxic acrylamide monomer. Results are presented in Figure 3.3.A. C_1-C_3 were separated with good resolution, while C_5 and C_6 were separated with lesser, but acceptable resolution. The saccharide sample on the gel appeared as "dots", opposed to an expected "band" sample configuration.

At high sample concentration, sample dots at the gel expended proportionally and very often started to interfere with the neighboring dots (Figure 3.3.B). This was undesired, and thus further investigations were conducted with 20 % polyacrylamide gels.
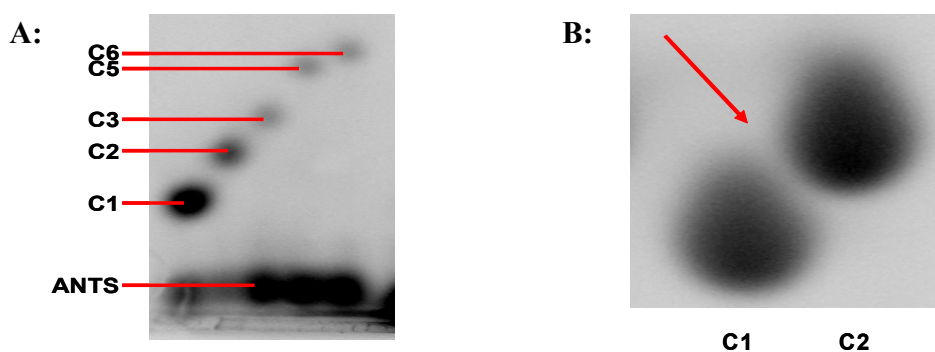
Figure 3.3: Evaluation of 4-12 % Bis-Tris mini-gel (Invitrogen). **A:** Separation of glucose (C_1), cellobiose (C_2), cellotriose (C_3), cellopentaose (C_5), and cellohexaose (C_6) on a 4-12 % Bis-Tris mini-gel (Invitrogen). Excess fluorophore (ANTS) is accumulated near the bottom of the gel. **B:** At high sample concentration, sample dots expended and started to interfere with the neighboring dots.

Evaluation of 20 % polyacrylamide gels

The procedure for preparation of polyacrylamide gel is described in Appendix 10.7. Three different electrophoresis buffers and buffer systems were tested:

A: Discontinuous buffer system consisting of Tris-HCl buffer in the gel and Tris-Glycine buffer in the buffer tank. Though it is very widely used (Jackson, 1990) it failed to separate sample saccharides in our lab (Figure 3.4.A).

B: Continuous buffer system with Tris-HCl in both the gel and the buffer tank. All saccharides tested showed identical pattern on the gel (Figure 3.4.B). Additionally, some of the bands had abnormal shapes (indicated by the arrow on Figure 3.4.B).

C: Continuous buffer system with Tris-Borate in both the gel and the buffer tank. On this gel all saccharides/standards appeared as "bands" and there was excellent separation for all saccharides tested, except for cellobiose, which migrated virtually the same distance as glucose (Figure 3.4.C). Excess ANTS appeared as multiple bands near the bottom of the gel and an extra band that migrated slower then the target band was observed in four out of five investigated saccharides (indicated by the arrows on Figure 3.4.C).
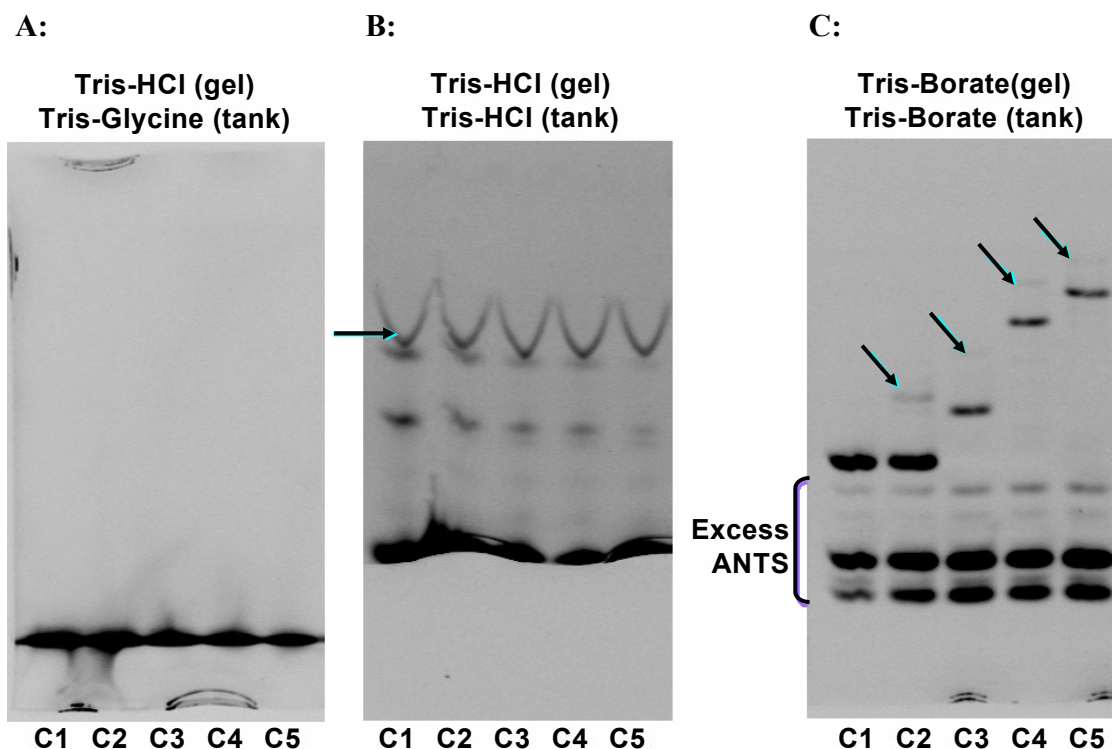
Figure 3.4: Evaluation of different electrophoresis buffers and buffer systems in 20 % polyacrylamide gels. **A:** Discontinuous Tris-HCl/Tris-Glycine**.** This system failed to separate the saccharides. **B:** Continuous Tris-HCl/Tris-HCl. All tested saccharides had the same band pattern. Furthermore, some bands hade unexpected shape (indicated by the arrow). **C:** Continuous Tris-Borate/Tris-Borate. This system detected and separated all saccharides except for cellobiose, which showed the same migration distance as glucose. In four out of five samples some extra bands have been observed (indicated by the arrow).

Quantification of samples detected on 20 % polyacrylamide gels

The procedure for gel imaging is presented in Appendix 10.8. Quantification of samples was performed using the analytical tool "Volume tool" available on Quantity One software. Cellotriose was chosen as the standard. Based on the eight investigated concentrations in the range from 0.05 to 1.5 mM, linear relationship (y=0.0006x + 0.0064, R^2=0.988) between concentration and sample fluorescence intensity (calculated by Quantity one software) was found.

The constructed standard curve was consequently used to predict concentrations of three known glucose and cellobiose concentrations. The average error of calculated glucose and cellobiose concentrations was 11.5 % and 14.6 % [mM], respectively.

Optimization of derivatization conditions

The most optimal amount of ANTS used for derivatization was determined to be: 10 µl of 20 µM ANTS and 0.1 M NaCNBH$_3$ for derivatization of 20 µl of hydrolysis sample. Derivatization was shown to be highly reproducible (tested on five glucose and cellobiose samples on 4-12 % Bis-Tris mini-gel) (data not shown).

Derivatization and detection of samples were tested on the hydrolysis of Avicel with the crude cellulases obtained from *Penicillium brasilianum* strain IBM 20888, Figure 3.5. This fungus is producing a number of cellulolytic and xylanolytic enzymes, including relatively high concentrations of β-glucosidase enzyme (Krogh et al., 2004). The results obtained are therefore not surprising (Figure 3.5). Analysis of the sample showed exclusively presence of glucose, as the main and final product of the hydrolysis. Additionally, the amount of the glucose in the sample was increasing with time.
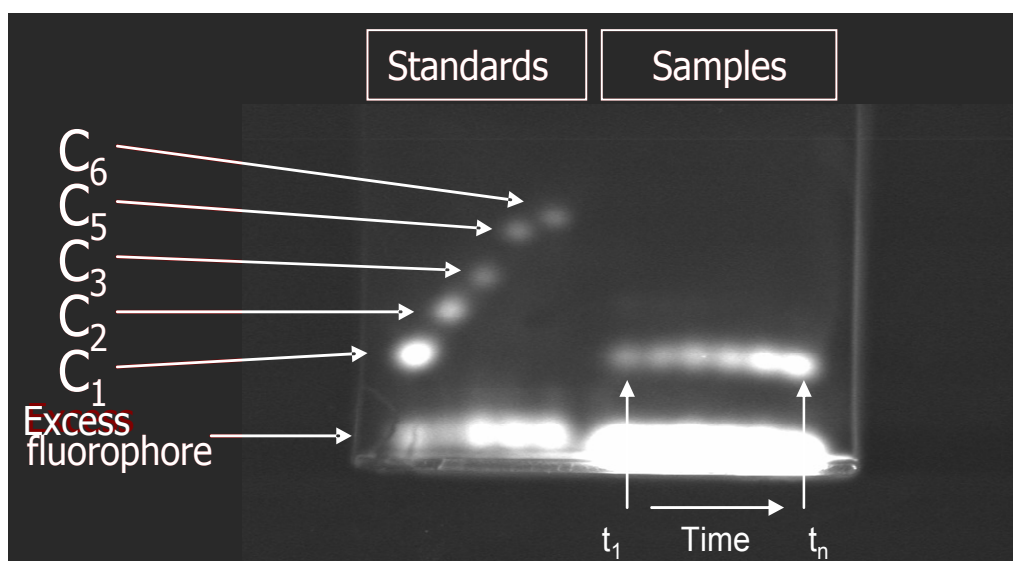
Figure 3.5: Hydrolysis of Avicel with *Penicillium brasilianum* IBM 20888 crude cellulases. Ten g/l of substrate was hydrolyzed with 0.25 FPU/ml of enzyme. Samples were collected during 48 hours (t$_l$=1.5 h, t$_n$=48 h), derivatized as described earlier and analyzed on a 4-12 % Bis-Tris mini-gel (Invitrogen). Excess fluorophore (ANTS) is accumulated near the bottom of the gel.

Construction of β-1,4-linked glucose oligomer ladder

As mentioned earlier, to be successfully identified, enzymatic hydrolysis products need to be compared to standards on gel. Unfortunately, only standards of DP of 1 to 6 are commercially available. Thus, in this study several methods were used to construct glucose oligomer ladder, with the purpose of using it as a standard for identification of unknown hydrolysis products (Appendix 10.9 and Table 3.2). The obtained results are presented in Figure 3.6 and will shortly be discussed here.

Table 3.2: Methods used to generate glucose oligomer ladder

Method	1	2-a	2-b	2-c	2-d	2-e	3	4
Substrate [mg/ml]	Starch 100	Avicel 100	Avicel 100	Avicel 100	Avicel 100	Avicel 100	Avicel 100	PASC 10
Procedure	0.1 M HCl 50 °C One hour	0.1 M HCl 50 °C One hour	0.5 M HCl 95 °C One hour	1.0 M HCl 95 °C One hour	1.5 M HCl 95 °C One hour	2.0 M HCl 95 °C One hour	2.0 M H_2SO_4 95 °C One hour	1.4 Bar 125 °C 20 minuttes

In literature, an often used method for generating glucose oligomers is hydrochloric acid (0.1 M) hydrolysis of starch. Bands produced and detected on the gel by this method are presented in Figure 3.6, Lane 1. As the acid hydrolysis of starch results in production of maltodextrines (low-molecular-weight carbohydrates, mixtures of linear α-(1,4)-linked D-glucose polymers) the alignment of the hydrolysis products did not correspond to the C_1-C_6 standards.

Another method used was hydrolysis of Avicel with HCl (2-a to 2-e in Table 3.2 and Figure 3.6). Some of the bands detected (indicated by stars on Figure 3.6) corresponded to the C_1-C_6 standards, but in none of the cases higher cellooligosaccharides were detected.

In addition to acid hydrolysis of Avicel with HCl, hydrolysis with 2.0 M H_2SO_4 was performed. Also here a few bands were detected (indicated by stars in the Figure 3.6) corresponded to the C_1-C_6 standards, but in general acid hydrolysis of Avicel was not successful in generating standards.

31
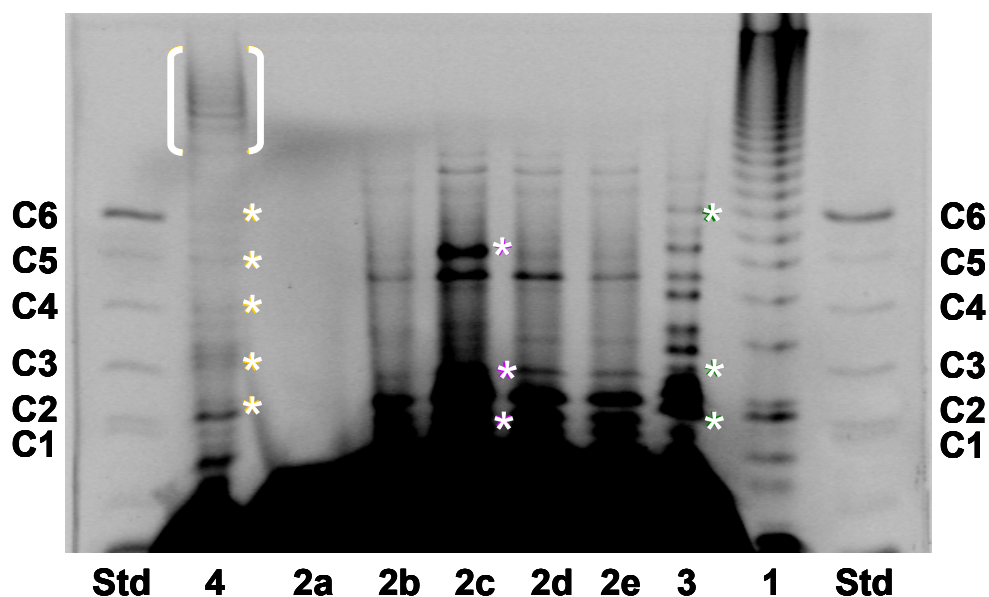
Figure 3.6: Different methods used to construct a β-1,4-linked glucose oligomer ladder. **(1)** Starch hydrolyzed with 0.1 M HCl; **(2a – 2e)** Avicel hydrolyzed with 0.1 M, 0.5 M, 1.0 M, 1.5 M and 2.0 M HCl, respectively; **(3)** Avicel hydrolyzed with 2.0 M H_2SO_4; **(4)** PASC autoclaved at 1.4 bar for 20 minutes; **Std:** Standard mixture of glucose (C_1), cellobiose (C_2), cellotriose (C_3), cellotetraose (C_4), cellopentaose (C_5) and cellohexaose (C_6). Stars indicate compounds that match the standards. Bracket indicates possible presence of large β-1,4-linked glucose oligomer.

The most promising procedure for production of large β-1,4-linked glucose oligomers investigated here was autoclavation of PASC. Short saccharides C_2-C_6 were identical to the standards, while larger cellooligosaccharides (indicated by the bracket on Figure 3.6) were also detected.

Summary of the results – Optimization of PACE for cellulose hydrolysis studies

The purpose of optimization of PACE for cellulose hydrolysis studies was to investigate and suggest fast and easy technique for detection and consequent quantification of *higher, insoluble* cellooligosaccharides. Commercially available gels did not show satisfactory results, thus gels needed to be manually casted, which was tedious, time consuming and potentially hazardous.

Quantification of the samples from the gel was possible but an average error was 11.5 % for C_1 and 14.6 % for C_2. Several methods aiming at production of large β-1,4-linked glucose oligomer ladder were investigated. Autoclavation of PASC seemed to be the most promising procedure. Nevertheless, PASC is not well defined, commercially

available substrate. The quality of PASC can vary depending on operation conditions such as the concentration of the acid, swelling time and temperature, blending time and severity, and efficiency in removing any cellulose lumps (Zhang et al., 2006). This will, consequently, influence reproducibility and quality of what was supposed to be a standard for quantification.

PACE method can potentially be useful for identification and quantification of enzymatic hydrolysis products, but further optimization of the process would be needed. Heaving in mind all the obstacles of the investigated process, and the aim of this PhD study, it was decided not to continue with further investigations.

3.4   Summary

Traditional enzyme assays based on the measurement of "reducing ends" are fast and easy techniques, but are prone to errors due to poor stoichiometric relationship between glucose (commonly used standard) and other cellooligosaccharides. Moreover, they can give us no information on the actual pattern of the hydrolysis. Here, a number of assays for determination of reducing ends were evaluated based on their detection range, and stoichiometric relationship between cellobiose and glucose standard. Nelson-Somogyi assay measured at 750 nm was shown to be the most appropriate, and was used in further experiments.

To be able to identify the hydrolysis pattern, chromatographic technique (HPAEC-PAD) was used. All soluble cellooligosaccharides (DP 1-6) from the hydrolysis broth were measured and quantified.

There is an eminent interest in being able to identify and measure higher, insoluble hydrolysis products. Therefore, attempts were made to optimize PACE method and transfer the experiences form the plant cell wall polysaccharide analysis to enzymatic hydrolysis. The critical step in identification of the unknown hydrolysis products is to be able to compare them with the known standards. As standards are not commercially available efforts were made in producing β-1,4-linked glucose oligomer ladder. The results were not fully satisfactory, but this method may be proven useful if efforts are made to further develop it.

3.5   References

Bach E, Schollmeyer E. 1992. An ultraviolet-spectrophotometric method with 2-cyanoacetamide for the determination of the enzymatic degradation of reducing polysaccharides. Anal. Biochem. 203: 335-339.

Cataldi TRI, Campa C, De Benedetto GE. 2000. Carbohydrate analysis by high-performance anion-exchange chomatography wiht pulsed amperometric detection: the potential is still growing. Fresenius J Anal Chem 368: 739-758.

Chan M, Breuil C, Schwald W, Saddler JN. 1989. Comparison of methods for quantifying the hydrolysis potential of cellulase enzymes. Appl Microbiol Biotech 31: 413-418.

Coward-Kelly G, Aiello-Mazzari C, Kim S, Granda C, Holtzapple M. 2003. Suggested improvements to the standard filter paper assay used to measure cellulase activity. Biotechnol. Bioeng. 82:745-749.

Ghose T. 1987. Measurement of cellulase activity. Pure Appl. Chem. 59:257-268.

Goubet F, Jackon P, Deery MJ, Dupree P. 2002. Polysaccharide analysis using carbohydrate gel electrophoresis: a method to study plant cell wall polysaccharides and polysaccharide hydrolases, Anal. Biochem. 300: 53-68.

Honda S, Nishimura Y, Takahashi M, Chiba H, Kakehi K. 1982. A manual method for the spectrophotometric determination of reducing carbohydrates with 2-cyanoacetamide. Anal. Biochem. 119: 194-199.

Jackson P. 1990. The use of polyacrylamide-gel electrophoresis for the high-resolution separation of reducing saccharide labeled with the fluorophore 8-aminonaphtalene-1,3,6-trisulphonic acid. Biochem. J. 270: 705-713.

Kabel MA, van der Maarel MJEC, Voragen AGJ, Schols HA. 2005. Standard assays do not predict the efficiency of commercial cellulase preparations towards plant material.   Biochem Bioeng 93:56-63.

Krogh KBR, Mørkeberg A, Jørgensen H, Frisvad JH, Olsson L. 2004. Screening genus *Penicillinm* for producers of cellulolytic and xylanolytic enzymes. Appl. Biochem. Biotechnol. 113-116: 389-401.

Lever M. 1972. A new reaction for the colorimetric determination of carbohydrates. Anal. Biochem. 47: 273-279.

Lever M. 1977. Carbohydrate determination with 4-hydroxybenzoic acid hydrazide (PAHBAH): effect of bismuth on the reaction. Anal. Biochem. 81: 21-27.

Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal. Chem. 31: 426-428.

Nelson N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153: 375-380.

Park JT, Johnson MJ. 1949. A submicrodetermination of glucose. J. Biol. Chem. 181: 149-151.

Somogyi M. 1952. Notes on glucose determination. J. Biol. Chem. 195: 19-23.

Starr CM, Masada IR, Hague C, Skop E, Klock JC. 1996. Fluorophore-assisted carbohydrate electrophoresis in the separation, analysis and sequencing of carbohydrates. J. Chrom. 720: 295-321.

Tolan JS, Foody B. 1999. Cellulase from submerged fermentation, Adv. Biochem. Eng. Biotechnol. 65: 41-67.

Wang NS. 2005. Experiment no. 4A, Glucose assay by dinitrosalicylic colorimetric method. Retrieved 14/5-2005 from the University of Maryland, Department of Chemical Engineering Web site: http://www.glue.umd.edu/~nsw/ench485/lab4a/htm.

Wood TM, Bhat KM. 1988. Method for measuring cellulase activities. In: Biomass – A: Cellulose and hemicellulose San Diago: Academic Press. p 87-112.

Zhang Y-H P, Himmel M E, Mielenz J R. 2006. Outlook for celulase improvement: screening and selection strategies. Biotechnol. Adv. 24: 452-481.

Zhang Y-H P, Lynd LR. 2005. Determination of the number-average degree of polymerization of cellodextrins and cellulose with application to enzymatic hydrolysis. Biomacromolecules. 6: 1510-1515.

CHAPTER 4

Cellulolytic enzymes

As noted in Chapter 2, natural cellulosic substrates (primarily plant cell wall polysaccharides) are heterogeneous materials composed of cellulose and hemicellulose embedded in lignin. It is often observed that microorganisms that degrade cellulose usually also degrade hemicellulose. Attempts to purify enzymes involved in degradation of lignocellulosic materials have led to the realization that microorganisms are producing a multiplicity of enzymes, referred to as an *enzymatic system* (Warren, 1996). Cellulases and hemicellulases are considered as components of the enzymatic system for the hydrolysis of plant cell walls (Warren, 1996). The discussion in this chapter focuses primarily on the action of hydrolytic enzyme systems on cellulose (cellulases). It should, however, be realized that enzyme systems active on cellulose also shows activity on hemicellulose, and enzymes active specifically on hemicellulose (hemicellulases) often show activity towards cellulose (Lynd et al., 2002).

Above described enzyme systems are complex on two quite different levels (Warren, 1996). First, the systems are complex as they are comprised of several enzyme classes (cellobiohydrolases, endoglucanases and β-glucosidase), each containing a number of similar enzymes from the same class (see section 4.3.1). Second, many of the individual enzymes are complex as they are modular proteins (see section 4.1). Moreover, even though enzymes are generally specific towards a particular substrate, cellulolytic enzymes often posses ability to react on a variety of similar substrates, thus showing broad substrate specificities.

Many microorganisms in the nature produce a set of enzymes capable of degrading native cellulose, but only a few microorganisms are particularly recognized for their efficiency. Above all *Trichoderma reesei* (also referred as *Hypocrea jecorina*), a soft-root fungi, have been extensively investigated since it was first identified during the Second World War (Reese, 1976). The interest in this fungus relates with its ability to

secret high levels of enzymes. Strains that produce up to 0.14-0.38 g of soluble protein/g carbon source have been developed over the last several decades (Esterbauer et al., 1991).

4.1 Molecular structure of cellulolytic enzymes

Cellulases are modular enzymes that are composed of independently folded, structurally and functionally discrete units, referred to as either domains or modules (Henrissat, et al., 1998). Most commonly, cellulases consist of one catalytic domain (CD) and one carbohydrate binding module (CBM), which is usually joined to the CD by a relatively long (30-44 amino acids), often glycosylated, linker peptide. Additionally, a few microorganisms with multi-catalytic domain enzymes, and, at the same time possessing several binding modules, have been identified (Zverlov et al., 1998; Gibbs et al., 1992).

By definition, a CBM is a contiguous amino acid sequence within a carbohydrate active enzyme with a restrained fold and independent carbohydrate-binding activity (http://www.cazy.org/fam/acc_CBM.html, Marts 2007). It is generally accepted that the primary role of CBM is to accommodate physical contact of the enzyme to the cellulose, increasing at the same time both the effective concentration of the enzyme, but also the time the enzyme will spend in the near proximity of the substrate. CBMs are currently distributed within 49 families, ranging from small (30-40 amino acids), family 1, peptides, to modules consisting of over 200 residues (in families 11 and 17).

All fungal CBMs (relevant for the enzymes used during this PhD study) belong to family 1. Those peptides primarily demonstrate affinity for crystalline cellulose. The cellulose binding surface has been shown to be a planar surface with three aromatic amino acids and few conserved polar residues (Kraulis et al., 1989; Linder et al., 1995; Mattinen et al., 1998; Reinikainen et al., 1992).

The binding sites of family 1 CBMs have been visualized by transmission electron microscopy (Lehtio et al., 2003), providing evidence of their preferred binding on the corner of cellulose microfibril (hydrophobic (110) plane of *Valonia* crystals) as illustrated in Figure 4.1. Such a binding specificity implies that, in perfect cellulose crystals, the surface area of the proposed binding site for the CBMs is very limited.
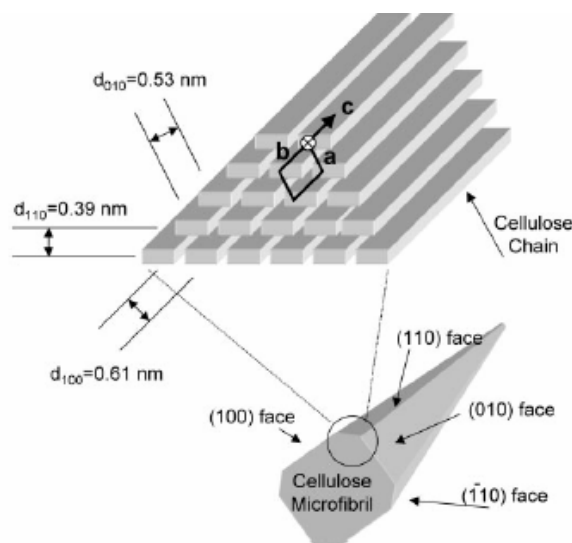
Figure 4.1: Schematic representation of the organization of the cellulose chains in the Iα allomorph of cellulose crystals (present in *Valonia* crystals) and the shape of the complete crystal formed. The d-spacing characteristic to the different crystalline planes are indicated. The corner (circled), which exposes the (110) face, is the proposed binding site for the CBMs. Picture from Lehtio et al., 2003.

Additionally, Lehtio et al. (2003) observed fully reversible binding of family 1 CBM to crystalline cellulose at 4 °C. Reversibility of CBM binding to the cellulose is an important issue as it will promote the hydrolysis reaction to proceed from another point on the crystal, i.e. enzyme loss due to unproductive binding is minimized. Nevertheless, the biological significance of experiments performed at this temperature (4 °C) should be further investigated as organisms producing cellulases, in particular *T. reesei*, naturally operate at tropical temperatures.

Enzymes lacking CBM, i.e. only having one module (catalytic domain), have been shown to still have the ability to absorb to cellulose, but often with lower affinity compared to the full length enzyme (Schulein, 1997; Karlsson et al., 2002). Sculein (1997) measured kinetic properties of ten purified cellulases produced by *H. insolens*, of which three enzymes were available in two forms; the full length enzyme (i.e. possessing both CBM and CD) and the "core" enzyme (lacking CBM). He observed increase of apparent K_M, but also increase in apparent k_{cat} when the enzyme possessed a CBM (Table 4.1). The later has been suggested to be related to the reduced mobility of the full length enzyme.

Table 4.1: Apparent kinetic constants for several purified *Humicola* cellulases on phosphoric acid swollen cellulose (PASC)

Enzyme	kcat (app.) [1/s]	Km (app.) [g/l]
Cel6A (CBH II)	6	0,9
Cel6A core (CBH II core)	22	3,3
Cel45A (EG V)	58	0,5
Cel45A (EG V core)	82	3,6
Cel6B (EG VI)	54	0,7
Cel6B (EG VI core)	56	3,3
Cel7B (EG I)	16	2,5
Cel5A (EG II)	22	1,1
Cel12A (EG III)	1	6,0

The results were obtained after incubation for 20 minutes at 40 °C and pH 8.5. After determination of the reducing sugars, the apparent kinetic constants were calculated using the Grafit program. Standard errors within 10 %. Data taken from Schulein, 1997.

CD and CBM are connected by linker peptide. The linker sequences from different enzymes rarely share any apparent sequence homology, but their amino acid composition is typically rich in proline and hydroxyl amino acids (Gilkes et al., 1991). It has been suggested that linkers represent extended, flexible hinges between the two domains facilitating their independent function (Burton et al., 1989; Bushuev et al., 1989). The role of the linker peptide of *T. reesei* CBH I on interaction and consequent hydrolysis of crystalline cellulose was studied by Srisodsuk et al. (1993). They introduced two internal deletions to the linker, and concluded that applying a longer deletion, i.e. removing practically all of the linker, dramatically reduced the rate of crystalline cellulose degradation (even though the enzyme still binds to the substrate). Thus, the sufficient spatial separation of the two domains (CD and CBM) is required for efficient function of the enzyme.

4.2   Mechanisms of cellulase activity

Cellulolytic enzymes, as glycosyl hydrolases, hydrolyze glycosidic bonds via the mechanism of general acid catalysis, which requires two critical residues: a proton donor (HA) and a nucleophile/base (B⁻). The catalytic activity is provided by two aspartic- or glutamic acid residues (Mosier et al., 1999).

Hydrolysis, as the name describes, involves breaking of bonds by adding water. Two different mechanisms can be distinguished: inverting and retaining mechanism, Figure 4.2. Inverting mechanism (Figure 4.2.A) is a single nuchleophile displacement mechanism in which the charged environment of the catalytic site is used to "activate" a water molecule to act as a nucleophile, while an acidic amino acid residue donates the required proton. The name, i.e. inverting mechanism, is related to the fact that the C_1 carbon of the sugar is inverted in the linkage from β to α configuration upon the hydrolysis.

The retaining mechanism (Figure 4.2.B) proceeds in two steps. First, a covalently bound intermediate is formed through nucleophilic attack of the charged amino acid on the glycosyl bond. In the second step, water molecule frees the hydrolysis product from the enzyme and recharges the proton donor (Mosier et al., 1999). Recently, a completely unrelated mechanism has been demonstrated for a family of glycosidases utilizing NAD+ as a cofactor (Rajan et al., 2004).

A: **B:**
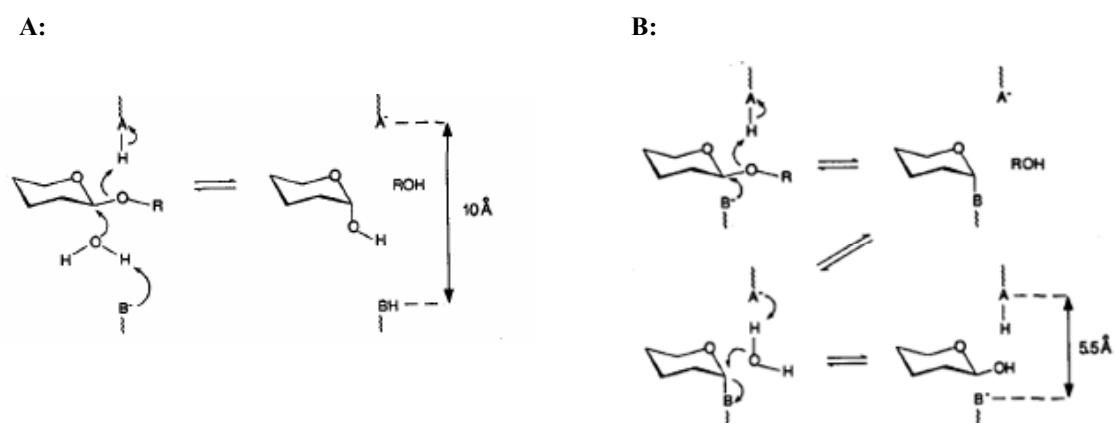
Figure 4.2: Schematic representation of the **(A)** inverting, and **(B)** retaining mechanism of enzymatic glycosidic bond hydrolysis. Figure from Davies and Henrissat (1995).

Enzymes that utilize the retaining mechanism are usually able to carry out transglycosylation, while inverting enzymes can not (Wilson and Irwin, 1999). Another major difference between inverting and retaining glucosidases is the distance between the two acidic residues. For the inverting enzymes the distance is ~ 10 Å, while the average difference between the catalytic residues for the retaining glucosidases is ~ 5.5 Å (McCarter and Withers, 1994; Mosier et al., 1999).

4.3   Classification of cellulases

Cellulases can be classified by different means, but the two modes of classification predominantly used are those based on the substrate specificities, and those based on the structural similarities of enzymes. In relation to the later, classification according to the reaction mechanism can also be made.

In general, all cellulolytic enzymes belong to the *O*-Glycosyl hydrolases (EC 3.2.1.-), a widespread group of enzymes which hydrolyze the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate. Traditionally, cellulases have been classified into two distinct classes: cellobiohydrolases (CBH) also called *exo*-1,4-β-D-glucanases (EC 3.2.1.91), and *endo*-1,4-β-D-glucanases (EG) (EC 3.2.1.4), based on their substrate specificities, i.e. affinity towards the chain ends or the interior of the glucose chain, respectively. The third class of enzymes working together and in synergy with CBH and EG, are β-glucosidases (BG). They are often not referred to as the "real" cellulases as they primarily hydrolyze glycosidic bonds of soluble cellooligosaccharides and cellobiose, thus not acting on cellulose itself.

This type of classification according to the substrate specificities is difficult, as the enzymes often have overlapping specificities. Therefore, in the early 1990s Henrissat and coworkers proposed a new classification of glycosyl hydrolases into families based on amino acid sequence similarities of the catalytic domain. The new designation for an enzyme determines its family and, because all members of a family have the amino acid

sequence in common, its three-dimensional fold and stereo-specificity of hydrolysis (retaining or inverting) can thus be predicted (Henrissat et al.,1998). Consequently, the one property that is completely conserved in all members of a family is stereochemistry of cleavage; e.g. all members of family 5 are retaining, and all members of family 6 inverting enzymes (Henrissat et al., 1998; http://www.cazy.org/fam/acc_fam.html, Marts 2007). The continuously updated information on the families of structurally-related catalytic and carbohydrate-binding modules of enzymes that degrade, modify, or create glycosidic bonds is available on Carbohydrate Active Enzyme Database server (http://www.cazy.org/).

4.3.1 The complete cellulolytic system (multiple cellulases)

Successful degradation of native cellulose requires cooperative action of multi-component enzyme system, such as those from genus *Trichoderma*, consisting of several *endo*- and *exo*-acting enzymes (Henrissat et al., 1998), and two β-glucosidases (Bhikhabhai et al., 1984; Chen et al., 1992). The enzyme combination produced varies between the species and depends on the microorganism that has produced them. The distribution of individual cellulose degrading enzymes in three crude extracts, those produced by *Trichoderma longibrachiatum* Rut 30, *Humicola insolens* DSM 1800, and *Penicillium brasilianum* IBT 20888 is summarized in Table 4.2.

Table 4.2: Distribution of cellulase components from representative strains of *Trichoderma*, *Humicola* and *Penicillium*

Component	*Trichoderma longibrachiatum* Rut C30 [%] [a]	*Humicola insolens* DSM 1800 [%] [a]	*Penicillium brasilianum* IBT 20888 [%] [b]
CBH I (Cel7A)	50	20	18
CBH II (Cel6A)	20	< 5	25
EG I (Cel7B)	10	50	4
EG II (Cel5A)	5	5	10
EG III (Cel12A)	< 5	5	21
EG V (Cel45A)	< 5	10	n.d.
BG (Cel3A)	~ 1-2	~ 1-2	n.d.
Other (non-cellulase)	10	10	n.d.

CBH: Cellobiohydrolase; EG: Endoglucanase; BG: β-glucosidase; n.d.: not determined; [a]: from Tolan and Foody, 1999; [b]: from Jørgensen et al., 2003.

Two major questions in cellulose research are, why multiple cellulases are produced when there is only one type of bond (β1-4 linkage) present in cellulose, and, why is there such a predominance of CBH, particularly in *Trichoderma* strain (Table 4.1). The presence of several EG may be due to the fact that different enzymes exhibit optimal performance under different environmental conditions (pH and temperature), or have different regulatory properties and kinetic parameters, and thus, the fungus has evolutionary developed these to ensure its survival. Additionally, several EG consisting only of CD have been isolated. Thus, it has also been speculated that enzymes lacking CBM, and consequently being smaller in size, would be able to penetrate more easily into fibrous material, compared to the other EGs, and thereby help in opening up the structure of the material (Soloheimo et al., 1994).

Two *exo*-cellulases, CBH I and CBH II, are performing hydrolysis reaction from reducing and non-reducing end of cellulose chain in a processive manner, respectively (Nidetsky et al., 1994; Harjunpaa et al., 1996; Boisset et al., 2000). This processive action, which includes "pulling" cellulose chain away from its neighboring chains, and, in addition, simultaneous multiple hydrolysis reaction without dissociating from the substrate, is considered to be more difficult task than a "simple" bond hydrolysis of *endo*-glucanases, and thus CBH enzymes dominate (Wilson and Irwin, 1999).

4.3.2 Endoglucanases

Endo-glucanases perform random cuts at internal amorphous sites of the cellulose chain, e.g. areas containing bent, flexible and hydrated disordered regions, generating oligosaccharides of various lengths (Boisset et al., 2000). They are, thus, primarily responsible for decreasing degree of polymerization of cellulosic substrates. Consequently, new cellulose chain ends susceptible to the cellobiohydrolase action are being generated (Lynd et al., 2002).

Both EGs and CBHs are subject to product (cellobiose and glucose) inhibition. Cellobiose has been shown to have stronger inhibitory effect on the enzymes than glucose (Holtzapple et al., 1990). Regardless of the number of studies done, the type of

inhibition exhibited by cellulases is still poorly characterized. Nonetheless, inhibition patterns (competitive, non-competitive or mixed type) has been shown to depend on the cellulase binding constant, enzyme concentration, cellulose surface area accessible to the enzymes, e.g. enzyme/substrate ratio, nature of the substrate, the range in which substrate concentration is varied and presence of BG activity (Gusakov and Sinitsyn, 1992; Gruno et al., 2004). Literature summary of cellulase inhibition can be found in Holtzapple et al. (1990).

The structure of several EGs has been resolved (Davies et al., 1993; Davies et al., 1996; Kleywegt et al., 1997; Sandgren et al., 2000) revealing the presence of short loops that create a groove. The groove presumable allows entry of the cellulose chain for subsequent cleavage. The structure of *H. insolens* Cel45A catalytic core have been determined by X-ray analysis indicating that enzyme has a flattened spherical shape with rough dimensions of 42 x 42 x 22 Å (Davies, et al., 1993; Davies et al., 1995; Davies et al., 1996), Figure 4.3.

A: **B:**
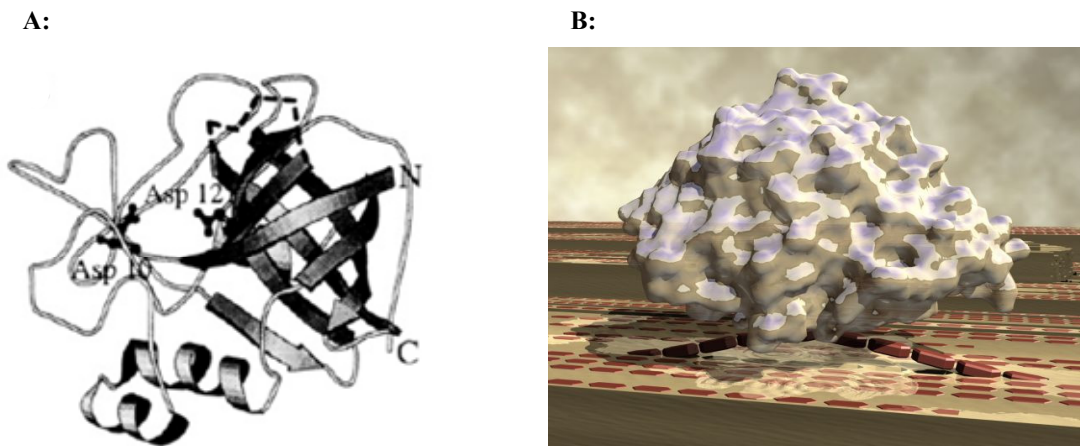
Figure 4.3: Structure of endoglucanases. **A:** Structure of endoglucanase V (Cel45A) from *H. insolens* (Kraulis, 1991). The catalytic core consists of 210 amino acids distributed to six-stranded β-barrel domain with long interconnecting loops. A 40 Å groove exists along the surface of the enzyme, and this contains the catalytic residues Asp 10 and Asp 121. The two catalytic aspartates sit to either side of the substrate binding groove in an ideal conformation for facilitating cleavage by inversion. Figure from Davies et al., 1995. **B:** Computer animation of endoglucanase on the surface of the cellulose chain. Picture from National Renewable Energy Laboratories (NREL) and Pixel Kitchen.

The hydrolysis patterns of several EGs have been studied on a wide range of the substrates (Karlsson et al., 2002), including microcrystalline (Avicel) and amorphous cellulose (Phosphoric Acid Swollen Cellulose - PASC) and soluble carbohydrate polymers (Carboxymethyl cellulose - CMC). In general, the results showed production of, primarily, cellobiose during hydrolysis with Cel7B (EG I), Cel5A (EG II) and Cel12A (EG III), and cellotetraose during hydrolysis with Cel45A (EG V) and the core enzyme, missing CBM, Cel45A core, Table 4.3 (Karlsson et al., 2002). Thus, a notable difference in product formation pattern relative to the other EGs has been observed for Cel45A. Moreover, Karlsson et al. (2002) observed no activity of *T. reesei* Cel45A core on cellooligosaccharides C3, C4 and C5, while, on the contrary, Schou et al. (1993) reported degradation of C4 and longer cellooligosaccharides by *H. insolens* Cel45A. Apparently, the Cel45A endoglucanases of *T. reesei* and *H. insolens* are significantly different in their hydrolysis pattern regardless of the fact that both belong to the same glycoside hydrolases family.

Table 4.3: Qualitative distribution of products formed after hydrolysis of Avicel, Phosphoric acid swollen cellulose (PASC) and Carboxymethyl cellulose (CMC) by five endoglucanases from *Trichoderma reesei.*

Enzyme	Avicel		PASC		CMC	
	Main product(s)	Secondary product(s)	Main product(s)	Secondary product(s)	Main product(s)	Secondary product(s)
Cel7B (EG I)	C2 [~200 µM]	C1	C2, C1 [~800, 650 µM]	C3	C1, C2 [~300 µM each]	-
Cel5A (EG II)	C2 [~110 µM]	C1, C3	C2, C1 [~500, 420 µM]	C3	C1, C2 [~300 µM each]	C3
Cel12A (EG III)	C2, C3, C1 [~40 µM each]	-	C2 [~350 µM]	C3, C1, C4	C1, C2 [~130 µM each]	C3, C4
Cel45A (EG V)	C4 [~14 µM]	C3, C5, C2	C4 [~32 µM]	C5, C3, C2	C4 [~40 µM]	C5, C3, C2
Cel45A core (EG V core)	C4 [~14 µM]	C5, C3	C4 [~42 µM]	C5, C3	C4 [~40 µM]	C5, C3

C_1: Glucose; C_2: Cellobiose; C_3: Cellotriose; C_4: Cellotetraose; C_5: Cellopentaose. The amount of main product(s) [µM of saccharide] is stated. Secondary product(s) are reported in the order of the descending saccharide concentration. Data taken from Karlsson et al., 2002.

4.3.3 Cellobiohydrolases

Exo-glucanases (or CBHs) act in a processive manner (Davies and Henrissat, 1995; Henrissat 1998; Rouvinen et al., 1990; and others) from the ends of cellulose polysaccharide chains, processing along the polymer chain while liberating cellobiose as major product. Occasionally, glucose and cellotriose are also produced in small quantities during initial stages of hydrolysis (Divne et al., 1994). One of the important features of all cellobiohydrolases is that they can act on microcrystalline cellulose (Terri, 1997).

The processivity of these enzymes appears to be related to the fine details of their three dimensional structure (Figure 4.4). Opposed to the open groove present in EGs, the active site of CBHs is located inside the tunnel. In CBH I (Cel7A) from *T. reesei* this tunnel is 50 Å long (Divne et al., 1994), while in CBH II (Cel6A) the tunnel is much shorter, only 20 Å long (Rouvinen et al., 1990). Cellobiohydrolases similar to the CBH II, which have shorter active site tunnels, may exhibit increasing degrees of *endo*-glucanase activity. Namely, the structure of *T. reesei* Cel6A (CBH II) in complex with oligosaccharides have shown that one of the loops have substantial mobility and that the resulting tunnel could be either more tightly closed or almost fully open, resembling, thus, the active site of EGs (Zou et al., 1999).

A: **B:**
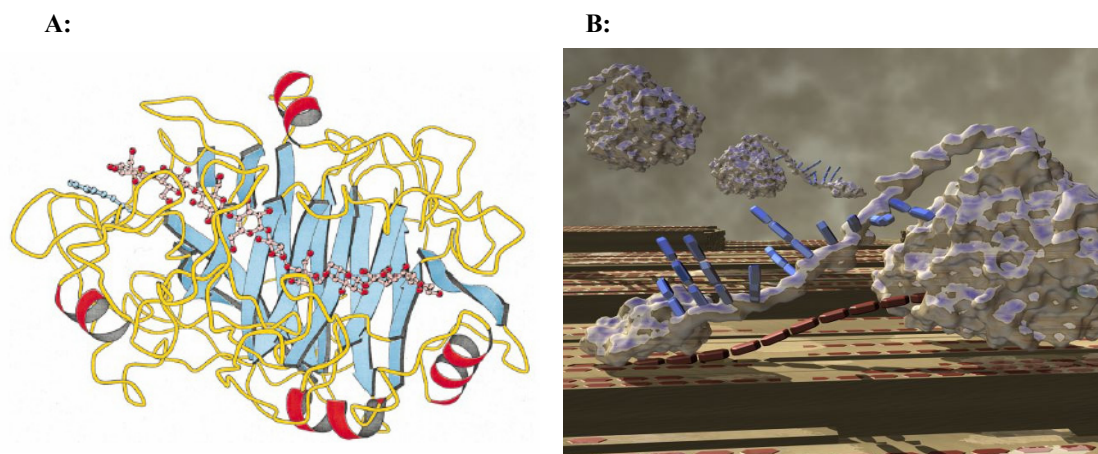
Figure 4.4: Structure of cellobiohydrolase. **A:** Schematic representation of the CBH I (Cel7A) catalytic domain from *T. reesei*. The catalytic domain consists of approximately 50 Å long cellulose binding tunnel (β-strands). Additionally, α helices and long loop regions coexist. The cellooligomer bound inside the tunnel is shown as a ball-and-stick object. From Divne et al.,1998. **B:** Computer animation of cellobiohydrolase on the cellulose surface. Enzyme consists of carbohydrate binding module (CBM) connected to the catalytic domain (CD) with the flexible linker. One cellulose chain is shown to enter the tunnel shaped active site of the enzyme. Picture from National Renewable Energy Laboratories (NREL) and Pixel Kitchen.

From the biochemical studies, it has been suggested that CBH I hydrolyze cellulose chain from the reducing towards the non-reducing end of the chain (Barr et al., 1996; Boisset et al., 2000), while hydrolysis proceeds from the non-reducing towards the reducing end during enzymatic degradation by CBH II (Moiser et al., 1999).

4.3.4 β-glucosidases

β-glucosidases hydrolyze cellobiose and short (soluble) cellooligosaccharides to glucose; e.g., the hydrolysis reaction is performed in the liquid phase, rather then on the surface of the insoluble cellulose particles, which is the case for the "real" cellulases, EGs and CBHs. The removal of cellobiose is an important step of the enzymatic hydrolysis process, as it assists in reduction of the inhibitory effect of cellobiose on EG and CBH. BG activity has often been found to be rate-limiting during enzymatic hydrolysis of cellulose (Duff and Murray, 1996; Tolan and Foody, 1999), and due to that the commercial cellulase enzyme preparations are often supplemented with BG activity.

Two BGs have been isolated from culture supernatants of *T. reesei* (Chen et al., 1992), however, studies on several *Trichoderma* species have shown that a large fraction of these enzymes remain cell wall bound (Messner et al., 1990; Usami et al., 1990). The evolutionary advantage of securing high glucose concentrations in the near proximity of fungal cell wall is obvious. BGs of *T. reesei* are subject to product (glucose) inhibition (Chen et al., 1992), whereas, those of *Aspergillus* are more glucose tolerant (Watanabe 1992; Gunata and Vallier, 1999; Decker et al., 2000). For example, BG I from *T. reesei* have a K_m=182 µM on *p*-nitrophenyl-β-glucoside as substrate, while the inhibition constant, K_I, increases to 624 µM when 5 mM of glucose is present in the hydrolysis broth (Chen et al., 1992).

4.3.5 Summary of the enzymes used in this study

One of the aims of this study was to identify and understand the most crucial aspects of the hydrolysis process, and consequently distinguish bottlenecks of the process by using a experimental studies (presented in Chapter 5 and Chapter 6) and mathematical modeling (Chapter 7). To be able to accurately describe the complex enrollment of all cellulolytic

enzymes, and cooperative action between them during the hydrolysis, a *mechanistic* mathematical model was desired. This type of model is particularly well designed for describing the mechanism of the process, as it incorporates the specific characteristics of all three enzyme classes needed for efficient hydrolysis. Accordingly, the experiments also needed to be performed with purified, mono-component, enzymes, so that those results could be appropriately evaluate against the mechanistic mathematical model. Therefore, during this PhD study a number of mono-component, highly purified enzymes isolated from either *Humicola insolens*, *Penicillium brasilianum* or *Aspergillus niger* were used. In some cases, the performance of the mixture consisting of three mono-component enzymes (including the representative from all of the three enzyme classes) was compared to the performance of the commercial enzyme mixture Celluclast 1.5 L and Novozym 188 (3:1, vol:vol). Table 4.4 summarizes some of the main enzyme characteristics.

Table 4.4: Summary of the enzymes used during this PhD study.

Mono-component enzymes							
	Class	Organism	MW [kDa]	CBM	Mechanism	pH optimum	Comments / Additional activity
Cel7B	EG I	*H. insolens*	50	No	Retaining	7,5	*Exo*-activity identified
Cel45A	EG V	*H. insolens*	43	Yes	Inverting	7,5	No *exo* activity
Cel5C	EG	*P. brasilianum*	65	Yes	Retaining	4,0	-
Cel7A	CBH I	*H. insolens*	70	Yes	Retaining	5.5 - 6.0	Processive, from reducing end
Cel6A	CBH II	*H. insolens*	51	Yes	Inverting	9,0	*Endo*-processive, from non-reducing end
Cel7B	CBH	*P. brasilianum*	60	No	Retaining	n.a.	-
Cel3A	BG	*P. brasilianum*	110	No	Retaining	4,8	-
MCN 188	BG	*A. niger*	91	No	Retaining	4,0	-

Commercial enzymes		
	Class	Organism
Celluclast 1.5 L	EG/CBH	*T. reesei*
Novozym 188	BG	*A. niger*

BG: β-glucosidase; EG: Endoglucanase: CBH: Cellobiohydrolase; CBM: Carbohydrate Binding Module; MCN 188: Mono-component Novozym 188 (purified β-glucosidase); n.a.: not available.

Additionally, some of the enzymes used in this study (e.g. Cel45A, see also Table 4.1) are available in two variants; with, and without carbohydrate binding module, which further opens a possibility of investigating the significance of enzyme adsorption.

4.4 **References**

Barr BK, Hsieh Y-L, Ganem B, Wilson DB. 1996. Identification of two functionally distinct classes of exocellulases. Biochem. 35: 586-592.

Bhikhabhai R, Johansson G, Pettersson G. 1984. Isolationof cellulolytic enzymes from *Trichoderma reesei* QM 9414. J. Appl. Biochem. 6:336-345.

Boisset C, Fraschini C, Schulein M, Henrissat B, Chanzy H. 2000. Imaging the enzymatic digestion of bacterial cellulose ribbons reveals endo character of the cellobiohydrolase Cel6A from *Humicola insolens* and its mode of synergy with cellobiohydrolase Cel7A. Appl. Environ. Microbio. 66: 1444-1452.

Burton J, Wood SG, Pedyczak A, Siemieon IZ. 1989. Conformational preferences of sequential fragments of the hinge region of human IgA1 immunoglobulin molecule: II. Biophys. Chem. 33: 39-45.

Bushuev VN, Gudkov AT, Liljas A, Sepetov NF. 1989. The flexible region of protein L12 from bacterial ribosomes studied by proton nuclear magnetic resonance. J. Biol. Chem. 264: 4498-4505.

Chen H, Hayn M, Esterbauer H. 1992. Purification and characterization of two extracellular beta-glucosidases from *Trichoderma reesei*. Biochem. Biophys. Acta 1121: 54-60.

Davies G, Henrissat B. 1995. Structures and mechanisms of glycosyl hydrolases. Structure. 3: 853-859.

Davies GJ, Dadson GG, Hubbart RE, Tolley SP, Dauter Z, Wilson KS, Hjort C, Mikkelsen JM, Rasmussen G, Schulein M. 1996. Structure and function of endoglucanase V. Nature. 365: 362-364.

Davies GJ, Dodson G, Moore MH, Tolley SP, Dauter Z, Wilson KS, Rasmussen G, Schulein M. 1993. Structure determination and refinement of the *Humicola insolens* endoglucanase V at 1.5 Å resolution. Acta. Cryst. D52: 7-17.

Davies GJ, Tollay SP, Henrissat B, Hjort C, Schulein M. 1995. stroctures of oligosaccharide-bound forms of the endoglucanase V from *Humicola insolens* at 1.9 Å resolution. Biochem. 34: 16210-16220.

Decker CH, Visser J, Schreier P. 2000. β-glucosidases from five black *Aspergillus* species: study of their physico-chemical and biocatalytic properties. J. Agric. Food Chem. 48: 4929-4936.

Divne C, Ståhlberg J, Reinikainen T, Ruohonen L, Pettersson G, Knowles JKC, Teeri TT, Jones TA. 1994. The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from *Trichoderma reesei*. Science 265: 524-528.

Divne C, Ståhlberg J, Teeri TT, Jones TA. 1998. High-resolution crystal structures reveal how a cellulose chain is bound in the 50 Å long tunnel of cellobiohydrolase I from *Trichoderma reesei*. J. Mol. Biol. 275: 309-325.

Duff SJB, Marray WD. 1996. Bioconversion of forest products industry waste cellulosics to fuel ethanol: a review. Bioresour. Technol. 55: 1-33.

Esterbauer H, Steiner W, Labudova I, Hermann A, Hayn M. 1991. Production of *Trichoderma* cellulase in laboratory and pilot scale. Biores. Technol. 36: 51-65.

Gibbs MD, Saul DJ, Luthi E, Bergquist PL. 1992. The β-mannanase from "*Caldocellum saccharolyticum*" is part of a multidomain enzyme. App. Environ. Microbiol. 58: 3864-3867.

Gilkes NR, Henrissat B, Kilburn DG, Miller RC, Warren RAJ. 1991. Domains in microbial beta-1, 4-glycanases: sequence conservation, function, and enzyme families. Microbiol. Rev. 55: 303-315.

Gruno M, Valjamae P, Pettersen G. Johansson G. 2004. Inhibition of the *Trichoderma reesei* cellulases by cellobiose is strongly dependent on the nature of the substrate. Biotechnol. Bioeng. 86: 503-511.

Gunata Z, Vallier MJ. 1999. Production of a highly glucose-tolerant extracellular β-glucosidase from three *Aspergillus* strains. Biotechnol. Lett. 21: 219-223.

Gusakov AV, Sinitsyn AP. 1992. A theoretical analysis of cellulase product inhibition: effect of cellulase binding constant, enzyme/substrate ratio, and β-glucosidase activity on the inhibition pattern. Biotechnol. Bioeng. 40: 663-671.

Harjunpaa V, Teleman A, Kiovula A, Ruohonen L, Teeri TT, Teleman O, Drakenberg T. 1996. Cello-oligosaccharide hydrolysis by cellobiohydrolase II from *Trichoderma reesei*. Association and rate constant derived from analysis of progress curves. Eur. J. Biochem. 240: 584-591.

Henrissat B, Teeri TT, Warren RAJ. 1998. A scheme for designating enzymes that hydrolyze the polysaccharides in the cell walls of plants. FEBS Lett. 425: 352-354.

Henrissat B. 1998. Enzymatic cellulose degradation. Cellulose Commun. 5: 84-90.

Holtzapple M, Cognata M, Shu Y, Hendrickson C. 1990. Inhibition of *Trichoderma reesei* cellulase by sugars and solvents. Biotechnol. Bioeng. 36: 275-287.

Jørgensen H, Eriksson T, Borjesson J, Tjerneld F, Olsson L. 2003. Purification and characterization of five cellulases and one xylanase from *Penicillium brasilianum* IBT 20888. Enz. Microb. Technol. 32: 851-861.

Karlsson J, Siika-aho M, Tenkanen M, Tjerneld F. 2002. Enzymatic properties of the low molecular mass endoglucanases Cel12A (EG III) and Cel45A (EG V) of *Trichoderma reesei*. J. Biotechnol. 99: 63-78.

Kleywegt GJ, Zou JY, Divne C, Davies GJ, Sinning I, Stahlberg J, Reinikainen T, Srisodsuk M. Teeri TT, Jones TA. 1997. The crystal structure of the catalytic core domain of endoglucanase I from *Trichoderma reesei* at 3.6 Å resolution, and a comparison with related enzymes. J. Mol. Biol. 272: 383-397.

Kraulis PJ. 1991. MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. J. Appl. Cryst. 24: 946-950.

KraulisJ, Clore GM, Nilges M, Jones TA, Pettersson G, Knowles J, Gronenborn AM. 1989. Determination of the three-dimensional solution structure of the C-terminal domain of cellobiohydrolase I from *Trichoderma reesei*. A study using nuclear magnetic resonance and hybrid distance deometry-dynamical simulated annealing. Biochemistry. 28: 7241-7257.

Lehtio J, Sugiyama J, Gustavsson M, Fransson L, Linder M, Teeri TT. 2003. The binding specificity and affinity determinants of family 1 and family 3 cellulose binding modules. PNAS. 100: 484-489.

Linder M, Lindeberg G, Reinikainen T, Teeri TT,Pettersson G. 1995. The difference in affinity between two fungal cellulose-binding domains is dominated by a single amino acid substitution. FEBS Lett. 372: 96-98.

Lynd LR, Weimer PJ, van ZylWH, Pretorius IS. 2002. Microbal cellulose utilization: fundamentals and biotechnology. Microbiol. Molec. Biol. Rev. 66: 506-577.

Mattinen ML, Linder M, Drakenberg T, Annila A. 1998. Solution structure of the cellulose-binding domain of endoglucanase I from *Trichoderma reesei* and its interaction with cello-oligosaccharides. Eur. J. Biochem. 256: 279-286.

McCarter JD, Withers SG. 1994. Mechanisms of enzymatic glycoside hydrolysis. Curr. Opin. Struct. Biol. 4: 885-892.

Messner R, Hagspiel K, Kubicek CP. 1990. Isolation of the β-glucosidase-binding and activating polysaccharide from cell walls of *Trichoderma reesei*. Arch. Microbiol. 154: 150-155.

Mosier NS, Hall P, Ladisch CM, Ladisch MR. 1999. Reaction kinetics, molecular action and mechanisms of cellulolytic proteins. Adv. Biochem. Eng. Biotechnol. 65: 23-40.

Rajan SS, Yang X, Collart F, Yip VL, Withers SG, Varrot A, Thompson J, Davies GJ, Anderson WF. 2004. Novel catalytic mechanism of glycoside hydrolysis based on the structure of an NAD+/Mn2+ -dependent phospho-alpha-glucosidase from Bacillus subtilis. Structure. 12:1619-1629.

Reese ET. 1976. History of the cellulase program at the US army Natick development center. Biotechnol. Bioeng. Symp. No. 6: 9-20.

Reinikainen T, Ruohonen L, Nevanen T, Laakosonen L, Kraulis P, Jones TA, Knowles JK, Teeri TT. 1992. Investigation of the function of mutated cellulose-binding domain of *Trichoderma reesei* cellobiohydrolase I. Proteins. 14: 475-482.

Rouvinen J, Bergfors T, Teeri T, Knowles JKC, Jones TA. 1990. Three-dimensional structure of cellobiohydrolase II from *Trichoderma reesei*. Science 249: 380-386.

Saloheimo A, Henrissat B, Hoffren AM, Toleman O, Penttila M. 1994. A novel, small endoglucanase gene, *egl5*, from *Trichoderma reesei* isolated by expression in yeast. Mol. Microbiol. 13: 219-228.

Sandgren M, Shaw A, Ropp TH, Wu S, Bott R, Cameron AD, Stålberg J, Mitchinson C, Jones TA. 2000. The X-ray crystal structure of the *Trichoderma reesei* family 12 endoglucanase 3, Cel12A, at 1.9 Å resolution. J. Mol. Biol. 308: 295-310.

Srisodsuk M, Rainikainen T, Panttila M, Teeri TT. 1993. Role of the interdomain linker peptide of *Trichoderma reesei* cellobiohydrolase I in its interaction with crystalline cellulose. J. Biol. Chem. 268: 20756-20761.

Terri TT. 1997. Crystalline cellulose degradation: a new insight into the function of cellobiohydrolases. Tibtech. 15: 160-167.

Tolan JS, Foody B. 1999. Cellulases from submerged fermentation. Adv. Biochem. Eng./Biotechnol. 65: 41-67.

Usami S, Kirimura K, Imura M, Morikawa S. 1990. Cellular localization of the constitutive β-glucosidase in *Trichoderma viride*. J. Ferment. Bioeng. 70: 185-187.

Warren RAJ. 1996. Microbial hydrolysis of polysaccharides. Annu. Rev. Microbial. 50: 183-212.

Watanabe T, Sata T, Yoshioka S, Koshijima T, Kuwahara M. 1992. Purification and properties of *Aspergillus niger* β-glucosidase. Eur. J. Biochem. 209: 651-659.

Wilson DB, Irwin DC. 1999. Genetics and properties of cellulases. Adv. Biochem. Eng. Biotechnol. 65:1-21.

Zou JY, Kleywegt GJ, Ståhlberg J, Driguez H, Nerinckx W, Claeysens M, Koivula A, Terri TT, Jones AT. 1999. Crystalographic evidence for substrate ring distortion and protein conformational changes during catalysis in cellobiohydrolase Cel6A from *Trichoderma reesei*. Structure. 7: 1035-1045.

Zverlov V, Mahr S, Riedel K. 1998. Properties and gene structure of a bifunctional cellulolytic enzyme (CelA) from extreme thermophile '*Anaerocellum thermophilum*' with separate glucosyl hydrolases family 9 and 48 catalytic domains. Microbiology 144: 457-465.

CHAPTER 5

Synergism between the cellulolytic enzymes

Cooperative action, often designated *synergy*, of the different enzyme classes involved in degradation of cellulose is essential for efficient enzymatic hydrolysis process. Synergy between cellulolytic enzymes occurs when the combined action of two or more enzymes leads to a higher rate of action than the sum of their individual actions (Mansfield et al., 1999). The two most often reported synergy types involve cooperative action of either *endo*-glucanses (EG) and *exo*-glucanses (CBH), in so called *endo-exo synergy* (Beldman et al., 1988; Nidetzky et al., 1993; Nidetzky et al., 1994; and many others), or the two complementary CBHs, i.e. acting from the reducing and the non-reducing end of the cellulose chain, in *exo-exo synergy* (Henrissat et al, 1985; Medve et al., 1994). *Cross synergism*, referring to the synergy observed between the endo- and exo-acting enzymes from different microbial origins, has also been report (Wood et al., 1980).

Numerous studies done on synergy between the purified cellulolytic enzymes confirm that the synergy is dependent on the ratio of the individual enzymes (Henrissat et al., 1985), the substrate saturation, i.e. enzyme concentration (Woodward et al., 1988; Nidetzky et al., 1993), and the physico-chemical properties of the substrate (Nidetzky et al., 1993; Henrissat et al., 1985). For example, Woodward et al. (1988) have investigated the role of cellulase concentrations in determining the degree of synergism in the hydrolysis of microcrystalline cellulose. The optimum DS (2.03) has been reached when 20 µg/ml of Cel5A (EG II), Cel7A (CBH I) and Cel6A (CBH II) from *T. reesei* was used. At 10 µg/ml of enzyme Woodward et al. (1988) reported decrease of DS to 1.49. Using higher enzyme loadings, a further decline in degree of synergy has been observed and it has been suggested that it is due to the competitive adsorption, where CBH was the more preferably adsorbed enzyme then EG.

As a general note, the higher cooperative effect between the different enzymes has been found during degradation of more crystalline substrates like bacterial microcrystalline cellulose (BMCC), whereas weaker or no synergism has been recorded

on cellulose microcrystals (Avicel) or the soluble carboxymethyl cellulose (Henrissat et al., 1985). Nevertheless, the cooperativity of enzymes depends, as above mentioned, on variety of factors and thus this general rule is not always valid.

In this chapter the manuscript entitled "Enzymatic hydrolysis of cellulose using mono-component enzymes show synergy during hydrolysis of Phosphoric Acid Swollen Cellulose (PASC), but competition on Avicel" is presented. This is a study of the synergy between the three classes of cellulolytic enzymes, i.e. EG, CBH and β-glucosidase (BG). In contrast to previous studies where BG was either not added or added in excess, we here focus on engineering binary, as well as, ternary cellulase mixtures for maximal total sugar production on two commonly used cellulose model substrates, Avicel and PASC. Better understanding of the mechanisms underlying synergism could facilitate the development of designer cellulases for more efficient hydrolysis process. Additionally, the data obtained by the experiments were later used in the modeling part of this study.

5.1 References

Beldman G, Voragen AGJ, Rombouts FM, Pilnik W. 1988. Synergism in cellulose hydrolysis by endoglucanases and exoglucanases purified from *Trichoderma viride*. Biotechnol. Bioeng. 31: 173-178.

Henrissat B, Drigues H, Viet C, Schulein M. 1985. Synergism of cellulases from *Trichoderma reesei* in the degradation of cellulose. Bio/Technology 3: 722-726.

Mansfielsd S, Mooney C, Saddler JN. 1999. Substrate and enzyme characteristics that limit cellulose hydrolysis, Biotechnol. Prog. 15: 804-816.

Medve J, Ståhlberg J, Tjerneld F. 1994. Adsorption and synergism of cellobiohydrolase I and II from *Trichoderma reesei* during hydrolysis of microcrystalline cellulose. Biotechnol. Bioeng. 44: 1064-1073.

Nydetzky B, Hayn M, Maccaron R, Steiner W. 1993. Synergism of *Trichoderma reesei* cellulases while degrading different celluloses. Biotechnol. Lett. 151: 71-76.

Nydetzky B, Steiner W, Hayn M, Claeyssens M. 1994. Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: A new model for synergistic interaction. Biochem. J. 298: 705-710.

Wood TM, McCrae SI, Macfarlane CC. 1980. The isolation, purification and properties of the cellobiohydrolase component of *Penicillium funiculosum* cellulase.. Biochem. J. 189: 51-65.

Woodward J, Lima M, Lee NE. 1988. The role of cellulase concentration in determining the degree of synergism in the hydrolysis of microcrystalline cellulose. Biochem J 255: 895-899.

5.2 Article A

Hydrolysis of cellulose using mono-component enzymes show synergy on Phosphoric Acid Swollen Cellulose (PASC), but competition on Avicel

Natalija Andersen, Katja S. Johansen, Michael Michelsen, Erling H. Stenby, Kristian B.R.M. Krogh, Lisbeth Olsson

Submitted for publication

Hydrolysis of cellulose using mono-component enzymes show synergy on Phosphoric Acid Swollen Cellulose (PASC), but competition on Avicel

Natalija Andersen[1,3], Katja S. Johansen[2], Michael Michelsen[3], Erling H. Stenby[3], Kristian B.R.M. Krogh[2], Lisbeth Olsson[1,*]

[1] Center for Microbial Biotechnology - CMB, BioCentrum-DTU, Building 223, Technical University of Denmark, 2800 Lyngby, Denmark
[2] Novozymes A/S, Smørmosevej 25, 2880 Bagsværd, Denmark
[3] The Engineering Research Center – IVC-SEP, Department of Chemical Engineering – DTU, Building 229, Technical University of Denmark, 2800 Lyngby, Denmark

[*] Correspondence to: L. Olsson: Center for Microbial Biotechnology, BioCentrum-DTU, Building 223, Technical University of Denmark, 2800 Lyngby, Denmark, Tel.: +45 4525 2677; e-mail: lo@biocentrum.dtu.dk

Abstract: To study the synergy between the three groups of cellulolytic enzymes, twenty mixtures of different mole percentage of *Humicola insolens* Cel45A (EG V) and Cel6A (CBH II), and *Penicillium brasilianum* Cel3A (β-glucosidase) were used to hydrolyze Avicel and phosphoric acid swollen cellulose/Avicel (PASC). In contrast to previous studies, where β-glucosidase was either not added or added in excess, we here focus on engineering binary, as well as, ternary cellulase mixtures (including a range of different mole % of Cel3A) for maximal total sugar production. Precise hydrolysis pattern based on the concentration of soluble hydrolysis products (glucose to cellohexaose measured by HPLC) was determined. Importance of proper assay selection for hydrolysis products detection was illustrated.

It was found that degree of synergy (DS) for degradation of PASC were generally larger than 1, (indicating cooperativity between the enzymes), increasing as the hydrolysis proceeded. DS of binary *exo-/endo*-glucanase mixtures, decreased as the mole % of Cel45A increased. In contrast to hydrolysis of PASC, DS values during degradation of Avicel were less then 1, indicating inhibition of the involved enzymes. Thus, our data point to competition for the same binding sites between *endo-* and *exo*-glucanases, and preferential absorbance of *exo*-glucanases on crystalline substrates.

INTRODUCTION

The pioneer work of Reese and coworkers [1] has established that the degradation of cellulose required a complex of enzymes, consisting of at least three classes of enzymes, working together. Exo-1,4-β-D-glucanases, also called cellobiohydrolases (CBH) (EC 3.2.1.91), cleave off cellobiose units from the ends of cellulose chains. Endo-1,4-β-D-glucanases (EG) (EC 3.2.1.4) hydrolyze internal β-1,4-glucosidic bonds in the cellulose chain, presumably acting mainly on the amorphous or disordered regions of cellulose. Hydrolysis to the final product is accomplished by 1,4-β-D-glucosidases (BG) (EC 3.2.1.21), which hydrolyze cellobiose to glucose, and also cleave off glucose units from the various soluble cellooligosaccharides (DP 1-6) [2]. BG activity has often been found to be rate-limiting in enzyme cocktails during enzymatic hydrolysis of cellulose [3, 4], and is thus frequently supplemented. For the extensive review of cellulolytic enzymes, microorganism producing cellulolytic enzymes and the enzymatic hydrolysis process see [5].

During an enzymatic hydrolysis process, in which the three enzyme classes are used to break down cellulose into sugars, typically, glucose is released quickly in the beginning of the process after which the hydrolysis rate is slowing down due to the high substrate recalcitrance [5]; if only one enzyme class was used for hydrolysis, the process would be hampered. Cooperative action, often designated *synergy*, of the three cellulolytic enzyme classes is essential for efficient enzymatic hydrolysis process. Synergy between cellulolytic enzymes is a term used for the observation that the overall degree of hydrolysis of a mixture of enzyme components is greater than the sum of the degrees of hydrolysis observed by the individual enzymes. A common way to quantify the extent of synergy is to calculate a "degree of synergy" (DS), which is defined as the ratio of activity exhibited by a mixture of components divided by the sum of the activities of separate components. The calculated DS can be based on product formation (e.g. sum of all soluble sugars), overall hydrolysis rates (d (product)/dt) [6], or based on the extent of substrate conversion [7].

Synergism between the various classes of the cellulose degrading enzymes have been reported and

investigated extensively (for review see [8]). Most studies reported up to date, have investigated binary or ternary enzyme mixtures focusing on *endo*(glucanase)-*exo*(glucanase) (e.g. EG-CBH) and/or *exo*(glucanase)-*exo*(glucanase) (e.g. CBH I-CBH II) type of cooperative action with or without the addition of external β-glucosidase activity [7, 8, 10, 11, 12, 13, 14, 15, and others]. To the best of our knowledge, no data have, so far, been published on the actual synergistic interactions between the three classes of cellulolytic enzymes, namely CBH, EG and BG. Therefore, in the present study we focus on engineering binary, as well as, ternary cellulase mixtures for maximal total sugar production by varying the relative content of *H. insolens* Cel45A (EG V) and Cel6A (CBH II), and *Penicillium brasilianum* Cel3A (β-glucosidase), thus introducing a novel approach to the traditional synergy studies.

In the current study, we selected to work with two cloned, expressed and purified enzymes produced by *Humicola insolens*. Cel6A from *H. insolens* has 65 % sequence similarity to Cel6A from *T. reesei* [16] and it has a fungal (family 1) carbohydrate binding module (CBM). It is acting from the non-reducing end of the cellulose chain and it has no activity towards soluble carboxymethyl cellulose (CMC) (i.e., no endoglucanase activity), the activity towards filter paper is relatively low, but the activity towards bacterial crystalline cellulose is high [17].

Cel45A has high activity on CMC with a predominant endo action and the main products of hydrolysis of filter paper are cellobiose, cellotriose and cellotetraose, while the main product during hydrolysis of PASC, Avicel and CMC by Cel45A is cellotetraose, with significant amounts of cellopentaose and cellotriose also being produced [18]. Cel45A also possess a family 1 CBM. Having in mind that β-glucosidases have an ability to degrade soluble cellooligosaccharides (DP 1-6), and that the aim of this study was to investigate synergy between the three enzyme classes, the above mentioned particular hydrolysis pattern of Cel45A was of high interest when selecting for an endoglucanase representative enzyme.

The novel β-glycosidase, Cel3A from *P. brasillianum* IBT 20888, was chosen as the third component of the cellulase system. Krogh, et al. [19] cloned and expressed at high level the β-glycosidase gene in *Aspergillus oryzae*, and reported that it is thermostable at 60 °C (after 24 hours incubation at pH 4-6 and 60 °C, 100 %

residual activity on *p*-nithrophenyl β-D-glucopyranoside was measured; in comparison, the commercial β-glycosidase preparation had 50 % residual activity after 24 hours of incubation). Both of these characteristics have high industrial importance, and were thus motivation for choosing Cel3A as the β-glucosidase representative enzyme for the present study.

The substrates chosen for this study are Avicel and Phosphoric Acid Swollen Cellulose (PASC), representing two model substrates with distinctive characteristics (Table I). As differences in the chemical composition and the fraction of crystalline cellulose in cellulosic materials have been shown to influence the potential of the material to be hydrolyzed [20], the direct comparison between selected cellulosic materials will provide us with a good platform for synergy studies.

MATERIALS AND METHODS

Cellulose
Avicel® PH-101 was purchased form Fluka BioChemika, (Ireland) (product no. 11365) and it has an average particle size of about 50 μm. The Avicel concentration in the experiments was 10.0 g/l. PASC was prepared from Avicel according to [16]. The concentration of PASC was determined by dry weight measurements to be 9.0 g/l.

Enzymes
Cel45A from *H. insolens* has a 98 % purity on SDS, MW of 43 kDa and the active protein concentration was 232 μM. Cel6A from *H. insolens* has a 95 % purity on SDS, MW of 65 kDa, an activity of 9.8 pNP-cellobioside U/ml and an active protein concentration of 94.8 μM. Both enzymes were purified from the broth of *Aspergillus* hosts by affinity chromatography using Avicel. The protein concentrations of the resulting pools were determined by calculation from the absorbance at 280 nm and the extinction coefficient of the molecules.

Cel3A has been isolated from *P. brasillianum* IBT 20888 and cloned and expressed in *Aspergillus oryzae* [19]. This enzyme has a MW of 110 kDa and an active protein concentration of 12.1 μM. The buffer used in all experiments was 0.1 M sodium acetate, pH=5.5. The total enzyme concentration was in all cases 0.1 μM.

Table I: Determination of degree of polymerization and summary of some physical properties of Avicel and PASC

Substrate	Reducing Saccharide Nelson-Somogyi Assay		Total Glucose Content Phenol-Sulfuric Acid Assay		DP	MW	CrI*	SSA*
	Glc. Eq. (mM)	Error	Glc. Eq. (mM)	Error		(g/mol)		(m2/g)
PASC	0,53	0,02	50,9	0,06	96	15570	0.00-0.04	240
Avicel	0,39	0,07	57,9	0,03	150	24320	0.5-0.6	20

PASC: Phosphoric Acid Swollen Cellulose; Error: calculated as (Standard deviation/Absorbance mean), from five independent experiments; Glc. Eq.: Glucose Equivalent; DP: Degree of Polymerization; MW: Molecular weight; CrI: Crystallinity index; SSA: Specific Surface Area.
* Reference: [8].

For comparison, a commercial cellulase mixture, Celluclast® 1.5L + Novozym® 188 (3:1, vol:vol), was also included in the experiments. Celluclast® 1.5L (Novozymes A/S) from *T. reesei* has activity of 700 Endo-Glucanase Units (EGU)/g and density of approximately 1.2 g/ml. Novozym® 188 (Novozymes A/S) from *A. niger* has activity of 250 Cellobiase Units (CBU)/g. The Filter Paper Activity (FPA) of Celluclast® 1.5L + Novozym® 188 (3:1, vol:vol) has been determined to be 60.2 U/ml (Christensen, unpublished data).

Hydrolysis studies with mono-component enzymes

Hydrolysis experiments were performed by varying the mole percentage of the three cellulases as presented in Table II. The total enzyme concentration was in all cases 0.1 µM. All experiments were carried out in duplicates at 50 °C and pH 5.5 and with 0.1 M sodium acetate buffer. Samples were taken over a 52 h time course at: 0, 1, 2, 3, 6, 25, 29 and 52 h. Samples at 2 h for experiments with Avicel were omitted.

Experiments with PASC (9 g/l) were performed in 3.6 ml NUNC plastic tubes using a volume of 2.5 ml by gentle inverting on SM 1 universal mixer (Sarstedt group). At the defined time points 200 µl of sample was withdrawn from the tube and the reaction was stopped by adding 50 µl of 2 N NaOH (increasing the pH to 12.5). All samples were diluted 10 times in MQ-water and filtrated trough a 0.45 µm syringe filters before analyzed on Dionex HPLC.

Experiments with Avicel (10 g/l) were performed in 12 ml plastic tubes using a volume of 10 ml by gentle inverting on SM 1 universal mixer (Sarstedt group). At the defined time points 1600 µl of sample was withdrawn from the tube and the reaction was stopped by adding 400 µl of 2 N NaOH (increasing the pH to 12.5). All samples were filtrated trough a 0.45 µm syringe filters before analyzed on Dionex HPLC.

Table II: Set-up of hydrolysis experiments.

Hydrolysis experiments Set-up		
Mole Percentage (%)		
Comb.	Cel3A	Cel6A	Cel45A
1	0	0	100
2	0	20	80
3	0	50	50
4	0	80	20
5	0	100	0
6	10	0	90
7	10	40	50
8	10	80	10
9	20	0	80
10	20	20	60
11	20	60	20
12	30	0	70
13	30	20	50
14	30	50	20
15	30	70	0
16	40	0	60
17	40	20	40
18	50	0	50
19	70	10	20
20	100	0	0

In all cases total enzyme concentration was 0.1 µM.

Hydrolysis studies with the commercial enzymes

Celluclast® 1.5L + Novozym® 188, 3:1 (vol:vol) mixture was prepared by diluting 20 µl of the Celluclast + Novozym 188 (3:1, vol:vol) mixture (60 x) in 1180 µl of sodium acetate buffer pH 5.5.

Hydrolysis of PASC (9 g/l) was performed by using 250 µl of prepared diluted enzyme mixture in the final volume of 2500 µl, thus the enzyme loading in experiments with PASC was 11.1 FPU/ g cellulose. Samples were taken at 0, 1, 2, 3, 5 and 24 h. To confirm that enzymes are still active, after 24 h of the primary hydrolysis, 1300 µl of fresh PASC (9 g/l) was added (the amount of extra substrate was equal to 100 % of the remaining substrate), and hydrolysis was followed for another 24 hours; samples being taken at 25, 30 and 48 h. Note that, as PASC is liquid substrate, addition of extra substrate will double the reaction volume, and at the same time reduce the enzyme loading to half of the initial amount. At the defined time points 200 µl of sample was withdrawn from the tube and the reaction was stopped by adding 50 µl of 2 N NaOH. All samples were appropriately diluted in MQ-water and filtrated trough a 0.45 µm syringe filters before analyzed on Dionex HPLC.

Hydrolysis of Avicel (10 g/l) was performed by using 2 ml of prepared diluted enzyme mixture in the final volume of 20 ml (i.e. enzyme loading was 10 FPU/g cellulose). Samples were taken at 0, 1, 3, 5 and 24 h. To confirm that enzymes are still active, after 24 h of the primary hydrolysis, 1.2 g Avicel was added leading to an additional concentration of Avicel in supplement to the remaining substrate from the first part of hydrolysis, and hydrolysis was followed for another 24 hours; samples being taken at 25, 30 and 48 h. At the defined time points 1.6 ml of sample was withdrawn from the tube and the reaction was stopped by adding 400 ml of 2 N NaOH. All samples were filtrated trough a 0.45 µm syringe filters before analyzed on Dionex HPLC.

Analytical procedures

Glucose (C_1), cellobiose (C_2), cellotriose (C_3), cellotetraose (C_4) and cellopentaose (C_5), and cellohexose (C_6) were determined by HPLC with pulsed amperometric detection (HPAEC-PAD) using CarboPac PA 1 column (Dionex). A gradient system was used for separation: solvent A: 40 mM NaOH and solvent B: 100 mM NaOH, both containing 75 mM NaAc. The following gradient was used: 100 % A and 0 % B (0-15 min), a linear decrease of A to 0 % and a linear increase of B to 100 % (15-40 min), a linear increase of A to 100 % and a linear decrease of B to 0 % (40-50 min) [21]. The flow rate was 1 ml/min. The products were quantified based on peak areas using standard sugars. Standard sugars, C_1-C_5, were purchased from Sigma-Aldrich (USA); C_6 was purchased form Seikagaku Corporation (Japan).

Determination of the DP

The number-average-degree of polymerization of Avicel and PASC were estimated from their total reducing

saccharide content, by Nielson-Somogyi assay at 750 nm [22], and their total glucose content, by Phenol-Sulfuric Acid assay [23].

Calculation of "% conversion"

The amounts (in μM) of C_1, C_2, C_3, C_4, C_5 and C_6 quantified as described above, were recalculated to C-mols and denoted (C-mols)$_{Cx}$, x=1-6. Consequently, the maximal theoretical amount of C-mols in the substrates, denoted (C-mols)$_{substrate}$, was calculated from determined DP values and initial substrate concentration. The "% conversion" (C-mol/C-mol) was defined as [(C-mols)$_{Cx}$ / (C-mols)$_{substrate}$]*100. "SUM % conversion" was defined as

$$\sum_{x=1}^{6}[(C\text{-}mols)_{Cx} / (C\text{-}mols)_{substrate}]*100.$$ In the

further text "% conversion" and "SUM % conversion" will be represented in units (C-mol/C-mol).

Calculation of degree of synergy

Degree of synergy of a ternary mixture used in this study was calculated as:

$$DS = \frac{(SUM\%conversion)_{Cel45A+Cel6A+CeBA}}{\alpha \cdot (SUM\%conversion)_{Cel45A} + \beta \cdot (SUM\%conversion)_{Cel6A} + \gamma \cdot (SUM\%conversion)_{CeBA}}$$

where: α, β and γ correspond to mole fraction of Cel45A, Cel6A and Cel3A, respectively, used in the particular enzyme combination. DS was calculated for 3-hour- and 52-hour hydrolysis points. For comparison (data not shown) DS was also calculated based on the sum of all soluble sugars in g/l, yielding satisfactory comparable values (difference on the second decimal).

RESULTS AND DISCUSSION

Low enzyme loading provided maximum insight into detailed hydrolysis pattern

Among microcrystalline celluloses, Avicel has often been chosen as a good model substrate as it is considered to be highly ordered and, commercially available in standardized form. PASC is produced from Avicel and is considered to be an amorphous substrate [16]. In order to better define their differences, the number-average-degree of polymerization of Avicel and PASC were determined, and furthermore, the molecular weights were estimated. Table I summarizes the most important physical characteristics of Avicel and PASC, showing that the treatment with phosphoric acid deceased the DP of the substrate by more then one third. Furthermore, these results showed that phosphoric acid treatment resulted in generation of more reducing ends in PASC. This would have, in turn, created more attackable sites for the exoglucanase (Cel6A).

In the experiments very low enzyme loadings e.g. 0.1 μM were used. An intermediate enzyme loading is commonly considered to be 300-400 mg protein/l [24], i.e. approximately 20 FPU/g cellulose or around 5.5 μM (calculated with the average cellulase MW of 70 kDa). The low enzyme loading was chosen to particularly promote production, and consequent detection, of higher soluble cellooligosaccharides (DP 3-6). If higher enzyme loadings had been used, conversion of insoluble substrate to soluble intermediate products would not have been detectable, thus resulting, in most cases, in detection of only glucose and/or cellobiose. Consequently, our experimental conditions were selected to provide maximum insight to the molecular mechanism of enzymatic hydrolysis of cellulose.

Mono-component Cel45A exhibited highest initial productivity among the investigated individual enzymes

All cellulase mixtures investigated here exhibited non-linear production of soluble saccharides with time as illustrated in Figures 1-4. The hydrolysis profile of Cel45A on PASC (Figure 1A) reveals production of C_2 and almost equimolar production of C_3 and C_4, while the main product of hydrolysis of Avicel with Cel45A was C_2 and a equimolar amounts of C_1 and C_3 (Figure 2A).

A:
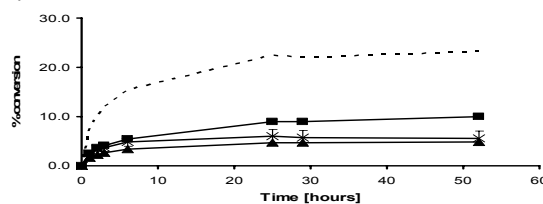
B:
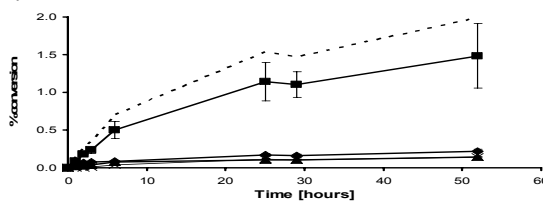
C:
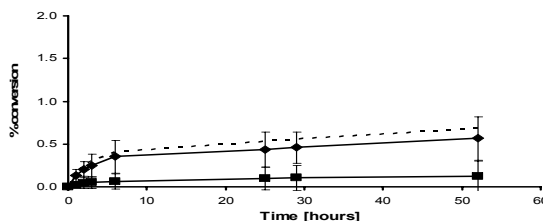
Figure 1: Hydrolysis profile of PASC with mono-component enzymes. **A:** 100 % (mole percentage) Cel45A; **B:** 100 % (mole percentage) Cel6A; **C:** 100 % (mole percentage) Cel3A. In all cases the enzyme concentration was 0.1 μM. All experiments were performed in duplicates, with 9 g/l PASC, at 50 °C, pH 5.5 and in 0.1 M sodium-acetate buffer. Error bars represent standard deviation of duplicate experiments. The conversion of the substrate is presented in "% conversion" (C-mol/C-mol) for each detected sugar. C_1: ♦; C_2: ■; C_3: ▲; C_4: X; Sum C_1-C_6: dashed line.

A:

B:

C:
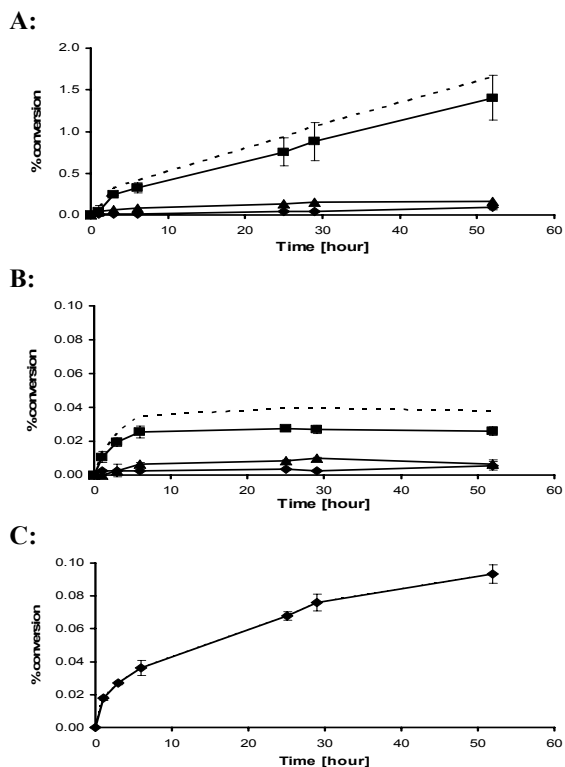
Figure 2: Hydrolysis profile of Avicel. **A:** 100 % (mole percentage) Cel45A; **B:** 100 % (mole percentage) Cel6A; **C:** 100 % (mole percentage) Cel3A. In all cases the enzyme concentration was 0.1 μM. All experiments were performed in duplicates, with 10 g/l of Avicel, at 50 °C, pH 5.5 and in 0.1 M sodium-acetate buffer. Error bars represent standard deviation of duplicate experiments. The conversion of the substrate is presented in "% conversion" (C-mol/C-mol) for each detected sugar. C_1: ♦; C_2: ■; C_3: ▲; Sum C_1-C_6: dashed line.

These results indicate an *exo-* behavior of an endoglucanase Cel45A. Similar hydrolysis pattern of three other endoglucanases from *T. reesei*, Cel7B, Cel5A and Cel12A have also been observed by Karlsson et al., 2002 [18], The extent of conversion after 52 h with the mono-component enzymes was highest with Cel45A; 20.9 % (C-mol/C-mol) of PASC and 1.7 % of Avicel, respectively, (Table III). Furthermore, Cel45A exhibited the highest initial productivity among the investigated individual enzymes, i.e. 6.85 ((C-mol/C-mol)/h) on PASC and 0.12 ((C-mol/C-mol)/h) on Avicel, respectively (Table III).

Hydrolysis profile of PASC using Cel6A and Cel3A is presented in Figure 1B and 1C, respectively, and a considerably lower substrate conversion was observed. Schou [17] reported high activity of Cel6A towards bacterial crystalline cellulose, which is considered to be highly ordered crystalline substrate. Our data show low activity of Cel6A on Avicel; reaching maximum % conversion of only 0.04 % after five hours, and stagnating at the same level for the rest of the experiment (Figure 2B). Hydrolysis of Avicel with Cel3A yielded 0.1 % conversion, increasing constantly through the experiment (Figure 2C).

At low enzyme loading the conversion of PASC was five to sixty fold higher than the conversion of Avicel

Significant difference in the extent of substrate conversion was observed for the two substrates; PASC in all cases being degraded to a much higher extent (five to sixty fold more) than Avicel. These differences in the hydrolysis rates of the cellulosic samples before (Avicel) and after phosphoric acid treatment (PASC) can to some extend be explained by observed decrease of DP (Table 1) and thus generation of more reducing ends in PASC then in Avicel. Similar results have also been reported by Zhang et al. [26].

Table III: Summary of results obtained by degradation of PASC and Avicel

| Comb.* | Hydrolysis of PASC | | | | | | Hydrolysis of Avicel | | | | | |
| | SUM % conv. | | DS (% conv.) | | DS change | I.P. | SUM % conv. | | DS (% conv.) | | DS change | I.P. |
	3 h.	52 h.	3 h.	52 h.	[%]	[(C-mol/C-mol)/h]	3 h.	52 h.	3 h.	52 h.	[%]	[(C-mol/C-mol)/h]
1	10.5	20.9	n.a.	n.a.	-----	6.85	0.3	1.7	n.a.	n.a.	-----	0.12
2	7.5	17.6	0.9	1.0	14.6	3.90	0.2	0.7	0.9	0.5	-78.7	0.11
3	6.4	16.9	1.2	1.5	20.6	3.49	0.2	0.3	0.9	0.4	-141.9	0.09
4	4.0	11.3	1.7	2.0	17.1	1.61	0.1	0.1	0.9	0.4	-145.0	0.04
5	0.3	1.8	n.a.	n.a.	-----	0.13	0.0	0.0	n.a.	n.a.	-----	0.01
6	12.6	27.4	1.3	1.5	8.8	7.33	0.1	0.4	0.4	0.2	-57.8	0.06
7	9.0	23.3	1.7	2.1	19.5	4.01	0.1	0.4	0.5	0.5	-9.8	0.06
8	3.2	11.2	2.4	3.1	23.7	1.03	0.0	0.2	0.6	0.8	28.4	0.02
9	9.3	19.3	1.1	1.1	4.3	4.17	0.1	0.3	0.4	0.2	-105.5	0.05
10	4.3	10.2	0.7	0.8	13.8	2.37	0.1	0.6	0.5	0.6	8.8	0.05
11	3.2	9.5	1.4	1.8	23.0	1.10	0.0	0.2	0.5	0.5	-7.3	0.03
12	9.5	19.9	1.3	1.3	5.3	4.02	0.1	0.4	0.4	0.3	-27.6	0.05
13	8.0	17.9	1.5	1.6	9.5	3.11	0.1	0.3	0.5	0.3	-53.8	0.04
14	4.6	14.7	1.9	2.8	30.5	1.24	0.0	0.2	0.5	0.5	-1.5	0.02
15	0.5	1.7	1.5	1.2	-22.9	0.26	0.0	0.1	0.9	1.1	20.3	0.01
16	9.9	23.1	1.5	1.8	14.7	2.96	0.1	0.4	0.3	0.4	6.8	0.04
17	7.9	22.2	1.8	2.5	27.3	2.06	0.1	0.2	0.4	0.3	-40.5	0.04
18	8.7	21.9	1.6	2.0	21.0	2.10	0.1	0.2	0.4	0.3	-52.2	0.04
19	4.6	14.9	2.0	3.1	35.9	0.87	0.0	0.2	0.5	0.4	-7.9	0.02
20	0.3	0.6	n.a.	n.a.	-----	0.13	0.0	0.1	n.a.	n.a.	-----	0.02

Comb.*: The enzyme mixtures are described in Table II. In all cases total enzyme concentration was 0.1 μM. All experiments were performed in duplicates, with 10 g/l of Avicel or 9 g/l PASC, at 50 °C, pH 5.5 and in 0.1 M sodium-acetate buffer. SUM % conv.: sum of all sugars produced (C_1 to C_6), calculated as % conversion (C-mol/C-mol); DS (% conv.): degree of synergy based on % conversion; 3 h.: samples taken out after 3 hours of hydrolysis; 52 h.: samples taken out after 52 hours of hydrolysis; DS change: % change of DS from 3- to 52-hour hydrolysis time points; I.P.: initial productivity, calculated as the rate of sum product formation (C_1-C_6) as % conversion (C-mol/C-mol) after 1-hour of hydrolysis; n.a.: not applicable.

However, they concluded that changes were due to the modifications in supra-molecular structures (accessibility), but not due to phosphoric acid hydrolysis during preparation of PASC.

Supplementary material (Appendix A and Appendix B), report on the precise hydrolysis patterns emerging from the degradation of PASC and Avicel, respectively. The SUM % conversion, DS, % increase of DS from 3 to 52 hours, and, initial productivities are listed in Table III.

Conversion of substrates with binary and ternary enzyme mixtures showed competition for the same binding sites and stereo-chemical blocking

The highest SUM % conversion of PASC was observed with a binary mixture of 10 % Cel3A + 90 % Cel45A (combination 6) (Table III), where 27.4 % of the substrate was converted within 52-hours hydrolysis. The second highest conversion, 23.3 % was observed with a ternary mixture composed of 10 % Cel3A + 40 % Cel6A + 50 % Cel45A (combination 7), Figure 3A.

A:

B:
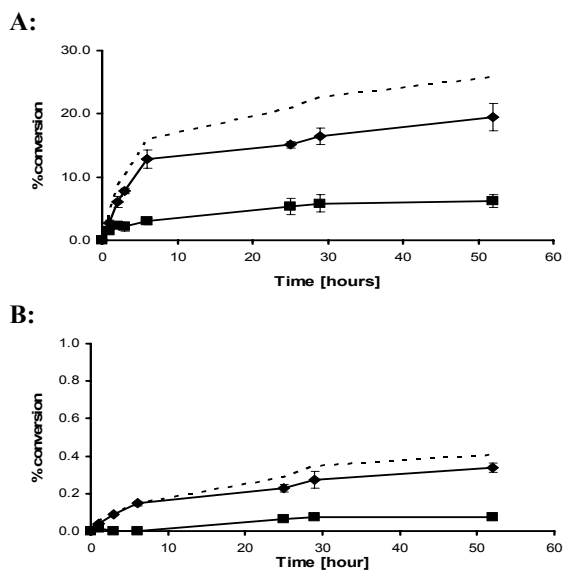
Figure 3: Hydrolysis profile of PASC and Avicel with the enzyme combination 7 (10 % Cel3A + 40 % Cel6A + 50 % Cel45A). **A:** PASC; **B:** Avicel. In all cases the enzyme concentration was 0.1 μM. All experiments were performed in duplicates, with 9 g/l PASC and 10 g/l Avicel at 50 °C, pH 5.5 and in 0.1 M sodium-acetate buffer. Error bars represent standard deviation of duplicate experiments. The conversion of the substrate is presented in "% conversion" (C-mol/C-mol) for each detected sugar. C_1:♦; C_2: ■; Sum C_1-C_6: dashed line.

This is an interesting result as the relatively similar % conversion (27.4 % and 23.3 %) was observed with markedly different amounts of Cel6A present in the enzyme mixtures, namely, 0 mole % and 40 mole % Cel6A respectively. These results indicate that the amount of

Cel6A is not the rate limiting factor in the cooperativity between the two enzymes, and that the two enzymes, Cel45A and Cel6A, are competing for the accessible binding sites on the substrate. A similar observation by Boisset et al. [26], however investigating degradation of bacterial cellulose ribbons (highly crystalline material) reported occasional *endo*-glucanase character of Cel6A in mixtures with Cel45A.

The performance of the used mono-component enzymes was evaluated against the commercially used cellulase mixture: Celluclast[®] 1.5L + Novozym[®] 188 (3:1, vol:vol). Already after 5 hours of hydrolysis of PASC using commercial cellulase mixture 100 % conversion was reached (Figure 4A).

A:

B:
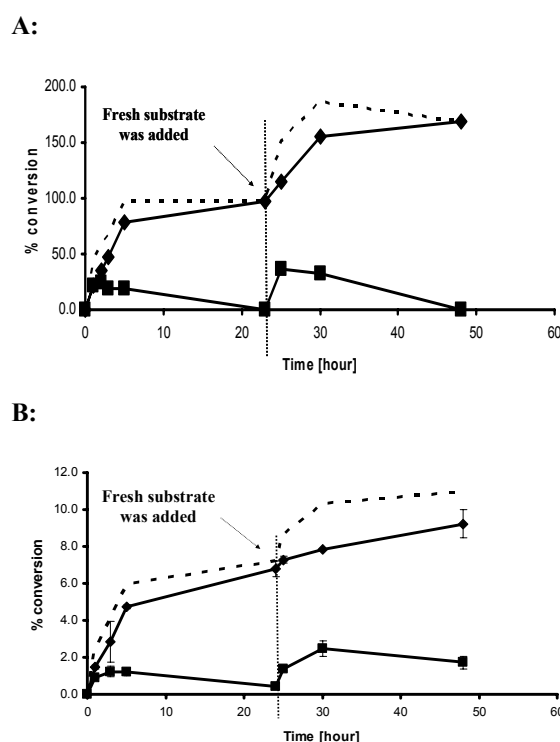
Figure 4: Hydrolysis profile of PASC and Avicel with the commercial enzyme mixture, Celluclast[®] 1.5L + Novozym[®] 188 (3:1, vol:vol). Experiments were performed in duplicates, with 9 g/l PASC or 10 g/l of Avicel, at 50 °C, pH 5.5 and in 0.1 M sodium-acetate buffer. Error bars represent standard deviation of duplicate experiments. The conversion of the substrate is presented in "% conversion" (C-mol/C-mol) for each detected sugar. After 24-hours of primary hydrolysis (noted at the graph), fresh substrate was added. **A:** PASC; **B:** Avicel; C1: ♦; C2: ■; Sum C1-C6: dashed line.

To confirm that the enzymes were still active, additional portion of the substrate was added after 24 h of primary hydrolysis. The same hydrolysis pattern, with a lower conversion level, was also observed for the second portion of PASC, indicating that the enzymes are still active, but possibly also inhibited by glucose (approximately 50 mM) present in the broth after 24 hours.

Markedly lower % conversion of Avicel with the commercial cellulases was observed; 7 % after 24 hours, and additional 4 % within the next 24 hours, after addition of fresh substrate (Figure 4B). Consistently, hydrolysis of Avicel with the mono-component enzymes, e.g. combination 7 (Figure 3B), reveals markedly lower conversion of this microcrystalline substrate compared to PASC.

Opposed to synergy observed during degradation of PASC, competition was observed during degradation of Avicel

The *exo/endo* synergy (i.e. CBH/EG synergy) is the most widely studied synergy type. DS values of up to 10 on bacterial cellulose have been reported [27] and it is highly dependent on the substrate properties (where DP appears to play an important role), the enzymes used in the study, but also the experimental conditions (such as enzyme loading). As a general rule, the highest DS values are reported on highly crystalline substrates (like bacterial cellulose and cotton) and are declining with the decrease of substrate crystallinity [8]. Consequently, DS values will also change during the hydrolysis process following the changes in physical structure of the substrate. In Table III values for calculated DS and % change of DS from 3 to 52 hours are listed. With one exception, DS during degradation of PASC with investigated enzymes generally increased as the hydrolysis proceeded from 3 to 52 hours. The highest value was 3.1 (dimensionless), observed after 52 hours of hydrolysis with enzyme mixtures 8 and 19, having predominantly Cel6A (80 mole %) or Cel3A (70 mole %) activities, respectively. Studies have shown that lower ratios of the major *endo*-glucanases (in mixtures with *exo*-glucanases) result in higher values of DS [9, 15]. Consistent with previous studies, the same was also observed in the present study for the binary mixtures of Cel45A/Cel6A (enzyme combinations 1-5), Figure 5A, but also for Cel45A/Cel3A, an *endo*-glucanase/β-glucosidase binary mixtures (enzyme combinations 1, 6, 9, 12, 16, 18, 20) with the exception for enzyme combination 9 (Figure 5B).

Opposed to the synergy effect observed during degradation of PASC, no synergy was observed during degradation of Avicel (DS values less then 1, Table III) indicating competition of individual enzyme activities in binary and ternary mixtures, Figure 6. Additionally, in most of the cases DS values decreased further throughout the hydrolysis.

The only enzyme mixture that showed minor synergistic effect (DS=1.1) during hydrolysis of Avicel was a binary mixture of Cel3A/Cel6A (the only binary mixture of these two enzymes investigated in the present study) (Table II). To understand these results we need to be familiar with the overall mechanisms of enzymatic degradation of cellulosic materials.
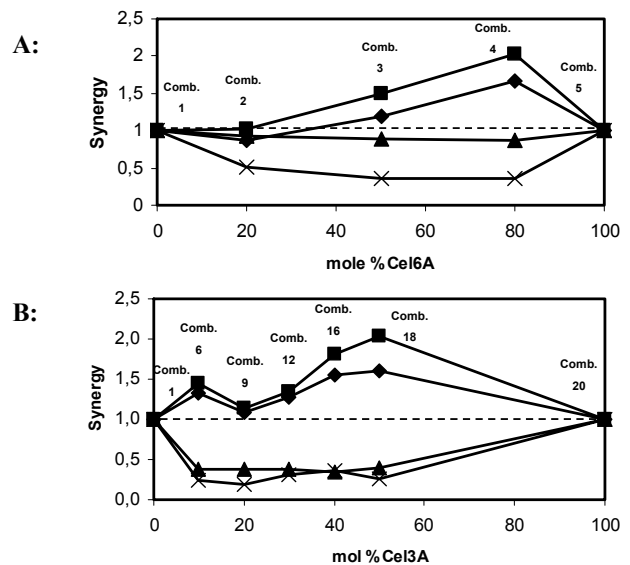
Figure 5: Synergy in binary enzyme mixtures on PASC and Avicel. **A:** Cel6A and Cel45A, and **B:** Cel3A and Cel45A. For convenience, the enzyme combination number (Comb.) corresponding to Table II, is stated. The dotted line represents the theoretical DS value for non-cooperative degradation (DS=1). Sum of all detected soluble sugars was used to calculate DS. ♦: 3 h, PASC; ■: 52 h, PASC; ▲: 3 h, Avicel; X: 52 h Avicel.

In general, upon binding of CBM to the available active site of the substrate, endo-glucanases perform single hydrolysis reaction while exo-cellulases carry out processive hydrolysis of cellulose, e.g performing multiple attacks from one end of cellulose chain without desorption of the enzyme. Desorption of the protein should follow, before further adsorption to a new available active site can occur. Studies have shown unusually strong and irreversible binding of CBH I to cellulose [27, 28]. Additionally, Chanzy et al. [30] showed that CBH I binds along the length of cellulose microfibrils rather then just at the chain ends, occupying therefore expected available binding sites for endoglucanase. Competition between *endo*-glucanases and *exo*-glucanases for cellulose binding sites has also been reported by Kyriacou et al. [29], who stated that fraction of CBH I was preferentially adsorbed (approaching levels of 100 %) when in competition with the other enzymes (EG I, EG II and EG III). Ryu et al. [28], moreover, observed that primarily absorbed EG was shown to desorb from cellulose upon addition of CBH. As it can be observed from these numerous studies, preferential absorbance and its slow desorption rate of *exo*-glucanases in the presence of *endo*-glucanases plays an important role.

Conversion of the substrate calculated based only on glucose production might significantly deviate from the values calculated based on all soluble sugars

The extent of enzymatic hydrolysis is most commonly evaluated by assays that involve the quantification of hydrolysis products, including reducing sugars and total sugars [31]. The most common reducing sugar assays include dinitrosalicylic acid (DNS) method and Nelson-Somogyi assay, where the amount of reducing ends of hydrolysis sample is estimated by comparison to the prepared glucose standard. However, there is poor stoichiometric relationship between cellodextrins and the glucose standards, often resulting in underestimation of cellulolytic activities if β-glucosidase activity is not in excess [31]. Our results were obtained by measurement of all soluble cellooligosaccharides on HPLC, overcoming, thus, the above mentioned obstacle. To illustrate the importance of correct assay selection, we present our result using two approaches. In Figure 7A all detected soluble sugars (glucose to cellohexaose) were used to calculate "SUM (C_1 to C_6) % conversion" of PASC. In Figure 7B the distribution of % conversion was calculated taking only into account produced glucose. Dark circles in Figure 7B represent experiments where the overall result deviated more then 15 % compared to Figure 7A. As expected, all investigated enzyme combinations with less then 20 mole % of Cel3A show large deviation in obtained results, underlining the importance of the exact experimental conditions under which the hydrolysis (and its products) are evaluated.

CONCLUSION

In this work we investigated enzymatic hydrolysis and the possible synergy between different cellulases by varying the mole percentage of three cellulases and reporting the conversion of the substrate, and production of soluble cellooligosaccharides, in relation to the ratio of the enzyme components. The precise hydrolysis pattern obtained by measuring soluble cellooligosaccharides during degradation of PASC and Avicel was determined. The highest substrate conversion (27.4 % (C-mol/C-mol)) obtained in this study was observed during degradation of PASC with 10 % Cel3A + 90 % Cel45A; a somewhat counterintuitive result, as this mixture does not include the cellobiohydrolase activity. Among the investigated ternary enzyme mixtures, the one consisted of 10 % Cel3A + 40 % Cel6A + 50 % Cel45A performed the best on PASC. *Endo* character of Cel6A, and its competition for the same binding sites as for the real endoglucanase, Cel45A, have been reported earlier [27] and this could explain our results. Thus, one major conclusion drawn is that, not only the presence of the

particular enzyme class, but the nature of the involved enzymes play an important role in cellulose degradation.
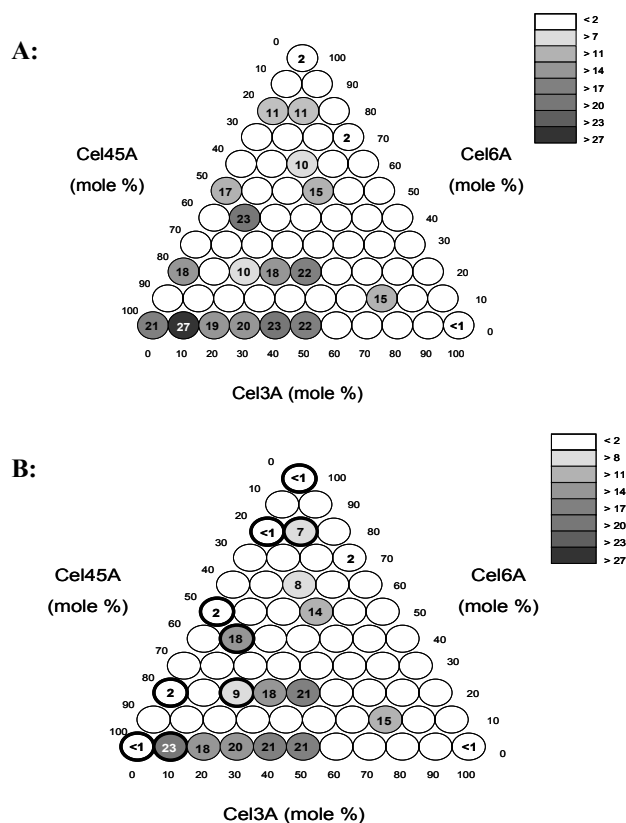
Figure 6: Conversion of PASC by ternary mixtures of Cel45A, Cel6A and Cel3A after 52-hour hydrolysis. In the diagram, the axes values are mole % of each enzyme of the ternary mixture. The extent of combined enzyme activity is represented as "% conversion" (C-mol/C-mol), and is stated inside each circle on the plot. Cycles are colored according to the color bar. **A:** Sum of all detected soluble sugars was used to calculate "% conversion" (C-mol/C-mol). **B:** "% conversion" (C-mol/C-mol) was calculated only based on detected glucose amounts. Black circles represent experiments where "% conversion" deviated for more then 15 % compared to Figure 6A.

The degree of synergy values for 3-h and 52-h hydrolysis time points reported here show evidence of synergy effect during the degradation of PASC, but, competitive effects during degradation of Avicel. Limited availability of accessible sites for adsorption of endoglucanase Cel45A on Avicel, compared to PASC, and, preferential absorbance and irreversible binding of exocellulases, can explain the lack of synergy observed during degradation of Avicel. Thus, our results demonstrate that the broad difference between the characteristics of two substrates used have predominant effect on the choice of an enzyme preparation.

REFERENCES

[1] Reese ET. History of the cellulase program at the U.S. Army Natick development center. Biotechnol Bioeng Symp 1976;6:9-20.

[2] Zhang Y-HP, Himmel ME, Mielenz JR. Outlook for cellulase improvement: screening and selection strategies. Biotech Adv 2006;24:452-81.

[3] Duff SJB, Marray WD. Bioconversion of forest products industry waste cellulosics to fuel ethanol: a review. Bioresour Technol 1996;55:1-33.

[4] Tolan JS, Foody B. Cellulase from submerged fermentation. Adv Biochem Eng Biotechnol 1999;65:41-67.

[5] Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol Mol Biol Rev 2002;66:506-77.

[6] Eriksson T, Karlsson J, Tjerneld F. A model explaining declining rate in hydrolysis of lignocellulose substrate with cellobiohydrolase I (Cel7A) and endoglucanase I (Cel7B) of *Trichoderma reesei*. Appl Biochem Biotechnol 2002;101:41-60.

[7] Jeoh T, Wilson DB, Walker LP. Effect of cellulase mol fraction and cellulose recalcitrance on synergism in cellulose hydrolysis and binding. Biotechnol Prog 2006;22: 270-7.

[8] Zhang Y-HP, Lynd LR. Towards an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems Biotechnol Bioeng 2004;88:797-824.

[9] Walker LP, Belair CD, Wilson DB, Irwin DC. Engineering cellulase mixtures by varying the mole fraction of the *Thermomonosporea fusca* E5 and E3, *Trichoderma reesei* CBHI, and *Caldocellum saccharolyticum* β-glucosidase. Biotechnol Bioeng 1993;42:1019-28.

[10] Nidetzky B, Steiner W, Hayn M, Claeyssens M. Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: a new model for synergistic interaction. Biochem J 1994;298:705-10.

[11] Woodward J, Lima M, Lee NE. The role of cellulase concentration in determining the degree of synergism in the hydrolysis of microcrystalline cellulose. Biochem J 1988;255:895-9.

[12] Medve J, Karlsson J, Lee D, Tjerneld F. Hydrolysis of microcrystalline cellulose by cellobiohydrolases I and endoglucanase II from *Trichoderma reesei*: adsorption sugar production pattern, and synergism of the enzymes. Biotechnol Bioeng 1998;59:621-34.

[13] Boisset C, Fraschini C, Schulein M, Henrissat B, Chanzy H. Imaging the enzymatic digestion of bacterial cellulose ribbons reveals endo character of the cellobiohydrolase Cel6A from *Humicola insolens* and its mode of synergy with cellobiohydrolase Cel7A. Appl Environ Microbiol 2000;66:1444-52.

[14] Jeoh T, Wilson DB, Walker LP. Cooperative and competitive binding in synergistic mixtures of *Thermofibida fusca* cellulases Cel5A, Cel6B and Cel9A. Biotechnol Prog 2002;18:760-9.

[15] Watson DL, Wilson DB, Walker LP. Synergism in binary mixtures of *Thermofibida fusca* cellulases Cel6B, Cel9A and Cel5A on BMCC and Avicel. Appl Biochem Biotechnol 2002;101:97-111.

[16] Schulein M. Enzymatic properties of cellulases from *Humicola insolens*. J Biotechnol 1997;57:71-81.

[17] Schou S. A study of cellulolytic enzymes of fungal and bacterial origin. Ph. D. Thesis. 1993.

[18] Karlsson J, Siika-aho M, Tenkanen M, Tjerneld F. Enzymatic properties of low molecular mass endoglucanases Cel12A (EG III) and Cel45A (EG V) of *Trichoderma reesei*. J Biotechnol 2002;99:63-78.

[19] Krogh KBRM, Harris PV, Olsen CL, Johansen KS, Hojer-Pedersen J, Tjerneld F, Olsson L. Cloning and characterization of GH3 β-glucosidase from *Penicillium brasilianum* including a novel method for measurement of glucose inhibition of cellobiose hydrolysis. Submitted for publication to Eur J Biochem.

[20] Teeri TT, Koivula A. Cellulase degradation by native and engineered fungal cellulases. Carbohyd Eur 1995;12: 28-33.

[21] Panagiotou G, Olsson L. Effect of compounds released during pretreatment of wheat straw on microbial growth and enzymatic hydrolysis rates. Biotechnol Bioeng 2006;96: 250-8.

[22] Hatanaka C and Kobara Y. Determination of glucose by a modification of Somogyi-Nielson method. Agr Biol Chem 1980;44:2943-9.

[23] Dubois B, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350-6.

[24] Zhang Y-HP, Lynd LR. A funtionally based model for hydrolysis of cellulose by fungal cellulase. Biotechnol Bioeng 2006;94:888-898.

[25] Zhang Y-HP, Cui J, Lynd LR, Kuang LR. A transition from cellulose swelling to cellulose dissolution by *o*-phosphoric acid: evidence from enzymatic hydrolysis and supramolecular structure. Biomacromolecules 2006;7:644-8.

[26] Boisset C, Pétrequin C, Chanzy H, Henrissat B. Optimized mixtures of recombinant *Humicola insolens* cellulases for the biodegradation of crystalline cellulose. Biotechnol Bioeng 2001;72:339-45.

[27] Samejima M, Sugiyama J, Igarashi K, Eriksson KEL. Enzymatic hydrolysis of bacterial cellulose. Carbohydr Res 1998;305:281-8.

[28] Ryu DD, Kim C, Mandels M. Competitive adsorption of cellulase components and its significance in a synergistic mechanism. Biotechnol Bioeng 1984;26:488-96.

[29] Kyriacou A, Neufeld RJ, MacKenzie CR. Reversibility and competition in the adsorption of *Trichoderma reesei* cellulases components. Biotechnol Bioeng 1989;33:631-7.

[30] Chanzy, H., Henrissat, B. and Vuong, R. Colloidal gold labeling of 1,4-β-D-glucan cellobiohydrolase adsorbed on cellulose substrates. FEBS Lett 1984;172:193−7.

[31] Zhang Y-HP, Himmel ME, Mielenz JR. Outlook for cellulase improvement: screening and selection strategies. Biotech Adv 2006;24:452-81.

SUPPLEMENTARY MATERIAL

Appendix A: Enzymatic hydrolysis products during degradation of PASC

Comb.	C₁ [mM]		C₂ [mM]		C₃ [mM]		C₄ [mM]		C₅ [mM]		C₆ [mM]	
	3 h.	52 h.	3 h.	52 h.	3 h.	52 h.	3 h.	52 h.	3 h.	52 h.	3 h.	52 h.
1	0.17	0.52	1.15	2.79	0.48	0.89	0.52	0.78	0.06	0.14	d.l.a.	d.l.a.
2	0.32	1.13	0.89	2.62	0.34	0.72	0.28	0.40	0.07	0.14	n.d.	n.d.
3	0.23	0.99	0.69	2.47	0.28	0.67	0.27	0.41	0.05	0.14	0.03	d.l.a.
4	0.12	0.50	0.39	1.68	0.16	0.47	0.17	0.33	d.l.a.	d.l.a.	0.03	d.l.a.
5	0.04	0.12	0.06	0.41	d.l.a.	0.03	d.l.a.	0.02	n.d.	n.d.	n.d.	n.d.
6	5.75	14.23	0.92	1.27	0.04	d.l.a.	d.l.a.	n.d.	n.d.	n.d.	n.d.	n.d.
7	4.31	10.79	0.60	1.72	d.l.a.	d.l.a.	d.l.a.	n.d.	n.d.	n.d.	n.d.	n.d.
8	1.60	4.57	0.17	1.05	d.l.a.	0.1	n.d.	d.l.a.	n.d.	n.d.	n.d.	n.d.
9	4.90	11.12	0.41	0.37	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10	1.92	5.21	0.32	0.43	d.l.a.	d.l.a.	d.l.a.	d.l.a.	n.d.	n.d.	n.d.	n.d.
11	1.72	5.12	0.10	0.27	d.l.a.	d.l.a.	d.l.a.	d.l.a.	n.d.	n.d.	n.d.	n.d.
12	5.32	12.21	0.24	d.l.a.	n.d.	n.d.	d.l.a.	n.d.	n.d.	n.d.	n.d.	n.d.
13	4.48	10.97	0.16	d.l.a.	d.l.a.	d.l.a.	n.d.	d.l.a.	n.d.	n.d.	n.d.	n.d.
14	2.61	8.71	0.08	0.14	d.l.a.	d.l.a.	d.l.a.	d.l.a.	n.d.	n.d.	n.d.	n.d.
15	0.20	0.99	d.l.a.	d.l.a.	n.d.	n.d.	d.l.a.	d.l.a.	d.l.a.	d.l.a.	n.d.	n.d.
16	5.54	13.10	0.29	0.58	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
17	4.53	12.89	0.17	0.38	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18	4.94	12.65	0.20	0.42	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
19	2.71	9.20	0.08	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20	0.14	0.31	d.l.a.	d.l.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Experiments were performed by varying the mole percentage of the three cellulases, Cel45A, Cel6A and Cel3A as described in the Materials and Methods section. The relative standard deviation of the duplicate experiments was below 15 %. Comb.: Combination as described in Table II; C_1: glucose; C_2: cellobiose; C_3: cellotriose; C_4: cellotetraose; C_5: cellopentaose; C_6: cellohexaose; n.d.: not detected; d.l.a.: detected in low amount; 3 h.: samples taken out after 3 hours of hydrolysis; 52 h.: samples taken out after 52 hours of hydrolysis.

Appendix B: Enzymatic hydrolysis products during degradation of Avicel

Comb.	C₁ [µM]		C₂ [µM]		C₃ [µM]		C₄ [µM]		C₅ [µM]		C₆ [µM]	
	3 h.	52 h.	3 h.	52 h.	3 h.	52 h.	3 h.	52 h.	3 h.	52 h.	3 h.	52 h.
1	d.l.a.	53.6	76.1	433.2	13.2	34.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2	d.l.a.	24.0	58.2	177.7	d.l.a.	15.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3	d.l.a.	d.l.a.	37.7	77.5	d.l.a.	d.l.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4	d.l.a.	d.l.a.	16.5	31.4	d.l.a.	d.l.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5	d.l.a.	340.0	d.l.a.	d.l.a.	d.l.a.	d.l.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	67.2	208.0	d.l.a.	d.l.a.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
7	54.9	207.9	d.l.a.	22.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8	21.2	82.0	d.l.a.	11.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
9	59.9	156.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10	68.9	373.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
11	26.9	102.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12	58.2	224.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
13	49.2	158.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
14	27.6	108.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15	14.5	39.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
16	42.6	236.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
17	38.0	131.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18	44.7	143.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
19	26.4	102.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20	16.7	57.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

See table legend of Appendix A.

CHAPTER 6

Factors affecting enzymatic hydrolysis of cellulose

Cellulose hydrolysis occurs in heterogeneous system of two phases; enzymes are dissolved in liquid phase and cellulose exists as a solid phase. Moreover, cellulose itself is a heterogeneous polymer, consisting of crystalline (including different types of crystal forms) and amorphous regions (discussed in Chapter 2) that requires the multiplicity of enzymes belonging to at least three different classes (discussed in Chapter 4) for its efficient hydrolysis. Thus, factors effecting enzymatic degradation of cellulose can be divided in two groups:

1. Enzyme related factors, and,
2. Substrate related factors.

Even though this division is made, it is important to stress that many factors are interrelated during a hydrolysis process, and, as a result, the influence of each factor is difficult to quantify in isolation. Consequently, digestibility of biomass is highly dependent on the combination of the composition of the substrate, type of pretreatment, and, dosage and efficiency of the enzymes used for hydrolysis.

Furthermore, these factors will also be discussed in the following chapter (Chapter 7), but seen from the modeling point of view. There I will introduce the consequences that major enzyme and substrate related factors have on the hydrolysis process, and, as a result, challenges for the development of the model describing the process; see the summary in Figure 7.1.

6.1 Enzyme related factors

Several factors associated with the nature of the cellulase enzyme system have been suggested to be influential during the hydrolysis process. These include:

1. Enzyme concentration,
2. Adsorption,
3. Synergism,
4. End-product inhibition,
5. Mechanical deactivation (fluid shear stress or gas-liquid interface),
6. Thermal inactivation, and,
7. Irreversible (non-productive) binding to lignin.

The extent of enzymatic hydrolysis depends, to a large degree, on the nature of the enzyme system employed. In previous chapters mostly fungal enzyme systems have been discussed, but it should be noted here that other systems, such as those from bacteria, consisting of enzyme complexes bound to the cell surface, i.e. cellulosomes, exist (for reference see Lynd et al. (2002)). In parallel with the nature of the enzyme system employed, the mode of action (*endo-* vs. *exo*-enzymes) and their stereochemical mechanism of hydrolysis (inverting vs. retaining) are interrelated. Furthermore, it has been shown that synergism between the enzymes can be of significant benefit in increasing the hydrolysis rates of complex substrate. However, as concluded in Article A (Chapter 5) synergism is also substrate-dependent, with some mixtures showing cooperative action on amorphous substrates, but not on microcrystalline cellulose. All this factors can collectively be called the efficiency of the enzymes used.

As a general rule the conversion of the substrate increases with increase of enzyme concentration/dosage (Settler et al., 1989; Godfrey and West, 1996), but, as shown in Figure 6.1, the increase of the conversion is not proportional to the increase of enzyme dosage. It is particularly important to notice the significant difference in the slope of progress curves at the very beginning of the reaction (initial stage), Figure 6.1.A, which is used for the determination of enzyme activities. If we now move focus from the initial rates to the level of conversion, the real data presented in Figure 6.1.B, shows that

70

doubling of enzyme dosage, e.g. from 5 to 10 FPU/g substrate, will result in approximately 50 % increase in product formation, i.e. ~40 and ~60 g glucan/100 g substrate, respectively, after 96 hours. Additional ten times increase in enzyme dosage, e.g. from 10 to 100 FPU/g substrate, results only in ~30 % increase of product formed, from ~60 to ~80 g glucan/100 g substrate, respectively.
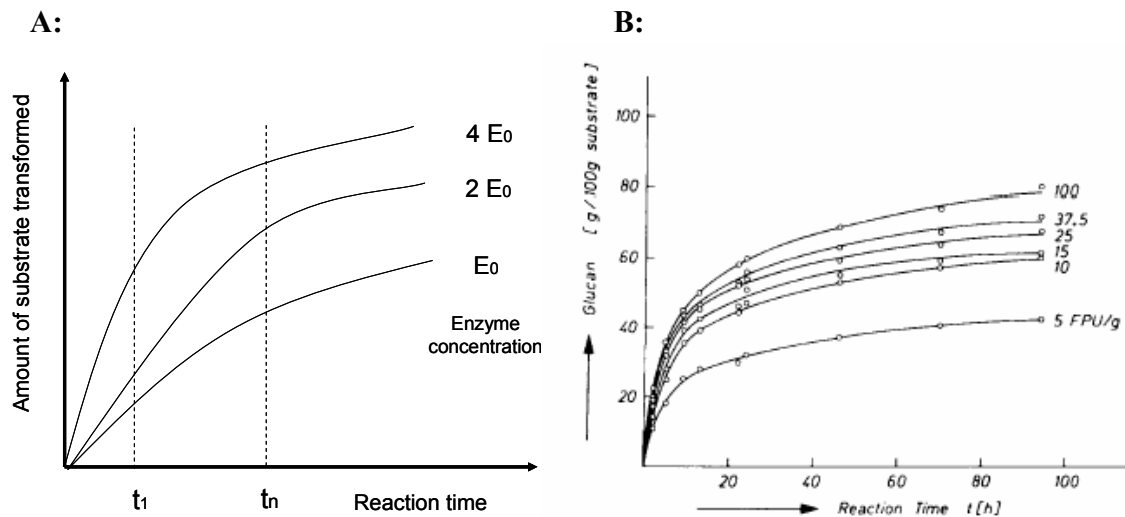
Figure 6.1: A: Effect of different initial enzyme concentrations on reaction progress. In particular the importance of measurement of initial reaction velocity $(0 – t_1)$ for the purpose of enzyme activity assays is demonstrated. Figure redrawn from Godfrey and West, 1996. **B:** Progress curves for hydrolysis of Sigmacell 50 with different enzyme dosages. Figure is taken from Sattler et al., 1989.

As mentioned earlier, the hydrolysis of cellulose differs from most other enzymatic reactions by the fact that substrate is insoluble; consequently, to ensure the reaction, the physical contact, i.e. adsorption of the enzymes to the substrate, is pre-requisite for cellulose hydrolysis. It has been shown that cellulase adsorption is facilitated by the carbohydrate binding modules (CBM), although, in some cases, catalytic domains can also specifically adsorb to cellulose independent of CBMs (as described in section 4.1). The efficiency of cellulases adsorption on the surface of the cellulose can be characterized by the partition coefficient K_p [L/g] of the enzyme between the substrate surface and the water phase (Klyosov, 1990). Adsorption experiments are usually performed at low temperatures (e.g. 2 °C) to minimize the change in the initial substrate concentration through degradation by the enzymes; cellulose degradation is very slow at

low temperatures and therefore does not interfere with the adsorption. Data on the adsorption ability of 26 endoglucanases, of which 10 were highly purified, showed great variations, in the range of more than 1000 times, but strong correlation between hydrolysis rates and values for the adsorption equilibrium constant have been found (Klyosov, 1990). The general conclusion was: the better adsorption, the better the catalysis.

Besides productive adsorption to cellulose, cellulase also adsorbs to lignin, which is thought to be non-specific (Ooshima et al., 1990; Tatsumoto et al., 1988). This adsorption is highly undesired as it is irreversible and accounts for the continuous loss of active enzyme during the hydrolysis process. Other factors such as mechanical deactivation (permanent change due to fluid shear stress or gas-liquid interface) and thermal inactivation (temporary/reversible change) of enzymes during the typical industrial lignocellulose hydrolysis process (T~50 °C, pH~5.5, t~7 days) additionally contribute to the enzyme loss.

Last but not least, end-product inhibition is often considered an important enzyme related factor influencing the hydrolysis process. Cellulase complex is inhibited by cellobiose, but also glucose, though to a minor extent. The effect of product inhibition have been studied extensively (Holtzapple et al., 1990; Gusakov and Sinitsyn, 1992; Väljamäe 2001; and others), but is still an area of debate as the actual inhibition mechanism as well as its magnitude depends strongly on the source of enzymes, the enzyme-substrate ratio (Gusakov and Synitsyn, 1992), and the nature of the substrate (Gruno et al., 2004). Holtzapple et al. (1990) summarized literature data of cellulase inhibition of which majority or the research characterized inhibition as a competitive type, although much convincing evidence of non-competitively inhibited enzymes also exists. The confusion and disagreements related to the determination of the type of inhibition are resulting from difficulty in conducting conclusive experiments. Namely, the use of inhibitor, i.e. cellobiose or glucose, often in high concentrations is interfering with the measurement of product release (also cellobiose and/or glucose) as a high sugar background prevents accurate measurements of hydrolysis rates. Recently a new, elegant, method for determining glucose inhibition of hydrolysis of cellobiose has been proposed. The assay involves using ^{13}C-labeled glucose as inhibitor and subsequent mass

spectrometry analysis to distinguish and quantify the amount of the hydrolysis product, i.e. naturally labeled glucose (Krogh et al., submitted for publication).

6.2 Physical properties of the substrate affecting the hydrolysis

The rate of enzymatic hydrolysis of lignocellulose is profoundly effected by the structural features of cellulose such as (Fan et al., 1981):

1. Crystallinity of cellulose,
2. Degree of polymerization (DP), i.e. molecular weight of cellulose,
3. Available/accessible surface area,
4. Structural organization, i.e. macro-structure (fiber) and micro-structure (elementary microfibril) and particle size, and,
5. Presence of associated materials such as hemicellulose and lignin.

The typical time course of the enzymatic hydrolysis of the lignocellulosic material is characterized by the rapid initial rate of hydrolysis followed by slower and incomplete hydrolysis, Figure. 6.2. Such a time course has been suggested to be due to the rapid hydrolysis of more easily available amorphous cellulose, with consequent increase of inherent degree of crystallinity, as the hydrolysis proceeds (Mansfield et al., 1999).
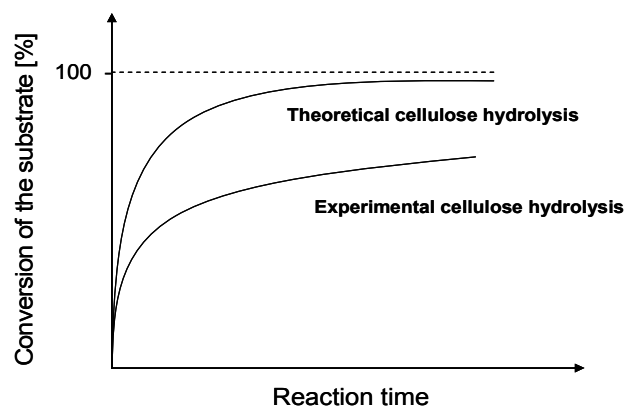
Figure 6.2: Typical time course of the enzymatic hydrolysis of the lignocellulosic material.

73

The effect of substrate crystallinity has been shown to play a major role in limiting hydrolysis in some studies (Fan et al., 1980, Fan et al., 1981), while other studies have shown that, when all other substrate factors are similar, the degree of crystallinity of the substrate has no effect on hydrolysis (Puri, 1984).

The effect of the degree of polymerization (DP, number of glycosyl residues per cellulose chain) is essentially related to other substrate characteristics, such as crystallinity. It has been shown that the depolymerization is largely a function of the nature of the cellulosic substrate being attacked. E*ndo*-glucanases (EGs) preferentially attacking less ordered, inside, regions of the cellulose chain contribute, thus, to a large extent to the rapid decrease of DP. On the contrary, *exo*-glucanases (CBHs) hydrolyzing substrate from the chain ends releasing cellobiose as a product have little effect on the change of DP through out the hydrolysis process. However, regardless of the substrate being hydrolyzed, there seems to be a "leveling off" of the cellulose DP, which is correlated with the increased recalcitrance of the residual (crystalline) cellulose, as shown in Figure 6.3.
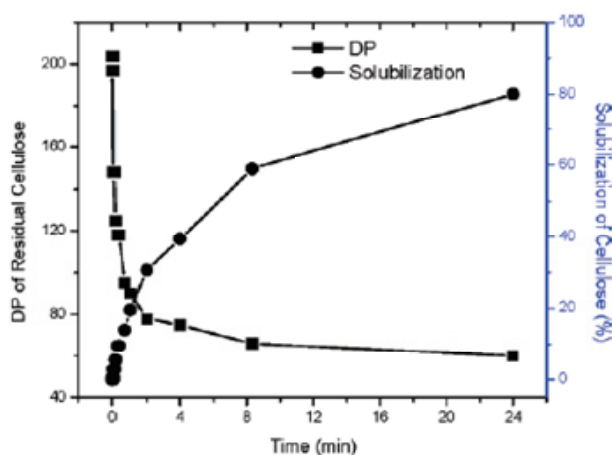
Figure 6.3: Decrease of cellulose DP with time during enzymatic hydrolysis of phosphoric acid swollen cellulose (PASC) by *Trichoderma reesei* cellulase complex. Figure is taken from Zhang and Lynd, 2005.

Another major substrate characteristic influencing hydrolysis process is accessibility of the substrate. Most often accessibility is measured by the BET (Bennet-Emmit-Teller) method, which measures the surface area available to the nitrogen molecule (Masamune and Smith, 1964). The drawbacks of the method are that it involves

the drying of the substrate, thus not allowing measurements on the material in its swollen state, and that the nitrogen molecule is substantially smaller in size compared to the enzyme molecule. As a consequence, specific surface area (SSA) can be overestimated as small nitrogen molecules have access to pores and cavities on the fiber surface that cellulases cannot enter. As it has been noted in the previous sections the size of the catalytic core of the cellulase molecule is approximately 40 x 40 x 20 Å, while the whole enzyme, including carbohydrate binding module, can reach 180 Å (Divne et al., 1993; Divne et al., 1994). At the same time, one glucose unit as a building block of cellulose is 5.15 Å in size. Consequently, enzyme attached to the cellulose surface cover hundreds of glucose units, i.e. potential hydrolysis sites (β-glucosidic bonds), while the hydrolysis reaction is proceeding, in the case of endoglucanases, only on one β-glucosidic bond (Figure 6.4). In other words, due to their size, enzymes are sterically blocking access for each other to otherwise potentially available hydrolysis sites on the surface of the substrate.
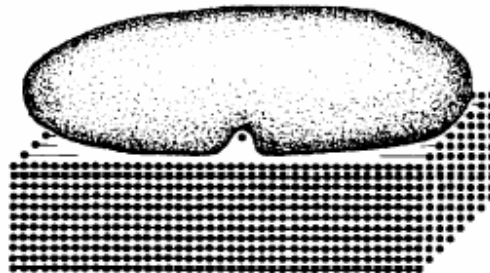
Figure 6.4: Schematic drawing of an enzyme catalyzed reaction of an insoluble substrate. The authors are suggesting that approximately 300 cellulose sites on the surface are covered by an absorbed enzyme since cellulase measures 200 x 35 Å and individual glucose molecules are 5.15 Å apart. Figure taken from Brown and Holtzapple, 1990.

External surface area is closely related to shape and particle size, and, thus, a higher surface area-to-weight ratio should mean more available adsorption sites per mass of substrate. Consequently, substrate pretreatment methods are often including cutting, i.e. reduction in size, of the lignocellulosic material to increase SSA. Also, removal of lignin and hemicellulose by the pretreatment methods causes extensive changes in the structure and accessibility of cellulose (complimentary to the desired effect of preventing

enzyme loss by unproductive binding to lignin). Their removal leaves the cellulose more accessible and more open to swelling on contact with cellulases (Grethlein et al., 1984).

As a general remark, it has to be acknowledged that the majority of the research has been conducted using relatively pure, model cellulosic substrates, which had been mechanically treated, and likely changed their chemical composition, and consequently, their characteristics. It is therefore often difficult and challenging to predict the effect of the various substrate characteristics on the hydrolysis of the "real", untreated, lignocellulosic material.

6.3   References

Brown RF, Holtzapple MT.1990. A comparison of the Michaelis-Menten and HCH-1 models. Biotechnol. Bioeng. 36: 1151-1154.

Divne C, Sinning I, Ståhlberg J, Pettersson G, Bailey A. 1993. Crystalization and preliminary x-ray studies on the core protein of cellobiohydrolaze I and endoglucanase I from *Trichoderma reesei*. J. Biol. Chem. 234: 905-907.

Divne C, Ståhlberg J, Reinikainen T, Ruohonen L, Pettersson G, Knowles JKC, Teeri TT, Jones TA. 1994. The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from *Trichoderma reesei*. Science 265: 524-528.

Fan LT, Lee Y-H, Beardmore DR. 1981. The influence of major structural features of cellulose on rate of enzymatic hydrolysis. Biotechnol. Bioeng. 23: 419-424.

Fan LT, Lee Y-H, Beardmore DR.1980. Mechanism of the enzymatic hydrolysis of cellulose: effects om major structural features of cellulose on enzymatic hydrolysis. Biotechnol. Bioeng. 22: 177-199.

Godfrey T, West S. 1996. Industrial Enzymology (2nd ed.) New York: Stockon Press. 609 p.

Grethlein HE, Allen DC, Converse AO. 1984. A comparative study of the enzymatic hydrolysis of acid pretreated whit pine and mixed hardwood. Biotechnol. Bioeng. 26: 1498-1505.

Gruno, M., Valjamae, P., Pettersen G. and Johansson, G. (2004) Inhibition of the *Trichoderma reesei* cellulases by cellobiose is strongly dependent on the nature of the substrate. Biotechnol. Bioeng. 86, 503-511.

Gusakov, A.V. and Sinitsyn, A.P. (1992) A theoretical analysis of cellulase product inhibition: effect of cellulase binding constant, enzyme/substrate ratio, and β-glucosidase activity on the inhibition pattern. Biotechnol. Bioeng. 40: 663-671.

Klyosov AA. 1990. Trends in biochemistry and enzymology of cellulose degradation. Biochem. 29: 10577-10585.

Krogh KBRM, Harris PV, Olsen CL, Johansen KS, Hojer-Pedersen J, Tjerneld F, Olsson L. Cloning and characterization of GH3 β-glucosidase from *Penicillium brasilianum* including a novel method for measurement of glucose inhibition of cellobiose hydrolysis. Submitted for publication.

Lynd LR, Weimer PJ, van ZylWH, Pretorius IS. 2002. Microbal cellulose utilization: fundamentals and biotechnology. Microbiol. Molec. Biol. Rev. 66: 506-577.

Mansfield SD, Mooney C, Saddler JN. 1999. Substrate and enzyme characteristics that limit cellulose hydrolysis. Biotechnol. Prog. 15: 804-816.

Masamune S, Smith JM. 1964. Adsorption rate studies - Significance of pore diffusion. AIChE J. 10: 246-252.

Ooshima H, Burns DS, Converse AO. 1990. adsorption of cellulase from *Trichoderma reesei* on cellulose and lignaceous residue in wood pretreated by dilute sulfuric acid with explosive decompression. Biotechnol. Bioeng. 36: 446-452.

Settler W, Esterbauer H, Glatter O, Steiner W.1989. The effect of enzyme concentration on the rate of the hydrolysis of cellulose. Biotechnol. Bioeng. 33: 1221-1234.

Tatsumoto K, Baker JO, Tucker MP, Oh KK, Mohagheghi A, Grohmann K, Himmel ME. 1988. Digestion of pretreated aspen substrates: hydrolysis rates and adsorptive loss of cellulase enzyme. Appl. Biochem. Biotechnol. 18: 159-174.

Zhang Y-H P, Lynd LR. 2004. Towards and aggregated understanding of enzymatic hydrolysis of cellulose: noncomplex cellulase systems. Biotechnol. Bioeng. 88: 797-824.

6.4 Article B

Enzymatic degradation of cellulose –

Investigation of declining hydrolysis rate

Natalija Andersen, Katja S. Johansen, Michael Michelsen, Erling H. Stenby,
Lisbeth Olsson

Enzymatic degradation of cellulose - Investigation of declining hydrolysis rate

Natalija Andersen[1,3], Katja S. Johansen[2], Michael Michelsen[3], Erling H. Stenby[3], Lisbeth Olsson[1,*]

[1] Center for Microbial Biotechnology, BioCentrum-DTU, Building 223, Technical University of Denmark, 2800 Lyngby, Denmark
[2] Novozymes A/S, Smørmosevej 25, 2880 Bagsværd, Denmark
[3] The Engineering Research Center – IVC-SEP, Department of Chemical Engineering – DTU, Building 229, Technical University of Denmark, 2800 Lyngby, Denmark

[*] Correspondence to: L. Olsson: Center for Microbial Biotechnology, BioCentrum-DTU, Building 223, Technical University of Denmark, 2800 Lyngby, Denmark, Tel.: +45 4525 2677; e-mail: lo@biocentrum.dtu.dk

Keywords: Avicel, PASC, *Humicola insolens*, *Penicillium brasilianum*

Abstract: In this study a series of experiments were performed in which either fresh substrate, or fresh enzymes were added to partially hydrolyzed model cellulosic substrates, Avicel and Phosphoric Acid Swollen Cellulose/Avicel (PASC) to determine factors influencing the hydrolysis rate. The composition of the optimal enzyme mixture used for hydrolysis was shown to be dependent on the substrate characteristics. The β-glucosidase enzyme, responsible for the reaction in the liquid phase, was shown to be rate limiting during degradation of PASC, while degradation of Avicel, as more crystalline substrate, was limited by the availability of substrate surface.

The effect of supplementing enzyme activities to partially hydrolyzed PASC was investigated. With the low enzyme loading used and supplements including only mono-component enzymes the largest increase in substrate conversion was observed upon addition of Cel7B, a novel cellobiohydrolase from *Penicillium brasilianum*. Additionally, multi-component enzyme mixtures and commercial enzyme mixture (CEM) were used. CEM supplemented in high concentrations showed major effect on the hydrolysis, while when both multi-component enzyme mixtures and CEM were supplemented in low concentrations no significant effect on the hydrolysis of partially hydrolyzed material was observed.

INTRODUCTION

Enzymatic degradation of cellulose has been extensively studied since the 1950's. This process is complex, as it incorporates not only a variety of different substrates and their characteristics (chemical composition, crystallinity, specific surface area, degree of polymerization; for review see Zhang and Lynd, 2004), but also the cooperative action of three enzyme classes, namely, endo-1,4-β-D-glucanases (EG), exo-1,4-β-D-glucanases (also called cellobiohydrolases, CBH) and 1,4-β-D-glucosidases (BG); for review see Lynd et al., 2002. One of the most important characteristics of this process is the declining hydrolysis rate with time (Zhang et al., 1999; Valjamae et al., 1998; and others). Product and substrate inhibition, together with inactivation of the enzymes, and, increase of substrate recalcitrance (through increase of substrate crystallinity and decrease of surface accessibility), are just some of the factors that has been claimed to contribute to the deceleration of hydrolysis rate (for review see Zhang and Lynd, 2004).

This study was designed to provide an increased understanding of factors resulting in declining hydrolysis rates. For that purpose, a series of experiments were performed in which either fresh substrate, or fresh enzymes were added to partially hydrolyzed model cellulosic substrates, Avicel and/or Phosphoric Acid Swollen Cellulose/Avicel (PASC). In particular, the effect of changes within the substrate and changes of enzyme characteristics throughout the hydrolysis, as well as, the importance of the composition of the enzyme mixture used for hydrolysis were investigated.

Cellulose is a chemically simple (linear polymer composed of β-1,4 bound D-anhydroglucopyranose units), but extremely recalcitrant substrate. Native cellulose has a degree of polymerization (DP) of up to 20 000 (O'Sullivan, 1997; Zhang and Lynd, 2004) and is composed of crystalline and amorphous regions. The two model cellulose substrates chosen for this study, Avicel and PASC, represent predominantly crystalline and amorphous material, respectively. PASC is prepared from Avicel, and its DP was determined to be 96, whereas DP of Avicel is 150 (Andersen, et al., submitted for publication). Crystallinity index (dimensionless) of PASC has been reported to be 0.00-0.04, and of Avicel 0.5-0.6, while specific surface area (SSA) [m^2 g^{-1}] is 240 and 20, respectively (Zhang and Lynd, 2004).

The second important component of the investigated system are the enzymes. During 48 hours of hydrolysis at 50 °C and pH=5.5, the protein molecules can be heat inactivated or, irreversibly or unproductively bound to cellulose, thus not further contributing to the hydrolysis process. Klyosov (1990) estimated the active time of enzymes on the surface of microcrystalline cellulose (Avicel) to be 25-35 minutes, while, on the surface of amorphous cellulose (PASC), 60-75 minutes. Moreover, turnover number (or the catalytic constant) of endoglucanases from various microbial sources are 0.01-0.7 s^{-1} on crystalline and 1.0-5.6 s^{-1} on amorphous
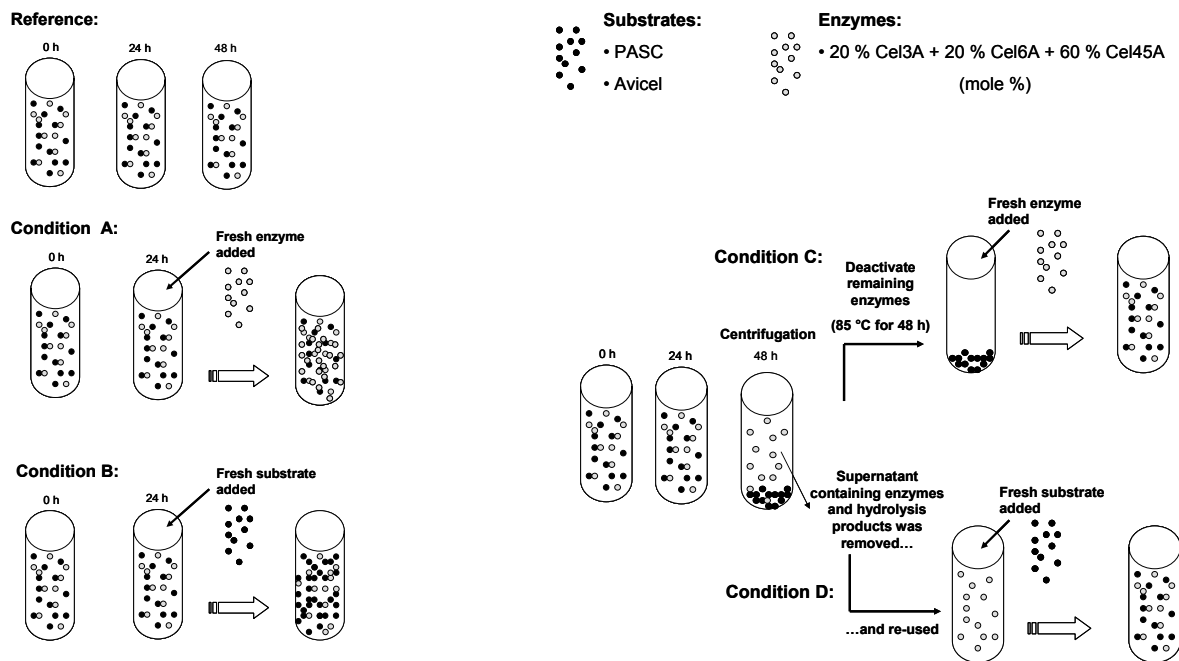
Figure 1: Illustrative diagram of <u>Investigation of bottlenecks in connection cellulose hydrolysis (Experiment 1)</u>. Two substrates: PASC and Avicel were hydrolyzed with Model Enzyme Mixture 1 (MEM 1) containing: 20 % Cel3A + 20 % Cel6A + 60 % Cel45A (mole %). Reference experiment and four experimental Conditions were performed. **In the reference experiment** hydrolysis was followed during 48 hours without introducing any changes to the experiment. **Condition A:** After 24 hours of hydrolysis the equimolar amount of fresh enzyme was added and hydrolysis was followed for another 24 hours. **Condition B:** After 24 hours of hydrolysis the fresh substrate was added and hydrolysis was followed for another 24 hours. Condition C and Condition D share the first 48 hours of hydrolysis (as in reference experiment), after what remaining (partially degraded) substrate was centrifuged and separated from supernatant. **Condition C:** Centrifuged substrate was washed and kept at 85 °C for 48 hours to deactivate remaining enzymes. After this treatment fresh enzymes were added and hydrolysis was followed for another eight hours. **Condition D:** Collected supernatant (containing soluble enzymes and hydrolysis products) was used to degrade fresh substrate; this hydrolysis reaction was followed for eight hours.

substrates (Klyosov, 1990). Thus, combining information given, approximately ten times more catalytic reactions are to be expected on PASC compared to Avicel.

Inactivation of *T. reesei* Cel6A (CBH II) during hydrolysis of short cellooligosaccharides, (DP 4-6) has been studied by Harjunpaa et al. (1996). They suggested that cellohexaose binding to a tunnel shaped active site of Cel6A may cause a twist, a minor conformational change, or a displacement of a sugar that will lead to slow, but time-dependent inactivation of the enzyme with a rate constant of 10^{-3} s^{-1}. Furthermore, they concluded that each Cel6A molecule on average processes 6000 cellohexaose molecules prior to inactivation. Moreover, Nidetsky et al. (1994), observed time-dependent inactivation of *T. reesei* CBH II with not only DP 6, but also with cellooligosaccharides of DP 4-8.

Inactivation, irreversible binding or unproductive binding of enzymes, have all been shown to have impact on the hydrolysis process. In this study, we are addressing the question: to which extent does the alteration of the enzyme characteristics, relative to the changes of the substrate characteristics, influence reduction of hydrolysis velocity.

Successful degradation of native cellulose requires cooperative action of multi-component enzyme system, such as those from genus *Trichoderma*, consisting of several *endo-* and *exo*-acting enzymes (Henrissat et al., 1998), and two β-glucosidase (Bhikhabhai et al., 1984;

Chen et al., 1992). Up to 70 % of the total cellulase protein in *T. longibrachiatum* RutC30 consists of *exo*-cellulases; CBH I (50 %) and CBH II (20 %) (Terri TT, 1997). Thus, two major questions in cellulose research are, why multiple cellulases are produced when there is only one type of bond (β1-4 linkage) present in cellulose, and, why is there such a predominance of CBH I? The presence of several EG may be due to the fact that different enzymes exhibit optimal performance under different environmental conditions (pH and temperature), or have different regulatory properties and kinetic parameters, and thus, the fungus has evolutionary developed to ensure its survival. Additionally, two *exo*-cellulases, CBH I and CBH II, are performing hydrolysis reaction from, reducing and non-reducing end of cellulose chain in a processive manner, respectively (Boisset, et al., 2000; Nidetsky et al., 1994; Harjunpaa et al., 1996). This processive action, which includes "pulling" cellulose chain away form its neighboring chains and simultaneous multiple hydrolysis reaction without dissociating from the substrate, is considered to be more difficult task than a "simple" bond hydrolysis of *endo*-glucanases, and thus CBH enzymes dominate (Wilson and Irwin, 1999).

The enzyme mixture used in this study consists of mono-component enzymes, endoglucanase Cel45A and cellobiohydrolase II Cel6A both from *H. insolens*, and, β-glucosidase Cel3A from *P. brasilianum*. Intentionally, mixture deficient in CBH I (the most abundant enzyme in

natural cellulolytic systems) was chosen. In order to try to increase the yield of soluble cellooligosaccharide production, an investigation of the effect of supplementing enzyme activities was carried out. Mono-component enzymes, multi-component enzyme mixture, commercial enzyme mixture (Celluclast 1.5 L and Novozym 188 in different concentrations), and fresh substrate were supplemented to partially hydrolyzed substrate in 16 different experiments. Such an experimental design is expected to enhance clarification of the observed hydrolysis rate stagnation, as well as, contribute to the mechanistic understanding of the enzymatic degradation of cellulose.

MATERIALS AND METHODS

Cellulose

Avicel® PH-101 was purchased from Fluka BioChemika, (Ireland) (product no. 11365) and it has an average particle size of about 50 μm. The Avicel concentration in the experiments was 10.0 g l^{-1}. PASC was prepared from Avicel according to Schülein (1997). The concentration of PASC was determined by dry weight measurements to be 9.0 g l^{-1}.

Enzymes and hydrolysis condition

Enzymes used in this study are summarized in Table I. All experiments were carried out in duplicates, at 50 °C, pH 5.5 and with 0.1 M sodium acetate buffer. Hydrolysis studies with PASC were performed in 3.6 ml NUNC plastic tubes with 2.5 ml final volume, while studies with Avicel were performed in 12 ml plastic tubes using a volume of 10 ml. Mixing was carried out by gentle inversion on SM 1 universal mixer (Sarstedt Condition).

Table I: Summary of enzymes used in this study

Mono-component enzymes				
	Class	Organism	MW [kDa]	CBM
Cel3A	BG	*P. brasilianum*	110	No
Cel5C	EG	*P. brasilianum*	65	Yes
Cel7B	CBH	*P. brasilianum*	60	No
Cel6A	CBH II	*H. insolens*	51	Yes
Cel45A	EG V	*H. insolens*	43	Yes
Cel7B	EG I	*H. insolens*	50	No
Cel7A	CBH I	*H. insolens*	70	Yes
MCN 188	BG	*A. niger*	91	No

Commercial enzymes		
	Class	Organism
Celluclast 1.5 L	EG/CBH	*T. reesei*
Novozym 188	BG	*A. niger*
CEM*	EG/CBH/BG	*T. reesei/A.niger*

BG: β-glucosidase; EG: Endoglucanase: CBH: Cellobiohydrolase; CBM: Carbohydrate Binding Module; MCN 188: Mono-component Novozym 188 (purified β-glucosidase); CEM*: Commercial Enzyme Mixture: Celluclast 1.5 L + Novozym 188 (3:1, v:v). The activity of CEM was determined to be 60.2 FPU/ml (Christensen, unpublished data). One unit (U) of activity is defined as the amount of enzyme hydrolyzing 1 μmol of substrate per minute; n.a.: not available.

Investigation of bottlenecks in connection to cellulose hydrolysis (Experiment 1)

PASC (9 g l^{-1}) and Avicel (10 g l^{-1}) were hydrolyzed with 0.1 μM Model Enzyme Mixture 1 (MEM 1) as described above. MEM 1 consisted of: 20 % Cel3A + 20 % Cel6A + 60 % Cel45A (mole %). Performed experiments can be divided in following conditions, see Figure 1:

Experimental conditions

Reference: Uninterrupted batch hydrolysis with substrate and enzyme for 48 hours.

Condition A: From zero to 24 hours hydrolysis was performed in the same way as in the reference experiment. At 24 hours fresh enzyme (0.1 μM MEM 1) was added.

Condition B: From zero to 24 hours hydrolysis was performed in the same way as in the reference experiment. At 24 hours fresh substrate (9 g l^{-1} PASC or 10 g l^{-1} Avicel) was added.

Condition C: From zero to 48 hours hydrolysis was performed in the same way as in the reference experiment. After 48 hours the remaining substrate was centrifuged (at 25 °C, 15000 g, and for 15 minutes) and supernatant was removed (and used in experimental Condition D). This partially degraded cellulose was washed with MQ-water several times to remove the main fraction of the absorbed enzymes, and it was kept in 0.1 M sodium acetate buffer at 85 °C for 48 hours to deactivate remaining enzymes. Subsequently, fresh enzymes (0.1 μM MEM 1) were added and hydrolysis was followed for another 8 hours.

A control experiment, containing only substrate (PASC or Avicel) in buffer at 85 °C for 48 hours was performed and it was confirmed that this treatment did not result in any hydrolysis of cellulose.

Condition D: From zero to 48 hours hydrolysis was performed in the same way as in the reference experiment. After 48 hours the remaining substrate was centrifuged, and the supernatant (containing enzymes and the 48-hour hydrolysis products) was used again to hydrolyze fresh substrate (9 g l^{-1} PASC or 10 g l^{-1} Avicel). This second part of hydrolysis was followed for another 8 hours.

Sampling: Samples were taken over a 48 h time course (for reference and experimental Conditions A-D) at: 0, 1, 2, 3, 5, 23, 25, 30 and 48 h. Samples at 2 hours for experiments with Avicel were omitted. Additional sampling (after the first 48 h) was performed at: 1, 3, 5, and 8 h for experimental Conditions C and D.

At the defined time points X μl of sample was withdrawn from the tube and the reaction was stopped by adding Y μl of 2 N NaOH (increasing the pH to 12.5). For hydrolysis of PASC X=200 and Y= 50. For hydrolysis of Avicel X=1600 and Y=400. Samples were appropriately diluted with MQ-water and filtered through a 0.45 μm syringe filter before being analyzed on Dionex HPLC.

Enzyme supplements to partially hydrolyzed substrates (Experiment 2)

To start with, 9.0 g l^{-1} of PASC was hydrolyzed by 0.1 μM of Model Enzyme Mixture 2 (MEM 2) in a total

volume of 50 ml. MEM 2 consisted of: 40 % Cel3A + 20 % Cel6A + 40 % Cel45A (mole %).

At 24 hours the remaining hydrolysis mixture was equally distributed to 17 reaction tubes and experiment was continued by adding extra enzymes or substrate as described in Table II.

Table II: Hydrolysis set-up for <u>Experiment 2</u> and results obtained during degradation of PASC

Hydrolysis set-up		Degradation of PASC	
ID	Supplements	Δ Conversion [C-mol C-mol-1] 24-31 h	St Dev [C-mol C-mol-1]
Reference	------	1.9	2.6
	Mono-component enzymes:		
# 1	0.1 µM Cel45A	3.2	0.9
# 2	0.04 µM Cel45A	2.3	1.6
# 3	0.1 µM Cel6A	1.3	1.4
# 4	0.02 µM Cel6A	3.4	1.0
# 5	0.1 µM Cel3A	1.6	0.2
# 6	0.04 µM Cel3A	2.3	0.7
# 7	0.1 µM Cel7B	4.4	1.0
# 8	0.1 µM Cel5C	1.5	0.1
# 9	0.1 µM Cel7A	0.8	1.0
# 10	0.1 µM Cel7B	0.7	0.4
# 11	0.1 µM MCN 188	0.2	1.3
	Enzyme mixtures:		
# 12	11.0 FPU g-1 cell. CEM	24.7	8.1
# 13	1.1 FPU g-1 cell. CEM	14.3	3.4
# 14	0.1 FPU g-1 cell. CEM	0.9	2.2
# 15	0.1 µM MEM 2	-1.0	2.0
	Substrate:		
# 16	9 g l-1 PASC	5.8	5.1

Hydrolysis set-up: Primary hydrolysis of PASC with 0.1 µM Model Enzyme Mixture 2 (MEM 2) was followed during 24 hours. MEM 2 consists of 40 % Cel3A + 20 % Cel6A + 40 % Cel45A (mole %). At 24 hours, primary hydrolysis was supplemented with: mono-component enzymes (see more details in Table I), enzyme mixtures (MEM 2 and Commercial Enzyme Mixture (CEM): Celluclast 1.5 L + Novozym 188 (3:1, v:v)) or substrate. MCN 188: Mono-component Novozym 188 (purified β-glucosidase). **Degradation of PASC**: Δ Conversion: difference in the conversion [C-mol C-mol-1]; St Dev: Standard deviation of the duplicate experiments.

Sampling: Hydrolysis was followed over 31 hours, samples being taken at: 0, 1, 2, 3, 5, 7, 23, 24, 25, 27, 29 and 31 h. At the defined time point 200 µl of sample was withdrawn from the tube and the reaction was stopped by adding 50 µl of 2 N NaOH (increasing the pH to 12.5). All samples were appropriately diluted in MQ-water and filtrated trough a 0.45 µm syringe filters before analyzed on Dionex HPLC.

Analytical procedures
Glucose (C_1), cellobiose (C_2), cellotriose (C_3), cellotetraose (C_4) and cellopentaose (C_5), and celloheksaose (C_6) were determined by HPLC with pulsed amperometric detection (HPAEC-PAD) using CarboPac PA 1 column (Dionex). A gradient system was used for separation: solvent A: 40 mM NaOH and solvent B: 100 mM NaOH, both containing 75 mM NaAc. The following gradient was used: 100 % A and 0 % B (0-15 min), a linear decrease of A to 0 % and a linear increase of B to 100 % (15-40 min), a linear increase of A to 100 % and a linear decrease of B to 0 % (40-50 min), Panagiotou and Olsson (2006). The flow rate was 1 ml min^{-1}. The products were quantified based on peak areas using standard sugars. Standard sugars, C_1-C_5, were purchased from Sigma-Aldrich (USA); C_6 was purchased form Seikagaku Corporation (Japan).

Calculation of "% conversion"
The amounts (in µM) of C_1, C_2, C_3, C_4, C_5 and C_6 determined as described above, were recalculated to C-mols and denoted (C-mols)$_{Cx}$, x=1-6. Consequently, the maximal theoretical amount of C-mols in the substrates, denoted (C-mols)$_{substrate}$, was calculated from determined DP values (Andersen et al., submitted for publication) and initial substrate concentration. The "% conversion" (C-mol C-mol^{-1}) was defined as $[(C-mols)_{Cx} / (C-mols)_{substrate}]*100$. "SUM % conversion" was defined as $\sum_{x=1}^{6}[(C-mols)_{Cx} / (C-mols)_{substrate}]*100$. In the further text "% conversion" and "SUM % conversion" will be represented in units (C-mol/C-mol).

In calculating % conversion, dilution of the hydrolysis broth due to addition of substrate or enzymes was taken into account.

RESULTS

The first part of this study, i.e., <u>Investigation of bottlenecks in connection to cellulose hydrolysis</u> (<u>Experiment 1</u>), utilize structural differences of the two substrates (Avicel and PASC) to explore suitability and/or limitations of the enzymes used, and consequently provide increased understanding of the factors resulting in decrease of hydrolysis rate.

The second part of this study focuses on investigation of rectitude (in terms of composition) of enzyme mixture used for hydrolysis by adding <u>Enzyme supplements to partially hydrolyzed substrate</u> (<u>Experiment 2</u>).

<u>Investigation of bottlenecks in connection to cellulose hydrolysis</u> (<u>Experiment 1</u>)

Under all hydrolysis conditions non-linear soluble sugar production with time was observed (Figures 2 and 3). During degradation of PASC the detectable products were glucose and cellobiose, while hydrolysis of Avicel generated exclusively glucose.

Conversion of PASC

Condition A and Condition B
Conversion of PASC in the reference experiment is presented in Figure 2A. From these results SUM % conversion was calculated to be 11.5 % [C-mol C-mol^{-1}] after 48 hours. When fresh enzyme was added after 24 hours of hydrolysis (Condition A), C_2 present in the hydrolysis broth was converted to C_1 (Figure 2B). SUM % conversion after 48 hours of hydrolysis reached 12.8 %.
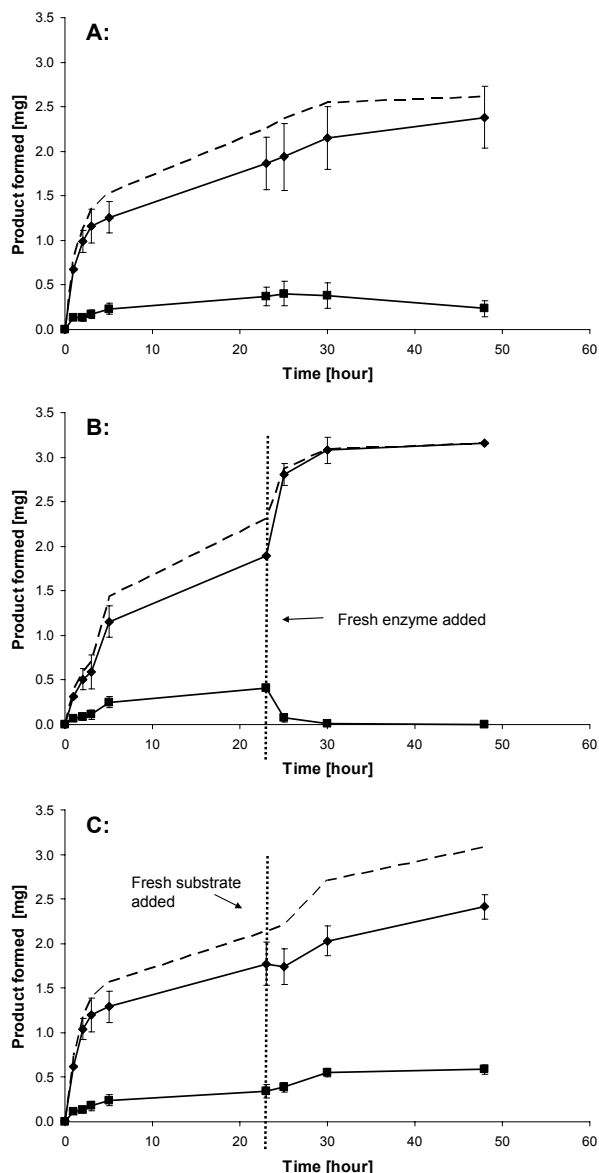
Figure 2: Hydrolysis profile of PASC with MEM 1 (20 % Cel3A + 20 % Cel6A + 60 % Cel45A (mole %)); Exp. 1. Total enzyme concentration was 0.1 μM. Experiments were performed in duplicates, with 9 g l^{-1} of substrate, in final volume of 2.5 ml, at 50 °C, pH 5.5 and in 0.1 M sodium-acetate buffer. The conversion of the substrate is presented in (mg l^{-1}) for each detected sugar, and represent the average of the two duplicate experiments. Error bars represent standard deviation of duplicate experiments. **A:** Reference experiment; **B:** Condition A experiments; **C:** Condition B experiments. Dotted lines in graphs B-E indicate the time point where the changes were introduced. C_1:♦; C_2: ■; Sum C_1-C_6: dashed line.

When fresh substrate was added to the 24-hour hydrolysis broth (Figure 2C, Condition B) intermediate increase of the conversion was observed; 15.6 % after 48 h (i.e. increase of 4.1 % relative to the reference sample), primarily emerging from the increase of C_2 concentration.

One hour hydrolysis rates after introducing the changes in experimental Condition A and B (24-25 h) were 0.41 and 0.42 [(C-mol C-mol^{-1}) h^{-1}], respectively; almost an order of magnitude smaller than initial (0-1 h) 1-h hydrolysis rate of the reference experiment, i.e. 3.9 [(C-mol C-mol^{-1}) h^{-1}] (Table III). Additionally, in Table III, difference in % conversion (Δ conversion) [C-mol C-mol^{-1}] for the relevant time points are reported.

Condition C and Condition D
When fresh enzyme was added to centrifuged, washed and for 48-hours hydrolyzed substrate (Condition C), only 100 mg l^{-1} of C_1 was additionally detected within 8 hours (data not shown); which was a analogue result to the one observed in experiments in Condition A. Results obtained with experimental Condition D show marginal increase in product formation; this time a small amount of C_2, was also produced (data not shown). In both cases the difference in % conversion between 48 and 55 h was similar (Table III). Nevertheless, the hydrolysis rate (48 to 49 h) of Condition D experiment is twice as fast as the rate of Condition C experiment, e.g 1.09 [(C-mol C-mol^{-1}) h^{-1}] and 0.56 [(C-mol C-mol^{-1}) h^{-1}], respectively.

Conversion of Avicel
The only hydrolysis product detected during degradation of Avicel with 0.1 μM MEM 1 (reference condition) was glucose. SUM % conversion yielded 0.2 % [C-mol C-mol^{-1}], or 26.3 [mg l^{-1}] after 48 hours of hydrolysis, data not shown. The initial hydrolysis rate (0-1 h) was found to be 0.06 [(C-mol C-mol^{-1}) h^{-1}], which is substantially slower (65 times) compared to the hydrolysis of PASC, Table III.

Condition A and Condition B
Addition of fresh enzyme to 24-hour hydrolyzed Avicel (Condition A), showed to be more efficient than addition of fresh substrate (Condition B), Figure 3A, with the Δ conversion (24-48 h) calculated to be 0.16 and 0.06 [C-mol C-mol^{-1}], respectively (Table III).

Condition C and Condition D
In contrast to the results obtained using PASC as substrate, results of the experimental Conditions C and D when Avicel was degraded (Figure 3B) both show moderate increase in product concentration relative to the initial 48-hour hydrolysis conversion. One hour hydrolysis rates at the time point where changes were introduced (i.e. 48-49 h) were 0.03 and 0.04 [(C-mo C-mol^{-1}) h^{-1}] for Condition C and D, respectively, e.g. at the same order of magnitude as the 0-1 h hydrolysis rate of the reference experiment. Interestingly, even though higher rate was observed in Condition D experiment, addition of fresh enzyme (Condition C) appears to have slightly higher effect on release of products obtained after 8 hours (Figure 3B).

Table III: <u>Experiment 1</u> – Hydrolysis rates and % conversion of the substrate.

ID	Degradation of PASC				Degradation of Avicel			
	Rate [(C-mol C-mol^{-1}) h^{-1}]	Δ Conversion [C-mol C-mol^{-1}]		St Dev [C-mol C-mol^{-1}]	Rate [(C-mol C-mol^{-1}) h^{-1}]	Δ Conversion [C-mol C-mol^{-1}]		St Dev [C-mol C-mol^{-1}]
		24-48 h	48-55 h			24-48 h	48-55 h	
Reference	3.88 (a)	0.50	n.a.	2.10	0.06 (a)	0.06	n.a.	0.06
Condition A	0.41 (b)	1.60	n.a.	0.20	0.02 (b)	0.16	n.a.	0.03
Condition B	0.42 (b)	5.20	n.a.	0.96	0.02 (b)	0.06	n.a.	0.00
Condition C	0.56 (c)	n.a.	1.00	1.90	0.03 (c)	n.a.	0.07	0.03
Condition D	1.09 (c)	n.a.	1.10	1.50	0.04 (c)	n.a.	0.01	0.02

Rate: One hour hydrolysis rate reported for the time point of the experiment where changes were introduced; (a): 0-1 h; (b): 24-25 h; (c): 48-49 h; Δ Conversion: difference in the conversion [C-mol C-mol^{-1}]; St Dev: Standard deviation of the duplicate experiments; n.a.: not applicable.
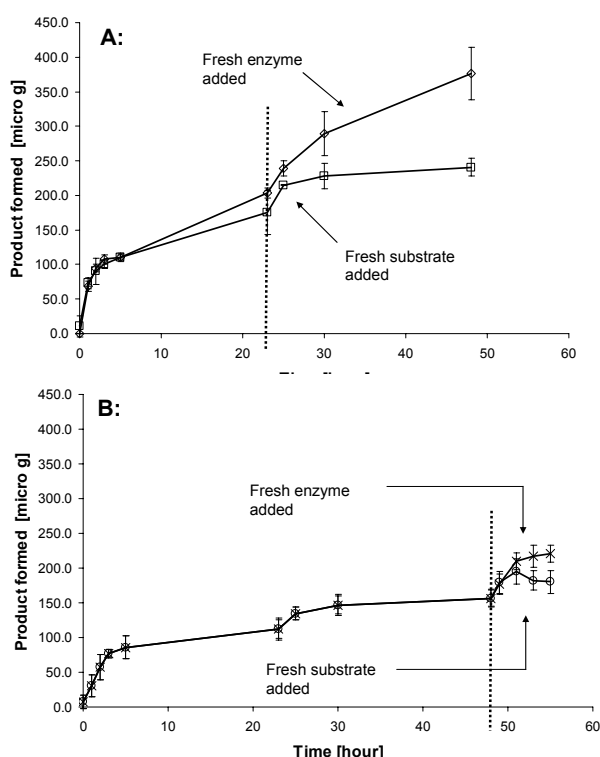
Figure 3: Hydrolysis profile of Avicel with MEM 1 (20 % Cel3A + 20 % Cel6A + 60 % Cel45A (mole %)); Exp. 1. Total enzyme concentration was 0.1 μM. Experiments were performed in duplicates, with 10 g l^{-1} of substrate, in final volume of 10 ml, at 50 °C, pH 5.5 and in 0.1 M sodium-acetate buffer. The only detected hydrolysis product was glucose. Error bars represent standard deviation of duplicate experiments. **A:** Condition A: ◊ and Condition B: □; **B:** Condition C: x and Condition D: ○. Vertical dotted lines indicate the points where the changes were introduced.

Enzyme supplements to partially hydrolyzed substrate (Experiment 2)

The particular enzyme mixture used in <u>Experiment 1</u> did not include the most abundant enzyme in natural cellulolytic systems, namely, CBH I. In order to try to increase the yield of soluble cellooligosaccharides production, <u>Experiment 2</u> was designed. This experiment focuses on investigation of the effect of supplementing primarily hydrolysis with: a) mono-component enzymes, b) multi-component enzyme mixture, or c) fresh substrate. Hydrolysis set-up of <u>Experiment 2</u> is explained in more details in Table II. Additionally, as it was concluded from <u>Experiment 1</u> that the amount of Cel3A in MEM 1 was insufficient for total removal of C_2 from the hydrolysis broth, in <u>Experiment 2</u> a new multi-component enzyme mixture, i.e. MEM 2 was used; Opposed to MEM 1 (20 % Cel3A + 20 % Cel6A + 60 % Cel45A, mole %), MEM 2 included higher mole percentage of β-glucosidase Cel3A (40 % Cel3A + 20 % Cel6A + 40 % Cel45A, mole %).

In the reference experiment 20.4 % [C-mol C-mol^{-1}] of substrate was converted within the 31 hour of hydrolysis; hydrolysis product was predominantly glucose with small amounts of cellobiose also being produced (Figure 4). The initial hydrolysis rate (0-1 h) was 7.1 [(C-mol C-mol^{-1}) h^{-1}] (data not shown); almost double as high as the rate observed in the reference condition of <u>Experiment 1</u> (Table III). The same substrate concentration and environmental conditions (pH, temperature and enzyme load) were used; the only difference was, as previously noticed, in the enzyme composition.
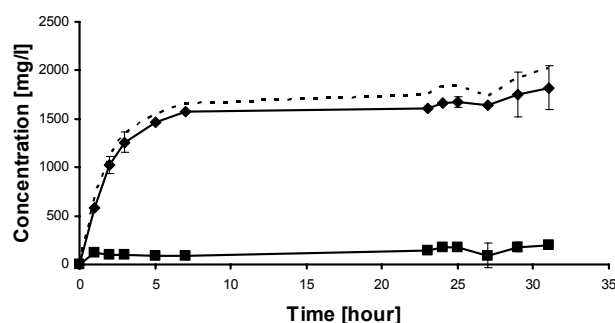
Figure 4: Hydrolysis of PASC with MEM 2 (40 % Cel3A + 20 % Cel6A + 40 % Cel45A (mole %)): Exp. 2, Reference hydrolysis. Total enzyme concentration was 0.1 μM. Experiments were performed in duplicates, with 9 g l^{-1} of substrate, at 50 °C, pH 5.5 and in 0.1 M sodium-acetate buffer. Error bars represent standard deviation of duplicate experiments. C_1:◆; C_2: ■; Sum C_1-C_6: dashed line.

Supplements with mono-component enzymes

In general, addition of mono-component enzymes in low concentrations (up to 0.1 μM) did not have any major effect on the hydrolysis, data not shown. Enzymes used in primarily hydrolysis, i.e. Cel45A, Cel6A and Cel3A were supplemented in two concentrations; one corresponding to the amount of the particular enzyme used in MEM 2 (i.e. 0.04 μM Cel45A, 0.02 μM Cel6A and 0.04 μM Cel3A), and, the other corresponding to the total enzyme concentration used (0.1 μM). Addition of 0.1 μM Cel45A contributed to slightly higher substrate conversion (21.7 %) compared to supplement with 0.04 μM, in which case 20.8 % of the substrate was converted. The opposite was observed for Cel6A and Cel3A; upon addition of higher amount (0.1 μM) of these two enzymes lower conversion of the substrate was observed then when 0.02 or 0.04 μM were added, respectively (Table II).

The highest % conversion of the substrate (22.9 %, data not shown) in experiments # 1-11 was observed by supplementing primarily hydrolysis with 0.1 μM Cel7B, a CBH from *P. brasilianum*. As expected, substantial increase in cellobiose production was observed. In Table II, difference in % conversion of the substrate from 24 to 31 h are reported. To our surprise, Cel7A (CBH I) *H. insolens* supplement did not have significant effect on substrate conversion. It is important to stress here that there is considerable difference in the quantitative distribution of cellulase components from the representatives of *Trichoderma* and *Humicola* strains. Namely, the quantitatively dominant enzyme (50 %) in *T. longibrachiatum* Rut C30 broth is Cel7A (CBH I), opposed to *H. insolens* broth which comprise of 50 % Cel7B (EG I), and only 20 % Cel7A (Tolan and Foody, 1999).

Supplements with enzyme mixtures or substrate

Supplementing primarily hydrolysis with 11.0 FPU g^{-1} cellulose CEM (Celluclast 1.5 L + Novozym 188; 3: 1, v:v), resulted in 1-h hydrolysis rate (24-25 h) of the same order of magnitude as the 1-h rate of reference experiment (0-1 h), data not shown. Additionally, more than a doubling of value reported for % conversion was observed within the 7 hours upon addition of supplement, e.g. conversion of the substrate reached 43.2 %, Figure 5A.

Figure 5B shows product formation after supplementing hydrolysis with 1.1 FPU g^{-1} cellulose CEM. The 1-h reaction rate was one tenth of the rate reported for experiment where 11.0 FPU g^{-1} cellulose CEM was supplemented, and final conversion of the substrate reached 32.8 %, Table II.

Results shown in Figures 5C and 5D represent addition of 0.1 FPU g^{-1} cellulose CEM and 0.1 μM MEM 2, respectively. No significant difference of the hydrolysis process with these two multi-component enzyme mixtures could be observed.

Upon supplementing primarily hydrolysis with 9 g l^{-1} of PASC, production of C$_2$ and small amounts of C$_3$ and C$_4$ was observed, while glucose concentration remained unchanged, data not shown. Final conversion of

the substrate increased slightly (reaching 24.3 %) compared to the reference sample, and calculated 1-h rate (24-25 h) was 2.4 [(C-mol C-mol^{-1}) h^{-1}], data not shown.
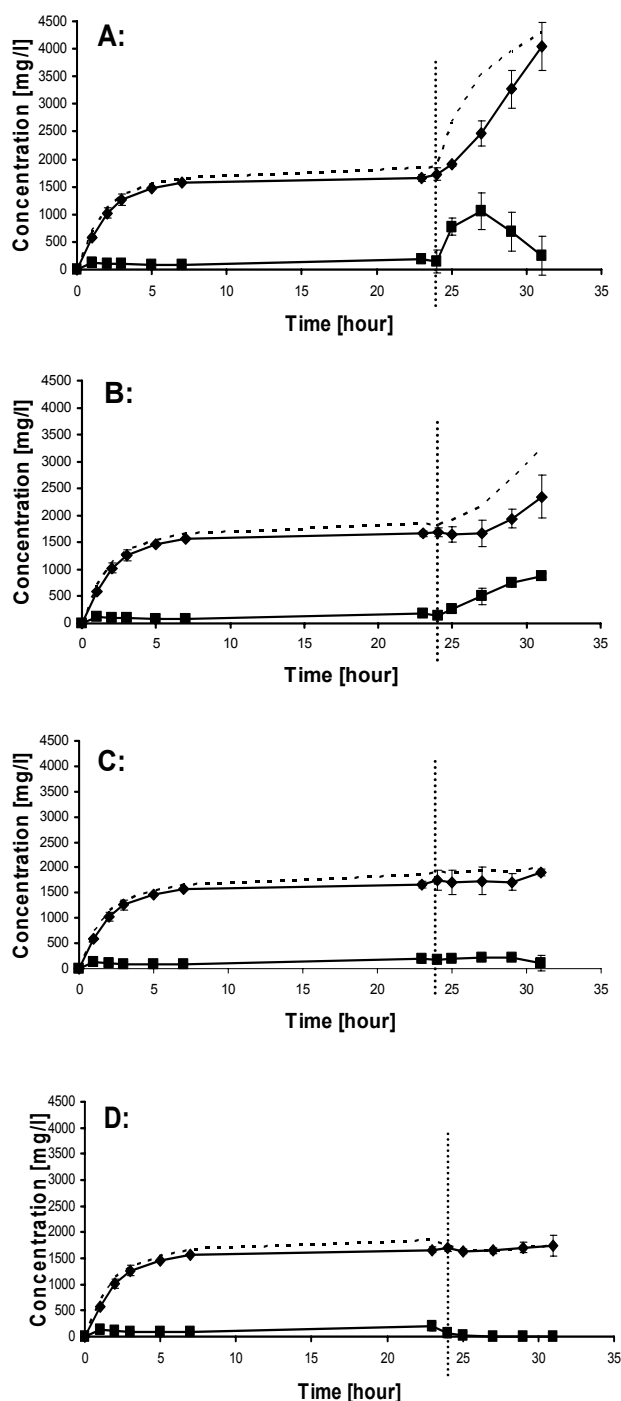
Figure 5: Hydrolysis of PASC with MEM 2 (40 % Cel3A + 20 % Cel6A + 40 % Cel45A (mole %)): Exp. 2, # 12- # 16. Experiments were performed in duplicates, with 9 g l^{-1} of substrate, at 50 °C, pH 5.5 and in 0.1 M sodium-acetate buffer. Error bars represent standard deviation of duplicate experiments. C$_1$:♦; C$_2$: ■; Sum C$_1$-C$_6$: dashed line. From 0 to 24 h enzyme concentration was 0.1 μM MEM 2. At 24 h hydrolysis was supplemented with: **A:** 11 FPU g^{-1} cellulose Commercial Enzyme Mixture (CEM): Celluclast 1.5 L + Novozym 188 (3:1, v:v); **B:** 1.1 FPU g^{-1} cellulose CEM; **C:** 0.11 FPU g^{-1} cellulose CEM; **D:** 0.1 μM MEM 2.

DISCUSSION

In this study we aimed at investigating to which extent is the declining hydrolysis rate is influenced by the changes related to the applied enzymes, and/or, the structural modifications of the substrate.

Experiments were intentionally performed with low enzyme loading, e.g., 0.1 μM, for several reasons: 1) Low enzyme loading provides maximum insight into detailed hydrolysis pattern as it particularly promotes production of higher soluble cellooligosaccharides (DP 3-6) (Andersen et al., submitted for publication); 2) In the industrial application of this process (e.g., production of biofuels), enzyme cost constitutes the largest share of the overall process cost, and it is thus of both industrial and academic interest to investigate the hydrolysis process under low enzyme loadings. Moreover, the mono-component enzymes we worked with were only available in limited amounts.

Both enzymes and substrate contribute to the declining hydrolysis rate

Experiments were performed on two cellulose substrates, Avicel and PASC and with the mono-component enzyme mixture (MEM 1) consisting of all three classes of cellulolytic enzymes, namely Cel3A (BG), Cel6A (CBH II) and Cel45A (EG V).

Addition of the fresh enzyme after 24 hours of hydrolysis of PASC enhanced degradation of C_2 to C_1, but only marginally influenced overall conversion of the substrate (Figure 2B). BG is generally recognized to be responsible for removal (degradation) of C_2 from hydrolysis broth. The particular BG used has been shown to be stable under the experimental conditions used (pH = 5.5, T = 50°C) and it is not prone to cellobiose inhibition at concentrations of 0.29 g/l (detected in the broth). Thus, our results demonstrate that Cel3A in the particular enzyme mixture used in the present study (MEM 1) is insufficient to remove all C_2 from the broth. The other two enzymes from MEM 1, Cel6A and Cel45A, additionally added after 24 hours of primarily hydrolysis does not seem to contribute to further degradation of PASC, as no increase of C_2 or higher cellooligosaccharides (DP 3-6) was detected. These enzymes (opposed to Cel3A, which reacts on the soluble cellooligosaccharides in the hydrolysis broth) react on the surface of substrate, and thus, need to absorb to the available/accessible binding sites prior to the hydrolysis reaction. Based on the literature data, concentrations of C_1 and C_2 present in the broth (Figure 2B) are not high enough to trigger product inhibition mechanism, for those two enzymes (Gusakov and Sinitsyn, 1992; Holtzapple et al., 1990; Valjamae 2001; and others).

Generalizing available data from the literature, cellulolytic enzymes are strongly inhibited by cellobiose and weakly inhibited by glucose, but the effect of product inhibition can not always be predicted. For example, Gruno et al. (2004) have studied the inhibition effect of cellobiose on the initial stage of hydrolysis of *T. reesei*

Cel7A (CBH I) and three endoglucanases, Cel7B (EG I), Cel5A (EG II) and Cel12A (EG III). They observed inhibition of Cel5A (K_i=34±6 mM, i.e. 11.6±2.0 g l^{-1}), and, activation of Cel12A by cellobiose during hydrolysis of amorphous cellulose. The results that we observed (Figure 2C), confirm that our enzymes are still active after 24 hours of hydrolysis, but the presence and extent of enzyme inhibition should be investigated in a separate study.

Besides the noticeable difference in the amount of products released when degradation of PASC and Avicel are compared, addition of fresh enzyme after 24 hours of hydrolysis of Avicel contributed to higher product release than addition of fresh substrate (Figure 3B). This result is opposite of what was observed for degradation of PASC (Figure 2A and 2B). The explanation of our results lays in the markedly different structure of the two cellulosic substrates. Approximately sixty percent of Avicel is crystalline cellulose, and its specific surface area is estimated to be ten times smaller compared to PASC (Zhang and Lynd, 2004). It is, thus, much more difficult for the enzymes to absorb to its surface and perform the hydrolysis reaction than if PASC is used as substrate. As the amount of released products during degradation of Avicel is less compared to degradation of PASC, the relative amount of Cel3A (compared to the amount of Cel45A and Cel6A) in the enzyme mixture used for degradation of both substrates is no longer the rate limiting factor. In other words, the reaction on the surface of the insoluble cellulose is restraining the overall hydrolysis of Avicel.

With Avicel as substrate (Figure 3C), the 1-h hydrolysis rate at the point where changes were introduced (48-49 h) was of the same order of magnitude as the rate at the beginning of the reaction (0-1 h) (Table III). Thus, the results indicate that the enzymes are still active, and moreover that there are more available binding sites on the substrate from where the hydrolysis reaction can proceed. Nevertheless, even though the progress on the relative basis seems to be considerable, the overall conversion of Avicel reached in these experiments is neglectable. On the other hand, when degradation of PASC is investigated, results indicate that neither enzymes nor substrate can substantially contribute to further hydrolysis (experimental Condition C and D, data not shown). A possible explanation may be that a portion of the enzymes might (still) be trapped in the bulky, swollen regions of PASC, influencing the results by sterically blocking the access of fresh enzymes (in Condition C experiments), or by decreasing the overall amount of the enzymes (i.e. substrate/enzyme ratio) that could perform hydrolysis (in Condition D experiments).

Mono-component enzyme supplements did not substantially increase substrate degradation

As it was concluded from Experiment 1 that the amount of Cel3A in MEM 1 was insufficient for total removal of C_2 from the hydrolysis broth, in Experiment 2 a new multi-component enzyme mixture, i.e. MEM 2 was used.

Deliberately, this mixture was not containing CHB I activity, the most abundant cellulase naturally excreted by cellulolytic organisms. Our expectations were, thus, to achieve elevated hydrolysis of the substrate upon subsequent addition of, in particular, CBH I, but also other investigated enzymes (Table II). With the low enzyme loadings used, none of the mono-component enzymes supplemented showed considerable increase in substrate conversion (Table II). Yet, the largest increase in product conversion was observed upon subsequent addition of Cel7B, novel CBH from *P. brasilianum*. This enzyme have been cloned and expressed in *Aspergillus* host, and preliminary sequence information indicates that it belongs to CBH I class of enzymes (Krogh, personal communication).

In Figure 5A the addition of 11 FPU g^{-1} cellulose of CEM clearly illustrates that the substrate can be degraded to much higher extent compared to degradation with MEM 2 (or MEM 1). The molar concentration of CEM can not be precisely calculated as the composition and amounts of the individual enzymes present are not publically available. For comparison, an intermediate enzyme loading is commonly considered to be 20 FPU g^{-1} cellulose or around 5.5 µM (calculated with the average cellulase MW of 70 kDa). Thus, not only the considerably higher enzyme load, but also the protein composition of CEM have an impact on the observed results. Besides expected cellulolytic activities (EG, CBH and BG) this commercial enzyme mixture exhibits ability to degrade hemicelluloses (Kabel et al., 2006) which makes it significantly superior compared to the three mono-component enzymes from MEM.

CONCLUSION

Depending on the substrate used, different enzyme components showed to be of different importance. PASC hydrolysis, due to its relatively high digestibility, resulted in high amounts of soluble intermediate products, that later needed to be degraded to glucose by Cel3A, which was shown to be present in insufficient amounts. Thus, the liquid phase reaction was limiting further degradation of PASC. Degradation of Avicel, as more crystalline substrate, was shown to be hindered by limited availability of the surface of the substrate. Under the condition of the present study we can, thus, conclude that different molar ratio of the three enzyme classes needs to be considered for the maximal conversion of different types of cellulosic substrates, e.g., more crystalline or more amorphous material. For industrial practice, the benefit of constructing "tailor made" enzyme mixtures for the particular use would be evident. Moreover, step-vice addition of the particular limited enzyme activity throughout the hydrolysis process could be suggested.

Mono-component enzymes supplemented in low amounts only marginally improved hydrolysis. In particular, addition of CBH I was expected to elevate hydrolysis. This was true for Cel7B from *P. brasilianum*, but did not apply to Cel7A, CBH I from *H. insolens*. Thus, our results indicate that absence of this particular enzyme activity was not the rate limiting factor in hydrolysis of PASC.

As expected, supplement of 11.0 FPU g^{-1} cellulose CEM showed sharp increase in hydrolysis. In low concentrations (0.1 FPU g^{-1} cellulose), CEM performed the same as MEM 2.

REFERENCES

Andersen, N., Johansen, K.S., Michelsen, M., Stenby, E.H., Krogh, K.B.R.M. and Olsson, L. Enzymatic hydrolysis of cellulose using mono-component enzymes show synergy during hydrolysis of Phosphoric Acid Swollen Cellulose (PASC), but competition on Avicel. Submitted for publication to Enz. Microbial Technol.

Bhikhabhai R., Johansson G. and Pettersson G. (1984) Isolationof cellulolytic enzymes from *Trichoderma reesei* QM 9414. J. Appl. Biochem. 6, 336-345.

Chen H., Hayn M. and Esterbauer H. (1992) Purification and characterization of two extracellular beta-glucosidases from *Trichoderma reesei*. Biochem. Biophys. Acta 1121, 54-60.

Gruno, M., Valjamae, P., Pettersen G. and Johansson, G. (2004) Inhibition of the *Trichoderma reesei* cellulases by cellobiose is strongly dependent on the nature of the substrate. Biotechnol. Bioeng. 86, 503-511.

Gusakov, A.V. and Sinitsyn, A.P. (1992) A theoretical analysis of cellulase product inhibition: effect of cellulase binding constant, enzyme/substrate ratio, and β-glucosidase activity on the inhibition pattern. Biotechnol. Bioeng. 40, 663-671.

Harjunpaa, V., Teleman, A., Kiovula, A., Ruohonen, L., Teeri, T.T., Teleman, O. and Drakenberg, T. (1996) Cello-oligosaccharide hydrolysis by cellobiohydrolase II from *Trichoderma reesei*. Association and rate constant derived from analysis of progress curves. Eur. J. Biochem. 240, 584-591.

Henrissat, B., Teeri, T.T. and Warren, R.A.J. (1998) A scheme for designating enzymes that hydrolyze the polysaccharides in the cell walls of planters. FEBS Lett. 425, 352-354.

Holtzapple, M., Cognata, M., Shu, Y.and Henrickson C. (1990) Inhibition of *Trichoderma reesei* cellulase by sugars and solvents. Biotechnol. Bioeng. 36, 275-287.

Karlsson, J., Siika-aho, M., Tenkanen, M. and Tjerneld F. (2002) Enzymatic properties of low molecular mass endoglucanases Cel12A (EG III) and Cel45A (EG V) of *Trichoderma reesei*. J Biotechnol 99: 63-78.

Klyosov, A.A. (1990) Trends in biochemistry and enzymology of cellulose degradation. Biochemistry 29, 10577-10585.

Krogh, K.B.R.M., personal communication.

Lynd, L.R., Weimer, P.J., van Zyl, W.H., Pretorius, I.S.(2002) Microbial cellulose utilization: fundamentals and biotechnology, Microbiol. Molecul. Biol. Rev. 66, 506-577.

Nidetzky, B., Zachariae, W., Gercken, G., Hyan, M. and Steiner, W. (1994) Hydrolysis of cellooligosaccharides by *Trichoderma reesei* cellobiohydrolases: experimental data and kinetic modeling. Enzyme Microb. Technol. 16, 43-52.

O'Sullivan, A.C. (1997) Cellulose: the structure slowly unravels. Cellulose 4, 173-207.

Panagiotou, G. and Olsson, L. (2006) Effect of compounds released during pretreatment of wheat straw on microbial growth and enzymatic hydrolysis rates. Biotechnol. Bioeng. 96, 250-258.

Schou, S. (1993) A study of cellulolytic enzymes of fungal and bacterial origin. Ph. D. Thesis.

Schulein, M. (1997) Enzymatic properties of cellulases from *Humicola insolens*. J. Biotech. 57, 71-81.

Terri, T.T. (1997) Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. Trends Biotechnol. 15, 160-167.

Tolan, J.S., Foody, B. (1999) Cellulases from submerged fermentation. Adv. Biochem. Eng./Biotechnol. 65, 41-67.

Valjamae, P., Pettersson, G. and Johansson G. (2001) Mechanism of substrate inhibition in cellulose synergistic degradation. Eur. J. Biochem. 268, 4520-4526.

Valjamae, P., Sild, V., Pettersson, G. and Johansson G. (1998) The initial kinetics of hydrolysis by cellobiohydrolases I and II is consistent with a cellulose surface-erosion model. Eur. J. Biochem. 153, 469-475.

Wilson, D.B. and Irwin, D.C. (1999) Genetics and properties of cellulases. Adv. Biochem. Eng./Biotech. 65, 1-21.

Zhang, S., Wolfgang, D.E. and Wilson D.B. (1999) Substrate heterogeneity causes the nonlinear kinetics of insoluble cellulose hydrolysis. Biotechnol. Bioeng. 66, 35-41.

Zhang, Y-H.P. and Lynd, L.R. (2004) Towards and aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. Biotechnol. Bioeng. 88, 797-824.

CHAPTER 7

Mathematical modeling of enzymatic degradation of cellulose

Per definition a mathematical model is "the *general* characterization of a process, object, or concept, in terms of mathematics, which enables the *relatively simple* manipulation of variables to be accomplished in order to determine how the process, object, or concept would behave in different situations" (www.oalj.dol.gov/public/dot/refrnc/glossary.htm, 26.10.2006). A model generally incorporates a number of parameters that are used to describe the desired process. The accuracy, to which the different parameters used in the model are experimentally determined, is usually an important issue. If parameters are difficult to determine, the introduction of errors in the model is inevitable. Thus, increasing the complexity of the model should be carefully evaluated as the uncertainty of the model can increase with increasing the number of parameters, as each parameter can introduce some additional variance into the system. Another important issue related to the parameter values is the physico-chemical space within which determined parameters can be applied. As the model should help us predict and understand the behavior of the system in a variety of different conditions it is desired that the parameters used in the model as well are applicable across a wide range of physico-chemical conditions. Thus, the task of mathematical modeling of enzymatic degradation of cellulose is highly challenging as it is necessary to balance complex biological process with many variables, with the basic requirement of a model, i.e. simplicity and robustness. It is therefore usually appropriate to make some approximations to reduce the model to a sensible size.

Given the complexity of enzymatic degradation of cellulose, multiple enzymes involved and continuously changing substrate features, it is not surprising that a big group of the models developed for describing this process are of empirical nature (King, 1966; Ghose, 1969; Brandt, et al., 1973; Lee, et al., 1980; Gharpuray, et al., 1983). An

additional group of the models describing enzymatic hydrolysis of cellulose in the literature can be defined as semi-empirical and involve one or only a few substrate variables and usually only one enzyme activity (representing lumped action of endoglucanases (EG), cellobiohydrolases (CBH) and β-glucosidases (BG)) (Huang, 1975; Howell and Mangat, 1978; Peiterson and Ross, 1979; Ryu, et al., 1982; Gan, et al., 2003). Only a few models involve a single substrate state variable and, at the same time, distinguish between the three enzyme classes involved (Okazaki and Moo-Young, 1978). The extensive summary of the models, dividing them in three groups, non-mechanistic, semi-mechanistic and functionally based models, is presented elsewhere (Zhang and Lynd, 2004). In Table 7.1, I present comparison of some of the above mentioned kinetic models.

Table 7.1: Comparison of some of the reported kinetic models of enzymatic hydrolysis of cellulose (adopted from Gan et al., 2003).

State of substrate	Enzyme system	Kinetic approach	Product inhibition	Reference
Homogenious material	E_{12}	QSS	Competitive	Howell and Stuck (1975)
Homogenious material	E_{123}	MM	Competitive	Huang (1975)
Degree of polymerization	E_1, E_2, E_3	MM	Non-competitive	Okazaki and Moo-Young (1978)
Homogenious material	E_{123}	QSS	Competitive	Howell and Mangat (1978)
Crysralline and amorphous	E_{123}	MM		Peitersen and Ross (1979)
Crysralline and amorphous	E_{123}	QSS	Competitive	Ryu et al. (1982)
Homogenious material	E_{12}, E_3		Non-competitive	Fan and Lee (1983)
Active and inert	E_{123}	QSS	Competitive	Gan et al. (2003)

E_1 – Endoglucanase; E_2 – Cellobiohydrolase; E_3 – β-glucosidase; E_{12} – Combined E_1 and E_2; E_{123} – Combined E_1, E_2 and E_3; QSS – Quasi-stady state; MM – Michaelis-Menten.

During this PhD study, I was working to obtain experimental data that could be evaluated against the mathematical model with the intention to:

- Identify and understand the most crucial aspects of the hydrolysis process,
- Contribute distinguishing bottlenecks of the process, and,
- Consequently lead us towards suggestions how to improve the hydrolysis process.

The desired model that could cover all of these requirements would need to be of mechanistic nature, e.g. appropriately describing the underlying mechanism of enzymatic degradation of cellulose, without introducing too many parameters. The model presented by Okazaki and Moo-Young (1978) was found to be appropriate for this purpose, in particular because all three enzyme classes involved in the hydrolysis process (e.g. cellobiohydrolases (CBH), endoglucanases (EG) and β-glucosidases (BG)) have been individually incorporated into the model they described. Additionally, minor improvements of the model were introduced, related, in particular, to the action of BG.

In the experimental studies (Chapter 5, Article A), the focus was particularly turned towards using highly purified, mono-component enzymes to degrade two model cellulose substrates (more crystalline and more amorph substrates, Avicel and PASC, respectively), while keeping constant physical conditions of the hydrolysis. With this experimental set-up, the unique hydrolysis pattern of each enzyme class used can be investigated in isolation, and in cooperation with other enzymes. Nevertheless, some limitations to this experimental set-up do exist. Purification of enzyme components is costly and tedious, and thus experiments were performed with low enzyme loadings. Additionally, we need to be aware of the fact that in some experiments a suboptimal enzyme mixture was used, as not all enzymes readily produced by cellulose degrading microorganisms were available in purified form. Thus, the reader is requested to keep this in mind when evaluating the quantitative results of the hydrolysis process.

If a mathematical model is designed in a way to appropriately describe the hydrolysis process, both quantitatively and qualitatively, the model could consequently be used for prediction purposes. For example, the behavior (outcome) of the system could be quickly and inexpensively evaluated/calculated if different substrates, enzymes or enzyme loading are used in the model.

7.1 Major aspects and challenges during modeling of hydrolysis process

Factors affecting enzymatic degradation of cellulose have been discussed in Chapter 6. In this chapter I would like to present some examples and give a short overview of how different authors have approached the problem of describing hydrolysis process and modeling by relating it to their observed results. Please note that examples presented here are not listed in the order of importance, nor that I attempt to make direct comparisons between them. Some frequently discussed factors that influence enzymatic hydrolysis of cellulose and their consequences on the modeling process are summarized in Table 7.2.

Table 7.2: Major factors influencing enzymatic degradation of cellulose and their consequence (marked as 1-5) on the hydrolysis and modeling of this process.

	1	2	3	4	5
Substrate related factors					
Cellulose crystallinity	x			x	
Degree of polymerization (DP)		x			
Available/accessible surface area	x	x	x	x	x
Structural organization (micro- and macro-structure)			x	x	
Particle size			x	x	
Porosity			x	x	x
Presence of lignin and hemicellulose	x			x	x
Enzyme related factors					
Nature of enzyme system employed (EG, CBH, BG)	x	x	x	x	x
Relative size of enzymes				x	
Enzyme concentration	x	x	x	x	
Adsorption	x	x	x	x	x
Synergism	x	x		x	
End-product inhibition				x	x
Mechanical deactivation					x
Thermal inactivation					x
Irreversible (non-productive) binding to lignin				x	x

1: Apparent increase of substrate crystallinity throughout the hydrolysis;
2: Decrease of DP;
3: Continuous changes in surface accessibility, and surface topology ("surface corrosion");
4: Continuous changes in substrate digestibility;
5: Decrease of active enzyme concentration;
EG: Endoglucanase; CBH: Cellobiohydrolase; BG: β-glucosidase

7.1.1 Specific surface area (SSA) and crystallinity index (CrI)

In a mechanistic model, it is essential to include the effect of the initial structural features of cellulose and their changes during hydrolysis. Fan et al. (1981) developed an empirical expression, based on the linear regression analysis of the experimental results, for hydrolysis of cellulose after eight hours, X_8:

$$X_8 = 0.380 \cdot (SSA)^{0.195} \cdot (100 - CrI)^{1.04}$$

Eq. 1

The only two structural features included in this model were SSA and CrI. From this expression, the rate of hydrolysis appears to be more sensitive to the crystallinity index, than to specific surface area. Experimentally, the authors have observed substantial increase in the SSA (based on unit weight of cellulose) during the first six hours of hydrolysis. This increase has been associated with fragmentation of the cellulose particles as hydrolysis proceeded. Subsequently, the observed value of SSA leveled off, without further significant change (Fan, et al. 1981). Therefore, the authors reported that it is unreasonable to conclude that the hydrolysis rate is proportional to the surface area throughout the extended hydrolysis times, as the hydrolysis rates decreases even when a substantial amount of surface area is available. It has, thus, been concluded that the surface area by itself is not a major contributing factor in the structural transformation of cellulose.

7.1.2 Available/accessible surface area

Further developing the concept of SSA, it is generally acknowledged that not only the area of the substrate is important, but this area has to be available/accessible to the enzymes. Thus, during the hydrolysis process, one of the very important factors related to the substrate is the limiting and/or unequal accessibility of all β-glucosidic bonds of the substrate to the enzymes. As already stated several times (Chapter 4), enzymes are much bigger in size then cellobiose, the repeating unit of cellulose chain. Therefore, the adsorbed enzyme will cover (and consequently contribute to their unavailability) numerous bonds (see also Figure 6.4, Chapter 6). Moreover, due to the three dimensional

structure of cellulose microfibril and their organization in "bundles", consisting of approximately 40 glucose chains (see Figure 4.1, Chapter 4), only the chains at the surface of the microfibril will be available for the reaction. Nevertheless, as the reaction proceeds, the cellulose structure will "corrode", as short cellooligosaccharides (DP 1-6) are being detached from the surface of the substrate and released to the hydrolysis broth. This continuous reaction on the surface of the substrate will consequently help "open up" the three dimensional structure of the substrate, and more and more enzymes will be able to infiltrate deeper into the substrate, and begin hydrolyzing it from the inside.

In their publication of a functionally based model for hydrolysis of cellulose by fungal cellulases, Zhang and Lynd (2006) introduced the F_a factor, corresponding to the fraction of β-glucosidic bonds accessible to cellulase relative to the total number of glucosidic bonds. F_a has been defined as:

$$F_a = 2 \cdot \alpha \cdot A_{max} \cdot MW_{anhydroglucose} = 2 \cdot N_0 \cdot MW_{anhydroglucose} \text{, as } \alpha = \frac{N_0}{A_{max}} \qquad \textbf{Eq. 2}$$

where $MW_{anhydroglucose}$=162 g/mol, N_0 represents the µmol accessible cellobiose lattice/g cellulose (Gilkes et al., 1992), A_{max} is the maximum adsorption capacity [µmol enzyme/g cellulose, or, mg/g], and α is cellobiose lattices occupied per bound cellulase molecule. It is easily noticed that the value of the F_a factor depends greatly on accuracy of the estimated N_0 value (as a substrate characteristic), or α value (as enzyme characteristic). Using numerous previously reported substrate and enzyme characteristic values, Zhang and Lynd (2004) estimated the value of F_a, i.e. fraction of β-glucosidic bonds accessible to the enzymes, to be 12 % for PASC, 6.0 % for bacterial microcrystalline cellulose (BMCC) and 0.62 % for microcrystalline cellulose (Avicel). Thus, the accessible surface area of three commonly used model cellulose substrates can be arranged in following ascending ordered: Avicel < BMCC < PASC. Moreover, due to the lack of information, the authors defined F_a factor as constant value, but ideally this should be a variable as the state of the substrate is continuously changing throughout the hydrolysis process.

Similar to the F_a factor used by Zhang and Lynd (2006), Gan et al. (2003) introduced a β coefficient, corresponding to the surface-active cellulose concentration. By

comparing the experimental data and the model simulation, the authors concluded that only 2 % of the cellulose on the surface has been active and available for enzyme binding and catalysis.

7.1.3 Deactivation of enzymes

The three previously discussed factors (SSA, CrI and accessible surface area) contributing to the declining of hydrolysis rate are all related to the substrate characteristics. But, according to Ohmine et al. (1983), the decline of hydrolysis rate could not be fully explained by changes in substrate characteristics. Thus, they concluded that other "rate retarding factor" must be in play, i.e. reversible, time dependent, inactivation of the adsorbed enzyme. Converse et al. (1988) observed reduction in the activity and the concentration of the adsorbed enzyme and presented a model for enzyme adsorption and hydrolysis of microcrystalline cellulose with slow deactivation of the adsorbed enzyme. The mechanism of the hydrolysis has been assumed to proceed as following:

$$E + S \Leftrightarrow E_a \Rightarrow P + E$$
$$E_a \Leftrightarrow E_d \Rightarrow E \qquad\qquad \textbf{Eq. 3}$$
$$E + P \Rightarrow E_p$$

where, free enzyme E combines with the adsorption site on the substrate S to form an active adsorbed enzyme E_a. This adsorbed enzyme can either promote the reaction of the substrate to product P in which case enzyme returns to the solution and contributes to the pool of free enzyme E, or it can slowly convert to an inactive form E_d. This inactivated enzyme E_d can either convert back to active enzyme E_a, or it can convert to free enzyme E at a rate proportional to the rate at which the substrate is solubilized; i.e. the enzyme is released from the cellulose surface, to form the free enzyme E, when the substrate "cage" around it is removed. The free enzyme can further on be inhibited E_p by reacting with the product of the hydrolysis.

97

The concept of reversible enzyme inactivation (in Eq. 3 presented as $E_a \Leftrightarrow E_d \Rightarrow E$) can be difficult to understand. The authors assume that the deactivated enzyme, E_d, is the enzyme that is trapped within the cellulose substrate due to its size and sterical interference with substrate and other enzymes, and that it would completely re-activate to its natural and active form upon release. Mechanical deactivation (due to shear stress or gal-liquid interface), irreversible, non-productive binding to lignin and thermal inactivation have not been accounted for in the Converse et al. (1988) model. Therefore, I suggest following modification of Converse et al. (1988) model:

$$
\begin{aligned}
&\quad E_d \\
&\quad \Uparrow \\
&E + S \Leftrightarrow E_a \Rightarrow P + E \\
&E + P \Rightarrow E_p
\end{aligned}
$$

Eq. 4

Besides the presented model for enzyme inactivation, Converse et al. (1988) experimentally observed that no matter how large the ratio of substrate to enzyme is examined, the fraction of enzyme that is adsorbed never exceeds 80 %. Hence, they assumed that 20 % of the protein cannot be adsorbed. The authors do not provide us with the explanation of the observed phenomenon, but, nevertheless, used this empirical value in the model. Thus the amount of free enzyme has been calculated as:

$$ E = (0.8E_{total} - E_a - E_d)/(1 - E_p) $$

Eq. 5

Inactivation of *T. reesei* Cel6A (CBH II) during hydrolysis of short cellooligosaccharides, (DP 4-6) has been studied by Harjunpaa et al. (1996). They suggested that cellohexaose binding to a tunnel shaped active site of Cel6A may cause a twist, a minor conformational change, or a displacement of a sugar that will lead to slow, but time-dependent inactivation of the enzyme with a rate constant of 10^{-3} s^{-1}. Furthermore, they concluded that each Cel6A molecule on average processes 6000 cellohexaose molecules prior to inactivation. Moreover, Nidetsky et al. (1994), observed time-dependent inactivation of *T. reesei* CBH II with not only DP 6, but also with cellooligosaccharides of DP 4-8. They presented a model based on the hypothetical assumption that the CBH II may form productive and non-productive enzyme-substrate

complexes. The effect of non-productive binding has been incorporated into the Nidetsky et al. (1994) model in a form of a proportionality constant α.

7.2 De-polymerization type of model

As discussed earlier, to be able to accurately describe, and predict, the hydrolysis process a mechanistic mathematical model, covering individual enzyme characteristics should be developed. The mathematical model presented here is based on the model developed by Okazaki and Moo-Young (1978). In following sections the outcome of the model (computer simulation of the hydrolysis process) will be presented and compared to the experimental data presented in Article A. Furthermore, the model, and in particular enzyme and substrate parameters incorporated into the model, will be discussed.

In the model, substrate concentration, degree of polymerization (DP) and number of β-glucosidic bonds within the substrate are employed as substrate variables. The substrate is hydrolyzed by cooperative action of three individually incorporated enzyme classes, i.e. E_1 (endoglucanase), E_2 (cellobiohydrolase) and E_3 (β-glucosidase). Moreover, inhibition by hydrolysis products, cellobiose (C_2) and glucose (C_1), is also integrated into the model. Schematic representation of the concept behind the model is presented in Figure 7.1.

The model presented here addresses a system in which the macromolecular starting material is an assembly of linear (unbranched) polymeric chains denoted as C_i, with the DP i; thus the name, de-polymerization type of model. After initial hydrolysis, hydrolyzed cellulose segments C_i', C_i'', C_{i-2}' and C_{i-2}'', having a degree of polymerization of DP_i' and DP_i'', respectively, are produced. Purely for clearness, those products are in Figure 7.1 separated in two independent boxes, i.e. products of E_1, and E_2, respectively. These insoluble intermediate products are further on degraded to soluble intermediate products (DP 3-6), and subsequently to cellobiose and glucose.

One of the assumptions in the model is that all enzyme reaction sites (β-glucosidic bonds) are equally available to all enzymes. It is at this point important to stress that the term "enzyme reaction site" refer to different site or part of the substrate, dependent on

the enzyme in question. For catalysis with EG, available β-glucosidic bonds on the surface of the substrate is necessary for the reaction to proceed, while, for the reaction of CBH, free chain end (reducing or non-reducing) need to interact with the tunnel shaped catalytic core, where the concurrent catalysis of the β-glucosidic bonds will take place. This assumption, i.e. equal availability of all β-glucosidic bonds at all times, is theoretically unrealistic, but necessary, as the requirement of maintaining the simplicity of the model should be fulfilled.
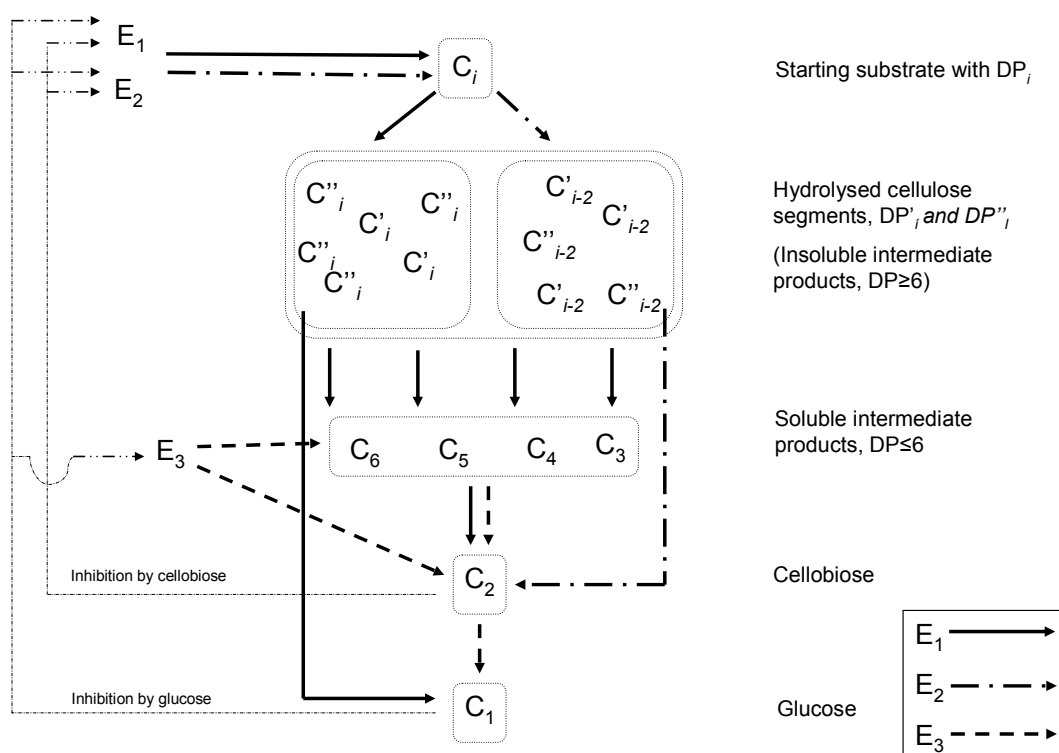
Figure 7.1: Schematic representation of the three cellulolytic enzymes E_1 (endoglucanase), E_2 (cellobiohydrolase) and E_3 (β-glucosidase) and their hydrolysis of different cellulose chain lengths. C_i, starting substrate with DP_i (i=96 for PASC and i=150 for Avicel, From Article A); C'_i, C''_i, C'_{i-2}, and C''_{i-2}, hydrolyzed cellulose segments of DP'_i and DP''_i (insoluble intermediate products, $DP \geq 6$); C_6, cellohexaose; C_5, cellopentaose; C_4, cellotetraose; C_3, cellotriose; C_2, cellobiose; C_1, glucose. Action of E_1 is represented by full arrow (⟶), of E_2 by dot-dash arrow (— · ➤) and of E_3 by dashed arrow (- - - ➤). Feedback inhibition of cellobiose and glucose is demonstrated in the figure.

The three enzyme classes incorporated in the model were assumed to have the following characteristics:

1. **E_1 - Endoglucanase** (corresponding to the Cel45A from *Humicola insolens* used in the experimental study, Article A) is degrading the substrate in a random manner and with equal affinity to different chain lengths. The main products of this reaction are hydrolyzed cellulose segments (C_i' and C_i''), but also higher soluble cellooligosaccharides (DP 3-6), cellobiose and glucose (Figure 7.1). This hydrolysis pattern of E_1 is in compliance with the experimental results (Medve et al., 1998; Karlsson et al., 2002; Article A). E_1 is inhibited by cellobiose and glucose.

2. **E_2 – Cellobiohydrolase** (corresponding to the Cel6A from *H. insolens* used in the experimental study, Article A) is cleaving off cellobiose units from the chain ends of either starting substrate C_i, or hydrolyzed segments C_i' and C_i''. *In vitro* this enzyme is attacking cellulose chain from the non-reducing end producing primarily cellobiose, but also some glucose and cellotriose (Schulein, 1997; Article A). In the model, there is no clear distinction between the reducing and the non-reducing cellulose chain end. Furthermore, E_2 has equal affinity to different chain lengths, and is inhibited by cellobiose and glucose.

3. **E_3 - β-glucosidase** (corresponding to the Cel3A from *Penicillium brasilianum* used in the experimental study, Article A). In previously presented models, β-glucosidase (if it was incorporated into the model as a separate enzyme) has been assumed to act exclusively on cellobiose (Okazaki and Moo-Young, 1978). Hydrolysis studies with Cel3A and other β-glucosidases show evidence that not only cellobiose, but also higher soluble cellooligosaccharides, up to cellohexaose (C_6), are being degraded to glucose. This feature was therefore incorporated into the model presented here, and the affinity of E_3 to the different substrate lengths, i.e. cellotriose to cellohexaose, can be adjusted by the means of the specially designed parameter – X, called C_3-C_6 reaction factor. E_3 is inhibited by glucose.

When enzyme and substrate interact with each other, enzyme-substrate complex (E_i*C_i) is initially formed. From a biological point of view this has an important meaning, representing productive binding of the enzyme to the substrate. Regardless of the existing experimental evidence that not all enzyme-substrate complexes lead to the release of

product (e.g. evidence of time dependent inactivation of the enzyme has been reported by Harjunpaa et al. (1996) and Converse et al. (1988), and existence of non-productive enzyme-substrate complexes has been suggested by Nidetzky, et al. (1994)), it is in this model assumed that all (E_i*C_i) complexes are dissociated to form free enzyme and product, e.g. steady-state approximation.

It is, furthermore, assumed that all enzymes are in the solution (dissolved in the aqueous phase) and that the concentration of the involved enzymes is constant during the whole hydrolysis process. Apart from that, enzymes can be present in three forms: 1) free enzymes, 2) enzymes productively adsorbed to the substrate, [E_i*C_i], and 3) inhibited enzymes, i.e., [E_i*C_1] and [E_i*C_2] (where C_1 denotes glucose and C_2 cellobiose). All three enzyme classes operate simultaneously to hydrolyze the substrate.

7.2.1 Individual enzyme kinetics for E_1, E_2, and E_3

In general terms, reactions of the individual enzymes were modeled as follows:

Enzyme E_1:

Using known enzyme kinetics the equations for E_1 attack on a cellulose molecule (C_i) of degree of polymerization i, is as follows:

$$E_1 + C_i \underset{k'_{s1}}{\overset{k_{s1}}{\rightleftharpoons}} [E_1 * C_i] \xrightarrow{k_1} E_1 + C'_i + C''_i \qquad \text{Eq. 6}$$
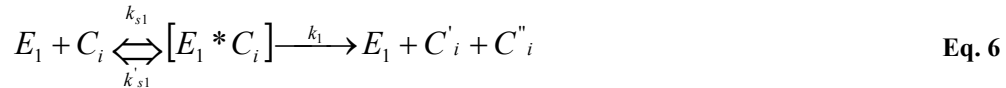
where C_i' and C_i'' are the hydrolyzed cellulose segments of DP_i' and DP_i''
(DP_i'+DP_i''=DP_i).

Enzyme E_1 can competitively be inhibited by the products of the reaction, e.g. C_2 and C_1. In the case of the competitive inhibition, inhibitor (I) binds to the same active site on the enzyme as substrate (C_i), and in that way I competes with C_i for a active site on the enzyme:

$$E_1 + C_1 \underset{k'_{g1}}{\overset{k_{g1}}{\rightleftharpoons}} [E_1 * C_1] \qquad \text{Eq. 7}$$

$$E_1 + C_2 \underset{k'_{c1}}{\overset{k_{c1}}{\rightleftharpoons}} [E_1 * C_2] \qquad \text{Eq. 8}$$

Total concentration of E_1 in the system is constant and can be represented as follows:

$$[E_1]_{free} = [E_1]_0 - \sum_{i=3}^{\max DP}[E_1 * C_i] - [E_1 * C_1] - [E_1 * C_2] \qquad \textbf{Eq. 9}$$

where $[E_1]_0$ is the initial total enzyme concentration (at t=0), and $[E_1]_{free}$ is the amount of free enzyme, e.g. not bound to the substrate or inhibitor.

Enzyme E_2:

Correspondingly, if we now focus on the enzyme E_2, which is cutting off cellobiose units from the end of the cellulose chain, producing C_2 and C_{i-2}, following reaction scheme can be proposed:

$$E_2 + C_i \underset{k'_{s2}}{\overset{k_{s2}}{\Longleftrightarrow}} [E_2 * C_i] \xrightarrow{k_2} E_2 + C'_{i-2} + C_2 \qquad \textbf{Eq. 10}$$

$$E_2 + C_1 \underset{k'_{g2}}{\overset{k_{g2}}{\Longleftrightarrow}} [E_2 * C_1] \qquad \textbf{Eq. 11}$$

$$E_2 + C_2 \underset{k'_{c2}}{\overset{k_{c2}}{\Longleftrightarrow}} [E_2 * C_2] \qquad \textbf{Eq. 12}$$
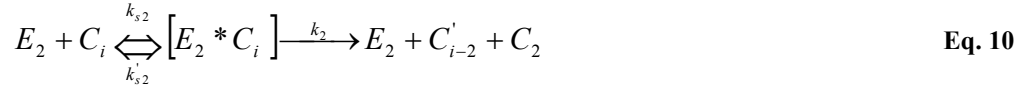
$$[E_2]_{free} = [E_2]_0 - \sum_{i=3}^{i}[E_2 * C_i] - [E_2 * C_1] - [E_2 * C_2] \qquad \textbf{Eq. 13}$$

Eq. 11 and Eq. 12 represent the competitive inhibition by glucose and cellobiose, respectively, and in Eq. 13 total concentration of E_2 is presented; $[E_2]_0$ is the initial total enzyme concentration (at t=0).

Enzyme E_3:

The third enzyme is acting on soluble cellooligosaccharides (DP 3 – DP 6) Cj, j=3-6 with the affinity factor X, and on cellobiose (C_2), degrading it to final product - glucose (C_1):

$$E_3 + C_j \underset{Xk'_{s3}}{\overset{Xk_{s3}}{\Longleftrightarrow}} [E_3 * C_j] \xrightarrow{Xk_3} E_3 + C_1 + C_{j-1} \qquad \textbf{Eq. 14}$$

$$E_3 + C_2 \underset{k'_{s3}}{\overset{k_{s3}}{\Longleftrightarrow}} [E_3 * C_2] \xrightarrow{k_3} E_3 + 2 \cdot C_1 \qquad \textbf{Eq. 15}$$

103
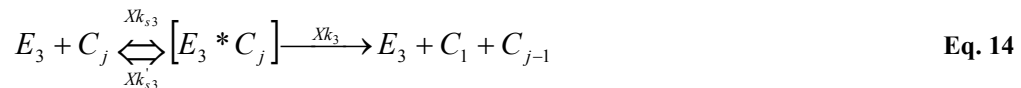
E_3 is inhibited by glucose (Eq. 16) and its total concentration is represented in Eq. 17.

$$E_3 + C_1 \underset{k'_{g3}}{\overset{k_{g3}}{\Longleftrightarrow}} [E_3 * C_1]$$

Eq. 16

$$[E_3]_{free} = [E_3]_0 - \sum_{j=3}^{6}[E_3 * C_j] - [E_3 * C_2] - [E_3 * C_1]$$

Eq. 17

Mass balances

Based on the Eq. 6 − Eq. 8 the mass balance of cellulose fragments of size i, $i \geq 3$ hydrolyzed with competitively inhibited E_1, can be written as follows:

$$\frac{dC_i}{dt} = -k_{s1}(i-1)[E_1]_f [C_i] + k'_{s1}[E_1 * C_i] + 2k_1 \sum_{i+3}^{\max DP} \frac{[E_1 * C_{i+1}]}{i}$$

Eq. 18

where, $(i\text{-}1)$ represents the number of available β-glucosidic bonds for enzyme cleavage, and the last term of the equation represents the actual production of species C_i, from higher polymers, e.g. C_{i+3}.

Applying the steady-state approximation, i.e. assuming that the rates of the formation of $[E_1 * C_i]$ complexes are equal to the rates of their decomposition:

$$\frac{d[E_1 * C_i]}{dt} \approx 0 \text{ , } \frac{d[E_1 * C_1]}{dt} \approx 0 \text{, and } \frac{d[E_1 * C_2]}{dt} \approx 0$$

Eq. 19

the mass balance from Eq. 18, can now be written as:

$$\frac{dC_i}{dt} = \frac{k_1[E_1]_0 \left(2\sum_{i+3}^{\max DP}[C_{i+3}] - (i-1)[C_i] \right)}{K_{M1}\left(1 + \frac{[C_1]}{K_{G1}} + \frac{[C_2]}{K_{C1}} + \sum_{i=3}^{\max DP}(i-1)[C_i] \right)}$$

Eq. 20

where $\dfrac{(k'_{s1} + k_1)}{k_{s1}} = K_{M1}$ represents the dissociation constant of the enzyme-substrate complex when $k_1 < k'_{s1}$, that is, when k_1 is (as we previously have assumed) the rate determining factor of the overall reaction represented in Eq. 6. Correspondingly, $\dfrac{k'_{g1}}{k_{g1}} = K_{G1}$, and $\dfrac{k'_{c1}}{k_{c1}} = K_{C1}$ represent the dissociation constant of the enzyme-inhibitor complex.

104

The obtained mass balance equation (Eq. 20) resembles the Michaelis-Menten enzyme kinetic reaction with mixed type of inhibition, which in general term can be written as:

$$\vartheta = -\frac{dS}{dt} = k_{cat}[E]_{Tot} \frac{[S]}{(K_M + [S])\left(1 + \frac{[I]}{K_I}\right)}$$

Eq. 21

A mixed inhibitor usually affects both K_M and V_{max} value of the enzyme, but while V_{max} decreases, K_M increases with increasing inhibitor concentration.

Using the same principals, mass balances of substrate degraded by the other two enzymes (E_2 and E_3), and consequently all three enzyme classes together can be obtained and are presented elsewhere (Okazaki and Moo-Young, 1978).

In summary, this model incorporates the action of all three cellulolytic enzyme classes necessary for efficient degradation of cellulose. Consequently, the mechanism of the hydrolysis process can be well defined. Nevertheless, although the model considers many inhibitory effects it does not incorporate any mechanism of irreversible degradation of enzyme, nor the existence of the unproductive enzyme-substrate complex. Such a mechanism of irreversible, time dependent, deactivation of the enzyme has not yet been studied in great details (although, as previously mentioned some experimental data does exist), and, thus, "setting a number on it" would be a pure speculation. Moreover, the number of unknown constants of the model would inevitably increase, affecting the complexity and increasing the uncertainty of the model.

7.2.2 Enzyme kinetic parameters used in the model

It has previously been reported (Zhang et al., 2006) that there is no clear relationship between the hydrolysis rates obtained on soluble substrates and those on insoluble substrates, mainly because of huge differences in substrate accessibility and DP. It is therefore highly important that researchers clearly state all parameters of their assay conditions, and resist temptation to compare their results to those of other researchers using different substrates, assay methods, etc. Unfortunately, measuring/determining all parameter values required for the model can be very tedious, difficult and sometimes impossible. For example, the rate constant of an enzyme (or Michaelis constant K_M)

depends on the size of the polymeric substrate. Consequently, as the reaction proceeds and DP of the substrate is decreasing, different values for the K_M should be supplied to the model. Experimentally, this would be extremely difficult, if not impossible, to obtain, as it will presume possession of a wide range of cellulosic substrates of different DP's. To illustrate the diversity of the kinetic values available in the literature, a summary of reported values for the rate constant of different enzymes is presented in Table 7.3.

Table 7.3: Michaelis constant (K_M) for the three enzyme classes.

Michaelis constant	Unit	Substrate	Enzyme	Organism	Conditions	Ref.
EG - K_{M1}						
2.00	mM	r DP 4[c]	EG 5 (Cel45A)	*Humicola insolens*	pH=7.5, T=40 C	Schulein et al., 1993
0.15	mM	r DP 5[c]	EG 5 (Cel45A)	*H. insolens*	pH=7.5, T=40 C	Schulein et al., 1993
0.05	mM	r DP 6[c]	EG 5 (Cel45A)	*H. insolens*	pH=7.5, T=40 C	Schulein et al., 1993
2.64 (64.30)	mM (mg/ml[a])	Avicel 20 g/l	EG 5	*T. viride*	pH=5.0, T=30 C	Beldman et al., 1987[d]
12.60	mM	PNP- cellobioside	EG 3 (Cel12A)	*Phanerochaete chrysosprum*		Nutt, 2006
3.46	mM	PNP- cellobioside	EG 1 (Cel7B)	*P. chrysosprum*		Nutt, 2006
1.61 (25.00)	mM (mg/ml[b])	PASC 10 g/l	EG 5 (Cel45A)	*H. insolens*	pH=5.5, T=50 C	Chang, unpublished data[e]
CBH - K_{M2}						
1.50	mM	r DP 3[c]	CBH 1 (Cel7A)	*H. insolens*	pH=7.5, T=40 C	Schulein et al., 1993
0.25	mM	r DP 4[c]	CBH 1(Cel7A)	*H. insolens*	pH=7.5, T=40 C	Schulein et al., 1993
0.06	mM	r DP 5[c]	CBH 1(Cel7A)	*H. insolens*	pH=7.5, T=40 C	Schulein et al., 1993
0.02	mM	r DP 6[c]	CBH 1(Cel7A)	*H. insolens*	pH=7.5, T=40 C	Schulein et al., 1993
0.47	mM	r DP 4[c]	CBH 2 (Cel6A)	*H. insolens*	pH=7.5, T=40 C	Schulein et al., 1993
0.003	mM	r DP 5[c]	CBH 2 (Cel6A)	*H. insolens*	pH=7.5, T=40 C	Schulein et al., 1993
0.005	mM	r DP 6[c]	CBH 2 (Cel6A)	*H. insolens*	pH=7.5, T=40 C	Schulein et al., 1993
1.81 (44.10)	mM (mg/ml[a])	Avicel 20 g/l	Exo 2	*T. viride*	pH=5.0, T=30 C	Beldman et al., 1987[c]
1.88 (29.30)	mM (mg/ml[b])	PASC 10 g/l	CBH 1 + CBH 2 (3:1)	*H. insolens*	pH=5.5, T=50 C	Chang, unpublished data[e]
BG - K_{M3}						
2.50	mM	cellobiose	BG (Cel3A)	*Penicillium brasilianum*		Krogh, personal communication
2.42	mM	cellobiose	Novozyme 188		pH=4.8, T=50 C	Calsavara et al., 1999
1.52 (0.52)	mM (mg/ml[b])	cellobiose	MCN 188	*Aspergillus niger*	pH=5.5, T=50 C	Chang, unpublished data

EG: Endoglucanase; CBH: Cellobiohydrolase; BG: β-glucosidase; MCN 188: : Mono-component Novozym 188 (purified β-glucosidase); [a] from Lineweaver-Burk plot; [b] from Hanes-Woolf plot; [c] reduced cellodextrins; [d] calculated with MW Avicel= 24320 g/mol; [e] calculated with MW PASC= 15570 g/mol.

In this work, experiments were performed with the model cellulose substrates providing us with the kinetic values for the three enzyme classes used, while missing information (K_I values) were supplemented with the complementary data available in the literature. The parameters that are needed for the model are summarized in Table 7.4.

Table 7.4: Parameters used in the model.

Parameter	Value*	Unit
Substrate chain lengnt (DP)	96	-
Substrat concentration (10 g/l)	0.64	mmol/L
EG activity ($k_1[E_1]$)	5.0	mmol/L/h
CBH activity ($k_2[E_2]$)	0.4	mmol/L/h
BG activity ($k_3[E_3]$)	1.0	mmol/L/h
Michaelis constant, EG (K_{M1})	1.6	mmol/L
Michaelis constant, CBH (K_{M2})	22.0	mmol/L
Michaelis constant, BG (K_{M3})	2.5	mmol/L
Glucose inhibiiton of EG (K_{G1})		mmol/L
Glucose inhibiiton of CBH (K_{G2})		mmol/L
Glucose inhibiiton of BG (K_{G3})		mmol/L
Cellobiose inhibiiton of EG (K_{C1})		mmol/L
Cellobiose inhibiiton of CBH (K_{C2})		mmol/L
C_3-C_6 reaction factor		-

*: Most often used values during the modeling study

7.2.3 Comparisons of model predictions and experimental results

Hydrolysis of the substrate with pure (mono-component) Cel6A (CBH II) is modeled so that the only product of this reaction would be cellobiose (Figure 7.1, Eq. 10, and Figure 7.2 A). During the experimental study a small amount of glucose, cellotriose and cellotetraose were, however, detected (Chapter 5, Article A; Figure 7.2 B). Nevertheless, under these relatively simple conditions, the model is describing the hydrolysis process well. The predicted conversion of PASC is at the same order of magnitude as what was experimentally measured (Figure 7.2).
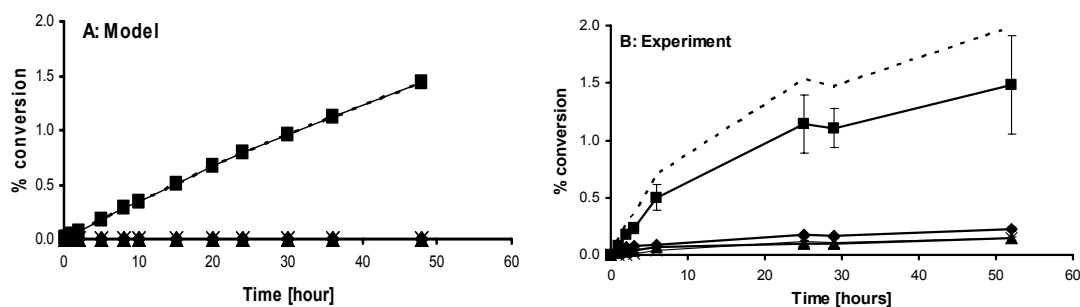
Figure 7.2: Hydrolysis of PASC with pure Cel6A (CBH II). **A:** Model prediction. Parameters used in the model are: CBH activity=0.4 mmol/L/h, K_{M2}=22.0 mmol/L, no inhibition. **B:** Obtained during hydrolysis of 9 g/L of PASC with 0.1 µM of Cel6A. Glucose: ♦; Cellobiose: ■; Cellotriose: ▲; Cellotetraose: ✕; Sum of all soluble sugars produced: dashed line.

The results showing comparison of the model and the experimental study during the condition where only Cel45A (EG) was used, are presented in Figure 7.3. Qualitatively, the model is able to describe the progress of the hydrolysis process reasonably well, but quantitative results are not satisfactory. While during the experimental study the overall conversion of the substrate reached approximately 25 %, in the model prediction the conversion is approaching 100 %. In other words, neither substrate nor the enzymes are restricting the hydrolysis process in the model, which is consequently proceeding to the point where there is no more substrate available for the hydrolysis.
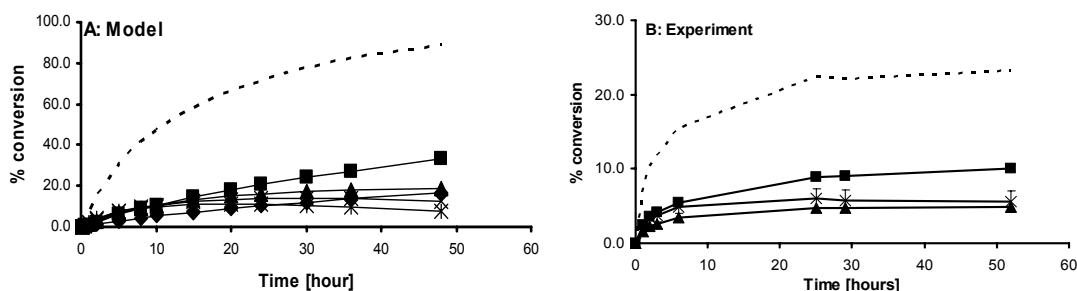
Figure 7.3: Hydrolysis of PASC with pure Cel45A (EG). **A:** Model prediction. Parameters used in the model are: EG activity=5.0 mmol/L/h, K_{M1}=1.6 mmol/L, no inhibition. **B:** Obtained during hydrolysis of 9 g/L of PASC with 0.1 µM of Cel45A. Glucose: ♦; Cellobiose: ■; Cellotriose: ▲; Cellotetraose: ✕; Cellopentaose: +; Sum of all soluble sugars produced: dashed line.

Inhibition of cellulolytic enzymes by cellobiose or glucose is commonly recognized as one of the reasons for the decline of the hydrolysis rate. This is, indeed, also possible to model, but the magnitude of either cellobiose or glucose inhibition, represented as K_I value, would need to be extremely low (K_I=0.025 mmol/L) to approach the conversion of the substrate corresponding to the experimentally determined values (Figure 7.4). For comparison, the apparent competitive inhibition constant K_i for Cel7A (CBH I) on bacterial cellulose was found to be 1.6±0.5 mM, 100 fold higher that that reported for Cel7A on low-molecular-weight model substrates (~20 μM). The hydrolysis of amorphous cellulose by EG was even less affected by cellobiose inhibition with apparent K_i values of 11±3 mM and 34±6 mM for Cel7B (EG I) and Cel5A (EG II), respectively Gruno, et al., 2004.
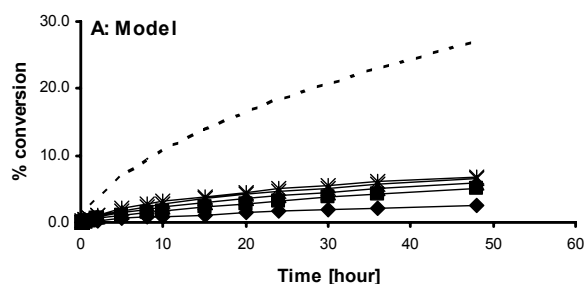

Figure 7.4: Hydrolysis of PASC with pure Cel45A (EG), model prediction. Parameters used in the model are: EG activity=5.0 mmol/L/h, K_{M1}=1.6 mmol/L, K_{C1}=0.025 mmol/L. Glucose: ♦; Cellobiose: ■; Cellotriose: ▲; Cellotetraose: ✕; Cellopentaose: +; Sum of all soluble sugars produced: dashed line.

In the model, the characteristics of the substrate are represented with substrate concentration, DP and, consequently, number of β-glucosidic bonds present in the substrate. DP for PASC and Avicel was determined to be 96 and 150, respectively (Chapter 5, Article A), while all experiments were performed with constant substrate concentration, 10 g/l. The above mentioned substrate characteristics incorporated into the model have, unfortunately, shown to be insufficient to properly describe the hydrolysis of Avicel. Namely, modeling of the time course of Avicel degradation with Cel45A result in both quantitatively and qualitatively similar prediction as shown in Figure 7.3 A (data not shown), while experimental results show dramatically lower conversion levels (Chapter 5, Article A). Thus, additional substrate features would need to be incorporated into the

model, if also quantitative results of the model prediction are to be satisfactory correlated with experimental results. Moreover, as we concluded in Article A, enzymatic hydrolysis of Avicel shows competition between the enzymes, opposed to observed synergy during degradation of PASC, and that is also an important point that would need to be taken into account.

In order to study the synergy between cellulolytic enzymes, experiments were performed with the variety of different ternary enzyme mixtures (combinations of CBH, EG and BG), Chapter 5, Article A. Here I will only present results of one of the enzyme combinations (consisting of 40 % Cel6A, 50 % Cel45A and 10 % Cel3A) compared to the corresponding model simulation (Figure 7.5). Yet again results show similarity in the product formation, but large disagreement in the overall conversion of the substrate. During the experimental study, the only detectable products of the reaction were glucose and cellobiose, while during the simulation study, soluble cellooligosaccharides of DP up to five were recorded. This indicates that the amount of BG in the model was insufficient to follow up with the high production of shorter cellooligosaccharides by EG, thus, pointing out to the bottleneck of this particular process.
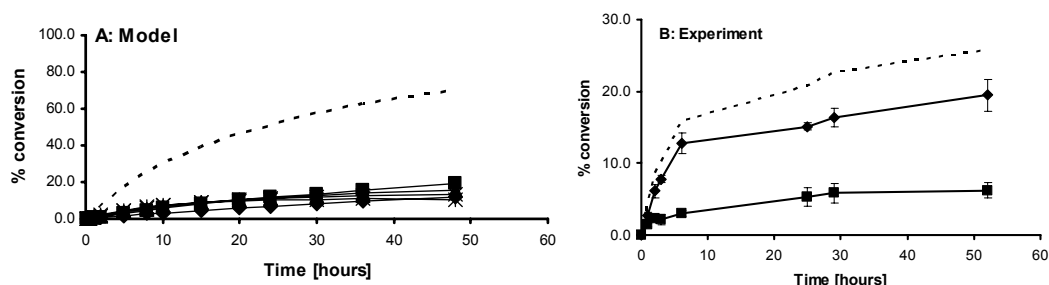
Figure 7.5: Hydrolysis of PASC with 50 % Cel45A (E_1) + 40 % Cel6A (E_2) + 10 % Cel3A (E_3) (mole %). **A:** Model prediction. Parameters used in the model are: EG activity=5.0*0.5 mmol/L/h, K_{M1}=1.6 mmol/L, CBH activity=0.4*0.4 mmol/L/h, K_{M2}=22.0 mmol/L, BG activity=1.0*0.1 mmol/L/h, K_{M3}=2.5 mmol/L, no inhibition. **B:** Obtained during hydrolysis of 9 g/L of PASC. Glucose: ♦; Cellobiose: ■; Cellotriose: ▲; Cellotetraose: ✕; Cellopentaose: +; Sum of all soluble sugars produced: dashed line.

7.3   Summary

The reaction between cellulose and cellulase enzyme is of heterogeneous nature and, thus, some of the suggested consequences are that the reaction rate should be proportional to the extent of cellulose surface that is accessible to the enzyme molecule. To incorporate that and other experimental observations in models describing enzymatic hydrolysis of cellulose, different authors have been suggesting either empirical equations describing the process, or constant factors and coefficients (see Chapter 7.1). However, trying to describe complex substrate and enzyme characteristics by incorporating a single or a few factors (summarized in Table 7.2) into the model is insufficient to achieve the appropriate description of the process. On the contrary, a large number of parameters, and consequent continuous (time dependent) change of both enzyme and substrate characteristics should, ideally, be taken into account. This makes modeling of the process significantly challenging, seen from two points of view:

> 1) A large number of parameters incorporated into the model can explicitly increase the uncertainty of the model, and,

> 2) Experimental measurements of time induced changes on both the substrate and the enzymes are difficult, tedious and prone to errors. Furthermore, experimental data often used to feed developed models has been obtained in different laboratories around the World, and in some cases with different enzymes, substrates or physical conditions (pH, temperature, enzyme load, etc.) of the hydrolysis.

Thus, the authors presenting the model of enzymatic degradation of cellulose have to find a delicate balance between the number and nature of parameters to be used in the model, while still maintaining model relatively simple and robust.

Mechanistic mathematical model presented by Okazaki and Moo-Young (1978) is incorporating specific enzyme kinetics for the three enzyme classes used during cellulose degradation. This is fundamental strength of the model as the enzymes used in the hydrolysis process exhibit distinct kinetics and modes of action, and thus need to be modeled separately. Moreover, these types of models are suitable for expressing synergy among the enzymes. The drawbacks are relatively large numbers of parameters that need

to be estimated and ignorance towards some substrate-enzyme interaction factors, such as mass transfer of enzyme and products, and the adsorption of enzymes.

The model presented here is able to qualitatively describe the hydrolysis process reasonably well. This comes as a result of a well defined mode of action of each of the enzymes used in the modeling study. The weakness of this specific model is that it does not take into account the irreversible degradation of enzymes, or the existence of the unproductive enzyme-substrate complexes. Consequently, regardless of the magnitude of the kinetic constants used in the model, the outcome of the model will always be complete conversion. Furthermore, the improvements of the model should be directed towards superior description of the substrate characteristics, in particular defining substrate not just as liner glucose chain, but as a assembly of 40 glucose chains (see Figure 4.1, Chapter 4), in which the accessibility of the inside chains will increase as the outer chains are being hydrolyzed.

7.4 References

Brandt D, Hontz L, Mandels M. 1973. Engineering aspects of the enzymatic conversion of waste cellulose to glucose. AIChE Symp Series 69: 127-133.

Chang CW. Unpublished data

Converse AO, Ryuichi M, TanakaM. 1988. A model of enzyme adsorption and hydrolysis of microcrystalline cellulose with slow deactivation of the adsorbed enzyme. Biotechnol. Bioeng. 32: 38-45.

Fan LT, Lee Y-H, Beardmore DH. 1981. The influence of major structural features of cellulose on rate of enzymatic hydrolysis. Biotechnol. Bioeng. 23: 419-424.

Gan Q, Allen SJ, Taylor G. 2003. Kinetic dynamics in heterogeneous enzymatic hydrolysis of cellulose: an overview, an experimental study and mathematical modeling. Bioprocess Biotechnol. 38: 1003-1018.

Gharpuray MM, Lee YH, Fan LT. 1983. Structural modification of lignocellulosics by pretreatments to enhance enzymatic hydrolysis. Biotechnol. Bioeng. 25: 157-172.

Ghose TK. 1969. Continuous enzymatic saccharification of cellulose with culture filtrates of *Trichoderma viride* QM 6a. Biotechnol. Bioeng. 11: 239-261.

Gilkes NR, Jervis E, Henrissat B, Tekant B, Miller RC Jr, Warren RAJ, Kilburn DG. 1992. The adsorption of a bacterial cellulase and its two isolated domains to crystalline cellulose. J. Biol. Chem. 267: 6743-6749.

Gruno M, Väljamäe P, Pettersson G, Johansson G. 2004. Inhibition of the *Trichoderma reesei* cellulases by cellobiose is strongly dependent on the nature of the substrate, Biotechnol. Bioeng. 86: 503-511.

Harjunpaa V, Teleman A, Kiovula A, Ruohonen L, Teeri TT, Teleman O, Drakenberg T. 1996. Cello-oligosaccharide hydrolysis by cellobiohydrolase II from *Trichoderma reesei*. Association and rate constant derived from analysis of progress curves. Eur. J. Biochem. 240: 584-591.

Howell JA, Mangat M. 1978. Enzyme deactivation during cellulose hydrolysis. Biotechnol. Bioeng. 20: 847-863.

Huang AA. 1975. Kinetic studies on insoluble cellulose-cellulase system. Biotechnol. Bioeng. 17: 1421-1433.

Karlsson J, Siika-aho M, Tenkanen M, Tjerneld F. 2002. Enzymatic properties of the low molecular mass endoglucanases Cel12A (EG III) and Cel45A (EG V) of *Trichoderma reesei*. J. Biotechnol. 99: 63-78.

King KW. 1966. Enzymatic degradation of crystalline hydrocellulose. Biochem. Biophys. Res. Comm. 24: 295-298.

Krogh KRBM. Personal communication.

Lee Y-H, Fan LT, Fan L-S. 1980. Kinetics of hydrolysis of insoluble cellulose by cellulase. Adv. Biochem. Eng. 17: 131-168.

Medve J, Karlsson J, Lee D, Tjerneld F. 1998. Hydrolysis of microcrystaline cellulose by cellobiohydrolases I and endoglucanase II from *Trichoderma reesei*: adsorption sugar production pattern, and synergism of the enzymes. Biotechnol. Bioeng. 59: 621-634.

Nidetzky B, Steiner W, Hayn M, Claeyssens M. 1994. Cellulose hydrolysis by the cellulases from *Trichoderma reesei*: a new model for synergistic interaction. Biochem. J. 298:705-710.

Ohmine K, Ooshima H, Harano Y. 1983. Kinetic study on enzymatic hydrolysis of cellulose by cellulose from *Trichoderma viride*. Biotechnol. Bioeng. 25: 2041-2053.

Okazaki M, Moo-Young M. 1978. Kinetics of enzymatic hydrolysis of cellulose: analytical description of a mechanistic model. Biotechnol. Bioeng. 20: 637-663.

Peiterson N, Ross EW. 1979. Mathematical model for enzymatic hydrolysis and fermentation of cellulose by *Trichoderma*. Biotechnol. Bioeng. 21: 997-1017.

Ryu DDY, Lee SB, Tassinari T, Macy C. 1982. Effect on compression milling on cellulose structure and on enzyme hydrolysis kinetics. Biotechnol. Bioeng. 24: 1047-1067.

Schülein M. 1997. Enzymatic properties of cellulases from *Humicola insolens.* J Biotech 57: 71-81.

Zhang Y-H P, Himmel M., Mielenz JR. 2006. Outlook for cellulase improvements: screening and selection strategies. Biotechnol. Adv. 26: 452-481.

Zhang Y-HP, Lynd LR. 2004. Towards and aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. Biotechnol. Bioeng. 88: 797-824.

113

Zhang Y-HP, Lynd LR. 2006. A functionally based model for hydrolysis of cellulose by fungal cellulase. Biotechnol. Bioeng. 94: 888-898.

7.5 Nomenclature

i	7-∞; number of glucose units present in the insoluble cellulose
j	3-6; number of glucose units present in soluble cellooligosaccharides
C_i	Insoluble cellulose of degree of polymerization i
C_j	Soluble cellooligosaccharides of degree of polymerization j
C_1	Glucose
C_2	Cellobiose
$C_i{}'$ and $C_i{}''$	Hydrolyzed cellulose segments of $DP_i{}'$ and $DP_i{}''$, where $DP_i{}' + DP_i{}'' = DP_i$
$C_{i-2}{}'$ and $C_{i-2}{}''$	Hydrolyzed cellulose segments after reaction with E_2
DP	Degree of polymerization
E_1	Enzyme endoglucanase
E_2	Enzyme cellobiohydrolase
E_3	Enzyme β-glucosidase
E_1*C_i	E_1-substrate complex
E_2*C_i	E_2-substrate complex
E_3*C_i	E_3-substrate complex
E_1*C_1	E_1-glucose complex (Glucose inhibited enzyme)
E_1*C_2	E_1-cellobiose complex (Cellobiose inhibited enzyme)
E_2*C_1	E_2-glucose complex (Glucose inhibited enzyme)
E_2*C_2	E_2-cellobiose complex (Cellobiose inhibited enzyme)
E_3*C_1	E_3-glucose complex (Glucose inhibited enzyme)
I_1	Inhibition by glucose
I_2	Inhibition by cellobiose
k_{S1}	Forward rate constant for formation of substrate-E_1 complex
k'_{S1}	Reverse rate constant for formation of substrate-E_1 complex
k_{S2}	Forward rate constant for formation of substrate-E_2 complex
k'_{S2}	Reverse rate constant for formation of substrate-E_2 complex
k_{S3}	Forward rate constant for formation of substrate-E_3 complex
k'_{S3}	Reverse rate constant for formation of substrate-E_3 complex
k_1	Rate constant of product formation for E_1
k_2	Rate constant of product formation for E_2
k_3	Rate constant of product formation for E_3
k_{G1}	Forward rate constant for formation of glucose-E_1 complex
k'_{G1}	Reverse rate constant for formation of glucose-E_1 complex
k_{C1}	Forward rate constant for formation of cellobiose-E_1 complex
k'_{C1}	Reverse rate constant for formation of cellobiose-E_1 complex
k_{G2}	Forward rate constant for formation of glucose-E_2 complex
k'_{G2}	Reverse rate constant for formation of glucose-E_2 complex
k_{C2}	Forward rate constant for formation of cellobiose-E_2 complex
k'_{C2}	Reverse rate constant for formation of cellobiose-E_2 complex
k_{G3}	Forward rate constant for formation of glucose-E_3 complex
k'_{G3}	Reverse rate constant for formation of glucose-E_3 complex
K_{G1}	Constant for glucose inhibition of E_1 [mmol/L]
K_{C1}	Constant for cellobiose inhibition of E_1 [mmol/L]
K_{G2}	Constant for glucose inhibition of E_2 [mmol/L]
K_{C2}	Constant for cellobiose inhibition of E_2 [mmol/L]
K_{G3}	Constant for glucose inhibition of E_3 [mmol/L]
X	Degree to which E_3 degrades soluble cellodextrins of DP 3 – 6
ϑ	Reaction rate (Michaelis-Menten type of kinetics)

CHAPTER 8

Application of Metabolic Control Analysis (MCA) theory to the enzymatic hydrolysis of cellulose

As it is seen from the previous chapters, the mechanism, and the optimal enzyme combination for efficient cellulose hydrolysis has been extensively studied by many research groups. In this chapter, I will take a novel approach and apply the principles and theory of metabolic control analysis (MCA) to enzymatic hydrolysis of cellulose, with the aim of identifying the most rate-controlling step(s) (enzymes) in the system.

MCA is a mathematical framework, originally developed to describe the control in metabolic pathways, but was subsequently extended to describe signaling and genetic networks. Although the MCA theory is applied in the framework of enzyme kinetics within a pathway (Figure 8.1), this theory has, to the best of my knowledge, not yet been applied to the specific topic of cellulose hydrolysis.
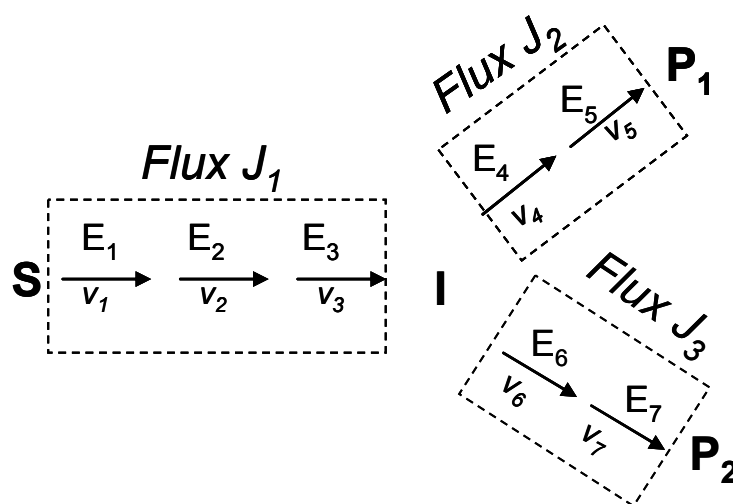
Figure 8.1: An example of branched metabolic pathway. Substrate S is converted to two products, P_1 and P_2, via intermediate (I). Enzyme activities are defined as E_1, E_2, etc. Flux, J, of a pathway is equal to the rates of the individual reactions (v_1, v_2, etc.) at steady state. $J_1 = J_2 + J_3$ at steady state.

In MCA one study how the control of fluxes (J) and intermediate concentrations (I) in a metabolic pathway is distributed among the different enzymes (E_1, E_2, etc.) that constitute the pathway (Figure 8.1). Contrary to other theories assuming existence of only one rate-limiting step, MCA theory assumes that the control is spread quantitatively among the enzymes. Namely, in MCA one studies the relative control exerted by each step (enzyme) on the system's variables (fluxes and metabolite concentrations). This control is measured by applying a perturbation to the step being studied and measuring the effect on the variable of interest after the system has settled to a new steady state (Heinrich and Schuster, 1996; Fell, 1997; Stephanopoulos et al., 1998).

To study MCA, one needs to build a kinetic model of studied network, which require profound knowledge of mathematics, and for many biologist and biochemists that can be rather challenging task. Fortunately, several user-friendly biochemical simulation softwares are available, and here I have used the free software Gepasi, developed by Pedro Mendes' team (Mandes, 1993; http://www.gepasi.org/, July 2007). Building a kinetic model on Gepasi only requires a list of the reactions in the pathway, type of kinetics for each reaction including the necessary kinetic constants, and the initial metabolite concentrations. Once a kinetic model has been established, the simulation software can perform MCA. To be able to correctly interpret the analysis results, it is necessary to understand the theory and principals on which MCA is developed.

8.1   MCA theory

One of the most important goals of metabolic engineering is to elucidate the parameters responsible for the *control of flux*. Flux control is important for keeping the rates of synthesis and conversion of metabolites closely balanced over a very wide range of external conditions. Additionally, understanding flux control is important for the rational modification of metabolic fluxes (Stephanopoulos et al., 1998). In the following text, I will shortly introduce the most important coefficients and theorems used in MCA, while, for more details, the reader is directed elsewhere (Heinrich and Schuster, 1996; Fell, 1997; Stephanopoulos et al., 1998).

8.1.1 Control coefficients and the summation theorem

MCA is strictly applied to steady-state (or pseudo-steady-state) conditions and it is under these conditions, the control coefficients are defined. The most important coefficients are the *flux control coefficients* (FCCs). They are defined by the *relative* change in the steady-state flux resulting from an infinitesimal change in the activity of an enzyme of the pathway divided by the *relative* change of the enzyme activity (Eq. 22).

$$C^J = \frac{E}{J}\frac{dJ}{dE} = \frac{d\ln J}{d\ln E}$$ **Eq. 22**

FCCs are dimensionless and they have values between 0 and 1 for a linear pathway. For a branched pathway, the equation becomes:

$$C_i^{J_k} = \frac{E_i}{J_k}\frac{dJ_k}{dE_i} = \frac{d\ln J_k}{d\ln E_i} \qquad i,k \in \{1,2,\text{K},L\}$$ **Eq. 23**

where J_k is the steady-state flux through the *k*-th reaction in the pathway, and E_i is the activity of the *i*-th enzyme. For such a system, FCCs may be either negative or positive.

From the definition of the FCCs, it is clear that the enzyme (and the reaction it catalyzes) with the largest FCC exerts the largest control of flux at the particular steady state because an increase in the activity of this enzyme results in the largest overall flux increase.

In addition, since the flux control coefficients are normalized with respect to each flux, they all must sum to unity. This theorem is known as the *flux-control summation theorem* (Eq. 24).

$$\sum_{i=1}^{L} C_i^{J_k} = 1 \qquad k \in \{1,2,\text{K},L\}$$ **Eq. 24**

From this equation, it should be clear that most FCCs may have small values in a long pathway compared to the FCCs in a short pathway. Hence, FCCs should only be compared with each other within the same pathway and never with FCCs of other pathways. Furthermore, a pathway is at its most efficient state when the flux control is evenly distributed among the enzymes that constitute the pathway.

Similar to FCCs are the concentration control coefficients (CCCs), where the variable affected by the enzyme activity (E_i) is a metabolite concentration (c_j).

119

$$C_i^{X_j} = \frac{E_i}{c_j} \frac{dc_j}{dE_i} = \frac{d \ln c_j}{d \ln E_i} \qquad i \in \{1,2,\mathrm{K}, L\}, j \in \{1,2,\mathrm{K}, L\} \qquad \textbf{Eq. 25}$$

or, more generally,

$$C_i^{X_j} = \frac{v_i}{c_j} \frac{dc_j}{dv_i} = \frac{d \ln c_j}{d \ln v_i} \qquad i \in \{1,2,\mathrm{K}, L\}, j \in \{1,2,\mathrm{K}, L\} \qquad \textbf{Eq. 26}$$

CCCs specify the relative change in the level of the j-th intermediate (Xj) when the activity of the i-th enzyme is changed. Consequently, as the level of any intermediate remains the same when all enzyme activities are altered by the same factor, the sum of all the CCCs for each of the K metabolites, must be equal to zero.

$$\sum_{i=1}^{L} C_i^{X_j} = 0 \qquad j \in \{1,2,\mathrm{K}, K\} \qquad \textbf{Eq. 27}$$

In this study, the focus will be mainly on the flux control coefficients.

8.1.2 Elasticity coefficients and the connectivity theorem

The control coefficients mentioned above are the so-called *systemic* properties of the overall metabolic system, meaning they reflect conditions concerning the entire system. Elasticity coefficients, on the other hand, are *local* properties of the individual enzyme in the metabolic network. The most common elasticity coefficients are the elasticities (ε) of the reaction rates (υ) with respect to metabolite concentrations (c_j).

$$\varepsilon_{X_j}^i = \frac{c_j}{v_i} \frac{\partial v_i}{\partial X_j} = \frac{\partial \ln v_i}{\partial \ln c_j} \qquad i \in \{1,2,\mathrm{K}, L\}, j \in \{1,2,\mathrm{K}, K\} \qquad \textbf{Eq. 28}$$

Elasticity coefficients have positive values for metabolites that stimulate a reaction, e.g. a substrate or an activator, but have negative values for metabolites that slow the reaction, e.g. a product or an inhibitor. Elasticity coefficients allow quantification of the influence of product concentrations, which usually have a significant effect due to the reversibility of cellular reactions. Furthermore, elasticity coefficients can be extended to include effectors that are not pathway intermediates.

The relationship between FCCs and elasticity coefficients is expressed in the *flux-control connectivity theorem* (Eq. 29).

$$\sum_{i=1}^{L} C_i^{J_k} \varepsilon_{X_j}^{i} = 0 \qquad i \in \{1,2,K,L\}, j \in \{1,2,K,K\} \qquad \textbf{Eq. 29}$$

The connectivity theorem has great importance in MCA because it provides the means to understand how local enzyme kinetics affects flux control. Furthermore, when determining FCCs, it is usually the elasticity coefficients that are measured experimentally. FCCs are usually calculated from the elasticity coefficients and the connectivity theorem.

8.2 MCA and enzymatic hydrolysis of cellulose

From the brief description of the theory behind MCA, it can be concluded that a change in an enzyme activity will have both a direct effect on the pathway flux, as well as, an indirect effect though changes in the metabolite concentrations. Thus, the idea behind this study was to apply the free simulation software Gepasi on the kinetic model that describes the enzymatic hydrolysis of cellulose, and consequently perform MCA. The outcome of the analysis will be a set of control coefficients and elasticity coefficients. The focus will, primarily, be on FCCs, which, by their magnitude can point out the most rate-controlling (rate-limiting) step(s) in the predefined system. Parallel to that, hydrolysis of model cellulose substrate with different combinations of mono-component cellulolytic enzymes was performed, providing us with "real data" platform for evaluating the results and conclusions from the MCA study. Schematic diagram of the experimental and computational studies performed and presented in this chapter is presented in Scheme 8.1.

121
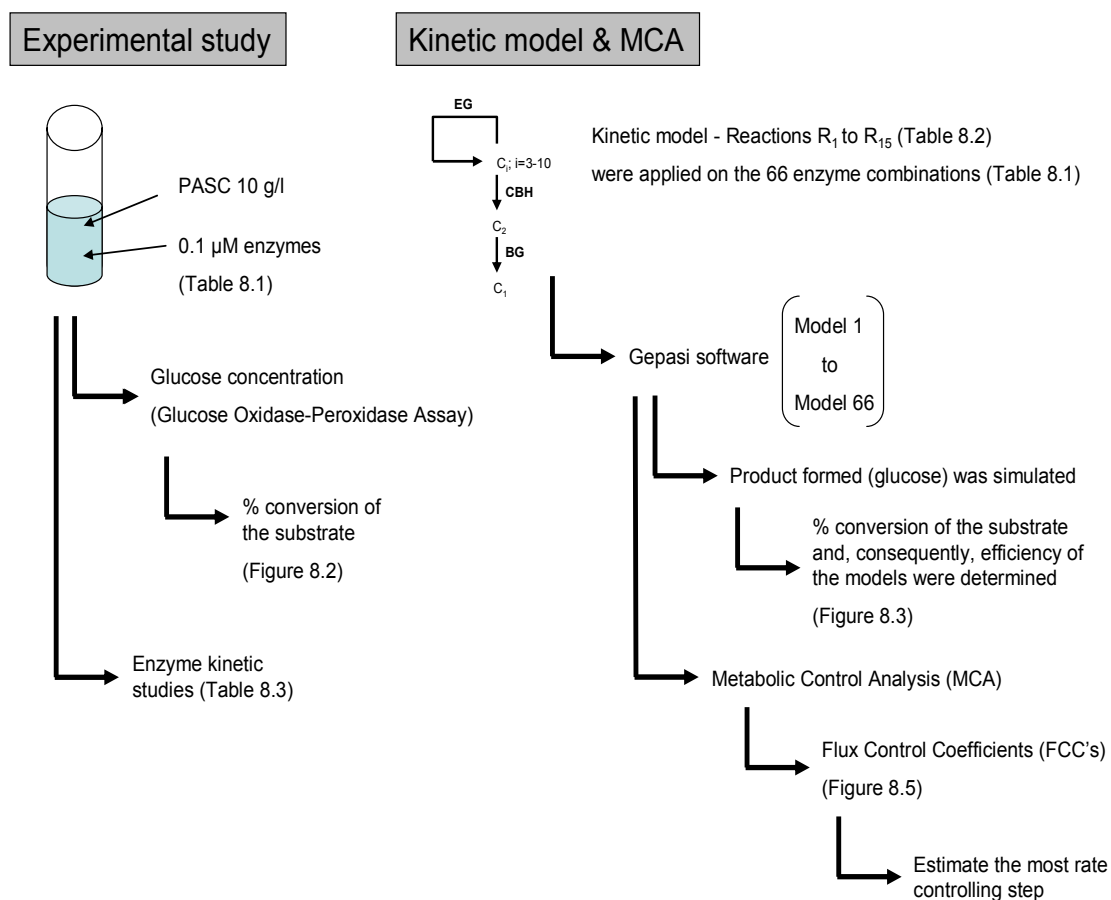
Scheme 8.1: Flow sheet of studies performed and presented in Chapter 8.

8.2.1 Experimental procedure

Model cellulose substrate, phosphoric acid swollen cellulose (PASC, 10 g/l) was hydrolyzed with a number of different enzyme combinations, consisting of endoglucanase (EG), Cel45A, a 1:3 molar ratio mixture of cellobiohydrolase (CBH) I and CBH II, Cel7A and Cel6A, respectively, and β-glucosidase (BG), Cel3A. The hydrolysis experiment set-up is presented in Table 8.1, while the detailed procedure can be found in Appendix 10.11.

Table 8.1: Hydrolysis experiment set-up.

	Mole Percentage (%)				Mole Percentage (%)				Mole Percentage (%)		
ID	BG	CBH	EG	**ID**	BG	CBH	EG	**ID**	BG	CBH	EG
1	0	0	100	**23**	20	10	70	**45**	40	60	0
2	0	10	90	**24**	20	20	60	**46**	50	0	50
3	0	20	80	**25**	20	30	50	**47**	50	10	40
4	0	30	70	**26**	20	40	40	**48**	50	20	30
5	0	40	60	**27**	20	50	30	**49**	50	30	20
6	0	50	50	**28**	20	60	20	**50**	50	40	10
7	0	60	40	**29**	20	70	10	**51**	50	50	0
8	0	70	30	**30**	20	80	0	**52**	60	0	40
9	0	80	20	**31**	30	0	70	**53**	60	10	30
10	0	90	10	**32**	30	10	60	**54**	60	20	20
11	0	100	0	**33**	30	20	50	**55**	60	30	10
12	10	0	90	**34**	30	30	40	**56**	60	40	0
13	10	10	80	**35**	30	40	30	**57**	70	0	30
14	10	20	70	**36**	30	50	20	**58**	70	10	20
15	10	30	60	**37**	30	60	10	**59**	70	20	10
16	10	40	50	**38**	30	70	0	**60**	70	30	0
17	10	50	40	**39**	40	0	60	**61**	80	0	20
18	10	60	30	**40**	40	10	50	**62**	80	10	10
19	10	70	20	**41**	40	20	40	**63**	80	20	0
20	10	80	10	**42**	40	30	30	**64**	90	0	10
21	10	90	0	**43**	40	40	20	**65**	90	10	0
22	20	0	80	**44**	40	50	10	**66**	100	0	0

Substrate=10 g/l PASC; Total enzyme concentration=0.1 μM; Incubation temperature=50 °C; Incubation time= 4 hours; BG: β-glucosidase; CBH: Cellobiohydrolase; EG: Endoglucanase. The highlighted enzyme combinations were omitted.

Contrary to similar hydrolysis experiments, described in more details in Chapter 5, Article A, analysis of the samples perform in these experiments were, due to the quantity of samples, restricted to the determination of glucose concentration by Glucose Oxidase-Peroxidse Assay (for more details see Appendix 10.12), instead of detailed quantification of all soluble sugars by HPLC.

8.2.2 Construction of the kinetic model on Gepasi
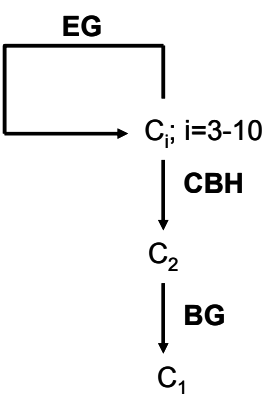
To construct a kinetic model on Gepasi, the user needs to define all possible reaction routes, from the starting molecule (substrate), trough intermediates, to the final product of the reaction (glucose). As the degree of polymerization (DP) of PASC, the model cellulose substrate used in this experiment, was determined to be 96 (Chapter 5, Article A), this would require definition of thousands of possible routes for degradation of such a large molecule. That task seemed to be too ambitious, so the kinetic model was simplified to represent degradation of C_{10} (a glucose oligomer consisting of ten glucose units) as the substrate (Table 8.2). In this model, EG was modeled to break down the larger oligomers

123

to smaller oligomers (e.g. C_{10} to C_6 and C_4). CBH will exclusively cut off cellobiose unit (C_2) from the oligomers, while BG will exclusively degrade C_2 to two glucose units (C_1). The kinetic constants used in the model, K_m and k_{cat}, were determined through enzyme kinetic studies, and are summarized in Table 8.3, while the inhibition constants were adopted from the literature. In the kinetic model, EG was assumed not to be inhibited, thus following classical Michaelis-Menten enzyme kinetics.

The designed kinetic model should, ideally, be compared to the experimental results obtained by hydrolysis of C_{10} molecule. This molecule is not commercially available, thus the experiments were performed on PASC.

Table 8.2: List of all possible reaction routes for degradation of C_{10} oligomer.

Rxn No.	S	P1	P2	Enzyme	Kinetics
1	C_{10}	C_8	C_2	CBH	CPI
2	C_{10}	C_7	C_3	EG	MM
3	C_{10}	C_6	C_4	EG	MM
4	C_{10}	C_5	C_5	EG	MM
5	C_8	C_6	C_2	CBH	CPI
6	C_8	C_5	C_3	EG	MM
7	C_8	C_4	C_4	EG	MM
8	C_7	C_5	C_2	CBH	CPI
9	C_7	C_4	C_3	EG	MM
10	C_6	C_4	C_2	CBH	CPI
11	C_6	C_3	C_3	EG	MM
12	C_5	C_3	C_2	CBH	CPI
13	C_4	C_2	C_2	CBH	CPI
14	C_3	C_2	C_1	CBH	CPI
15	C_2	C_1	C_1	BG	CPI

Rxn No.: Reaction number; S: Substrate; P1: Product 1; P2: Product 2; C_i: Glucose oligomer containing i glucose units; BG: β-glucosidase; CBH: Cellobiohydrolase; EG: Endoglucanase; CPI: Competitive product inhibition; MM: Michaelis-Menten.

The kinetic model (Table 8.2) was applied on the 66 different enzyme combinations described in Table 8.1 (i.e. the mole percentage of each enzyme was systematically changed between 0 % and 100 % (mole %) with a 10 % interval) resulting in 66 Gepasi models. In all models (Model 1 to Model 66), the total amount of enzyme used for simulations on Gepasi was set to be 0.1 μM, but varying in the percent of the

three cellulose enzymes, just as all experiments were performed with the same total amount of enzyme, using enzyme combinations described in Table 8.1.

All 66 models were simulated for a certain period of time, and the amount of glucose formed at the end of the simulation was used to determine how efficient one particular enzyme combination was at hydrolyzing the substrate. Consequently, the simulation results were compared with the real hydrolysis experiments results.

Furthermore, MCA was performed on the 66 kinetic models, resulting in a set of FCCs. The values of these coefficients were used to estimate the most rate-controlling step(s) in the simulated kinetic model, and additionally compare them with other system parameters, such as the enzyme combination.

Table 8.3: Summary of the kinetic constants used in the model.

| Enzyme	Enzyme class	k_{cat} [1/s]	K_m [g/l]	K_I [µM]
Cel45A	EG	0.085	25.0 (PASC)	
Cel7A + Cel6A, 3:1, vol:vol	CBH	0.02	29.3 (PASC)	650 (cellotriose)[1]
Cel3A	BG	0.12	0.52 (cellobiose)	3000 (cellobiose)[2]

[1]: Schulein, 1997; [2]: Calsavara et al., 1999; In parentheses, substrate used for determination of kinetic constants is stated.

8.2.3 Enzymatic hydrolysis of PASC - Results

Hydrolysis experiments as described in section 8.2.1 and Appendix 10.11 were performed on 43 enzyme combinations (Table 8.1) and the amount of glucose produced was determined using Glucose Oxidase-Peroxidase Assay (Appendix 10.12). Subsequently, the glucose concentration was converted to *percentage conversion*, and the results of these experiments are presented in Figure 8.2.

Within the four hours of hydrolysis maximal observed conversion of the substrate was 21.9 % with the enzyme combination consisting of 10 % BG, 40 % CBH and 50 % EG (enzyme combination ID 16, Table 8.1). As expected, enzyme combinations consisting of only one enzyme class (e.g. ID 1, 11 and 66, Table 8.1) showed poor substrate conversion, Figure 8.2. In more general terms it can be concluded that EG had substantial influence to the hydrolysis as all enzyme combinations consisting of 0 % or

10 % EG showed low percentage conversions which was not the case when percentage of CBH or BG was kept at that level (0 % or 10 %).
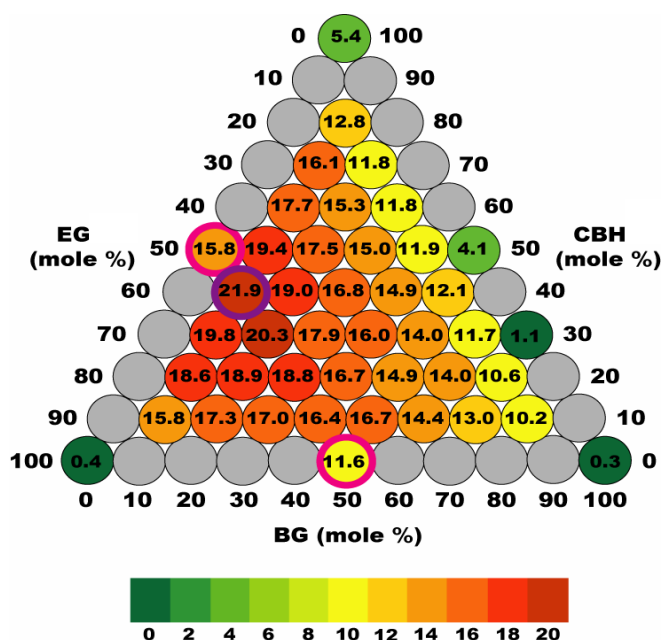
Figure 8.2: Experimental results of enzymatic hydrolysis of PASC. 1 ml of 10 g/l of PASC was hydrolyzed with 0.1 µM of enzyme of various combinations. The hydrolysis was performed at 50 °C, pH 5.5 for 4 hours. The *percentage conversion* is stated inside each circle on the plot, while the circle is colored according to the color bar below. A red circle indicates efficient hydrolysis and good enzyme combination while a green circle indicates inefficient hydrolysis. Hydrolysis performed with only one enzyme or without EG gained only small amounts of glucose (green circles). The lack of BG and CBH, respectively, did not hinder the hydrolysis efficiency much (circles with red border). The *in situ* optimal enzyme combination was determined to be 10% of BG, 40% of CBH and 50 % of EG (circle with purple border). The grey circles indicate experiments that were not performed.

8.2.4 Kinetic model of enzymatic hydrolysis of cellulose - Results

The kinetic model was defined as described in section 8.2.2 and simulated using the 66 different enzyme combinations (Table 8.1). Glucose concentration at the end of six different simulation times (4, 28, 139, 278 hours, and, 59 and 116 days) were recorded, and consequently, percentage conversion was calculated. Additionally, reaction rate change over time was simulated. The results are presented in Figure 8.3.

126
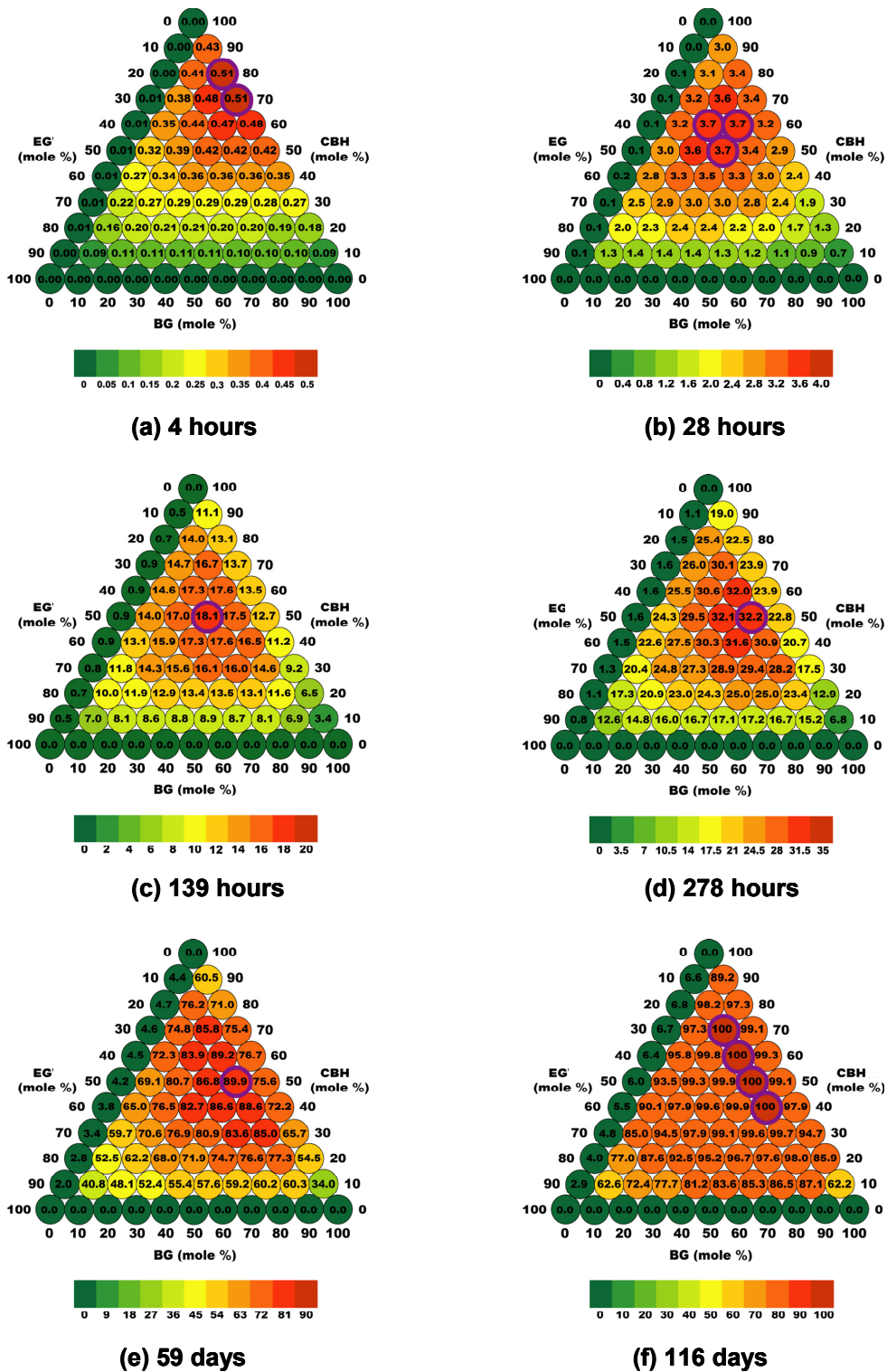
Figure 8.3: Kinetic model of enzymatic hydrolysis of cellulose – Results. Percentage conversions of C_{10} as substrate were calculated based on the simulated glucose concentrations and are stated inside the circles. The circles are colored based on their relative efficiency (see color bar). The *in silico* optimal enzyme combinations of each simulation are circled in purple.

127

The results obtained in this way (Figure 8.3) are rater different from what was observed during experimental study (Figure 8.2). Namely, while EG was identified to be the most important enzyme in the experimental study, CBH and BG were predominant enzymes during simulation study. The optimal *in silico* enzyme combination was shown to vary throughout the course of the simulation (from four hours to 2777 hours), and the general optimal enzyme combination consisted of 25-40 % BG, 50-75 % CBH and 0-20 % EG (Figure 8.4). Additionally, the glucose formation rate over time was simulated, and is presented in Figure 8.4.
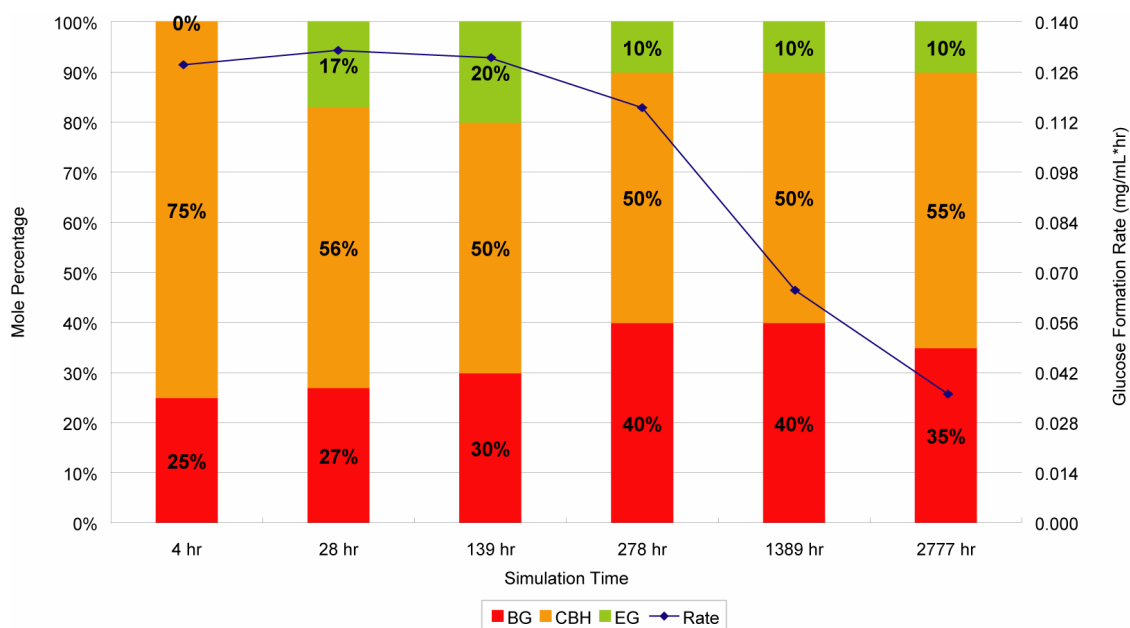
Figure 8.4: Distribution of *in silico* optimal enzyme combination for six simulated time periods and the rate of glucose formation over time.

Moreover, maximal conversion of the substrate obtained after four hours of simulation was 0.51 %, substantially lower compared to the experimental study result obtained after the same period (four hours) of hydrolysis which showed conversion of 21.9 % of the substrate. To obtain compatible substrate conversion, hydrolysis, according to the simulation study, would need to proceed for at least 139 hours.

128

8.2.5 MCA of the kinetic models - Results

MCA was performed on the results obtained by the 66 kinetic models mentioned earlier. FCCs, describing how much control each of the i-th reaction (R_i) had on the Reaction 15 (R_{15}), in which cellobiose was broken down to form two glucose units (see Table 8.2), i.e. $C(J(R_{15}), R_i)$, were calculated.

The positive value of FCC suggests that the large reaction rate of reaction R_i would increase the rate of reaction R_{15} (i.e. the glucose formation rate), while the negative value of FCC suggest that the large reaction rate of R_i would actually decrease the rate of reaction R_{15}. In other words, FCC are quantitative expressions of how much control each of the i-th reactions have over the rate of glucose formation. Moreover, if the flux control is distributed evenly among all the reactions (enzymes), the system is well-balanced.

The results showing distribution of FCCs of all kinetic models (Model 1 to Model 66) after simulation time of 139 h are presented in Figure 8.5.

Figure 8.5: Distribution of flux control coefficients (FCCs) among 66 kinetic models after the simulation time of 139 h. On the X-axis, 66 kinetic models are lined up from the least efficient one on the far left to the most efficient one on the far right. FCCs are color-coded to illustrate the distribution of the flux control. The flux control was distributed exclusively on reactions (Rxn) 1, 2, 3, 4, and 15 (see Table 8.2).

The efficiency of the kinetic models (Model 1 to Model 66) was defined based on the amount of simulated glucose production (using Gepasi software) after a defined time period, and consequently calculating the percentage conversion of the substrate. In Figure 8.3 models with low efficiency (color coded in green) are those where low amounts of glucose were predicted to be produced, thus resulting in low substrate conversion. Consequently, models with high efficiency (color coded in red) were those where high substrate conversions were calculated.

In Figure 8.5, the models are lined up from the least efficient on the left, to the most efficient on the right side. For some models, the FCC could not be determined, as steady state was not reached (e.g. in Model 1 (0 % BG, 0 % CBH and 100 % EG) glucose concentration was zero, resulting from the proposed kinetic model (Chapter 8.2.2), according to which EG will only produce shorter oligomers).

As earlier mentioned in Chapter 8.1.1, all FCC's sum to unity (Eq. 24), and furthermore, for the well balanced system the magnitude of FCC's within the reactions that constitute the pathway, and in our case between the fifteen reactions (R_1 to R_{15}) defined in Table 8.2, should be close to equal. This is unfortunately not the case as shown in Figure 8.5. First of all, FCC's seem to be distributed within only five (R_1, R_2, R_3, R_4 and R_{15}) of total fifteen reactions. Secondly, no trend of magnitude of those five FCC's getting equal as approaching to the high efficiency models was observed.

8.2.6 Summary and discussion of the results

The optimal enzyme combination evaluated based on the maximal conversion of the substrate in the experiment and the kinetic model is summarized in Table 8.4. There were two major differences between the kinetic model and hydrolysis experimental results regarding the optimal enzyme combination (Table 8.4). First, comparing the optimal enzyme combinations, BG was in less demand while EG was in much higher demand in the hydrolysis experiments than in the model. Second, the amount of conversion after four hours was 21.9 % in the experiment and only 0.51 % in the simulation, more than 40

times difference. Comparable conversion rates in the simulation study were achieved after simulating the hydrolysis period of 139 hours (Table 8.4).

Table 8.4: Summary of the optimal enzyme combinations obtained in the experiment and by modeling.

	Experiment	Kinetic model I	Kinetic model II
Time (hour)	4	4	139
Model number	16	30 and 38	36
BG:CBH:EG (mole %)	10:40:50	25:75:0	30:50:20
Conversion (mole %)	21.9	0.51	18.1

BG: β-glucosidase; CBH: Cellobiohydrolase; EG: Endoglucanase.

Based on the theory of MCA, it was assumed that well-balanced and efficient cellulose degrading enzyme system is one that has its flux control distributed evenly among the reactions, or the enzymes constituting the pathway. As shown in Figure 8.5, FCCs are dependent only on five reactions (R_1-R_4 and R_{15}) and, furthermore, there is no direct correlation between the magnitude of FCCs and efficiency of the models. For example, Model 29 and Model 37 have both similar and almost equally distributed FCCs, but are contrary to our expectations not standing beside each other on the X-axes of Figure 8.5.

8.2.7 Conclusions

The MCA have never before been applied to a pathway outside the living cell, and moreover in the classic examples of MCA the pathways are consisting of a certain number of reactions and the same number of enzymes governing them, i.e. each enzyme is catalyzing only one reaction. In the kinetic model that was proposed here one enzyme (EG or CBH) was assumed to catalyze several reactions (see Table 8.2).

The optimal enzyme combination for the hydrolysis of cellulosic substrate was shown to vary throughout the course of the simulation, underlining the importance of different enzymes at different stages of the hydrolysis. Nevertheless, large differences in the optimal enzyme combination and percentage conversion of the substrate obtained in

the experiment and the kinetic study was observed. This disagreement can be due to three factors; substrate, model assumptions and kinetic constants used in the model.

Substrate used in the kinetic model is linear C_{10} molecule while in the experiment, PASC was used. Besides having much higher DP (96), PASC is still assumed to have the three dimensional microfibril structure, as schematically represented in Figure 4.1. The hydrolysis of such a complex substrate was not properly represented by the oversimplified kinetic model. Additionally, kinetic constants used in this study were obtained from two different sources. K_m and k_{cat} were determined experimentally, in the physical conditions under which the hydrolysis was performed (pH=5.5 and T=50 °C), while inhibition constants were adopted from the literature.

No direct correlation between the FCCs and experimental results were found, but considering the large disagreement between the model assumptions and real experiment results, the final conclusion on weather MCA could be used as a tool for optimizing the hydrolysis process is still to be determined. With establishing new, properly representative kinetic model, some more promising results could be expected.

8.3   References

Calsavara LPV, de Moraes FF, Zanin GM. 1999. Modeling cellobiose hydrolysis with integrated kinetic models. App. Microbiol. Biotechnol. 77-79: 789-806.

Fell D. 1997. Understanding the control of metabolism. London: Portland Press. 300 p.

Heinrich R, Schuster S. 1996. The Regulation of Cellular Systems. New York: Springler. 400 p.

Mendes P. 1993. GEPASI: A software package for modelling the dynamics, steady states and control of biochemical and other systems. Comput. Applic. Biosci. 9: 563-571.

Schulein M.1997. Enzymatic properties of cellulases from *Humicola insolens*. J Biotechnol 57: 71-81.

Stephanopoulos GN, Aristidou AA, Nielsen N. 1998. Metabolic engineering. Principles and methodologies. San Diego: Academic press. 725 p.

CHAPTER 9

Conclusions and future perspectives

The interest in cellulose degrading microorganism has been rising since the 1950's, turning focus, particularly in the recent years, towards production of bioethanol from lignocellulosic materials, due to the growing concern about global warming and forecast of shortage of oil within the few decades. As the process of fuel production using biotechnology (i.e. bioethanol) is not in its fully developed stage and still not economically feasible, compared to fuel from oil, it is believed that this technique have large potential for improvement and cost reduction (Sheehan and Himmel, 2001). A lot has been done in improving the efficiency, and thus lowering the cost of enzymes used in the hydrolysis process (Sheehan and Himmel, 1999), but the hydrolysis process, and consequently the cost of bioethanol production, is to a large extent governed by significant compositional diversity of the lignocellulosic biomass.

In this thesis I have summarized and discussed the most important factors influencing enzymatic hydrolysis of cellulose, such as some physicochemical properties of the substrate (e.g. crystallinity, degree of polymerization, available/accessible surface area, and, macro- and micro- substrate structure), as well as, some enzyme related factors, in particular the importance of properly selected enzyme mixture, and cooperativity between the enzymes. In addition to the experimental studies, the hydrolysis process was also approached from the modeling point of view. Mathematical modeling and consequent model predictions can be used as a valuable tool for fundamental understanding of the process, but also for highlighting the points where improvements of the process could be beneficial. In this thesis two different types of modeling approaches have been applied; A mechanistic, de-polymerization type of model, relying heavily on the description of enzyme kinetics, and, a novel approach in which the application of the metabolic control analysis theory to the enzymatic hydrolysis of cellulose was investigated.

Both specific enzyme-, but also, to a great extent, substrate related factors have excessive influence on the course of the hydrolysis process. To be able to distinguish among them, during the experimental study, I choose to work with highly purified, mono-component, enzymes, and relatively well defined model substrates. Following was found:

- The composition of the optimal enzyme mixture used for hydrolysis was shown to be dependent on the substrate characteristics. The β-glucosidase enzyme, responsible for the reaction in the liquid phase, was shown to be rate limiting during degradation of more amorphous substrate (PASC), while degradation of Avicel, as more crystalline substrate, was hindered by limited availability of substrate surface. Thus, depending on the characteristics of the substrate used during hydrolysis, different enzyme components showed to be of different importance.

- Substrate characteristics also governed the cooperativity between the enzymes. Degree of synergy (DS) (as a quantitative measure of the extent of synergy between the enzymes) was found to be generally larger then one during degradation of PASC (indicating cooperativity between the enzymes, on this chemically treated, amorph, and thus, more easier accessible cellulosic substrate). Contrary to degradation of PASC, DS values less then one were observed during degradation of Avicel, demonstrating competition between the enzymes for the available/accessible binding sites on this crystalline substrate.

Opposed to tedious and time consuming experimental determination of optimal enzyme mixture for maximal substrate degradation, the advantages of fast and easy predictions of hydrolysis development available through mathematical modeling are appealing. The criteria of any mathematical model are simplicity and robustness, and if valuable process forecasts are to be expected, those need to be correlated with the requirements for accurate description of both substrate and enzyme characteristics in the model. In this thesis two model types were investigated:

- Mechanistic, de-polymerization type of model describing enzymatic degradation of cellulose was shown to be qualitative, i.e. describing reasonably well products of the hydrolysis process, but from a quantitative aspect, the model will, in almost all cases, irrelevant on the magnitude of kinetic constants used, predict 100 % conversion of the substrate. Therefore, concerning future perspectives of developing mechanistic mathematical model, a term describing irreversible enzyme degradation should be incorporated into the model. Moreover, more sufficient description of substrate characteristics, in particular explicit mathematical formulation of the three dimensional, bundle like, structure of cellulosic substrate would be essential if the improvements of the model are to be expected.

- A novel approach, based on application of metabolic control analysis (MCA) theory to the enzymatic hydrolysis of cellulose, was investigated in respect to determining the most rate controlling step(s)/enzyme of the process. Simple kinetic model describing degradation of ten glucose units long substrate (C_{10}) was evaluated against numerous experimental results from experiments performed using PASC. Flux control coefficients (FCCs) were consequently calculated, with the hypothesis that the magnitude of FCCs will point out to the rate-controlling step in the hydrolysis process. No direct correlation between the FCCs and experimental results were found, but considering the large disagreement between the model assumptions and real hydrolysis conditions, the final conclusion on weather MCA could be used as a tool for optimizing hydrolysis process is still to be examined. Future work could include designing kinetic model that will properly describe degradation of complex cellulosic substrates, corresponding to the model substrates such as Avicel and/or PASC. Such a modelling design could be expected to correlate with the experimental result performed on the model substrates to a much higher extent.

9.1 References

Sheehan JS, Himmel ME. 2001. Outlook for bioethanol production from lignocellulosic feedstocks: technology hurtles. Agro-Food-Industry Hi-Tech. September/October: 54-56

Sheehan J, Himmel M. 1999. Enzymes, energy, and the environment: a strategic perspective on the U.S. department of energy's research and development activities for bioethanol. Biotechnol. Prog. 15: 817-827.

APPENDIX

10.1 Nelson-Somogyi assay

Reagents	Apparatus
Potassium sodium tartrate	2-mL Eppendorf tube with safety cap
Sodium carbonate	Water bath (100°C)
Sodium hydrogen carbonate	Disposable cuvette
Sodium sulfate	Spectrophotometer
Sodium benzoate	
Copper (II) sulfate pentahydrate	
Ammonium molybdate	
Sodium arsenate dibasic heptahydrate	
Deionized water	

Procedure

1. Copper reagent (A) was prepared by dissolving 12 grams of potassium sodium tartrate, 24 grams of sodium carbonate, 16 grams of sodium hydrogen carbonate, 144 grams of sodium sulfate and 4 gram of sodium benzoate in ca. 500 mL of deionized water. The solution was heated until all chemicals were dissolved. The final volume was adjusted to 800 mL with deionized water. This solution can be stored in room temperature.

2. Copper reagent (B) was prepared by dissolving 4 grams of copper sulfate pentahydrate and 36 grams of sodium sulfate in ca. 150 mL of deionized water. The solution was heated until all chemicals were dissolved. The final volume was adjusted to 200 mL with deionized water. This solution can be stored in room temperature.

3. 25 grams of ammonium molybdate was dissolved in 450 mL of deionized water. 21 mL of concentrated sulfuric acid was added into the molybdate solution. 3 gram of sodium arsenate dibasic heptahydrate was dissolved in 25 mL of deionized water. Finally, the color reagent was prepared by mixing the acidic molybdate solution with the arsenate solution. The solution was kept in a brown bottle and was incubated at 37°C for 48 hours before use.

4. Glucose and cellobiose standards were prepared at 0.001 mM, 0.005 mM, 0.01 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.5 mM, 0.8 ,M, 1 mM, 5 mM, 10 mM, 15 mM, 20 mM,

30 mM, 40 mM, 50 mM, 60 mM and 70 mM in deionized water.

5. 100 µL of sample or standard was mixed with 80 µL of copper reagent (A) and 20 µL of copper reagent (B) in an eppendorf tube.

6. The mixture was thoroughly mixed before being heated in a boiling water bath for 10 minutes.

7. The mixture was then cooled in an ice/water bath for 1-2 minutes.

8. 100 µL of color reagent was then added to the mixture and mixed well.

9. 950 µL of deionized water was also added to the mixture and mixed well.

10. Once all carbon dioxide had escaped from the solution, absorbance was measured against a blank at 500 nm, 520 nm, 660 nm and 750 nm.

Note (1): Occasionally, there might be precipitation in the color reagent. Precipitation can be filtered out with any 0.45 µM or 0.22 µM filter.

Note (2): When using 750 nm, samples should be left for at least 1.5 hour for full color development before measurement.

References:

Hatanaka and Kobara. 1980. Determination of glucose by a modification of Somogyi-Nelson method. Agric. Biol. Chem. 44: 2943-2949.

Marais JP, de Wit JL, Quicke GV. 1966. A critical examination of the Nelson-Somogyi method for the determination of the reducing sugars. Anal. Biochem. 15: 373-381.

Nelson N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153: 375-380.

Somogyi M. 1952. Notes on glucose determination. J. Biol. Chem. 195: 19-23.

10.2 4-Hydroxybenzoic acid hydrazide (PAHBAH) Assay

Reagents	Apparatus
4-Hydroxybenzoic acid hydrazide	5-mL test tubes
0.5 M HCl	Water bath (70°C)
0.5 M NaOH	Disposable cuvette
Bismuth nitrate	Spectrophotometer
Potassium sodium tartrate	
Sodium hydroxide (solid)	
Glucose	
Cellobiose	

Procedure

1. 5 % (w/v) 4-hydroxybenzoic acid hydrazide was prepared in 0.5 M HCl. This solution can be stable for at least one month.

2. In every liter of the bismuth reagent, there was 1 mole of bismuth nitrate, 1 mole of potassium sodium tartrate and 3 moles of sodium hydroxide. The reagent was prepared with deionized water.

3. The acid hydrazide solution from (1) was mixed with bismuth reagent and diluted with 0.5 M NaOH to give 1 mM of bismuth and 0.5 % or 1 % (w/v) solution of hydrazide in alkali. This solution should be prepared right before use and is only stable through a normal working day.

4. Glucose and cellobiose standards were prepared at 0.001 mM, 0.005 mM, 0.01 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.5 mM, 0.8 ,M, 1 mM, 5 mM, 10 mM, 15 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM and 70 mM in deionized water.

5. For saccharide solution of greater than 1 mM, 10 μL of sample or standard was mixed with 3 mL of 0.5 % hydrazide in alkali, and heated at 70° C for 5 minutes.

6. For saccharide solution of less than 1 mM, 0.5 mL of sample or standard was mixed with 1.5 mL of 1 % hydrazide in alkali, and heated at 70° C for 5 minutes.

7. After cooling in an ice/water bath, absorbance was read at 410 nm against a blank.

References:

Lever M. 1972. A new reaction for the colorimetric determination of carbohydrates. Anal. Biochem. 47: 273-279.

Lever M. 1977. Carbohydrate determination with 4-hydroxybenzoic acid hydrazide (PAHBAH): effect of bismuth on the reaction. Anal. Biochem. 81: 21-27.

10.3 *Dinitrosalicylic acid (DNS) assay*

Reagents	Apparatus
Dinitrosalicylic acid	5-mL test tubes
Phenol	Water bath (100°C)
Sodium sulfite	Disposable cuvette
Sodium hydroxide	Spectrophotometer
Potassium sodium tartrate	
Deionized water	
Glucose	
Cellobiose	

Procedure

1. 1 % (w/v) dinitrosalicylic acid reagent solution was prepared by dissolving 10 grams of dinitrosalicylic acid, 2 grams of phenol, 0.5 gram of sodium sulfite and 10 grams of sodium hydroxide in 1 liter of deionized water.

2. 40 % (w/v) potassium sodium tartrate solution was prepared in deionized water.

3. Glucose and cellobiose standards were prepared at 0.001 mM, 0.005 mM, 0.01 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.5 mM, 0.8 ,M, 1 mM, 5 mM, 10 mM, 15 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM and 70 mM in deionized water.

4. 0.5 mL of sample or standard and 0.5 mL of 1 % dinitrosalicylic acid reagent were mix together in a test tube and heated in a boiling water bath for 10 minutes or 60 minutes.

5. 300 μL of 40 % potassium sodium tartrate solution was added into the mixture to stabilize the color.

6. The mixture was then cooled in an ice/water bath.

7. After cooling, absorbance was read at 575 nm against a blank.

Note (1): As a standard procedure, color development should be done in a boiling water bath for 10 minutes.

Note (2): Phenol was added to increase the color intensity. It could be omitted if desired.

References:

Wang NS. 2005. Experiment no. 4A, Glucose assay by dinitrosalicylic colorimetric method. Retrieved 14/5-2005 from the University of Maryland, Department of Chemical Engineering Web site: http://www.glue.umd.edu/~nsw/ench485/lab4a/htm.

10.4 2-Cyanoacetamide assay

Reagents	Apparatus
2-Cyanoacetamide	5-mL test tube
0.1 M Borate buffer, pH 9.0	Water bath (100°C)
Water	Ampertured quartz cuvette
Glucose	Spectrophotometer
Cellobiose	

Procedure

1. 1% (w/v) 2-cyanoacetamide reagent was prepared in deionized water. This reagent should be store in the dark at 4°C and used within one month.

2. Glucose and cellobiose standards were prepared at 0.001 mM, 0.005 mM, 0.01 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.5 mM, 0.8 ,M, 1 mM, 5 mM, 10 mM, 15 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM and 70 mM in deionized water.

3. 250 µL of sample or standard was mixed with 250 µL of 1 % 2-cyanoacetamide reagent and 500 µL of 0.1 M borate buffer (pH 9.0).

4. The mixture was heated in a boiling water bath for 5 minutes and cooled in an ice/water bath.

5. Absorbance was measured at 274 nm in a quartz cuvette against a blank.

References:

Honda S, Nishimura Y, Takahashi M, Chiba H, Kakehi K. 1982. A manual method for the spectrophotometric determination of reducing carbohydrates with 2-cyanoacetamide. Anal. Biochem. 119: 194-199.

Bach E, Schollmeyer E. 1992. An ultraviolet-spectrophotometric method with 2-cyanoacetamide for the determination of the enzymatic degradation of reducing polysaccharides. Anal. Biochem. 203: 335-339.

10.5 Ferricyanide assay

Reagents	Apparatus
Potassium ferricyanide	5-mL test tube
Sodium carbonate	Water bath (100°C)
Potassium cyanide	Disposable cuvette
Ferric ammonium sulfate	Spectrophotometer
Sodium dodecyl sulfate	
Concentrated sulfuric acid	
Deionized water	
Glucose	
Cellobiose	

Procedure

1. Ferricyanide reagent was prepared by dissolving 0.5 gram of potassium ferricyanide in one liter of deionized water and stored in a brown bottle.

2. Carbonate-cyanide reagent was prepared by dissolving 5.3 grams of sodium carbonate and 0.65 gram of potassium cyanide in one liter of deionized water.

3. Ferric iron reagent was prepared by dissolving 1.5 gram of ferric ammonium sulfate and 2 gram of sodium dodecyl sulfate in 0.2 M of sulfuric acid.

4. Glucose and cellobiose standards were prepared at 0.001 mM, 0.005 mM, 0.01 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.5 mM, 0.8 ,M, 1 mM, 5 mM, 10 mM, 15 mM, 20 mM, 30 mM, 40 mM, 50 mM, 60 mM and 70 mM in deionized water.

5. 300 μL of sample or standard was mixed with 100 μL of ferricyanide reagent and 100 μL of carbonate-cyanide reagent.

6. The mixture was heated in a boiling water bath for 10 minutes and cooled in an ice/water bath.

7. 500 μL of ferric iron reagent was then added to the mixture and 15 minutes or 60 minutes were allowed for color development.

8. Absorbance was measured at 690 nm against a blank.

References:

Park JT, Johnson MJ. 1949. A submicro determination of glucose. J. Biol. Chem. 181: 149-151.

Raju TS, Nayak N, Briggs J, O'Conner JV, Lerner L. 1999. A convenient micro-scale colorimetric method for determination of terminal galactose on immunoglobulins. Biocem. Biophys. Res. Commun. 261: 196-201.

10.6 8-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS) derivatization

Reagent	**Apparatus**
ANTS	Rotational vacuum concentrator
Acetic acid	
Sodium cyanoborohydride	
Dimethyl sulfoxide (DMSO)	
Urea	

Procedure

1. 0.2 M and 20 mM of ANTS was prepared in water/acetic acid (17:3 v/v). ANTS solution was split into small quantities and stored in -20˚C, which can be stored for at least 2 months.

2. 1.0 M and 0.1 M of sodium cyanoborohydride was prepared in DMSO. This solution was prepared right before the derivatization reaction.

3. 6 M of urea was prepared with distilled water.

4. Saccharide samples were dried in a rotational vacuum concentrator at 40˚C for 60 minutes or until samples were dried.

5. For saccharides with known concentration, each mole of saccharide was derivatized with 10 moles of ANTS and 10 moles of $NaCNBH_3$.

6. For samples of unknown concentration (the enzymatic hydrolysates), 20 µL of the hydrolysate was derivatized with 10 µL of 20 mM ANTS in acetic acid/water (3:17 v/v) and 10 µL of 0.1 M $NaCNBH_3$ in DMSO.

7. The mixture was incubated at 37 °C for 15 hours and dried in a rotational vacuum concentrator at 40 °C for 2 hours.

8. 6M Urea was added to each sample either to its original volume or to a different volume in order to dilute or concentrate the samples.

9. All samples were stored at -20 °C until analysis.

10.7 Polyacrylamide gel preparation

Reagent	Apparatus
ProtoGel, 30 % (37.5:1) acrylamide/bisacrylamide	Glass plates (16 x 18 cm)
Tris base	Spacer (1.5 mm thickness)
Hydrochloric acid	Comb (10 teeth, 1.0 cm width each)
Glycine	Vacuum pump
Borate	
Ethanol	
Ammonium persulfate	
N,N,N',N'-Tetramethylethylenediamine (TEMED)	
n-Butanol	
Urea	

Procedure

1. Glass plates, spacers and combs were cleaned twice with detergent, rinsed with distilled water and polished with 60 % ethanol.

2. Gel cassette was assembled according to manufacture instruction.

3. Stock gel buffer and stock running buffer were prepared according to Table 10.1.

4. 10 % (w/v) ammonium persulfate was prepared with deionized water. This solution was split into small quantities and stored at -20 °C.

5. 40 mL n-butanol was mixed with 4 mL of deionized water to make water-saturated n-butanol. This solution was kept at 4 °C.

6. Resolving gel solution was prepared with 3 mL of 10X stock gel buffer, 20 mL of ProtoGel solution, and 6.7 mL of deionized water. Gel solution was vacuum degassed for 5 minutes.

7. Degassed resolving gel solution was polymerized by adding 300 µL of 10 % ammonium persulfate and 30 µL of TEMED and poured into gel cassette. Immediately after pouring, a layer of water-saturated n-butanol was laid over the gel. Gel was allowed to set for at least 10 minutes.

8. Stacking gel solution was prepared with 0.5 mL of 10X stock gel buffer, 1.3 mL of ProtoGel solution, and 3.2 mL of deionized water. It was polymerized by adding 25 µL of 10 % ammonium persulfate and 5 µL of TEMED and poured onto the resolving gel. Gel was allowed to polymerize for at least one hour.

9. Gel was used immediately or covered with wet kitchen towel, wrapped in aluminium foil and stored at 4 °C. Gel can be kept for one week.

10. A special urea gel was prepared based on step 1 to 9, but including 30 % (w/v) of urea in the resolving gel and 46 % (w/v) of urea in the stacking gel.

Table 10.1: Different gel buffers and running buffers used in this study.

Stock Gel Buffer	pH	Stock Running Buffer	pH
20 % Polyacrylamile gel			
4X: 1.5 M Tris-HCl	8.8	10X: 1.92 M Glycine, 0.25 M Tris base	8.5
4X: 1.5 M Tris-HCl	8.8	10X: 1.0 M Tris-HCl	8.2
10X: 1.0 M Tris-Borate	8.2	10X: 1.0 M Tris-Borate	8.2
Invitrogen NuPage 4-12 % Gel			
Tris-HCl*	6.4	10X: 1.0 M Tris-HCl	8.2

* Concentration not stated by the manufacturer.

10.8 Gel imaging

Apparatus
Gel Doc 2000 UV Transilluminator
Quantity One software (version 4.0.2)

Procedure

1. Electrophorezed gels were viewed with Gel Doc 2000 UV transilluminator and Quantity One software (version 4.0.2) (Bio Rad). Both the shutter on the camera and the exposure setting on the software were adjusted so that the strongest possible signals could be obtained without saturating the image.

2. Background noise was removed by Filter Wizard at 3x3 pixels filter size.

3. For 4-12 % mini-gel, data of an "Intensity vs. Relative Front" plot was obtained using the "Lane Tool". Data were exported as Excel file. Quantification of samples on a 4-12 % mini-gel was defined in this project and explained in section 5-1.

4. For 20 % gel, "Volume" of each sample band was calculated by the software using the "Volume Tool" and sample concentration was calculated automatically from that. Concentration data were exported as Excel file.

10.9 Glucose oligomer ladder preparation

Reagent	Apparatus
Starch	Mini autoclaving pot
Avicel PH101	
Phosphoric acid swolle cellulose (PASC)	
Hydrochloric acid	
Phosphoric acid	

Procedure

1. Starch in distilled water (100 mg/mL) was hydrolyzed with 0.1 M HCl in 50 °C water bath for one hour.

2. Avicel in distilled water (100 mg/mL) was hydrolyzed with 0.1 M HCl in 50 °C water bath for one hour.

3. Avicel in distilled water (100 mg/mL) was hydrolyzed with 0.5 M, 1.0 M, 1.5 M and 2.0 M HCl in 95 °C water bath for one hour.

4. Avicel in distilled water (100 mg/mL) was hydrolyzed with 0.5 M H_2SO_4 in 95 °C water bath for one hour.

5. PASC (10 mg/mL) was autoclaved at 1.4 bar, 125 °C for 20 minutes.

10.10 *Electrophoresis*

Reagent	Apparatus
Tris base	Power supply
Glycine	
Hydrochloric acid	
Borate	

Procedure

1.   1.5 µL of the derivatized saccharide (resuspended in 6 M Urea) was loaded directly into each well.

2.   Derivatized cellotriose at 0.05 mM, 0.1 mM and 0.5 mM was used as concentration standards for each gel.

3.   NuPage 4-12 percent gel (Invitrogen) was electrophoresized at 100 V until the excess ANTS reached the bottom of the gel (roughly 2 hours).

4.   Large 20 % polyacrylamide gels were electrophorezed at 116 V for 20 minutes and at 586 V for 60 minutes or until the excess ANTS reached the bottom of the gel. The progress of the electrophoresis could be monitored using a hand-held UV light lamp without removing the gel cassette from the system, though excess exposure of the gel to UV light should be avoided.

5.   The entire electrophoresis was carried out in an ice bath. The lower buffer chamber of the electrophoresis system contained a set of cooling tubes and was circulated with ice-cold water during the entire electrophoresis.

10.11 Enzymatic hydrolysis

Reagent	Apparatus
Avicel PH101	5-mL test tubes
Phosphoric acid swollen cellulose (PASC)	Caps for 5-mL test tubes
Enzymes (see Table 4.4, Chapter 4)	Eppendorf tube with secure caps
0.1M NaOAc, pH 4.0 (enzyme buffer for Cel3A)	50˚C Oven with shaking tables
0.1M NaOAc, pH 5.5 (enzyme buffer for Cel7A)	
0.1M NaMOPS, pH 7.5 (enzyme buffer for Cel7B and Cel45A)	
0.1M Glycine, pH 9.0 (enzyme buffer for Cel6A)	
Penicillin V	

Procedure

1. Enzyme buffers were prepared and adjusted to the desired pH. 100 mmol of penicillin V was added for every liter of buffer to prevent bacterial growth.

2. PASC was precipitated by centrifugation at 5000 rpm for 20 minutes. The pellet was washed twice with enzyme buffer and finally resuspended with enzyme buffer to the original volume.

3. Hydrolysis experiment was performed according to Table 8.1 (Chapter 8)

4. In each 5-mL test tube, 3 mL of substrate/enzyme mixture was prepared, containing 10 mg/mL of substrate and 0.1 µL of enzyme.

5. All test tubes were kept in a 50˚C oven with gentle shaking on a shaking table.

6. At 1.5-hour, 3-hour, 6-hour, 12-hour, 24-hour, 48-hour, 72-hour and 96-hour, 100 µL of the Avicel samples was drawn and boiled for 10 minutes in an Eppendorf tube with secured cap to quench enzyme activity.

7. At 0.25-hour, 0.5-hour, 0.75-hour, 1-hour, 1.5-hour, 2-hour, 3-hour and 4-hour, 10.5-hour and 24-hour, 100 µL of the PASC samples was drawn and boiled for 10 minutes in an Eppendorf tube with secured cap to quench enzyme activity.

8. All hydrolysates were kept at 4˚C until ready for further analysis.

10.12 Glucose oxidase-peroxidase (GO) Assay

Reagents	Apparatus
Glucose oxidase-peroxidase reagent (GO kit)	Spectrophotometer
o-Dianisidene reagent (GO kit)	Cuvettes
Glucose standard solution (GO kit)	Test tubes
12 N Sulfuric acid	Water bath (100°C)

Procedure

1. Glucose oxidase/peroxidase reagent was prepared by dissolving the content of the capsule in the provided amber bottle with 39.2 mL of deionized water.

2. o-Dianisidine reagent was prepared by mixing the content in the o-dianisidine vial with 1 mL of deionized water. The vial was inverted several times to fully dissolve the content.

3. Assay reagent was prepared by adding 0.8 mL of o-Dianisidine reagent to the amber bottle containing 39.2 mL of glucose oxidase/peroxidase reagent. The reagent was mixed by inverting the bottle several times. The reagent was protected from light exposure and could be kept at 4°C for up to one month.

4. Glucose standard solutions of 20, 40, 60 and 80 ug/mL were constructed from the provided glucose standard solution (1 mg/mL).

5. 0.5 mL of water or glucose standard solution or sample was mixed with 1 mL of assay reagent in a test tube and mixed well.

6. The test tubes were transferred to a 37°C water bath and incubated for exactly 30 minutes.

7. After incubation, the reaction was stopped by adding 1 mL of 12 N sulfuric acid.

8. After careful mixing and transferring to a cuvette, all samples were read on a spectrophotometer at 540 nm against the reagent blank.

9. The amount of glucose was estimated from the glucose standard curve.

Note: It is important to properly dilute the samples so that the final glucose concentration falls within the range of the standard solutions.

SAMMENFATNING PÅ DANSK

Cellulose er det mest udbredte materiale på Jorden. Det findes primært i planter, hvor det sammen med hemicellulose og lignin opbygger så kaldt lignocellulose. Interessen for lignocellulose, som billig og let tilgængelig råmateriale for fremstilling af bioethanol, til brug som additiv i benzin har været stærkt stigende i de sidste år. I 2001 foreslog EU-Kommissionen et direktiv, der skal fremme brugen af biobrændsel i transportsektoren med et fælleds mål som fokuserer på at mindske CO_2-udslip, og er stærk forbundet med den øgede opmærksomhed omkring globalt opvarmning.

Fremstilling af bioethanol kan, meget groft, opdeles i to trin; nedbrydning af cellulose og hemicellulose til sukrer ved hjælp af enzymer, i en proces som kaldes enzymatisk hydrolyse, og efterfølgende gæring af sukkeret hvor ethanol produceres. Denne proces er på nuværende tidspunkt ikke økonomisk rentabel, men der er trods alt blevet gjort meget for at sænke prisen på enzymerne, og samtidig øge deres effektivitet. Andre retningslinier for forbedring af processen fokuser på bedre forståelse af den komplekse biomasse (lignocellulose) som bruges som råmateriale til fremstilling af bioethanol, og forståelse af det sammenspil mellem enzymerne som sikrer effektiv hydrolyse. Denne PhD afhandling går tæt på de vigtigste faktorer, der er forbundet med enzymatisk hydrolyse (nedbrydning) af cellulose; enzymrelaterede faktorer såsom synergi og enzymblandinger, og substratrelaterede faktorer som struktur, krystalinitet og tilgængelighed af substrat for enzymerne. Desuden, undersøges der muligheder for bedre procesforståelse, og derved optimering af enzymblandinger for hydrolyse af specifike biomassetyper ved hjælp af matematisk modellering.

Et bredt spektrum af mikroorganismer, primært bakterier og skimmelsvampe, producerer specifikke enzymer, cellulaser og hemicellulaser som kan, mere eller mindre effektiv nedbryde, hhv. cellulose og hemicellulose. I dette arbejde fokuser jeg på cellulaser, som kan videre inddeles i tre underklasser: endoglukanaser (EG), cellobiohydrolaser (CBH) og β-glukosidaser (BG). Disse tre enzymklasser arbejder i synergi (hjælpes ad) for at nedbryde cellulose, som er, trods sin simple kemiske struktur (lange kæder af glukoseenheder) meget svært nedbrydelig materiale. Da forskelle i

struktur og kvalitet af de substrater som bruges til hydrolyse eksperimenter kan have betydelig indflydelse på resultatet, har jeg valgt at arbejde med de så kaldte model-substrater, Avicel og fosforsyre behandlet Avicel (phosporic acid swollen cellulose, PASC). Deres karakteristikker afspejler de vigtige substratrelaterede faktorer i hydrolyseprocessen, som krystalinitet og tilgængelighed af substratet. I det eksperimentale arbejde som er blevet lavet i forbindelse men denne afhandling blev der fundet indbyrdes synergier afhængigt af hvilket substrat der har været brugt i hydrolyseprocessen. Graden af enzym synergi (degree of synergy, DS), viste sig at være positiv for hydrolyse af let tilgængelig PASC, men, derimod negativ for højkrystalinisk Avicel. Dette endvidere indikerer at vise stereokemiske processer på substratets overflade har stor betydning for hydrolysen. BG enzymet, som spalter cellobiose og andre små opløselig polymere til glukose, processens egentlig produkt, har stor betydning for hydrolysens forløb når substratet er PASC, hvor derimod, de andre to enzymklasser, EG og CBH, som primært katalysere reaktioner på substratet overflade, har omfattende betydning for hydrolyse af Avicel. Den ideelle enzymblanding skal derfor "skræddersyes" til det substrat som ønskes nedbrudt.

Matematiske modeller kan med større eller mindre succesgrad anvendes til beskrivelse af biologiske processer. En tilfredsstillende model burde være simpel og robust, samtidig med at den tager tilstrækkelig højde for de vigtigste systemparametre. Matematisk modellering af enzymatisk hydrolyse af cellulose kan være udfordrende og krævende da flere substrat- samt enzymkarakteristikker ønskes inkluderet i modellen, som efterfølgende kraftigt øger antal af parametre i modellen og dermed dens kompleksitet. En mekanistisk, de-polymeriseringstype model blev udviklet, og dens forudsigelse af hydrolyse forløbet blev sammenlignet med de eksperimentelle data. Resultater har vist at bedre (matematisk) beskrivelse af irreversibelt enzym degradering samt tilstedeværelse af uproduktivt enzym-substrat bindinger burde inkluderes i modellen for bedre hydrolyse forudsigelse.

Sidst men ikke mindst, har jeg undersøgt muligheder for at anvende en relativt ny metode, metabolic control anlysis (MCA), for enzymhydrolyse processen. I de lukkede systemer kan denne metode identificere de enzymer som har højst betydning for reaktionshastigheden ("flaskehalsenzymer"), men den har ikke været anvendt i

forbindelse med systemer udenfor cellen (såsom enzymatisk hydrolyse). En relativt simpel kinetisk model blev benyttet her, og der blev fundet at modelresultatet kunne ikke i tilstrækkeligt grad sammenlignes med eksperimentaldata og videre udvikling af modellen er nødvendig hvis denne metode skulle anvendes til udvikling og optimering af enzymhydrolyse processen.