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Activity and synergistic action on cellulosic substrates of engineered product-site variants of *Hypocrea jecorina* Cel7A

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Abstract

Cellobiohydrolase Cel7A (Hje_Cel7A) from the ascomycete fungus *Hypocrea jecorina* is the major component of enzyme cocktails for degradation of plant biomass to soluble sugars for conversion to biofuels. It's active site is enclosed in a cellulose-binding tunnel and it is able to processively cleave off several cellobiose units from the end of a cellulose chain before being released. However, Cel7A is sensitive to product inhibition due to strong binding of cellobiose in subsites +1 and +2 in the active site. Prior to this study, variants of Hje Cel7A, aimed at both stronger and weaker cellobiose binding, have been designed and expressed. Large increase of k_{cat} and K_M and reduced cellobiose inhibition were found on soluble substrates. The purpose of the present study is to complement the previous results with evaluation of the performance of the Cel7A variants on "real" insoluble cellulosic substrates, and also compare with the corresponding major enzymes from two basidiomycete fungi, *Phanerochaete chrysosporium* Cel7D (Pch Cel7D) and *Heterobasidion irregulare* Cel7A (Hir Cel7A). Activity was measured at 40°C, pH 5.0, on Avicel cellulose (5g/l) incubated for 2 hours, untreated spruce saw dust powder (30 g/l) incubated for 20 hours, and industrially pretreated spruce material (50 g/l) incubated for 20 hours with 50 mg/l of enzyme, either Cel7 enzymes alone, or together with a commercial cellulase enzyme cocktail, or mixed with the same cocktail where the Hje Cel7A wildtype enzyme had been selectively removed. The differences in activity were less dramatic than previously observed with soluble substrates. On Avicel, the AAA variant alone showed slightly higher activity, but in all other cases Hje Cel7A WT was most active, closely followed by the 2CC variant. On untreated spruce, the 2CC variant gave most reducing sugar when added to Cel7A-free Accellerase and Accellerase 1500. Interestingly, Hir Cel7A was much more active than the others when acting alone on untreated spruce. However, it was not analysed which sugars were actually released. No conclusions could be drawn with thermo-chemically pretreated spruce, because the amount of soluble sugar was rather high already before adding the enzymes, only small amounts of sugars were released by the enzymes, and the variation was high between samples. The possible presence of enzyme inhibitors need to be further investigated. Overall, the results indicate that a more open active site and weaker cellobiose binding is not beneficial for the enzyme performance on insoluble lignocellulose. Rather it is an advantage with a more closed tunnel.

Keywords

Hypocrea jecorina; biofuels; cellulase; Avicel; untreated spruce; Thermo-chemically pretreated spruce; Cellobiose

Abbreviations

CBH, Cellobiohydrolase: **CBM**, cellulose-binding module: **Hje Cel7A**, *Hypocrea jecorina* cellobiohydrolase 7A: **Pch Cel7D**, *phanerochaete chrysosporium* cellobiohydrolase 7D: **Hir Cel7A**, *Heterobasidion irregulare* Cellobiohydrolase 7A: **TCP**, Thermo-chemically pretreated: **DM**, Dry matter: **EG**, Endoglucanases: **SDS**, Sodium dodecyl sulphate: **GH**, Glycoside hydrolase: **DEAE**, Diethylaminoethyl: **PAHBAH**, p-hydroxybenzoic acid hydrazide reagent: **BGL**, Beta-glucosidase: **IEX**, Ion-exchange chromatography: **HPAEC-PAD**, High performance anion-exchange chromatography with pulsed amperometric detection.

Contents

Objective

The purpose of the project was to complement previous studies of a set of engineered product site variants of *Hypocrea jecorina* Cel7A (Hje Cel7A) with evaluation of their performance on "real" insoluble cellulosic substrates in comparison with the wildtype enzyme and also with the corresponding major enzymes from two basidiomycete fungi, *Phanerochaete chrysosporium* Cel7D (Pch Cel7D) and *Heterobasidion irregulare* Cel7A (Hir Cel7A). Since Cel7 enzymes have evolved to cooperate synergistically with other biomass degrading enzymes in the host's secretome, activities were also measured when the Cel7 enzyme was added to a commercial cellulase enzyme cocktail in order to evaluate any possible further enhancement of the activity by any of the Cel7 variants. Three different substrates were tested, pure microcrystalline cellulose (Avicel), untreated spruce wood powder, and industrially relevant thermo-chemically pretreated spruce material, intended to represent distinctly different substrate complexities and presumably also different requirements on enzyme properties.

1 Introduction

1.1 Conversion of plant biomass to biofuels

A serious threat to humanity is the depletion of world fossil fuel resources. According to recent predictions, if the use of fossil fuel resources will continue like the present situation, than these resources will be exhausted in near future [Global Reports and Publications, June 2007]. Rising cost and the need to stop the increasing level of CO_2, which is the main source of climatic change and global warming on our planet, motivates the scientists to consider and figure out other alternative energy resources. In a longer perspective there is an urgent need to develop biorefineries not only for production of biofuels, but also for replacing the organic chemicals that are today supplied by the petrochemical industry. In the future such biorefineries will produce both fuels and other co-products (chemicals, heat and power). Heat produced during some processes inside the biorefinery could be utilized to meet the requirements of other processes in the system [Demirbas, 2009].

The production of bioethanol is rapidly increasing. According to one estimate, 50 billion liters of bioethanol is produced annually, which indicates that bioethanol seems to be an important renewable liquid biofuel within the next couple of decades [Cherubini and Stromman, 2010]. Nowadays the bioethanol is mainly produced from sucrose and starch materials, so called first generation bioethanol [Furlan *et al*., 2012]. First generation biofuels can help in decreasing global warming by balancing the level of CO_2, but a major drawback is the use of food feedstocks (sugars and vegetable oils), which results in shortage of food and rising of food prices due to the increase in the production of these fuels [Laursen, 2006].

In order to avoid competition with the global food supply, attention is instead turning towards utilizing lignocellulose of plant biomass as raw material for biofuel production, so called second generation biofuels. However there are some barriers in using lignocellulosic materials that need to be eliminated [Eisberg, 2006]. The production of second-generation biofuels has not been fully commercialized yet, but pilot scale production is under development.

The lignocellulose of plant biomass is the most abundant organic material on earth and is abundantly available throughout the world. Examples of lignocellulosic feedstocks for the production of biofuels include agricultural residues (wheat straw, rice straw, hull, woodchips), municipal solid waste (paper mill sludge, pulp and paper industry waste) and herbaceous energy crops, e.g. Switch grass, Alfalfa fiber, Red canary [Wyman, 1994].

Lignocellulosic biomass consists of three major components, which are cellulose, hemicellulose, lignin and smaller amounts of other components like pectin, proteins etc. The percentage of each component in wood is shown in (Table 1).

Table 1. Examples of lignocellulose biomass composition, as given in: [Miller, 1999].

Constituents	Hardwood%	Softwood%
Cellulose	40-50%	40-50%
Hemicellulose	25-35%	25-30%
Lignin	20-25%	25-35%
Pectin	1-2%	1-2%

Cellulose is a major component of plant cell walls, generally constituting 40-50% of the dry weight [Joshi *et al*., 2011]. Cellulose is a homopolysaccharide composed of repeating D-glucose units linked together by β-1,4 glycosidic bonds to linear cellulose chains that may be several thousands of glucose units long (Figure 1). The cellulose chains coalesce to form rigid, highly ordered crystalline microfibrils, which in turn associate into larger fibril bundles [Hon, 1994], which build up strength of structure and maintain the shape of the cell. The properties of cellulose depend on its chain length and degree of polymerization, but are also strongly influenced by the synthesis process. The simultaneous secretion of other components into the cell wall affects how cellulose chains pack with each other. Properties like extent of polymerization and crystallinity of cellulose differ between cell wall layers, cell types, tissues and species, which in result have major impacts on acidic and enzymatic hydrolysis [Zhang *et al*., 2004].

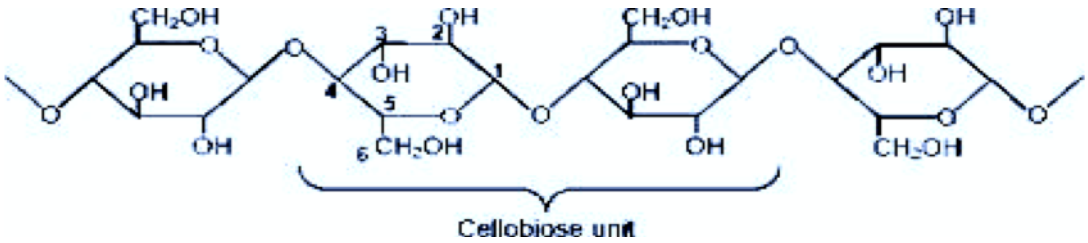


Figure 1: Structure of cellulose chain in which glucose units linked by β- 1,4 glycosidic bonds, cellobiose is a repeating unit, illustration taken from: [Kumar *et al*., 2009].

Hemicellulose is a collective name for heterogeneous non-cellulose polysaccharides within the cell wall, built up of several different monosaccahrides, mainly pentoses (D-xylose and L-arabinose) and hexoses (D-mannose, D-glucose and D-galactose) accompanied with different kinds of sugar acids like hexuronic acids [Balan *et al*., 2009]. Hemicellulose generally constitutes about 25 to 35% of the lignocellulosic biomass [Saha and Cotta, 2007]. Softwood (conifers) and hardwood (leave trees) differ in hemicellulose composition. In softwoods, O-acetyl galactoglucomannan and arabino-4-O-methylglucoroxylan dominate, whereas acetyl-4-O-methylglucoronoxylan and glucomannan are most common in hardwood [Sjostrom, 1993]. Hemicellulose is less ordered and unlike cellulose is non-crystalline and has an amorphous structure. It is more readily hydrolyzed by dilute acid, alkali, or by hydrolytic enzymes compared to cellulose, which has a crystalline structure and hence is more difficult to degrade [Fengel and Wegener, 1984].

The third major component, which constitutes about 20-25% of lignocellulosic biomass is lignin that is an aromatic polymer made up of monolignol monomers (coniferyl, coumaryl and sinapyl alcohol) [Joshi *et al*., 2011]. Lignin acts as strengthening agent and provides an impermeable barrier to protect woody plants from microbial degradation and enzymatic attack [Howard *et al*., 2003].

In nature, plant biomass is degraded and recycled by microorganisms using extracellular enzymes. The three major groups of enzymes which are used for the hydrolysis of cellulose are: 1) 1,4 β-D-glucanglucanohydrolase (EC 3.2.1.3), 2) 1,4 β-D-glucancellobiohydrolase (EC 3.2.1.91), and 3) β-D-glucosidase (EC 3.2.1.21) [Ladisch *et al*., 1983; Wright *et al*., 1988], also known as endoglucanase, exoglucanase and cellobiase respectively. However, it has been demonstrated that CBHs of both family GH6 and GH7 are able to perform endolytic cleavage and they are thus not true exocellulase enzymes [Boisset *et al*., 2000]. The mechanism of action of these enzymes is in following steps: 1) Endoglucanases randomly cut the cellulose chains to form new cellulose chain ends; 2) Exoglucanases act on the ends of cellulose chains and processively cleave off cellobiose units; 3) Finally cellobiase hydrolyses cellobiose into D-glucose that can be fermented to ethanol or converted to other chemicals.

However, lignocellulose is designed by nature to resist degradation, and requires harsh thermo-chemical pretreatment (TCP) prior to the enzymatic depolymerisation. Otherwise, less than 10-20% of the sugar content will be released within reasonable time limits [Eggeman and Elander, 2005]. The prior and essential goal of TCP of the lignocellulosic biomass is to disrupt its compact composite structure, i.e. to loosen up the matrix of hemicellulose and lignin in which the cellulose microfibrils are embedded in order for the enzymes to gain access to the structural polysaccharides (Figure 2) [Kumar *et al*., 2009]. The pretreatment is energy consuming and bears a high share of the production cost and is currently subject to intensive research and development [da Costa Sousa *et al*., 2009]. The TCP stage typically involves a combination of different treatments for example including mechanical size reduction (chipping, milling, grinding), high temperature, alkali or acids, organic solvents and/or oxidizing agents.

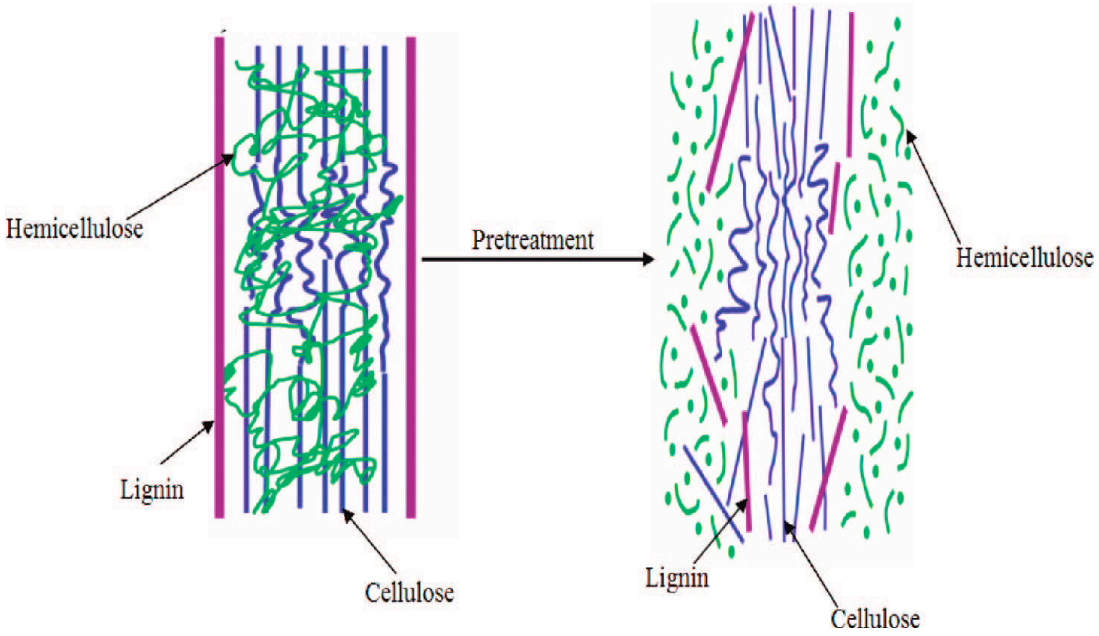


Figure 2. Schematic representation of pretreatment on lignocellulosic substrates.Illustration from: [Hsu *et al*., 1980]

One promising method applied by several investigators is so called steam explosion, usually after impregnation of the material with dilute sulphuric acid (~1%). Chipped or grinded biomass is heated with steam to 160-240°C in a pressurized vessel. After some 5-15 minutes of reaction time the pressure is released instantly and the material is allowed to expand into a flash tank [Sun *et al*., 2002]. The application of high temperature and low pH helps in the degradation of hemicellulose, solubilization of xylan and partial solubilization of lignin, and the sudden pressure drop disrupts the compact structure and increases the accessible surface area for enzyme action [Duff *et al*., 1996]. The two most commonly used acids in steam explosion are H_2SO_4 and SO_2, but also water itself acts as an acid at high temperature, and acid-free steam explosion has also been successfully applied [Morjanoff *et al*., 1987]. Important factors that influence the steam explosion are residence time, temperature, particle size and moisture content. The temperature and residence time shows an inverse relationship, i.e. substantial solubilization and hydrolysis of hemicellulose can be gained either at high temperature and at shorter residence time (270 °C, 1 minute) or at lower temperature and longer residence time (190 °C for 10 minutes) [Duff *et al*., 1996].

1.2 *Hypocrea jecorina*

The ascomycete *Hypocrea jecorina* (anamorph *Trichoderma reesei*) is a well-studied mesophilic soft-rot fungus that is widely used in industry as a source of cellulases and hemicellulases for the hydrolysis of lignocellulosic components of plant biomass [Martinez *et al*, 2008]. It is also the predominant source of cellulase enzymes for industrial use [Geddes, 2011]. *Hypocrea jecorina* is among the most prolific protein producers known. Certain industrial strains can make as much as 100 g/l of extracellular protein in submerged culture [Cherry and Fidantsef, 2003].

The genome of *Hypocrea jecorina* has been sequenced and transcriptome analysis revealed ~35 genes coding for known or putative biomass degrading activities that were up-regulated on cellulosic medium [Foreman *et al*., 2003]. Cellulase enzymes dominate in quantity. Major components are two cellobiohydrolases (CBH), Cel7A (40-50%) and Cel6A (~20%), characterized by having their active sites enclosed in a cellulose-binding tunnel [Divne *et al*., 1994; Rouvinen *et al*., 1990] and the ability to processively cleave off several cellobiose units from the end of a cellulose chain before releasing the substrate [Boisset *et al*., 2000; Kurasin and Väljamäe, 2011; Igarashi *et al*., 2011]. The enzymes cooperate synergistically and act preferentially from opposite ends of cellulose chains, Cel7A from the reducing end and Cel6A from the non-reducing end. They also utilize different reaction mechanisms. Cel7A hydrolyses glycosidic bonds with net retention of the β-anomeric configuration, whereas Cel6A is an inverting enzyme [Knowles *et al*., 1988].

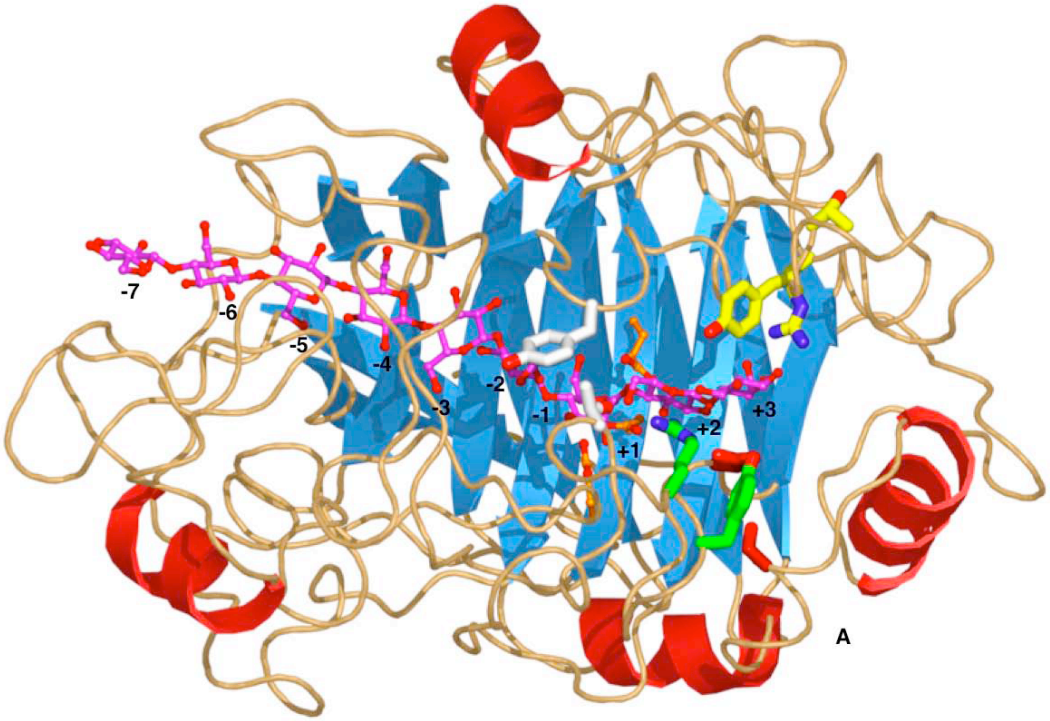
Several different endoglucanases (EG) are also produced at lower levels. They have their active sites in a more open cleft and show a higher tendency to cut internal bonds and dissociate from the substrate after hydrolysis. Generally they exhibit higher catalytic rates on soluble substrates, but are less efficient than CBHs on crystalline cellulose [Kleywegt *et al*., 1997]. Almost all the cellulase enzymes, except Cel12A, have a small (~35 amino acids) highly conserved family 1 carbohydrate-binding module (CBM1) attached to the catalytic domain with a flexible linker peptide [Tomme *et al*., 1995].

1.3 Cellobiohydrolase Hje Cel7A

Cellobiohydrolase Cel7A belongs to glycoside hydrolase family 7 (GH7). It is the major enzyme of *H. jecorina* and consitutes almost half of the total amount of secreted protein under cellulase-inducing conditions. As shown by gene knockout studies it is a key rate limiting factor in cellulose degradation [Ilmen *et al*., 1997]. The mature protein consists of 498 amino acids and has a bimodular domain organisation with an N-terminal catalytic module (434 aa), a flexible linker peptide (28 aa) and a C-terminal CBM1 module (35 aa). The function of the CBM is to bind on

the surface of cellulose and provide anchorage for the enzyme and to keep it strongly adsorbed on cellulose surface. [Ståhlberg *et al*., 1988]. The linker helps in keeping the two domains apart by restricting their movements so that the catalytic domain is always within close distance to the CBM, which binds on the surface of the cellulose fibre. The importance of the linker region is evident from the fact that by removing part of the linker region the degradation of crystalline cellulose is reduced [Srisodsuk *et al*., 1993].

The structure of the catalytic domain of Cel7A has been determined by X-ray crystallography [Divne *et al*., 1994] and the structure of the CBM by NMR [Kraulis *et al*., 1989]. Structure studies of cellodextrin binding to Hje Cel7A revealed that the active site can harbour a total of 11 glucose residues of a cellulose chain, with 7 subsites (-1 to -7) from the catalytic center towards the non-reducing end of cellulose chain, and 4 subsites (+1 to +4) towards the reducing end (Figure 3A) [Divne, 1998; von Ossowski, 2003]. Four tryptophan residues (W40, 38, 367, 376) act as sugar binding platforms at subsites -7, -4, -2, and +1. These tryptophans are highly conserved among the known CBHs in family GH7. During processive action, the cellobiose unit to be cleaved off is bound in subsites +1 and +2, which are thus called the product binding sites. The enzyme binds cellobiose strongly (Kd ~20 uM [Claeyssens *et al.,* 1989]), and is therefore sensitive to product inhibition. This has been proposed as a serious issue in enzymatic lignocellulose depolymerisation, and has been the subject of previous research [von Ossowski *et al*., 2003; Lintao *et al.,* 2012].



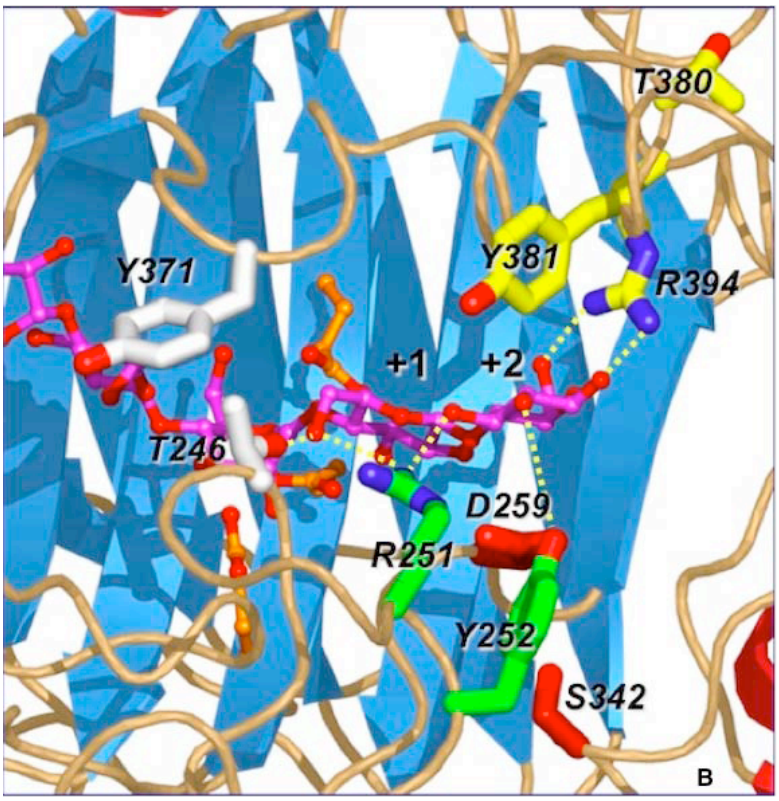


Figure 3. A) Secondary structure representation of *Hypocrea jecorina* Cel7A. B) Model of a cellulose chain bound in the active site tunnel [PDB code 8CEL; Divne *et al.,* 1994]. Residues are indicated around the cellobiose product binding sites +1/+2 that were mutated in the engineered Hje Cel7A variants 2CYS (T246C/Y371C), QWY (Y252Q/D259W/S342Y), AAA (T246A/R251A/Y252A) and GDA (T380G/Y381D/R394A).

Table 2. Engineered product site variants of *Hypocrea jecorina* (CBH1 from *Trichoderma reesei*). [Requadt *et al.,* unpublished]

Name	Mutated A.A	Purpose of Mutation	Binding sites
2CYS	T246C/Y371C	Increased processivity and product binding	-2,-1,+1,+2
QWY	Y252Q/D259W/S342Y	Increased processivity and product binding	+2
AAA	T246A/R251A/Y252A	Decreased product binding	+1, +2
GDA	T380G/Y381D/R394A	Decreased product binding	+2

In order to investigate the influence of affinity for cellobiose at the product binding subsites of Cel7A, on the enzyme's activity, mutants have been designed with the purpose of changing the affinity for cellobiose at these sites. This work was done prior to this study in collaboration between SLU and the company DuPont™ Genencor® Science. Four variants of Cel7A were designed, two that were aimed for stronger binding (called 2CYS and QWY), and two for weaker

binding (AAA and GDA). The mutated residues are listed in Table 2 and their positions in the protein structure in Figure 3B.

In the 2CYS variant (T246C/Y371C), two residues at the tips of two opposing loops (T246 and Y371; white in Fig 3B) enclosing the active site tunnel at the catalytic centre were replaced by cysteine residues. The purpose was to connect the loops covalently by a disulphide bridge. This should keep the loops closed and slow down release of the product and presumably also increase the processivity of the enzyme. The purpose of variant QWY (Y252Q/D259W/S342Y) was to introduce a new sugar-binding platform at subsite +2 by replacing Asp 259 (red in Fig 3B) with tryptophan. In order to accommodate a bulky Trp sidechain here, Y252 (green in Fig 3B) had to be reduced in size and was replaced by Gln. Furthermore, Ser 342 (red in Fig 3B) was replaced by Tyr to provide a hydrophobic support for the Trp sidechain to lean against.

In the AAA variant (T246A/R251A/Y252A), three residues that form hydrogen bonds with "upper" sugar hydroxyls in sites +1/2, were replaced with alanines, in order to remove those hydrogen bonds and result in weaker product binding. The yellow color in Fig 3B represents the residues mutated in the GDA variant (T380G/Y381D/R394A). The primary purpose was to decrease the affinity by removing the side chain of Arg 394 that binds OH-1 and OH-6 in site +2 at the bottom of the active site. This residue was replaced with Ala. At the same time Tyr 381 was replaced by Asp to decrease van der Waals and possible water mediated H-bonding with the sugar. The T380G mutation was introduced to increase the flexibility of the backbone and facilitate possible structural adjustments necessary to accomodate the other two mutations.

The Cel7A product site variants were constructed and homologously expressed in a *Hypocrea jecorina* host strain lacking the genes Cel7A, Cel6A, Cel7B and Cel5A. Activity measurements, enzyme kinetics and inhibition studies have been made using soluble substrates, and indeed revealed large increase in k_{cat} (~10-fold for AAA; ~60-fold for GDA), K_M (~7-fold for AAA; ~100-fold for GDA) and K_i (~7-fold for AAA; ~60-fold for GDA) for the mutants aiming at decreased product binding. However, on insoluble amorphous cellulose substrate (phosphoric acid swollen Avicel), only the 2CYS variant was comparable in activity to Hje Cel7A WT. This work was done prior to the present study (Requadt *et al.*, unpublished data).

1.4 *Phanerochate chrysosporium* and *Heterobasidion irregulare*

It is noteworthy that most of the fungal enzymes studied are from ascomycete fungi, whereas wood decomposition in Nature is predominantly conducted by fungi of the basidiomycete phylum [Fernandez-Fueyo *et al.*, 2012]. *Phanerochaete chrysosporum* is a white-rot fungus (basidiomycete) isolated from a wood chip pile in north of Sweden, able to produce cellobiohydrolase 58 (Cel7D), a major enzyme that is 10 % of the total secreted protein in liquid culture on cellulose [Szabo *et al.*, 1996] and the major enzyme under most growth conditions [Eriksson *et al.*, 1975]. This enzyme also belongs to family 7 of glycoside hydrolases (GH7). Pch Cel7D shows 55% sequence identity with Hje Cel7A and shows very similar structure, but several loop deletions make the cellulose-binding tunnel more open [Munoz *et al.*, 2001]. Comparison of the activity on cellulose indicate that Pch Cel7D is more active than Hje Cel7A, at least on amorphous cellulose and bacterial microcrystalline cellulose [von Ossowski *et al.*, 2003].

Heterobasidion annosum is an aggressive pathogen that causes root and butt rot mainly in conifers [Korhonen *et al.* 1998]. It is not only important due to its biological perspective, but also it is the most serious pathogen in conifer forests throughout the world. This fungus causes white rot decay in the wood and has the ability to penetrate through the roots and grow within live and dead trees. According to one estimate the economic loss in the forest industry is around 790 million € annually in Europe only [Woodward *et al*, 1998]. This white rot fungus *Heterobasidian annosum sensus lato S.l.* is a species complex including *H. irregulare, H. acidentale, H. annosum,*

H. parviporum and H. abietinum. The North American strains *H. irregulare* and *H. acidentale* have recently been classified as discrete biological species [Otrosina and Garbelotto, 2010]. The complete genome of *H. irregulare* (formerly called *H. annosum* North American P-strain) has been sequenced [Fernandez-Fueyo *et al.*, 2012] and is publically available at the Joint Genome Institute [http://genome.jgi-psf.org/Hetan2/Hetan2.home.html]. 282 carbohydrate active enzyme genes (CAZY) have been predicted in the genome, 59 glycosyl transferases, 179 glycoside hydrolases, 8 carbohydrate binding modules, 10 EXP (Expansion-like protein), 7 polysaccharide lyases and 19 carbohydrate esterases. It was noted that the most abundant protein when the fungus (*Heterobasidion irregulare*) was grown on spruce powder as carbon source was Hir Cel7A, encoded by the only gene in the genome coding for a GH7 enzyme [Wang, 2009]. Hir Cel7A lacks a linker and carbohydrate-binding module, in contrast to many other GH7 cellulases such as Hje Cel7A and Pch Cel7D. It consists of a single GH7 catalytic module of 440 amino acids, that shows 56% and 69% sequence identity with Hje Cel7A and Pch Cel7D, respectively, and overall very similar structure (Momeni *et al*, unpublished). However, differences in length and sequence of tunnel enclosing loops make the active site of Hir Cel7A more open than in Hje Cel7A and more closed than in Pch Cel7D, suggesting intermediate enzymatic properties (Figure 4).

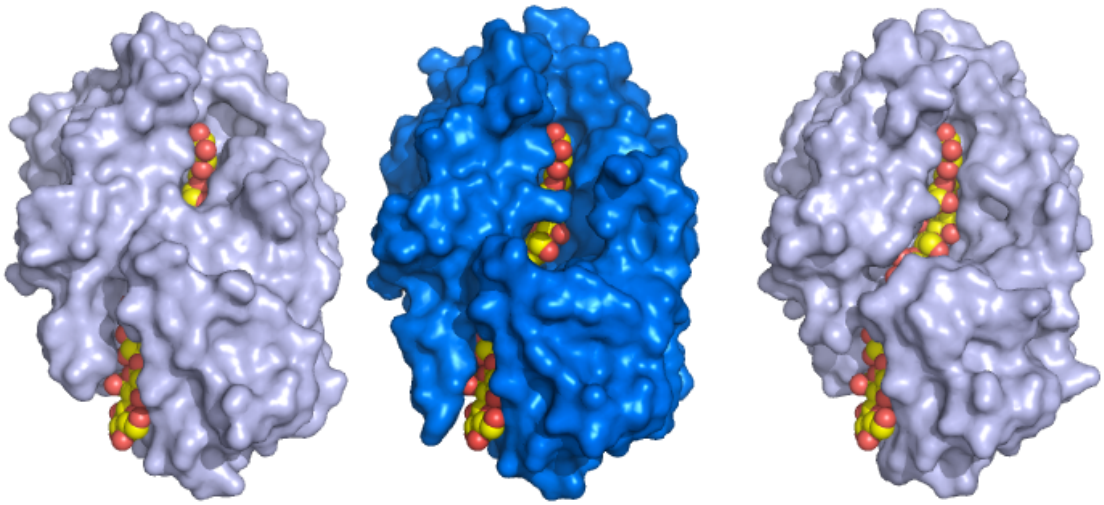


Figure 4. Structure representation (From left to right) of catalytic domains of Hje Cel7A, Hir Cel7A and Pch Cel7D respectively.

2 Materials and Method

2.1 Preparation of enzymes

2.1.1 Wildtype Hje_Cel7A

Wildtype Hje Cel7A was purified in this study from a commercial enzyme cocktail, Accellerase 1500™, containing *H. jecorina* enzymes (including Cel7A, Cel7B, Cel6A, Cel5A) complemented with extra beta-glucosidase, kindly provided by Genencor Intl – A Danisco Division, Palo Alto, CA, USA.

2.1.1.1 Purification of Wild type Cel7A

Wildtype Cel7A purification was performed on ion-exchange chromatography media from GE Health Care, using an Äkta Purifier 10/100 UV-900 chromatographic system (GE Health Care); Figure 5)

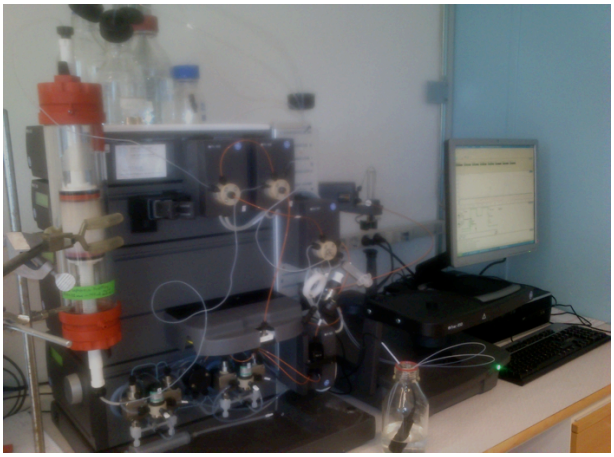


Figure 5. Äkta purifier 10/100 UV-900 chromatographic system (GE health care) used for purification of *Hypocrea jecorina* WT Cel7A along with DEAE Sepharose fast flow column.

Step 1. 45 ml of Accellerase 1500 was centrifuged and the supernatant was filtered through a 0.2 um syringe filter (polyether sulfone; Sartorius), followed by buffer exchange through repeated concentration and dilution with 20 mM sodium acetate, pH 5.0, in Vivaspin centrifugation concentrators (10 kDa cutoff; Sartorius Stedim Biotech). The sample was loaded on a 140 ml DEAE-Sepharose Fast Flow column (weak anion-exchanger; GE Health Care) equilibrated with 20 mM NaAc pH 5.0. After 1 column volume of equilibration buffer, proteins were eluted with a 180 ml salt gradient to 20 mM sodium acetate, pH 5.0, + 1 M NaCl. The flow rate was 1 ml/min.

Step 2. Pooled fractions (62-87) from step 1 (total volume 75 ml) was buffer exchanged as described above, to 50 mM sodium acetate, pH 4.0, and loaded onto the same DEAE-Sepharose Fast Flow column used above, equilibrated with 50 mM sodium acetate, pH 4.0. After 1 column volume of equilibration buffer, proteins were eluted with a 500 ml gradient to 500 mM sodium acetate, pH 4.0. Fractions of the Cel7A peak were pooled (180 ml volume) and was stored at 4 °C for the next purification step.

Step 3. Half of the material (90 ml) from step 2 was diluted 5 times with water and loaded on a Source 30Q column (20 ml; 1.5 ml/min; GE Health Care) equilibrated with 50 mM sodium acetate, pH 4.0. After two column volumes of equilibration buffer, a 350 ml gradient to 500 mM sodium acetate, pH 4.0, was applied. Fractions C1-D1 (16 fractions) were pooled together. This gave 50 ml purified protein sample. The same procedure was repeated with the remaining 90 ml protein sample from step 2, yielding a total of 100 ml purified Hje Cel7A WT. An aliquot was desalted to 0.1 M sodium acetate, pH 5.0, for use in enzyme reactions.

2.1.1.2 Measurement of Purified WT Hje_Cel7A protein concentration

Protein concentration was determined by measuring the absorbance at 280 nm. The extinction coefficient 1.65 l g^{-1} cm^{-1} of wildtype Hje Cel7A was calculated using Protparam software (http://web.expasy.org/protparam/).

2.1.2 Engineered variants of Hje_Cel7

The engineered variants 2CC (T246C/Y371C), QWY (Y252Q/D259W/S342Y) and AAA (T246A/R251A/Y252A) were constructed, recombinantly expressed in a fungal host, purified, and kindly provided by Genencor Intl, Palo Alto, CA, USA. The GDA mutant was not available at the time of study and could not be included. The concentrations of the variants (Appendix A) were measured using UV spectrophotometer at 280 nm, and Protparam-calculated extinction coefficients 1.606, 1.74 and 1.61 l g^{-1} cm^{-1} for 2CC, QWY, and AAA, respectively. Stock solutions of 500 µl and 1 mg/ml in 10 mM sodium acetate, pH 5.0 were prepared for use in the reactions. The concentrations were determined by measuring absorbance at 280 nm in a Nanodrop 1000 spectrophotometer (Thermo Scientific).

2.1.3 Pch Cel7D and Hir Cel7A

Pch Cel7D enzyme was provided by Jerry Ståhlberg, and purified Hir Cel7A by Majid Hadad Momeni (Dept Molecular Biology, SLU). Both enzymes were purified from culture filtrate of the respective fungus cultivated on cellulose-containing media. The concentrations of the provided enzyme solutions were measured spectrophotometircally at 280 nm, using Protparam extinction coefficients of 1.48 and 1.3 l g^{-1} cm^{-1} for Pch Cel7D and Hir Cel7A, respectively. Stock solutions of 1 mg/ml Pch Cel7D and Hir Cel7A in 10 mM sodium acetate, pH 5.0 were prepared and their concentrations checked at 280 nm in the Nanodrop spectrophotometer (Appendix A).

2.1.4 Accellerase 1500 and Cel7A-free Accellerase

Accellerase 1500 was prepared for activity reactions by centrifugation, filtration and buffer exchange as described for the purification of Cel7A WT above. A preparation of Accellerase 1500 where the Cel7A enzyme had been selectively removed was provided by Majid Haddad Momeni (Dept Molecular Biology, SLU). In short this Cel7A-free Accellerase was prepared by fractionation of Accellerase 1500 on a DEAE Sepharose column, similar to the description above. The fractions of the Cel7A peak were removed, and all the other fractions were pooled together and concentrated. The concentrations of the desalted Accellerase 1500 and Cel7A-free Accellerase solutions were estimated spectrophotometrically at 280 nm, using an assumed average extinction coefficient of 1.50 l g^{-1} cm^{-1}, and stock solutions were prepared of 1.0 and 0.64 mg/ml, respectively (Appendix A).

2.1.5 Beta-glucosidase

Almond β-glucosidase (lyophilized powder, 9.46 units/mg; Sigma-Aldrich), 11.5 mg, was dissolved and desalted to 10 mM sodium acetate, pH 5.0, and diluted to a final concentration of 100 µg/ml in the same buffer. By mistake this was 10 times higher than the intended concentration (10 µg/ml). The extinction coefficient 7.06 provided by the manufacturer was for a 1% solution and not 1 mg/ml as initially assumed. The procedure of desalting is described in Appendix B.

2.1.6 SDS-PAGE analysis

The purity of Hje Cel7A WT and mutants was checked by running SDS-PAGE in BioRad MiniPROTEAN® TGX™ precast 4-20% gel.

2.2 Preparation of cellulosic substrates

Three cellulosic substrates were used in this study, pure microcrystalline cellulose (Avicel), untreated spruce sawdust and thermo-chemically pretreated spruce material (TCP-spruce), as shown in Figure 6.

2.2.1 Avicel

Avicel is microcrystalline cellulose (in powder form) obtained from pulp. Avicel stock solution (FLUKA) 1% was prepared by dispersing 1 g of Avicel powder in 100 ml Milli-Q water in a flask while stirring using magnetic stirrer for one hour at room temperature and then sonicated for 2-3 minutes in order to obtain a homogeneous suspension without clumps of Avicel. The suspension was stored at 4 °C. For 300 µl enzymatic reactions, 150 µl of the suspension was pipetted to Eppendorff tubes using regular 200 µl pipette tips, giving a final concentration of 5 g/l Avicel.

2.2.2 Untreated spruce sawdust

Saw dust of spruce heartwood was a kind gift from a saw mill north of Uppsala. The dry matter content (DM) was analyzed three times using Precisa XM-60 Moisture analyzer (Precisa) and determined to be 92 %. For 500 µl enzymatic reactions 16.3 mg of spruce saw dust (4mm) weighed in Eppendorff tubes, giving final concentrations of 30 g/l DM.

2.2.3 Pretreated spruce (TCP-spruce)

Thermo-chemically pretreated spruce material (sulphuric acid impregnated and steam exploded, exact conditions not concealed) was a kind gift from the company SEKAB, Örnsköldsvik, Sweden, and was provided as a slurry at 27 % dry matter content and pH ~2. The slurry was diluted 3 times with Milli-Q water to 10 % DM, and the pH was adjusted to 5.0 with 0.1 M NaOH added slowly with continuous magnetic stirring. The moisture content of the diluted and pH-adjusted slurry was analyzed three times in the moisture analyzer, which gave a dry matter content of 6.8 %. The slurry was stored at 4 °C. For 500 µl enzyme reactions, 370 µl of the slurry was pipetted into Eppendorff tubes using 1 ml pipette tips cut at the very tip, to prevent clogging, giving a final concentration of 50 g/l DM.

Figure 6. Cellulosic substrates from left to right 1) Untreated spruce (sawdust), 2) TCP-spruce and 3) Avicel cellulose powder, used in the experimental study.

2.3 Activity measurements

Enzyme reactions with cellulosic substrates were done in triplicate at 40 °C in Eppendorff tubes under shaking (180 rpm; Unitron), in 0.1 M sodium acetate buffer, pH 5.0. The total enzyme dosage was 50 µg/ml, unless otherwise stated, in three different scenarios: i) 50 µg/ml Cel7 enzymes alone (Hje Cel7A WT, 2CC, QWY, AAA; Hir Cel7A; Pch Cel7D); ii) 25 µg/ml Cel7 + 25 µg/ml Cel7A-free Accellerase, or iii) 15 µg/ml Cel7 + 35 µg/ml Accellerase 1500. Reactions were also included with 50 µg/ml of Cel7A-free Accellerase (without added Cel7), and 35 and 50 µg/ml of Accellerase 1500, respectively. Samples with only buffer instead of enzymes added to the substrate were included as controls for determination of the amount of soluble sugar released from the substrate in the absence of enzymatic action. Details of enzyme reaction preparations are given in Appendix C-H.

2.3.1 Enzyme reactions with Avicel

Avicel cellulose (5 g/l) was incubated for 2 h with enzymes in Eppendorff tubes (reaction volume 300 µl), and thereafter immediately filtrated in 96-well plates with 1 um glass fiber filters (Whatman) on a vacuum filtration unit (Porvair Sciences). 50 µl of the filtrate was mixed with 100 µl of beta-glucosidase solution (to hydrolyse cellobiose to glucose) and was incubated for 1 h at 37 °C, whereafter 300 µl of Parahydroxy benzoic acid hydrazide reagent PAHBAH reagent was added for determination of soluble reducing sugar.

2.3.2 Enzyme reactions with untreated spruce

Untreated spruce (30 g/l) was incubated for 20 h with enzymes in Eppendorff tubes (reaction volume 500 µl), and thereafter immediately filtrated as above. For samples with Cel7 alone or together with Cel7A-free Accellerase, 50 µl of the filtrate was mixed with 100 µl of the 100 µg/ml beta-glucosidase solution (to hydrolyse cellobiose to glucose) and was incubated for 1 h at 37 °C. For samples with Accellerase 1500 (with or without added Cel7 enzymes), 100 µl water instead of beta-glucosidase was added to 50 µl of the filtrate, because it was assumed that the beta-glucosidase already present in Accellerase 1500 would efficiently hydrolyze the produced cellobiose to glucose. 300 µl of PAHBAH reagent was added for determination of soluble reducing sugar.

2.3.3 Enzyme reactions with TCP-spruce

Thermo-chemically pretreated and pH-adjusted spruce material (50 g/l) was incubated for 20 h with enzymes in Eppendorff tubes (reaction volume 500 µl), and thereafter immediately filtrated as above. The filtrates were stored at -20°C until later carbohydrate analysis by HPAE-PAD as described below.

2.3.4 Reducing sugar assay

A PAHBAH assay was used for measuring reducing sugars in order to determine enzymatic activity in samples. The principle of the PAHBAH reaction is that as a result of reaction with reducing carbohydrates in alkaline solutions, acid hydrides give yellow anions [Lever, 1972]. Low reagent blank values were shown by PAHBAH and at 100 °C color development reached a maximum after 5 minutes and then remained stable for at least 5 minutes. The PAHBAH reagent solution (50ml) contains 0.2 M potassium sodium tartrate (2.82 %), 0.1 M PAHBAH (0.76 %) and 0.5 M NaOH (2 %) in Milli-Q water. The solution was vortexed for proper mixing and sonicated in order to make a homogeneous solution. The PAHBAH was kept on icebox and was prepared the same day as the reactions in order to be used as fresh as possible.

2 volumes (300 µl) of PAHBAH reagent was mixed with 1 volume (150 µl) of each triplicate sample (enzyme reaction or glucose standard) in Eppendorff tubes, and was heated for 10 minutes in a boiling water bath. After cooling 300 µl of the reaction mix was transferred to a 96-well microtiterplate, as well as 100 µl of the remainder that was serially diluted 3 times with 200 µl Milli-Q water on the microtiterplate. The absorbance at 405 nm was recorded in a microtiterplate reader (Envision 2104, Perkin-Elmer). If the absorbance was greater than 2, then 9 times dilution was done by taking 100 µl from the 3 times diluted samples into 200 µl water-containing wells on the microtiterplate and the absorbance was read again.

Glucose standards of 0, 0.1, 0.2, 0.3, 0.4, 0.7, 1.0, 1.2, 1.5, 2, 3 and 4 mM concentrations in Milli-Q water were prepared from 20 mM stock solution of glucose. The same PAHBAH reactions was performed with the glucose concentrations incubated with β-glucosidase (10 µg/ml) by taking 150 µl from the β-glucosidase incubated concentrations and added to 300 µl PAHBAH solution in eppendorf tubes followed by boiling and measuring the absorbance of the sample at 405 nm, as described above. Glucose standard curves were made both without and with beta-glucosidase incubation and were used for the respective assays (Figures 12 and 13).

2.3.5 Carbohydrate analysis

Carbohydrate analysis was performed for thermo-chemically pretreated spruce as a substrate, using HPAE-PAD (high-performance anion exchange chromatography with pulsed amperometric detection) on a Dionex ICS-3000 HPLC system, Thermo Scientific (Figure 7). The frozen filtrates were thawed, and each triplicate sample was placed in the autosampler at 4 °C. Control samples without enzyme (only substrate) were diluted 20 and 100 times, with Cel7 alone 50 and 100 times, and all others 100 and 200 times. In most cases, both dilutions were analyzed, but in all cases the results of the 100 times dilution was used in the final results.

The separation and analysis was performed as described in Dererie *et al*., 2011, except that a 2x250 mm (instead of 4x250 mm) CarboPac PA10 column (Thermo Scientific) was used, a flow rate of 0.4 ml/min for elution, 29 mM sodium acetate in 0.2 M NaOH for the second gradient step, and 0.2 ml/min for the post-column flow. 5 standards with different combinations of concentrations of arabinose, galactose, glucose, xylose, mannose and cellobiose, were run between each series of 25 samples, for construction of standard curves for quantification of the respective soluble sugars in the analyzed samples.

Figure 7. Dionex ICS-3000 HPAE-PAD (Thermo Scientific) system used for carbohydrate analysis of samples.

3 Results & Discussion:

3.1 Purification of Hje_Cel7A Wild type

Cel7A WT from *Hypocrea jecorina* Cel7A was purified from 45 ml of Accellerase 1500 by ion-exchange chromatography in 3 steps. In the first step on DEAE- Sepharose, pH 5.0, the Cel7A peak was not well separated from the peak containing Cel7B (Figure 8), due to the use of a too steep salt gradient, but was well separated in the second step on DEAE-Sepharose at pH 4.0 (Figure 9). Finally, the material had to be divided in two portions for the last purification step on Source 30Q at pH 4.0, which gave one large peak (Figure 10). Fractions of the central part of the peak from both portions were pooled together and gave a total of 100 ml purified Cel7A with a concentration of 2.1 mg/ml. Thus, the yield from 45 ml Accellerase 1500 was 210 mg of pure Cel7A.

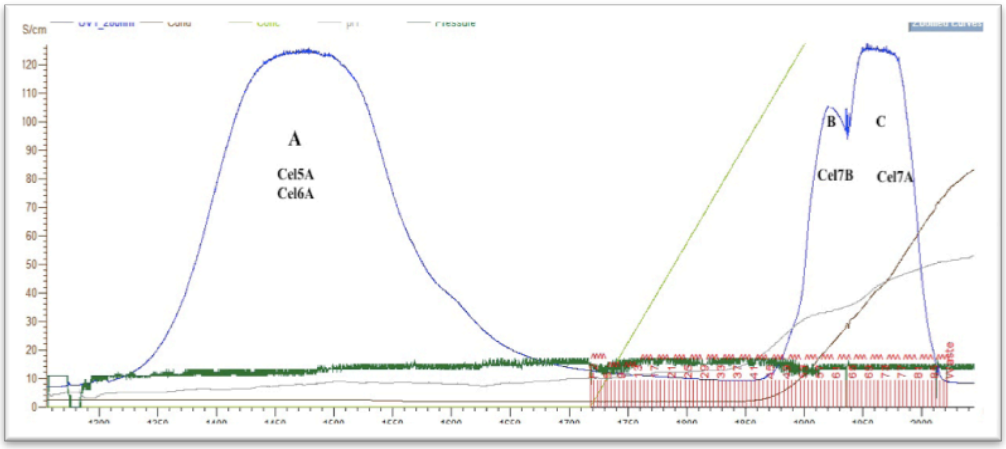


Figure 8: Chromatogram of purification step 1, fractionation of Accellerase 1500 on a DEAE Sepharose Fast Flow column at pH 5.0. Peak A represents the flow-through containing Cel5A, Cel6A and other proteins. The major components in Peak B and C are Cel7B and Cel7A, respectively. Fractions 62-87 were pooled and used in step 2.

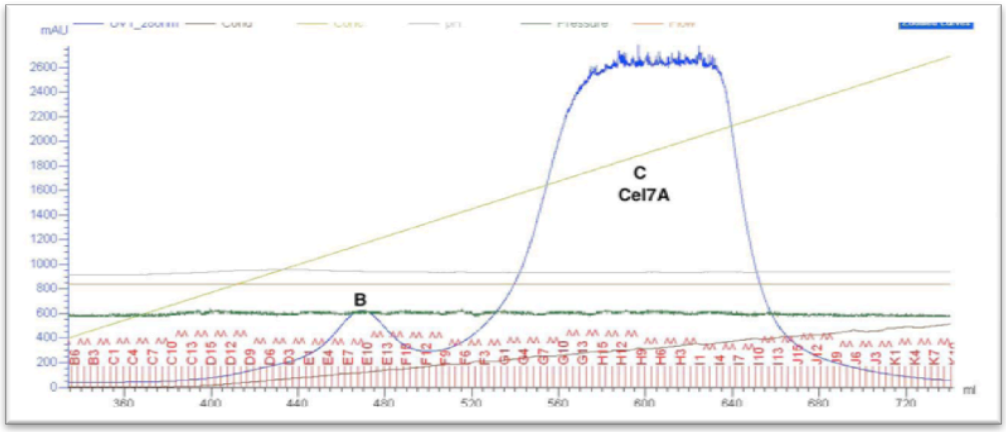


Figure 9: Chromatogram of purification step 2, the separation of Peak C from step 1 on a DEAE Sepharose Fast Flow column at pH 4.0. The major components in Peak B and C are Cel7B and Cel7A, respectively. Fractions of the Cel7A peak were pooled and used in step 3.

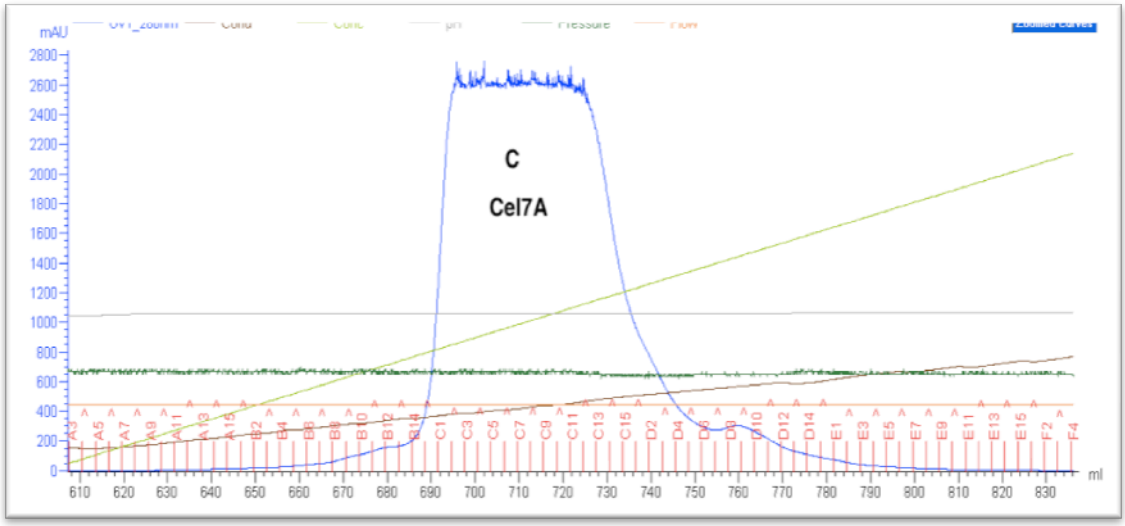


Figure 10. Chromatogram of purification step 3 on a Source 30Q column at pH 4.0. Fractions C1-D2 were pooled together to get 50 ml (x2) of purified wild type Cel7A with concentration of 2.1 mg/ml.

The purity of Cel7A WT and variants was checked by SDS-PAGE (Figure 11). The Cel7A WT appears to be very pure since only one band was visible at the expected position on the gel (~55 kDa), whereas minor contaminants of lower molecular weight (35-40 kDa) were visible in the three Cel7A mutant samples.

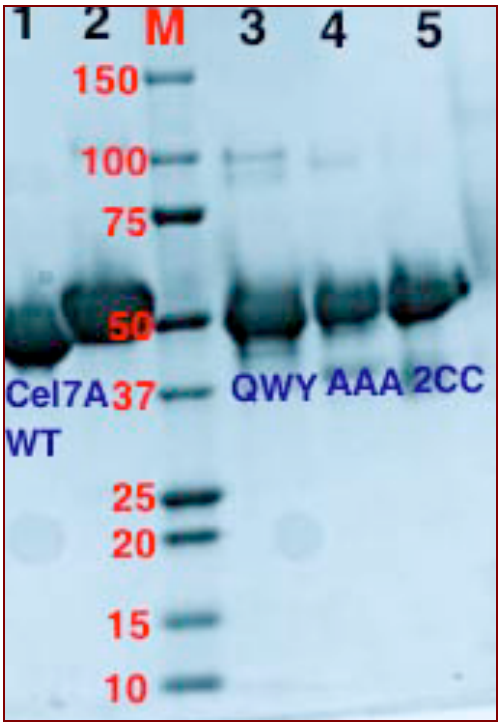


Figure 11. SDS-PAGE analysis of *H. jecorina* Cel7A variants on a 4-20 % gradient gel. Lane 1) WT Hje Cel7A; 2) Other protein not included in this study; 3) Cel7A variant QWY (Y252Q/D259W/S342Y); 4) AAA (T246A/R251A/Y252A); 5) 2CC (T246C/Y371C) along with Bio-Rad molecular weight markers.

3.2 Glucose standard curves for reducing sugar assays

It was found that the beta-glucosidase contributed significantly to the absorbance in the reducing sugar assay, probably due to the use of 10 times higher concentration than intended (100 instead of 10 µg/ml). Thus two sets of glucose standard curves were prepared, one without and one with dilution of standards with beta-glucosidase prior to addition of PAHBAH reagent, to use as standards for the respective enzyme reaction samples (without and with beta-glucosidase addition). Furthermore, it was found that the absorbance of the PAHBAH reaction with soluble sugar was linearly proportional to the sugar concentration only up to an absorbance around 2. For samples that gave higher absorbance than 2, the PAHBAH reaction mixture was diluted 3 times, and if the absorbance was still higher than 2 it was diluted 3 times further (9 times dilution). However, the absorbance was found not to be linearly proportional to the dilution factor. Thus it was necessary to use standard curves with same dilution factor of the PAHBAH reaction mixture as in the assayed enzyme reaction samples. As an example, for quantification of the amount of soluble reducing sugar obtained from Avicel after incubation with 25 µg/ml Cel7A WT + 25 ug/ml of Cel7A-free Accellerase, the filtrate was first diluted 3 times with beta-glucosidase followed by PAHBAH reaction. Direct measurement of the PAHBAH reaction mix (3x1) gave an absorbance above 2, that's why it was diluted further 3 times (3x3). In this case the 3x3 glucose standard curve was used for quantification, i.e. with beta-glucosidase addition and the same dilution factor of the PAHBAH reaction mix. The obtained and used glucose standard curves are shown in Figure 12 and 13.

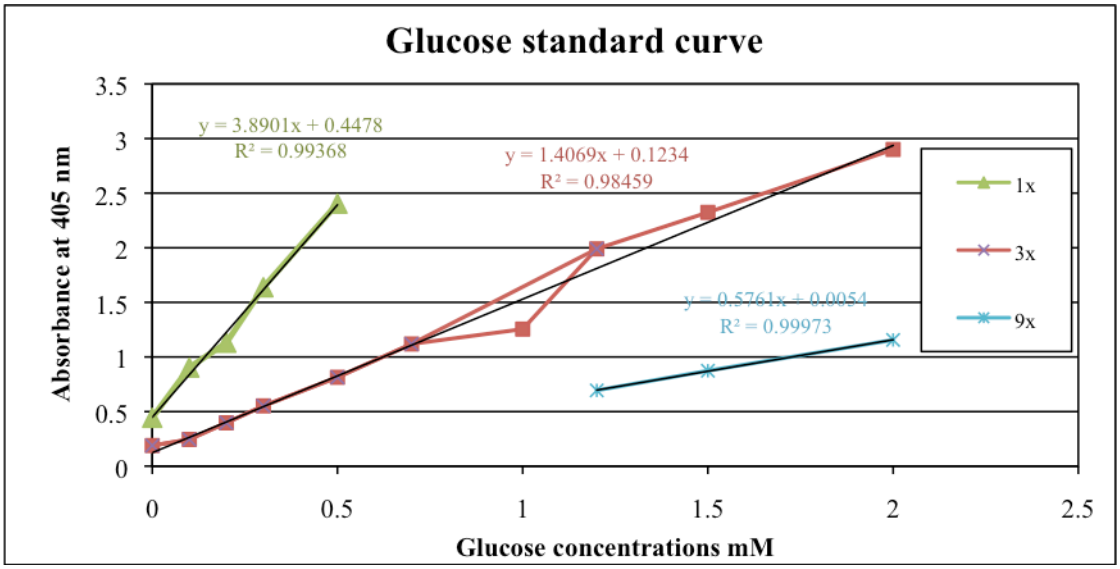


Figure 12. Glucose standard curves without β glucosidase incubation. 1x (Green), 3x (Red), 9x (Blue) represents no dilution (direct), 3 times dilution and 9 times dilution of samples respectively. Black line represents trendline.

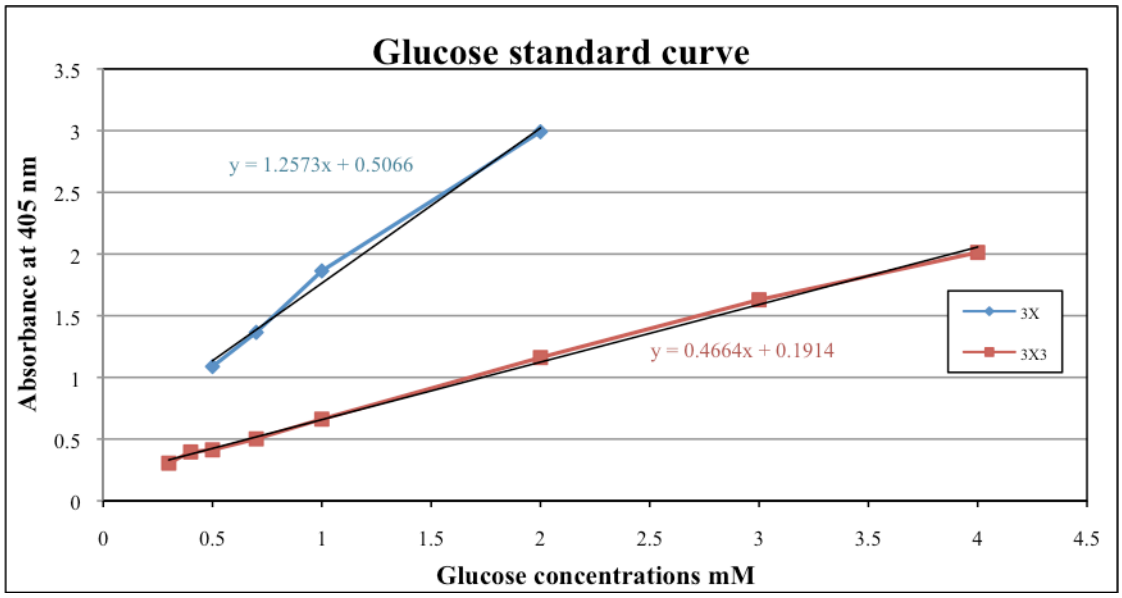


Figure 13. Glucose standard curve with β-glucosidase incubation. 3x1 (Blue), 3x3 (Red) represents 3 and 9 times dilution of samples, respectively. Black line represents trendline.

3.3 Activity of Cel7 variants on insoluble cellulosic substrates

Due to small amounts available of the engineered variants of Hje Cel7A we had to carefully select a limited number of experiments that could be performed. Three different substrates and conditions were chosen, presumably addressing different aspects of activity. Avicel at relatively low substrate concentration (5 g/l), high enzyme/substrate ratio (10 mg/g) and short incubation time (2 h), was used to compare activity on pure micro-crystalline cellulose. As an industrially relevant substrate, thermo-chemically pretreated spruce material was used at 50 g dry matter/l. Untreated spruce wood powder represented a native lignocellulose substrate, but the biomass load had to be limited at 30 g dry matter/l to ensure sufficient mixing. Both of the spruce materials were incubated for 20 h with enzymes. Activity was in all cases measured at 40 °C and pH 5.0.

A total enzyme concentration of 50 mg/l was chosen, corresponding to an enzyme/biomass ratio of 0.74 and 1.7 mg/g for pretreated and untreated spruce, respectively. In addition to comparing the activity of Cel7 enzymes alone we wanted to monitor their performance in the context of a biomass-degrading enzyme cocktail, either as supplement added to a *Hypocrea jecorina* enzyme cocktail that already contains the wild-type Hje Cel7A, or as replacement for Hje Cel7A wildtype. For the latter, replacement experiments, a batch of Cel7A-free Accellerase was prepared by IEC fractionation and selective removal of Cel7A containing fractions. Enzyme incubations were thus done with respectively 1) 50 mg/l Cel7 alone; 2) 25 mg/l of Cel7 + 25 mg/l of Cel7A-free Accellerase; 3) 15 mg/l of added Cel7 + 35 mg/l of Accellerase 1500.

3.3.1 Activity on Avicel cellulose

Avicel (5 g/l) was incubated 2 h with enzymes at 40 °C, pH 5.0, followed by filtration and determination of soluble sugar using PAHBAH reducing sugar assay. The average results of triplicate samples are shown in Figure 14.

Categories 1-6 show the amount of soluble reducing sugar with Cel7 alone (blue bars), Cel7 added to Cel7A-free Accellerase (red bars) and added to complete Accellerase 1500 (green bars), respectively. Category 7 (No Cel7) shows results with only substrate incubated without enzyme (blue bar), and 35 µg/ml of Accellerase 1500 without Cel7 addition (green bar). Category 8 (50

µg/ml) shows the results with 50 µg/ml of Cel7A-free Accellerase (red bar) and 50 µg/ml of Accellerase 1500 (green bar), i.e. without addition of Cel7 variants.

The amounts of soluble reducing sugar released by the enzymes correspond to a degradation of substrate in the order of 1-2% for Cel7 enzymes acting alone, and up to ~10% in the synergism experiments. When acting alone (blue bars), all Cel7 enzymes released more soluble sugar than without any enzyme addition. The AAA variant was slightly more efficient (+10%) than Hje Cel7A WT, while all produced less soluble sugar (2CC and QWY ~90%; Pch Cel7D ~80%; Hir Cel7A ~60%). When added to Cel7A-free Accellerase (red bars), all Cel7 variants gave less soluble sugar than Hje Cel7A WT (Pch Cel7D, 2CC, AAA ~90%; QWY ~75%; Hir Cel7A ~50%). Together with Accellerase 1500, variant 2CC was as efficient as Hje Cel7A WT. Variant AAA, Pch Cel7D and QWY gave ~90% as much, while Hir Cel7A gave ~70%. Thus, when acting on Avicel cellulose in synergy with other enzymes in the *H jecorina* cocktail, none of the other Cel7s were more efficient than Hje Cel7A WT. Among the engineered Hje Cel7A variants, 2CC was performing best.

It is noteworthy that 50 ug/ml of Accellerase 1500 (8, green bar) produces much more soluble sugar than 25 ug/ml Hje Cel7A WT + 25 ug/ml Cel7A-free Accellerase. They should contain about the same proportions of Hje Cel7A WT and other enzymes. One reason for this discrepancy might be that the same assumed extinction coefficient (1.5 liter g^{-1} cm^{-1}) was used for both Accellerase 1500 and the Cel7A-free Accellerase, although their compositions differ and they contain different amounts of pigments that contribute to the absorbance at 280 nm. Another possibility is that other essential enzyme components were removed together with Cel7A in the preparation of Cel7A-free Accellerase. Therefore, comparisons should be avoided between the results with Accellerase 1500 and the Cel7A-free Accellerase. Still the Cel7s can be compared with each other within each set of experiments.

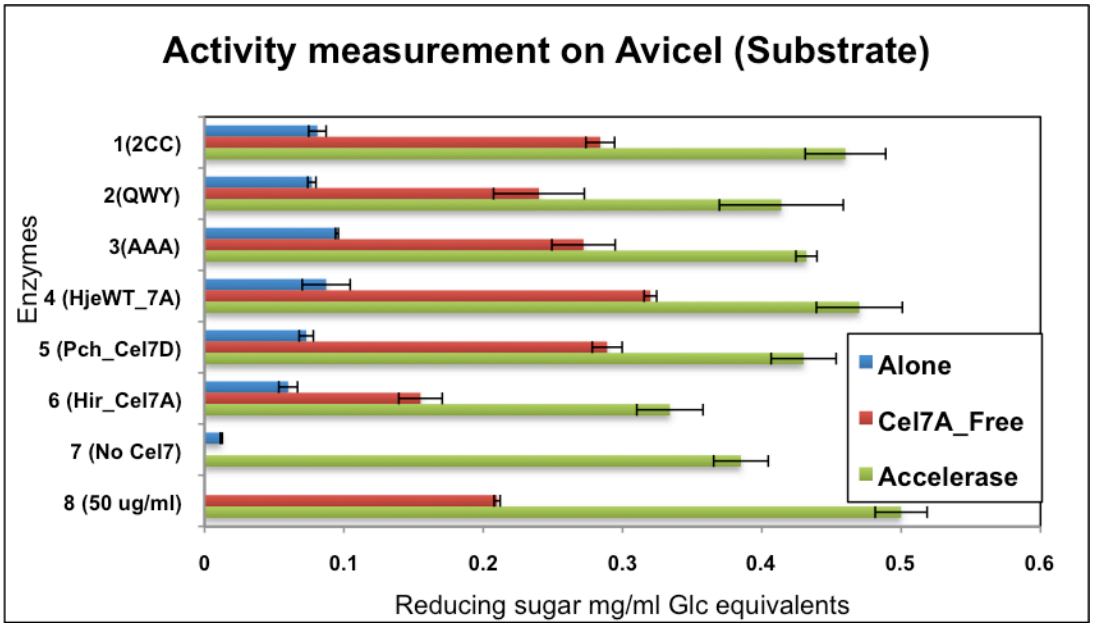


Figure 14: Soluble reducing sugar released from 5 g/l of Avicel cellulose after 2 h incubation at 40 °C, pH 5.0, using 50 µg/ml of Cel7A alone (blue), 25 µg/ml Cel7 + 25 µg/ml Cel7A-free Accellerase (red), and 15 µg/ml Cel7 + 35 µg/ml Accellerase 1500 (green). 1) 2CC; 2) QWY; 3) AAA; 4) WT Hje Cel7A; 5) Pch Cel7D; 6) Hir Cel7A (50 µg/ml); 7) No Cel7 (substrate without enzyme, blue; 35 µg/ml Accellerase 1500, green); 8) 50 µg/ml Cel7A-Free Accellerase (red), 50 µg/ml Accellerase 1500 (green). Error bars show standard deviation of triplicate samples.

3.3.2 *Activity on untreated spruce (sawdust)*

Spruce sawdust (30 g/l) was incubated 20 h with enzymes at 40 °C, pH 5.0, followed by filtration and determination of soluble sugar using PAHBAH reducing sugar assay. The average results of triplicate samples are shown in Figure 15, organized in the same way as for the Avicel experiments above.

As expected, very small amounts of the untreated spruce material was degraded, only up to 2.5 % in the synergism experiments. When acting alone (blue bars), the Hir Cel7A enzyme stood out in producing more than twice as much soluble sugar (250%), while Pch Cel7D (~85%) and all the engineered variants gave lower yields than Hje Cel7A WT (AAA ~70%; 2CC, QWY ~50%). Small amounts of reducing sugar was released from the spruce material also in the absence of enzyme (~0.07 mg/ml; Figure 15, 7 (No Cel7), blue bar). When added to Cel7A-free Accellerase (red bars), the 2CC variant was more efficient (~130%) than Hje Cel7A WT, while the other enzymes were less efficient (74-84%). The 2CC variant was also more efficient (~110%) when added to Accellerase 1500 (green bars). AAA gave about the same yield as Hje Cel7A WT, while the others gave slightly less (~90%).

It is interesting that the samples with Cel7A-free Accellerase gave higher yields on untreated spruce than Accellerase 1500, while the order was the opposite on Avicel cellulose. It should be remembered that the substrates are quite different and so are the compositions of the enzyme cocktails. The Avicel consists only of cellulose, whereas the spruce material also contains hemicellulose polysaccharides. The reducing sugar assay reacts also with soluble sugars released from the hemicellulose components, such as xylose and mannose monomers and oligomers. In the synergism experiments with Cel7A-free Accellerase the amount of other enzymes than Cel7 amount to 25 µg/ml. But in the experiments where Accellerase 1500 was used (35 µg/ml) around half is Hje Cel7A WT, meaning that there was less of the other enzymes present (~17 µg/ml). This also means that the amount of hemicellulose active enzymes was higher with Cel7A-free Accellerase, which might be the reason for higher activity on the hemicellulose-containing spruce material. It may thus be so that the soluble sugar comes primarily from degradation of hemicellulose in the spruce material. Furthermore it can be speculated that Hir Cel7A shows higher activity due to an ability to degrade some hemicellulose component(s). However, this needs further investigation, e.g. by carbohydrate analysis of the reaction filtrates.

Conversely, the synergism experiments with Accellerase 1500 contain ~30-35 µg/ml of Cel7 enzyme (15 µg/ml added Cel7 + ~17 µg/ml Hje Cel7A) compared to the 25 µg/ml Cel7 added when Cel7A-free Accellerase was used. A higher total amount of Cel7 enzyme may be the reason for higher activity on this pure cellulose substrate.

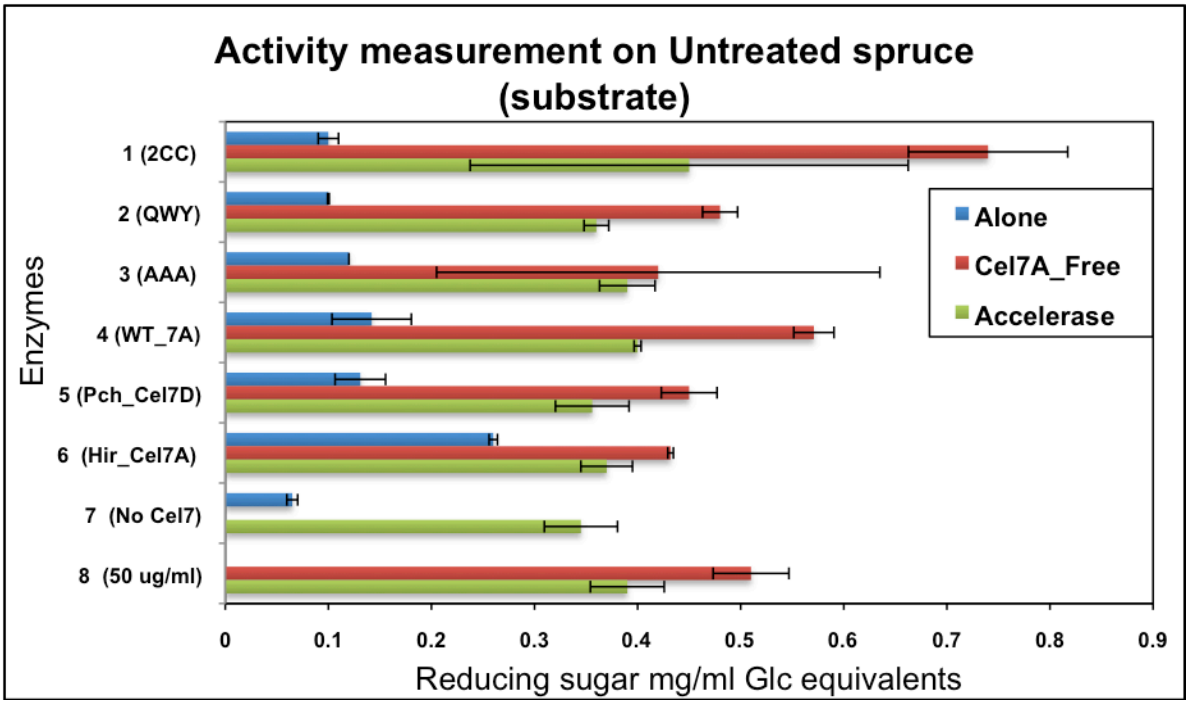


Figure 15. Soluble reducing sugar released from 30 g/l of spruce sawdust after 20 h incubation at 40 °C, pH 5.0, using 50 µg/ml of Cel7A alone (blue), 25 µg/ml Cel7 + 25 µg/ml Cel7A-free Accellerase (red), and 15 µg/ml Cel7 + 35 µg/ml Accellerase 1500 (green). 1) 2CC; 2) QWY; 3) AAA; 4) WT Hje Cel7A; 5) Pch Cel7D; 6) Hir Cel7A (50 µg/ml); 7) No Cel7 (substrate without enzyme, blue; 35 µg/ml Accellerase 1500, green); 8) 50 µg/ml Cel7A-Free Accellerase (red), 50 µg/ml Accellerase 1500 (green). Error bars show standard deviation of triplicate samples.

3.3.3 Activity on thermo-chemically pretreated spruce material

Thermo-chemically pretreated spruce material (50 g/l) was incubated 20 h with enzymes at 40 °C, pH 5.0, followed by filtration and carbohydrate analysis by HPAE-PAD. The average results of triplicate samples are shown in Figure 16, organized in the same way as for the Avicel experiments above. Due to uncertainities in the quantification of some of the monosaccharide components, as explained further below, only the total amount of glucose + cellobiose is included in the results in Figure 16. A complete table of the amount of each analyzed sugar in each sample is provided in Appendix I and J.

First it should be noted that the amount of soluble sugars was rather high already without enzyme addition, corresponding to about 13% of the substrate material. In most of the enzyme incubations there was very little increase in yield. Some samples even gave lower amounts than in the absence of enzymes, indicating a large variation between experiments that will be further discussed below. A substantial increase was observed only with Cel7A-free Accellerase + Hje Cel7A (→19% conversion), + AAA, or + 2CC (→16% conversion).

The degradation is still much lower than expected for a thermo-chemically pretreated industrial material. One possibility is that the enzymes are inhibited by some components in the material, either in the soluble or the solid fraction. To investigate this, further experiments may be performed after separation of the solids and the liquid. Another plausible explanation is that the pH has decreased after pH adjustment and dropped to a level where the enzymes are less active, due to slow titration of acidic components within the solid lignocellulose composite. The pH was not checked after incubation with enzymes. That should be done in further experiments.

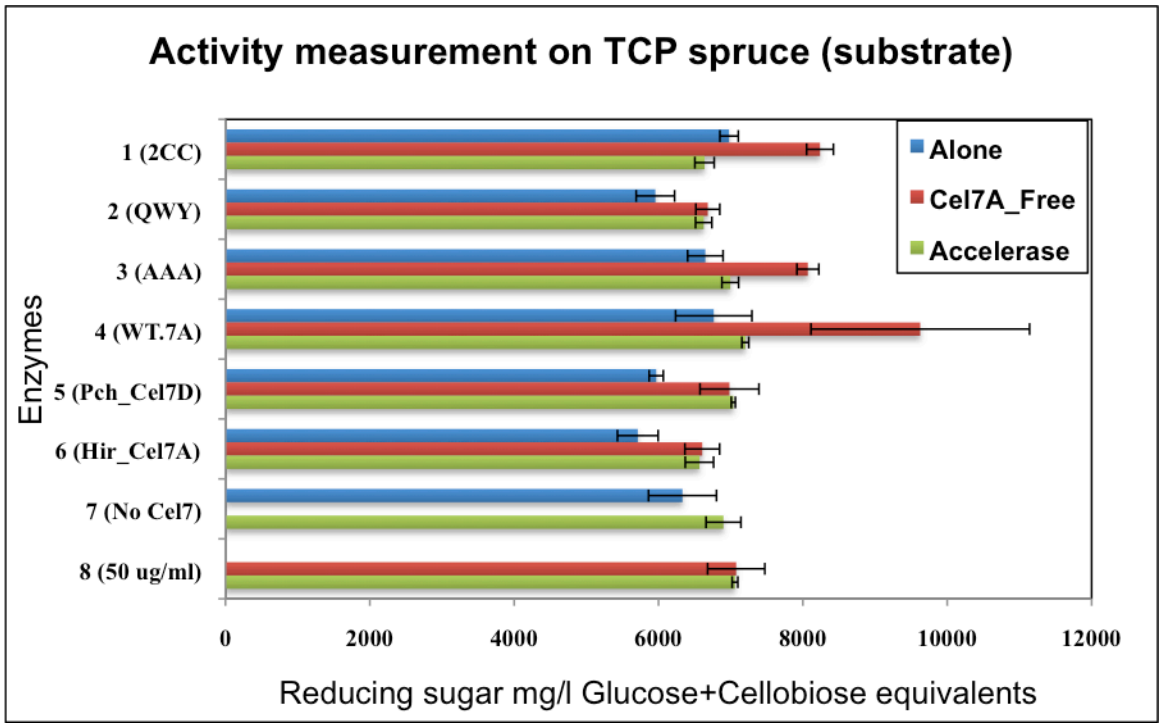
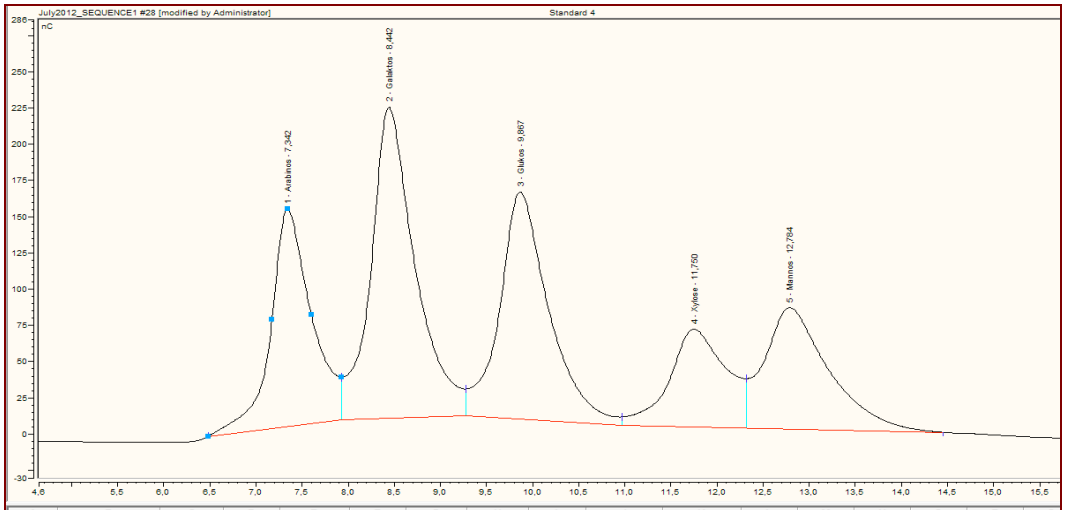


Figure 16. Soluble reducing sugar released from 50g/l of thermo-chemically pretreated spruce after 20 h incubation at 40 °C, pH 5.0, using 50 µg/ml of Cel7A alone (blue), 25 µg/ml Cel7 + 25 µg/ml Cel7A-free Accellerase (red), and 15 µg/ml Cel7 + 35 µg/ml Accellerase 1500 (green). 1) 2CC; 2) QWY; 3) AAA; 4) WT Hje_Cel7A; 5) Pch_Cel7D; 6) Hir_Cel7A (50 ug/ml); 7) No Cel7 (substrate without enzyme, blue; 35 µg/ml Accellerase 1500, green); 8) 50 µg/ml Cel7A-Free Accellerase (red), 50 µg/ml Accellerase 1500 (green). Error bars show standard deviation of triplicate samples.

Carbohydrate analysis of the samples on the Dionex HPLC system did not give as good separation as has been obtained previously with the same column and chromatography protocol. The chromatogram in (Figure 17) of one of the carbohydrate standards used, shows that adjacent monosaccharide peaks overlap with each other without baseline separation. The figure also shows how the Chromeleon chromatography software assigns the baseline for integration under the peaks. That may work fine and give reasonable accuracy in the quantification of the mono-saccharides, but only as long as the relative amounts of the mono-saccharides in the analyzed samples are similar to the standard samples, which was not the case. In the hydrolysates of spruce material (Figure 18) glucose dominates followed by mannose, xylose and much smaller amounts of arabinose and galactose. The quantification (peak integration) of one mono-saccharide will be highly influenced by the amounts of adjacent sugars in the chromatogram. For example, the amount of Gal is likely to be overestimated due to the overlap with the large Glc peak. At the same time Glc may be underestimated, because the overlap with surrounding peaks "lifts" up the baseline and gives a smaller area under the peak.

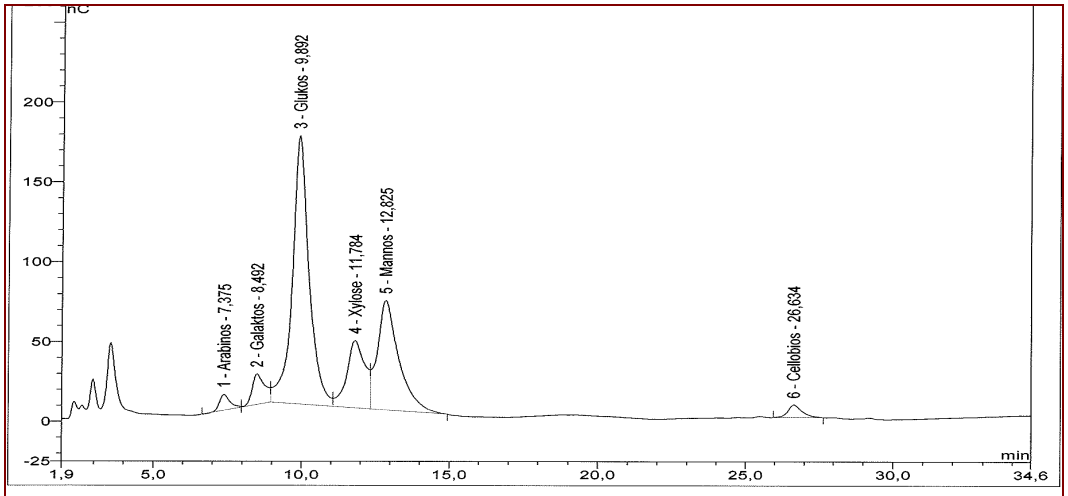
In order to overcome these problems, non-default baseline assignments were tried. The first strategy was to apply a straight baseline under all the peaks. However, this resulted in badly correlated calibration curves for the standards. In another attempt, the standards were divided in two groups, and two different QNT files were made, one for each group. The baselines were manually adjusted until the calibration curves for each group gave more accurate quantification of the sugars in the other group of standards. Then the baselines were adjusted in similar way for the samples as for the standards. Although this is likely to improve the accuracy of the quantification,

uncertainty may still be high due to peak overlap. Therefore it was decided to use only the amounts of the dominating monosaccharide glucose, plus cellobiose (where peak overlap does not seem to be a problem) as a measure of the efficiency of enzymatic hydrolysis.



No.	Peakname	Ret.Time min	Area nC*min	Amount mg/l	Type	Height nC	Rel.Area %	Resolution
1	Arabinos	7,342	76,5811	54,8363	BM *	149,828	16,60	1,46
2	Galaktos	8,442	120,0264	86,2020	M *	214,536	26,01	1,71
3	Glukos	9,867	96,9444	58,0406	M *	156,487	21,01	n.a.
4	Xylose	11,750	48,0866	87,2338	M *	67,174	10,42	n.a.
5	Mannos	12,784	67,8741	58,8387	MB*	83,582	14,71	12,90
6	Cellobios	26,592	51,8661	77,6360	BMB*	79,347	11,24	n.a.
Total:			461,3786	422,7875		750,955	100,00	

Figure 17. HPAE-PAD chromatogram of one of the carbohydrate standards used, baseline, peak integration zones, and peak integration table after base line adjustments. Note the overlap of monosaccharide peaks and lack of baseline separation.



No.	Ret.Time min	Peak Name	Type	Height nC	Area nC*min	Rel.Area %	Amount mg/l
1	7,38	Arabinos	BM *	10,001	4,045	1,85	230,4
2	8,49	Galaktos	M *	19,193	9,508	4,35	1011,9
3	9,89	Glukos	M *	168,235	111,003	50,73	6883,6
4	11,78	Xylose	M *	42,260	30,385	13,89	5437,2
5	12,83	Mannos	MB*	68,729	59,207	27,06	4950,4
6	26,63	Cellobios	BMB	8,068	4,647	2,12	471,9
Total:				316,487	218,80	100,0	18985,4

Figure 18. HPAE-PAD chromatogram of one analyzed sample of filtrate from TCP-spruce after incubation with enzymes, showing the relative sizes of monosaccharide peaks and the adjusted baseline.

The problems with peak overlap, sample variation, and low yield of solubilized sugar against a high background, together make the results on TCP-spruce very uncertain. Furthermore, the TCP-spruce slurry had been stored more than half a year in the cold room, and the pH in the slurry was around pH 2. Further reactions may have taken place during the storage time so that the material is no longer representative for an industrial process. Therefore, no conclusions will be drawn from these results, except that further experiments are needed to obtain reliable information about the performance of these Cel7 enzymes on thermo-chemically pretreated lignocellulose.

4 Conclusions

The changes introduced by the mutations in the engineered *H. jecorina* Cel7A variants did not result in dramatic changes in activity compared to WT Cel7A, at least not on the cellulose-only substrate Avicel. On untreated spruce, the 2CC variant was more efficient than wildtype, when added to the enzyme cocktails (both Cel7A-free and Accellerase 1500). The Pch Cel7D enzyme was not more active than Hje Cel7A WT on any of the substrates. Both Pch Cel7D and the AAA variant of Hje Cel7A, are more open at the product binding site, show higher k_{cat} on soluble substrates and weaker cellobiose product binding. The results indicate that these properties are not beneficial for cellulose degradation, at least not with the tested substrates and conditions. Rather it appeared that a closed active-site tunnel is better, as in the 2CC variant. However, it should be noted that the substrate conversion was relatively low, meaning that the results are a measure of rate of degradation at an early stage, rather than the total yield of soluble sugar that may be obtained from the substrate.

The *Heterobasidion* Cel7A enzyme was the only single-domain enzyme, while all the others have a CBM unit attached. As expected, Hir Cel7A showed the lowest activity of the tested enzymes on Avicel substrate. Interestingly, with untreated spruce, Hir Cel7A acting alone gave much more soluble reducing sugar than wildtype or any of the other variants. However, the reducing sugar assay that was used does not discriminate between different aldose sugars. Therefore it would be interesting to analyze which saccharides are actually released from the untreated spruce by the respective enzymes.

As explained above, no clear conclusions could be drawn from the experiments with TCP-spruce, other than that new trials need to be done with changed conditions.

5 Future Perspectives

Further experiments are needed to obtain reliable information about the performance of these Cel7 enzymes on thermo-chemically pretreated lignocellulose. Preferably a more fresh substrate should be used in that case. Also it would be desirable to increase the number of replicates in the experiments, for example five replicates, in order to get better statistic significance. A problem with experiments on insoluble substrates is that lignocellulose materials are inherently heterogeneous, and when using small sample volumes, there may be substantial variations in the composition and structure of the particles in the samples. As explained above it would also be interesting to analyze which sugars that are actually released from lignocellulose substrates by the different Cel7 enzymes and mixtures. Finally, it would be interesting to further investigate whether the low activity obtained with TCP-spruce material is due to the presence of inhibitory compounds in the soluble or insoluble fraction of the material.

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8 Popular Science Summary

Have you ever thought about the devastating challenge facing the world today because of the depletion of world's fossil fuel resources? If the answer is 'No' then it's a time to think about it!

Scientists have been trying to figure out best possible alternatives for the fossil fuels resources (which according to one estimate will be diminished now or later) to prevent increasing level of CO_2 and other detrimental gases (NO_2, CO) in the atmosphere, which is the main cause of global warming on our planet. Biofuel (whose energy is derived from biological carbon fixation) is a good alternative, but nowadays its production depends on first generation feed stocks (sugar and starch crops and vegetable oils) which in itself is a challenge due to the excessive use of food feedstocks resulting in shortage of food and increase in food prices. What should be the alternative then? Yes, you are right. We can also think to use second-generation feedstocks (lignocellulosic materials) instead of first generation feedstocks for the production of biofuels. However, the complexity of lignocellulosic materials hinder access to the structural polysaccharides for their conversion to soluble sugars for the production of biofuels, which is the main obstacle against commercialization of biofuels from lignocellulosic materials. There is a need to use best possible ways in order to disrupt these complex lignocellulosic structures in order to utilize them for biofuel production.

In nature microorganisms are used for the degradation of plant materials (lignocellulosic materials) through the action of enzymes. There is a need to use active enzymes in order to get effective conversion of lignocellulosic materials to soluble sugars for the production of biofuels. The major source of such biomass degrading enzymes, is a filamentous fungus called *Hypocrea jecorina*, isolated from a tropical island in the Pacific Ocean. It produces among the most efficient mixture of biomass degrading enzymes known today. Still the degradation process of plant biomass is slow and it would be desirable if the enzymes were more efficient.

In a previous study, the major cellulose-degrading enzyme of *Hypocrea jecorina*, called Hje Cel7A, has been modified by site-directed mutagenesis close to the active site of the enzyme, and the activity was investigated on soluble substrates. Large changes in activity properties were indeed obtained. However, it would also be interesting to know how efficient these enzyme variants are on "real" insoluble lignocellulosic substrates. To investigate this, was the major purpose of this Master thesis project.

In this study we wanted to know how the enzymes perform, both when acting alone, and in combination with the other biomass-degrading enzymes from *Hypocrea jecorina*. Furthermore, two enzymes of the same type from two other wood-degrading fungi were included in the study, *Heterobasidion irregulare*, which is a major pathogen in conifer (spruce and pine) forests, and *Phanerochaete chrysosporium*, that was isolated from a wood-chip pile in the north of Sweden and is claimed to be able to degrade all components of wood including lignin.

The activity on three different cellulosic substrates was investigated: i) a powder of pure cellulose called Avicel, ii) untreated saw dust from spruce wood, and iii) thermo-chemically pretreated forestry residues of spruce (TCP-spruce; treated with dilute sulphuric acid at high temperature and pressure). Unfortunately, with TCP-spruce the amount of soluble sugar was rather high already before adding the enzymes, and only small amounts of sugars were released by the enzymes, and the variation was high between samples. Therefore, no conclusions could be drawn from these experiments.

Overall the differences in activity between the Cel7 enzymes were less dramatic on the other two insoluble substrates, than seen previously with soluble substrates. The variants that had a more open cellulose binding tunnel were not more active than the native (wildtype) Hje Cel7A

enzyme, but the variant 2CC where the tunnel was physically closed by the introduction of a disulphide bridge over the active site was equally or more efficient than the wildtype enzyme.
All the tested Cel7 enzymes, except *Heterobasidion* Cel7A, are bimodular, which means that they have a small cellulose binding module attached by a flexible linker to the catalytic module that performs the actual cleavage of the cellulose chains. The *Heterobasidion* Cel7A consists only of a single catalytic module. Interestingly, this enzyme gave more soluble product from untreated spruce sawdust, than any of the others when acting alone. The analytical method used does not tell which components of the spruce wood that were degraded, and this would be interesting to investigate in further studies. Maybe the *Heterobasidion* has different properties than the other Cel7 enzymes because the need is different in a pathogenic fungus, than in the other fungi which are believed to be saprophytic.

9 Appendices

Appendix A. Stock concentrations of Cel7 enzymes used, Accellerase 1500 and Cel7A free Accellerase along with their extinction coefficients.

Engineered variants Hje_Cel7A	Extinction coefficients	Main stocks concentrations mg/ml	Reaction stocks Concentrations.
2CC (T246C/Y371C)	1.606	2.475	900 µg/ml
QWY (Y252Q/D259W/S342Y)	1.735	1.385	1 mg/ml
AAA (T246A/R251A/Y252A)	1.611	1.94	900 µg/ml
Pch Cel7D	1.4785	4.4	1 mg/ml
Hir Cel7A	1.276	2.35	1 mg/ml
WT Hje Cel7A	1.662	-	730 µg/ml
Cel7A free Accellerase	1.5		460 µg/ml
Accellerase 1500	1.5	-	1 mg/ml

Appendix B. Desalting procedure of enzyme samples using BioRad 10-DG Column:

1) The 10-DG column was equilibrated with 25-30 ml of 10 mM NaAc, pH 5.0.
2) 3.0 ml of protein sample was applied on the column and the outflow was allowed to go to waste.
3) 4.0 ml of Buffer (10 mM NaAC, pH 5.0) was applied and the outflow was collected in a new test tube.

Appendix C. Calculations of enzyme stock solution volumes (50ug/ml and 25ug/ml) for 300 ul reaction volume and preparation of enzyme cocktails to run 1:1 mix with Cel7A free Accellerase on Avicel

Enzymes	Volume = 50 ug/ml for 300 ul	Volume = 25 ug/ml for 300 ul	Total Enzyme volume. X3
2CC	16.67	8.33	25+48.9=73.9
QWY	15	7.5	22.5+48.9=71.4
AAA	16.67	8.33	25+48.9=73.9
Pch Cel7D	15	7.5	22.5+48.9=71.4
WT Hje Cel 7A	20.54	10.27	30.81+48.9=79.1
Hir Cel7A	15	7.5	22.5+48.9=71.4
Cel7A free Accellerase	32.6	16.3	

Reaction volume 300ul	Enzymes cocktail	Substrate (5g/l) ul	Buffer ul	Water ul	Enzyme ul (100%)
	2CC	*150*	*30*	*103.33*	*16.67*
	50-50%	*150*	*30*	*95.37*	*16.3+8.33*
	QWY	150	30	105	*15*
	50-50%	150	30	96.2	*7.5+16.3*
	AAA	*150*	*30*	*103.33*	*16.67*
	50-50%	*150*	*30*	*95.37*	*16.3+8.33*
	Pch_Cel7D	150	30	105	15
	50-50%	150	30	96.2	7.5+16.3
	WT Hje Cel 7A	150	30	99.46	20.54
	50-50%	150	30	93.43	10.27+16.3
	Hir Cel7A	*150*	*30*	*105*	*15*
	50-50%	*150*	*30*	*96.2*	*7.5+16.3*
	Cel7A free Accellerase	150	30	87.4	32.6

Appendix D. Calculations of enzyme stock solution volumes (50ug/ml and 25ug/ml) for 500 ul reaction volume and preparation of enzyme cocktails to run 1:1 mix with Cel7A free Accellerase on thermo-chemically pretreated spruce material.

Enzyme	Volume = 50 ug/ml for 500 ul	Volume = 25 ug/ml for 500 ul	Total Enzyme volume. X3
2CC	27.78	13.89x3	41.67+81.51=123.18
QWY	25	12.5x3	37.5+81.51=119.01
AAA	27.78	13.89x3	41.67+81.51=123.18
Pch Cel7D	25	12.5x3	37.5+81.51=119.01
WT Hje Cel7A	34.2	17.1x3	51.3+81.51=132.81
Hir Cel7A	25	12.5x3	37.5+81.51=119.01
Cel7A free Accellerase	54.35	27.17	

500 µl Reaction volume	Enzyme cocktails	Substrate (50g/l) µl	Buffer µl	Water µl	Enzyme µl
	2CC 100%	*370*	*30*	*72.22*	27.78
	50-50%	*370*	*30*	*58.94*	13.89+27.17=41.06
	QWY 100%	*370*	*30*	*75*	25
	50-50%	*370*	*30*	*60.33*	12.5+27.17=39.67
	AAA 100%	*370*	*30*	*72.22*	27.78
	50-50%	*370*	*30*	*58.94*	13.89+27.17=41.06
	Pch Cel7D 100%	370	*30*	*75*	25
	50-50%	370	*30*	*60.33*	12.5+27.17=39.67
	Hje Cel7A	370	*30*	65.8	34.2
	50-50%	370	*30*	55.73	17.1+27.17=44.27
	Hir Cel7A *100%*	*370*	*30*	*75*	*25*
	50-50%	*370*	*30*	*60.33*	*12.5+27.17=39.67*
	Cel7A free Accellerase 100%	370	30	45.65	54.35

Appendix E. Calculations of enzyme solution volumes (50 µg/ml and 25 µg/ml) for 500 µl reaction volume and preparation of enzyme cocktails to run 1:1 mix with Cel7A free Accellerase on untreated spruce powder

Enzyme	Volume = 50 µg/ml for 500 µl	Volume = 25 µg/ml for 500 µl	Total Enzyme volume. X3 (triplicates)
2CC	27.78	13.89x3	41.67+81.51=123.18
QWY	25	12.5x3	37.5+81.51=119.01
AAA	27.78	13.89x3	41.67+81.51=123.18
Pch Cel7D	25	12.5x3	37.5+81.51=119.01
Hje Cel7A	34.2	17.1x3	51.3+81.51=132.81
Hir Cel7A	25	12.5x3	37.5+81.51=119.01
Cel7A Free Accellerase	54.35	27.17	

500 µl Reaction volume	Enzyme cocktails	Substrate (30g/l) mg	Buffer µl	Water µl	Enzyme µl
	2CC 100%	*16.3*	*50*	*332.22*	27.78
	50-50%	*16.3*	*50*	*319*	13.89+27.17=41.06
	QWY 100%	*16.3*	*50*	*335*	25
	50-50%	*16.3*	*50*	*320*	12.5+27.17=39.67
	AAA 100%	*16.3*	*50*	*332.2*	27.78
	50-50%	*16.3*	*50*	*319*	13.89+27.17=41.06
	Pch Cel7D 100%	16.3	*50*	*335*	25
	50-50%	16.3	*50*	*320*	12.5+27.17=39.67
	Hje Cel7A	16.3	*50*	325.8	34.2
	50-50%	16.3	*50*	315.73	17.1+27.17=44.27
	Hir Cel7A *100%*	*16.3*	*50*	*335*	*25*
	50-50%	*16.3*	*50*	*320*	*12.5+27.17=39.67*
	Cel7A free Accellerase 100%	16.3	50	305.65	54.35

Appendix F. Calculations for enzyme cocktail preparations for enzyme synergism experiments with Avicel powder in 300ul total reaction volume.

Enzymes	Volume = 15 µg/ml for 300ul	Volume =15 µg/ml*3 (triplicates)	Total Enzyme volume
2CC	5	15	15+31.5=46.5
QWY	4.5	13.5	13.5+31.5=45
AAA	5	15	15+31.5=46.5
Pch Cel7D	4.5	13.5	13.5+31.5=45
Hje Cel7A	6.16	18.5	18.5+31.5=49.98
Hir Cel7A	4.5	13.5	13.5+31.5=45
Acc -1500 (35 µg/ml)	10.5	31.5	31.5
Acc-1500 (50 µg/ml)	15	45	45

Reaction volume 300 µl.	Substrate (5 g/l) µl	Buffer µl	Water µl	Enzyme µl
2CC	150	30	104.5	15.5
QWY	150	30	105	15
AAA	150	30	104.5	15.5
Pch Cel7D	150	30	105	15
Hje Cel7A	150	30	103.34	16.66
Hir Cel7A	150	30	105	15
Acc-1500 (100%) 35 µg/ml	150	30	102.5	10.5
Acc-1500 (100%E) 50 µg/ml	150	30	105	15
Blank (x3)	150	30	120	------

Appendix G. Calculations for enzyme cocktail preparations for enzyme synergism experiments with thermo-chemically pretreated spruce in 500ul total reaction volume.

Enzymes	Volume = 15 µg/ml for 500 µl	Volume = 15 µg/ml (X3)	Total Enzyme volume. X3
2CC	8.33	8.33x3	25+52.5=77.5
QWY	7.5	7.5x3	22.5+52.5=75
AAA	8.33	8.33x3	25+52.5=77.5
Pch Cel7D	7.5	7.5x3	22.5+52.5=75
WT Hje Cel7A	10.27	10.27x3	30.81+52.5=83.31
Hir Cel7A	7.5	7.5x3	22.5+52.5=75
Acc1500 15 µg/ml	17.5	17.5x3	52.5
Acc1500 35 µg/ml	25	25x3	75

Reaction volume 500 µl	Enzymes µl	Substrate µl	Buffer µl	Water µl	Enzyme µl
	2CC	370	50	54.14	25.83
	QWY	370	50	55	25
	AAA	370	50	54.14	25.83
	Pch Cel7D	370	50	55	25
	WT Hje Cel7A	370	50	52.2	27.8
	Hir Cel7A	*370*	50	55	25
	Acc1500	370	50	62.5	17.5
	Acc1500	370	50	55	25

Appendix H. Calculations for enzyme cocktail preparations for enzyme synergism experiment with untreated spruce powder in 500ul total reaction volume.

Enzymes	Volume 15 µg/ml	Volume *3	Total Enzyme volume
2CC	8.33	25	25+52.5=77.5
QWY	7.5	22.5	22.5+52.5=75
AAA	8.33	25	25+52.5=77.5
Pch Cel7D	7.5	22.5	22.5+52.5=75
Hje Cel7A	10.27	30.81	30.81+52.5=83.31
Hir Cel7A	7.5	22.5	22.5+52.5=75
Accellerase1500 35ug/ml	17.5	52.5	
Accellerase 1500 50ug/ml	25	75	

Reaction volume 500 µl	Substrate 30g/l mg	Buffer µl	Water µl	Enzyme µl
2CC	16.3	50	334.2	25.83
QWY	16.3	50	335	25
AAA	16.3	50	334.2	25.83
Pch Cel7D	16.3	50	335	25
Hje Cel7A	16.3	50	332.2	27.8
Hir Cel7A	16.3	50	335	25
Acc 1500 35µg/ml	16.3	50	342.5	17.5
Acc 1500 50 µg/ml	16.3	50	335	25
Blanks (X3)	16.3	50	360	----------

Appendix I. Results of Dionex carbohydrate analysis of TCP-spruce experiments, showing the concentration of each analysed sugar, for each individual triplicate sample. D.F = dilution factor; C6 = hexoses; C5 = pentoses; % LCB = percentage of lignocellulose biomass converted to soluble sugars.

Samples	Enzymes	D.F	Ara	Gal	Glc	Xyl	Man	Cel	Total	TotalC6	TotalC5	%LCB	Glc+Cel	Average
			mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	sugars	mg/l	mg/l		mg/l	Glc+Cel
1	B1	100	294	1325	6811	5248	4266	40.5	17983.6	12441.7	5541.9	23.6%	6167.6	
2	B2	100	319	1381	7706	6027	4705	39.3	20178.8	13831.6	6347.2	26.5%	6972.8	6332.1
3	B3	100	208	1128	6467	5326	4127	37.2	17294.7	11759.6	5535.1	22.7%	5855.9	
4	Acc-7A	200	247	1317	7265	5305	3837	161.6	18132.9	12580.6	5552.3	23.8%	6691.5	
5	Acc-7A	200	265	1296	8052	5349	4146	126.5	19234.5	13620.2	5614.3	25.3%	7366.3	7076.1
6	Acc-7A	200	313	1311	7786	5442	4501	172.5	19523.6	13769.3	5754.3	25.7%	7170.5	
7	Acc-7A	100	226	1004	8262	5762	4566	151.8	19970.4	13983	5987.4	26.3%	7578.9	
8	Acc-7A	100	228	1157	7566	4618	4098	160.4	17828.8	12982.5	4846.3	23.5%	6961.3	7054.0
9	Acc-7A	100	291	1007	7185	5141	4524	163.9	18310.8	12880.2	5430.6	24.1%	6621.7	
10	100% WT	100	264	1052	6254	4713	4153	560.7	16995.2	12018.7	4976.5	22.4%	6159.3	
11	100% WT	100	272	1214	7603	5566	4715	638.5	20008.6	14170.6	5838	26.4%	7447.7	6764.8
12	100% WT	100	222	876	6797	5362	5128	602.1	18986.6	13403.9	5582.7	25.0%	6687.6	
13	50%WT	100	283	1186	8464	5518	4906	703.8	21060.3	15259.3	5801	27.8%	8283.8	
14	50%WT	100	6018	8924	6241	9017	6336	6466.2	43001.4	27966.8	15034.6	56.9%	11742.9	9626.3
15	50%WT	100	316	1329	9097	5765	4872	701.1	22081	15999.1	6081.9	29.1%	8851.9	
16	100% 2CC	100	247	1095	7214	5363	4878	416.2	19212.2	13602.9	5609.3	25.3%	6886.7	
17	100% 2CC	100	299	1129	7213	6885	6249	419.2	22194.4	15010	7184.4	29.2%	6888.4	6977.6
18	100% 2CC	100	276	1197	7541	5714	5276	392.3	20396.9	14406.3	5990.6	26.8%	7157.8	
19	50% 2CC	100	322	1311	8830	6124	5507	518	22612.1	16166.6	6445.5	29.8%	8437.7	
20	50% 2CC	100	251	1135	8299	5188	4892	549.6	20314.6	14875.6	5439	26.8%	7990.2	8237.7
21	50% 2CC	100	231	1045	8628	5684	5094	548.9	21230.5	15316.4	5914.1	28.0%	8285.2	
22	100% AAA	100	256	1121	7208	5523	4918	490.4	19516.1	13737.3	5778.8	25.7%	6952.3	
23	100% AAA	100	231	1012	6884	5437	4951	471.9	18985.4	13317.8	5667.6	25.0%	6642.3	6648.7
24	100% AAA	100	213	995	6538	5742	4807	492.9	18788.9	12834.2	5954.7	24.7%	6351.6	
25	50% AAA	100	305	1191	8262	5598	4897	562.6	20815.2	14912.5	5902.7	27.4%	7968.78	
26	50% AAA	100	288	1050	8233	5454	5106	575.8	20706.7	14964.9	5741.8	27.3%	7954.7	8069.2
27	50% AAA	100	228	1046	8563	5853	5261	609.8	21559.2	15478.4	6080.8	28.4%	8283.8	
28	100% QWY	100	282	1061	6278	4615	4335	351.1	16923.3	12026	4897.3	22.3%	5983.5	
29	100% QWY	100	263	974	5893	4412	4149	331.7	16023.6	11348.6	4675	21.1%	5618.2	5956.6
30	100% QWY	100	279	1032	6473	4887	4466	467	17604.6	12438.3	5166.3	23.2%	6268.4	
31	50% QWY	100	259	1087	7198	4863	4138	442.9	17989.4	12868	5121.4	23.7%	6898.4	
32	50% QWY	100	273	948	6971	4417	4198	402.9	17209.9	12519.2	4690.7	22.7%	6654.8	6683.1
33	50% QWY	100	245	945	6797	4347	4042	399.5	16775	12183	4592	21.8%	6495.8	
34	100% Pc7D	100	278	1007	6222	4557	4196	535	16796	11961.3	4834.7	21.9%	6106.7	
35	100 %Pc7D	100	246	954	5901	4440	3946	608.9	16094.6	11409	4685.6	21.0%	5887.2	5968.5
36	100% Pc7D	100	264	976	5967	4288	3740	571.5	15807.4	11255	4552.4	20.6%	5911.8	
37	50%Pc7D	100	255	904	6504	4123	3628	607.1	16021.3	11643.9	4377.4	20.9%	6428.5	
38	50%Pc7D	100	266	983	7619	4589	4163	572.7	18194	13338.2	4855.8	23.7%	7399.9	6982.9
39	50%Pc7D	100	265	969	7302	4297	3897	578.9	17310.2	12748.3	4561.9	22.6%	7120.3	
40	100%HIR	100	242	902	5786	4581	4096	322.3	15929.8	11106.6	4823.2	20.7%	5512.5	
41	100%HIR	100	245	915	5822	4372	4041	289.8	15684.4	11067.9	4616.5	20.4%	5514.5	5712.9
42	100%HIR	100	279	1054	6459	4828	4418	314.7	17354.4	12246.8	5107.6	22.6%	6111.6	
43	50% Hir	100	279	869	6697	4607.9	4151	253.6	16857.6	11970.6	4887	21.9%	6267.7	
44	50% Hir	100	248	1020	7204	5474.5	4651	277.1	18876.1	13152.8	5723.3	24.5%	6746.4	6606.1

45	50% Hir	100	294	1035	7245	4851.7	4621	298.9	18346.7	13200.5	5146.2	23.9%	6804.1

Appendix J. Results of Dionex carbohydrate analysis of TCP-spruce experiments, showing the concentration of each analyzed sugar, for each individual triplicate sample. D.F = dilution factor; C6 = hexoses; C5 = pentoses; % LCB = percentage of lignocellulose biomass converted to soluble sugars.

Samples	Enzymes	D.F	Ara	Gal	Glu	Xyl	Man	Cel	Tot. Soluble Sugars	C6	C5	% LCB	Glc+Cel	Glc+Cel
			mg/l	mg/l	mg/l	mg/l	mg/l	mg/l						Average
1	Blank 1	100	294.6	1171.2	6769.7	5036.4	4433.4	37.8	17743.1	12412.1	5331	23.3%	6128.5	
2	Blank 2	100	289.8	1106.3	6509.2	5101	4379.3	31.1	17416.7	12025.9	5390.8	22.9%	5887.7	5848
3	Blank 3	100	282.9	1060	6113.7	4639.7	4188.3	27.5	16312.1	11389.5	4922.6	21.4%	5528.4	
4	Acc 50ug/ml	200	186.7	703	5665.8	4105.2	3343	346	14349.7	10057.8	4291.9	18.4%	5427.0	
5	Acc 50ug/ml	200	217.6	987.5	5823.9	4255.5	3372.9	354.9	15012.3	10539.2	4473.1	19.3%	5577.7	5586
6	Acc 50ug/ml	200	298.5	1057.7	6025.6	4569.1	3564.6	348.2	15863.7	10996.1	4867.6	20.4%	5752.9	
7	Acc 50ug/ml	100	238.8	998	7725.5	4782	4258.2	411.3	18413.8	13393	5020.8	23.7%	7342.6	
8	Acc 50ug/ml	100	235.9	1017.9	7464.6	4864.3	4051.9	384.9	18019.5	12919.3	5100.2	23.2%	7082.7	7059
9	Acc 50ug/ml	100	234.4	978.6	7122.4	4554.1	3851	360	17100.5	12312	4788.5	22.0%	6751.2	
10	Acc 35 ug/ml	100	279.1	1050.3	7279.7	4870.1	4120.7	414.5	18014.4	12865.2	5149.2	23.2%	6944.4	
11	Acc 35 ug/ml	100	237.4	1039.7	7237.7	4752.1	4181.8	414.6	17863.3	12873.8	4989.5	23.0%	6906.7	6899
12	Acc 35 ug/ml	100	259.2	1056.4	7148.1	4794.7	4140.5	437.6	17836.5	12782.6	5053.9	22.9%	6847.8	
13	30% WT	100	273.5	1058.3	7494.3	4908.1	4152.7	545.4	18432.3	13250.7	5181.6	23.5%	7261.5	
14	30% WT	100	276.7	1068	7409.2	4930.3	4209.6	563.4	18457.2	13250.2	5207	23.5%	7202.0	7203
15	30% WT	100	247.7	1018.8	7346.5	4822.6	4113.5	560.9	18110	13039.7	5070.3	23.1%	7143.2	
16	30% 2CC	100	195.8	1015.2	6715.6	4135.5	3575.2	431.4	16068.7	11737.4	4331.3	20.6%	6452.7	
17	30% 2CC	100	270.5	1108.8	6967.9	4903.2	4051.1	445.9	17747.4	12573.7	5173.7	22.8%	6693.5	6637
18	30% 2CC	100	241.2	1099.3	7063.2	4789.9	4044.9	432	17670.5	12639.4	5031.1	22.7%	6766.2	
19	30%AAA	100	277.8	1119.3	7093.9	5032.6	4132.2	470.7	18126.5	12816.1	5310.4	23.2%	6830.4	
20	30%AAA	100	293.5	1156.8	7354	5288.9	4393	494.4	18980.6	13398.2	5582.4	24.3%	7086.9	6993.8
21	30%AAA	100	299	1116.5	7333.5	5064.3	4307.8	489.5	18610.6	13247.3	5363.3	23.8%	7063.8	
22	30% QWY	100	292.7	1177.1	6974.3	4916.2	4198.2	441.3	17999.8	12790.9	5208.9	23.1%	6694.9	
23	30%QWY	100	262.6	1061.5	6735.2	4956.5	4137	427.3	17580.1	12361	5219.1	22.6%	6466.5	6626
24	30%QWY	100	255.2	1092.6	7000.3	5053.7	4406.9	439.9	18248.6	12939.7	5308.9	23.4%	6717.1	
25	30%Pch	100	270.5	1114.9	7315	5255.8	4416.9	519.6	18892.7	13366.4	5526.3	24.2%	7075.7	
26	30%PC	100	273.7	1101.9	7263.3	5148.3	4400.3	510.7	18698.2	13276.2	5422	23.9%	7020.8	7036
27	30%Pch	100	275.8	1082.4	7237.8	5213	4396.2	526.6	18731.8	13243	5488.8	24.0%	7012.9	
28	30%Hir	100	258.2	1089.8	6625	4982.5	4234.6	395.2	17585.3	12344.6	5240.7	22.6%	6336.9	
29	30%Hir	100	274.1	1094.4	6849	5045.6	4211.4	404.3	17878.8	12559.1	5319.7	23.0%	6547.2	6567
30	30%Hir	100	285.4	1097.3	7145.1	5231.2	4319.7	406.9	18485.6	12969	5516.6	23.8%	6816.1	