PROJECT FINAL REPORT

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4.1 Final publishable summary report

4.1.1 Summary

Exploitation of plant biomass as an alternative for fossil carbon sources in fuel and chemical production is one of the major challenges in the current world. The DISCO project aimed at developing more efficient and cost-effective enzymes for conversion of plant biomass into fermentable sugars and further to value added fuels and chemicals. For this goal, massive screening based on classical as well as more modern techniques (genome mining and metagenomics) was carried out for discovery of novel cellulase and hemicellulase enzymes. The project also focused on understanding of the mechanisms required for enzymatic degradation of complex plant polysaccharides and the inhibitory role of lignin. The plant biomasses chosen for the project were wheat straw, wheat bran, corn cob and spruce. All the selected biomasses represent abundant and sustainable European raw materials for the future biorefinereis.

The plant biomass mainly consists of three plant cell wall polymers: cellulose, hemicelluloses and lignin. Enzymatic hydrolysis alone is insufficient for degradation of the structurally resistant plant biomasses and thus suitable pretreatment methods are needed. The industrial project partners developed and optimised thermomechanical pretreatment methods for the selected biomasses. The effects of the pretreatment on the structure, chemistry and enzymatic digestibility of the plant biomasses were extensively characterized. The novel enzymes for the hydrolysis of lingocellulosics were searched from the unique fungal culture and soil sample collections, enzyme collections, and metagenomic libraries. Also the genome of *Myceliophthora thermophila* C1 a proprietary organism of the industrial partner Dyadic was used for enzyme discovery.

Various screening methods for cellulase and hemicellulase activities were developed within the project and used for the screening. Totally about 950 lignocellulolytic strains, of which more than 50% were from new soil isolates were screened for hemicellulases and cellulases suitable for an SSF (simultaneous saccharification and fermentation) process. Approximately 40 novel cellulases and hemicellulases were discovered and characterized in the project. Two highly (hemi)cellulolytic filamentous fungi were selected for total sequencing. Their genomes were sequenced and partially annotated within the project. Novel (hemi)cellulolytic activities were also in silico screened from these genomes. Several novel and unique cellulases and hemicellulases were also discovered from the unique enriched metagenomic libraries. Genome mining of the Dyadic's propertionary fungus C1 resulted in discovery of various interesting novel cellulases and hemicellulases.

The factors affecting enzymatic hydrolysis of xylans, the major hemicelluloses in the agricultural wastes were elucidated. The importance of hemicellulolytic and accessory enzymes in the hydrolysis of lignocellulosics was clearly shown. It was seen that the structural features of xylan had various effects on the mode of action of the hemicellulases. Novel specific hemicellulose structures which required specific enzyme activities were identified. Lignin typically has an inhibitory effect on the (hemi)cellulolytic enzymes. This phenomenon was elucidated in the project using isolated lignin rich residues. It was observed that inhibition of cellulase activity took place via enzyme adsorption onto lignin and that it was temperature dependent. At lower temperatures, for instance at temperatures typical for an SSF process effects of lignin were dimished. In addition, it was seen that the soluble compounds released from lignin affect cellulase and xylanase activities in an enzyme dependent manner.

The novel enzymes discovered within the DISCO project were evaluated in the hydrolysis of pretreated DISCO raw material and in SSF. The enzymes were tested in the enzyme mixtures of the *M. thermophila* C1 and the commercial reference enzyme mixtures Celluclast 1.5L and Novozym 188 (Novozymes, Denmark) in shake flask experiments, lab scale fermenters and in the SEKAB pilot plant in Örnskjöldsvik, Sweden. The results indicated that effectiveness of the enzyme mixtures in lignocellulose conversion is strongly dependent on substrate and process design. The greatest improvements in both total hydrolysis and ethanol yields were obtained by an addition of novel xylanases.

4.1.2.Project context and objectives

Exploitation of plant biomass as an alternative for fossil carbon sources in fuel and chemical production is one of the major challenges in the current world with diminishing amounts of fossil carbon and impact of their usage on carbon dioxide emissions. Utilization the non-food plant bio mass such as agricultural wastes is preferred to avoid conflicts between food and energy/chemical production. Lignocellulosic plant biomass is abundant, renewable and ubiquitous. Compared to fossil fuel the net emission of carbon dioxide is low(1), since the carbon dioxide released during combustion is assimilated when new biomass is produced. Sources of lignocellulosic biomass include waste materials from agriculture (straw), forestry (thinning wood, residuals), wood-based industries (saw dust, 'black liquor' from the pulp and paper industry), from specific energy crops such as short rotation forestry, and municipal solid waste. The estimated annual net yield of photosynthesis is 1.3 x 10^{10} metric tons of wood, which is equivalent of 4.4 x 10^{9} metric tons of crude oil, or about 40 % of the world's energy requirements(2), (http://ec.europa.en/dgs/energy_transport). Nevertheless, although lignocellulose is considered a potentially highly promising raw material, its exploitation presents many challenges for instance due to the difficulties in developing efficient conversion technologies, able to overcome its resistant structure.

The plant lignocellulosic biomass typically consist of structurally inhomogeneous plant cell wall fragments originating from different plant tissues and cell types and contain sugar polymers cellulose and hemicelluloses and lignin as the major components. Utilization of all major biomass components is essential in developing sustainable biorefinery concepts. Enzymatic hydrolysis is considered the most environmentally sustainable technology for the degradation of the sugar polymers. This is because the approach promises higher yields under mild reaction conditions as compared with chemical hydrolysis which generates higher amounts of inhibitory compounds and pollutants. The enzymatic hydrolysis technology is however, not yet used in an industrial scale although there are several operational lignocellulose to ethanol pilot and demonstration units worldwide, also in Europe (e.g. Italy, Spain, France, Denmark, Sweden).

Production of ethanol from starch containing annual crops is well established technology. The processes using lignocellulosic feedstock for bioethanol production are more challenging due to compactness and complexity of lignocellulose as compared to starch which makes it resistant to enzymatic hydrolysis. The susceptibility of lignocelluloses to enzymatic hydrolysis can be increased by pretreatment methods, which typically have to be optimized for the structurally variant biomasses. Sugar composition and lignin structure and the amount vary between lignocelluloses from different sources (**Table 1**), which in turn affects the requirements for the enzyme cocktail used for the hydrolysis step. The traditional fermenting yeast strains only utilize hexose sugars released from cellulose polymers, but for increased economic value, pentose yeast strains which also can consume the hemicellulosic sugars are under development. Thus, to improve the overall process economy in lignocellulosic ethanol production, improvements in the three major steps in the conversion process, pre-treatment, enzymatic hydrolysis and yeast fermentation, are still needed (Lynd et al. 2002).

Table 1. Typical compositions of different lignocellulosic raw materials, Biomass Feedstock Composition and Property Database (http://www.eere.energy.gov/biomass/progs/)

Source	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwood	40-50	25-35	**20-25**
Softwood	40-50	25-30	**25-35**
Wheat straw	40-45	20-30	**20-25**
Sugarcane bagasse	30-40	12-25	**20-30**
Corn stover	**30-40**	**20-25**	**15-25**

Cost of enzymes is one of the restrictions to economic production of cellulosic ethanol. Reduction of the enzyme cost is being aimed by development of the industrially relevant enzyme production hosts, production medium and down-stream processing as well as discovery and development of more efficient cellulases. A further approach is to add enzymes active on the other polymers in the lignocellulose matrix, such as hemicellulases, or other rate-limiting enzymes enhancing the hydrolysis. Enzymes that alter the interactions between lignin, hemicellulose and cellulose can be added as accessory enzymes. They improve the availability of cellulose to cellulases by exposing the matrix and making available new cellulose layers to the action of cellulases. The amount of hemicellulose in agricultural residues e.g. wheat straw is about 30% and in wood 20-30%. Considering the process economy, high yield in the conversion of the hemicellulosic sugars has a large impact on the overall process economy. Hemicellulose is not only a source of sugars for bioethanol or chemical production but it can be exploited in design of novel materials e.g. hydrogels, oxygen barriers (Kabel et al. 2002, Granström et al. 2004). Lignin has also been shown to negatively influence hydrolysis by adsorbing cellulases and thus decreasing their availability for cellulose hydrolysis (Sewalt et al., 1997; Berlin et al., 2006; Nakagame et al, 2010; Rahikainen et al, 2011). However, the sensitivity of enzymes to lignin varies (Berlin et al. 2006) which sets attractive target in finding enzymes with lower sensitivity to lignin caused inhibition**.** Very limited data is available related to the effects of lignin on the action of hemicellulases.

This project focused on development of enzyme tools for lignocellulose hydrolysis and elucidation of the underlying mechanisms of rate limiting factors in lignocellulose hydrolysis. The project approach was not limited to one type of lignocellulosic feedstock; instead the aim was to develop enzymes to more general use for the relevant European feedstock namely agricultural by-products or dedicated crops and soft wood. Although the lignocellulosic substrates vary in their composition and accessibility to the enzymes, they also have general features essential in the enzymatic hydrolysis. They all contain hydrolysis resistant crystalline cellulose, lignin and even after pre-treatment complex hemicelluloses, hydrolysis of .requires synergistically acting hemicellulases.

Hydrolysis of crystalline cellulose requires concerted action of several enzymes, which can be classified into endo-β-1,4-glucanases (EC 3.2.1.4), cellobiohydrolases (CBH, EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21). Cellobiohydrolases releasing cellobiose units sequentially from the ends of cellulose chains are the key enzymes in total hydrolysis of crystalline cellulose. They constitute up to 80 % of the total cellulolytic activity secreted by the filamentous fungus *Trichoderma reesei*, which has one the most well-known and industrially applied cellulolytic system. Endoglucanases are needed to cut the cellulose chains in the middle, thus creating free chain ends for cellobiohydrolases. The complementary activities of different cellulases are responsible for the synergistic action (e.g. endo-exo synergy). Recently an oxidative activity related to the degradation of cellulose polymer has been discovered (GH61, (Harris et al., 2010). This GH61 enzyme works on an oxidative matter makeing nicks to crystalline cellulose, thus providing free ends for the CBH enzymes to proceed.

Although the structure of hemicellulose is plant specific and may include complex xylans, (galacto/gluco)mannans, xyloglucans, galactans and arabinans, xylans are the most important hemicellulose in many feed-stocks for the second generation bioethanol. Xylans interlinked in the cellulose matrix will prevent cellulases from completely degrading the cellulosic material and as mentioned earlier provide significant source of carbohydrates in lignocellulosic plant biomass. Hemicellulases required for complex xylan hydrolysis include endo-xylanases (EC 3.2.1.8), **α**-arabinofuranosidases (EC 3.2.1.55) acetyl- (EC 3.1.1.72) or feruloyl esterases (3.1.1.73), α-glucuronidases (EC 3.2.1.131) and β-xylosidases (EC 3.2.1.37). Within their classification, the enzymes characterized in these groups have strict substrate specificities regarding degree of polymerisation and substitution of the hemicellulosic substrate. Depending of pre-treatment

conditions, xylans will be more or less modified in the process, which alters the demands for their enzymatic hydrolysis. Under harsh pre-treatment conditions, xylan may removed from the cellulose rather efficiently (Kabel et al. 2007). However, such a treatment will also result in a high levels of degradation and Maillard reaction products (furfural like compounds, acetic acid, etc) which inhibits the yeast during fermentation.

The rate limiting factors in enzymatic hydrolysis of crystalline cellulose have traditionally been divided into two categories: those related to the structure of the substrate and those related to enzymes and their interactions. The factors related to the substrates are the crystallinity of cellulose (Walseth et al. 1952), accessible surface area, particle size (Kim et al. 2002) and lignin distribution (Mooney et al. 1998). The rate limiting factors related to enzymes include end-product inhibition, inactivation of the enzymes during hydrolysis (Eklund et al. 1990) and irreversible or non-specific binding of cellulases (Ooshima et al. 1990). Adsorption of the enzyme on the substrate is essential mechanism in cellulose hydrolysis. Lignin has also been implicated as a competitive cellulose adsorbent which reduces the amount of enzyme available to catalyze cellulose and also to some extent hemicellulose hydrolysis (Ooshima et al. 1990, Bernandetz et al. 1993, Berlin et al. 2006). In addition, it has been suggested that residual lignin blocks the progress of cellulases down the cellulose chain (Mansfield et al. 1999, Erikson et al. 2002).

In the simultaneous saccharification and fermentation process, the hydrolytic enzymes and yeast are acting simultaneously in conversion of the plant biomass. In ethanol production SSF has been considered as the preferred choice in many studies because of high ethanol yields, short residence time and sustainable chemical production. This project focused on development of more efficient cellulases and hemicellulases specially selected and designed for SSF conditions. The focus was on enzymes having increased catalytic activity on various types of lignocellulosic biomass. In addition enzymes with lower sensitivity to lignin were searched. Such enzymes would increase the effective amount of cellulases for cellulose hydrolysis. The approach in this project was to discover the desired activities by combining classical and modern screening technologies, detailed knowledge on the raw materials and profound understanding on the enzymatic reaction mechanisms.

4.1.3 Description of the main S&T results

The DISCO project had 11 project partners representing different fields in science and industry (**Table 2**). The partners, both academic and industrial, had expertise and complementary knowledge on lignocellulosic biomass this included supply of the feedstock, excellent facilities and modern techniques to analyze lignocellulosic raw matrial (IFR, WU, VTT). Academic groups supported by an industrial enzyme producer had expertise on lignocellulosic enzymes from various aspects i.e. screening, molecular biology, protein production, enzymology, bioinformatics (BUTE, INBI, UH, VTT, Dyadic, WU) as well as expertise on lignocellulose hydrolysis techniques (VTT, UH, WU, Biogold, E-Tech). The industrial partners of the DISCO project had expertise in applying the technologies to a large scale and further commercialization. The whole value chain was covered in the project: from raw material (GRW, Cehave, Biogold) to industrial enzyme development and production (DNL) and large scale saccharification and fermentation (Biogold, E-Tech). The project approach is summarized in **Figure 1**. The efforts in each of the research field in the DISCO project are summarized below.

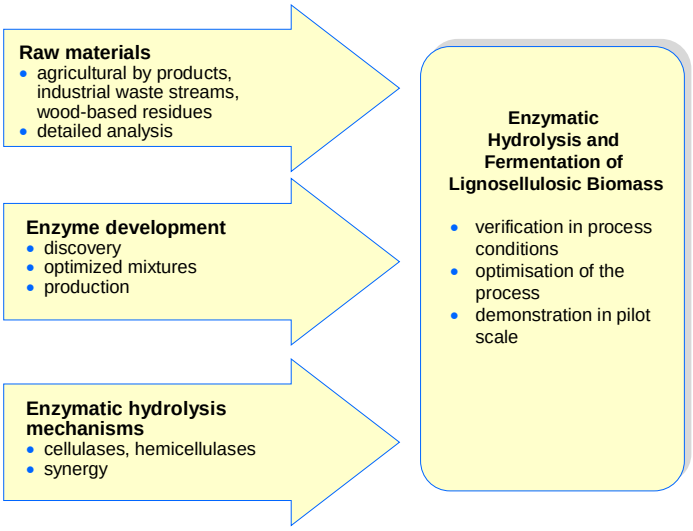

Figure 1. Project approach.

Table 2. List of DISCO participants.

Participant no.	Type*	Participant organisation name	Country
1 VTT	RES	Technical Research Centre of Finland VTT	**Finland**
2 WU	UNI	Wageningen University	**Netherlands**
3 IFR	RES	Institute of Food Research	**UK**
4 UH	UNI	University of Helsinki	**Finland**
5 INBI	RES	Institute of Biochemistry	**Russia**
6 BUTE	UNI	Budapest University of Technology and Economics	**Hungary**
7 DNL	IND	Dyadic Nederland BV	**Netherlands**
8 Biogold	IND	Oy Biogold	**Estonia**
9 GRW	IND	GR Wright & Sons Ltd.	**UK**
10 E-Tech	IND	SEKAB E-Technology	**Sweden**
11 Cehave	IND	Agrifirm (previously Cehave Landbouwbelang)	**The Netherlands**

*RES, Research Insitute; UNI, University; IND, Industry.

Lignocellulosic feedstocks investigated in the project

Wheat straw, wheat bran, corn-cobs and spruce were selected as lignocellulosic raw materials to be investigated in DISCO project (**Figure 2**). The current global wheat production is 644 million tons, making it the second most-produced cereal after corn (824 million tons) and before rice (456 million tons). An overall output of 134 million tons is expected from EU wheat producers and this is likely to increase (Farmers Guardian, 2010). In most EU member countries, wheat straw is an abundant by-product from wheat production and the sheer volume of unused straw is too large to ignore. The husk of the grain, separated when milling white flour, is wheat bran and industrial wheat bran usually accounts for 14-19 % of the grain and comprises of the outer aleurone and the starchy endosperm. Wheat bran is potential to serve as low cost feedstock to increase the production of fuel ethanol. Utilisation of the bran fraction i.e., both the starch and hemicellulose/cellulose part would increase the ethanol yield considerably. Corn (*Zea mays*) is the largest crop produced globally (824 million tons). On a dry matter basis, corn generates in the field a similar amount of grain to the amount of stems and stripped cobs (Somerville, 2010). One of the advantages of corn cobs as a biofuel feedstock is that they are by-products of corn grain production. Corn cobs are used on a limited basis for industrial purposes in the United States for bedding, oil sorbents, and polishing agents. Recently corn cobs are also seen as a potential biofuel feedstock for direct combustion, gasification, and cellulosic ethanol. They have many advantages over other competing feedstocks. Corn cobs are one of the most abundant agro residues. They have a high hemicellulose content (40%), and they have been utilized for xylose, xylitol and xylo-oligosaccharide production (Lia et al. 2010). After removal of the hemicellulose fraction the cellulose content increases to 56-60%. Corn cobs are dense and relatively uniform. They have a high heat value, low nitrogen and sulphur contents, and can be collected during corn grain harvest. Harvesting cobs has little impact on soil residue, soil carbon, or the nutrient requirements of subsequent crops. Therefore corn cobs are a sustainable, however relatively low-yielding feedstock that could be used in cellulosic ethanol production. The need for a significant local resource and the development of special harvesting equipment are the limiting factors for exploitation of corn cobs (http://www.extension.org/main/about). Corn cobs are abundant raw material available globally except in Africa and Australia. The annual supply of corn cobs for fuel purposes is 78 million metric tons, which equals 6 000 million gallons of cellulosic ethanol. As comparison annual availability of wheat straw is 413 milloin metric tons (29400 million gal cellulosic ethanol) (http://www.bioenergy.novozymes.com/biofuels). Norway spruce (*Picea abies*) is the most common and economically important conifer species in Europe utilized both by the pulp and paper and the construction industry. The wood of Norway spruce has long fibers, which makes it especially suitable for paper making. In economically feasible bioethanol production, the usage of spruce is restricted e.g. by its recalcitrance to enzymatic digestion and relatively slow growth rate compared to e.g. many annual plants. Furthermore the good fibre properties of spruce make it suitable for more valuable applications and products.

Figure 2 Lignocellulosic plant biomasses studied in the project

Pretreatment technologies

The compactness and complexity of lignocellulosic plant biomass makes it as such relatively resistant to enzymatic hydrolysis. Therefore pretreatment methods have been developed for lignocellulosics in order to enhance their susceptibility to enzymatic hydrolysis (**Figure 3**). Pretreatment technologies for the selected lignocellulosic feedstocks for enhanced digestibility were developed by industrial project partners Biogold OU (Estonia), Agrifirm (Netherlands) and SEKAB E-Technology (Sweden). The pretreatment technique developed by Biogold OU for wheat straw and wheat bran was a hydrothermal method, where hot pressurized water without additional chemical catalysts was used for partial chemical hydrolysis of the cell wall polymers and breaking the biomass structure. Similarly, the Agrifirm's pretreatment method tested for wheat straw was based on elevated temperature but utilized dilute organic acids for enhanced chemical hydrolysis. The pretreatment method used at SEKAB E-Technology included high pressure steam explosion aided by acid catalyst. The SEKAB's pretreatment technology can be used for processing various lignocellulosic biomasses from spruce wood to corn cobs.

Figure 3 Breakdown of wood structure for cellulosic ethanol production.

Chemical and structural variation in plant biomasses and pretreated lignocelluloses

The structure and chemical composition of plant biomasses vary depending of the plant species and which part of the plant is collected. Furthermore, the pretreatments modify both structure and chemistry of the plant biomasses. Both natural and pretreated biomasses were analysed in the project in detail. Complex nature of studied biomasses required combination of different analytical methods and optimization and development of the analytics. Institute of Food Research (IFR) applied combination of traditional carbohydrate, lignin and phenolic analysis to sequential extraction, NMR, FTIR spectroscopy, size exclusion chromatography and various microscopy techniques in the analysis of the structural chemistry of lignocelluloses (Merali et al. submitted) and effects of the pretreatment on the lignocellulose features (Merali et al. submitted). Cellulose comprised ca 20-50% of the dry matter of the biomasses studied in the project. The hemicellulose content in the studied feedstocks varied from 25% (DM) to almost 60 % (DM) quantified from wheat bran. The main hemicelluloses in studied lignocellulosic biomasses were xylans and mannans. Typical effects of pretreatment techniques used in the project were partial solubilisation of hemicelluloses thus causing enrichment of cellulose in the insoluble residue. Lignin typically is also enriched in the insoluble residue, but may undergo structural modifications and relocalizations.

Structure of hemicelluloses is complex and the amount and structure of hemicelluloses greatly vary between biomasses (**Figure 4**). Analysis of hemicellulose structure require combination of different methods and analytical tools which were developed by project partners University of Wageningen (WU) and University of Helsinki (UH). These methods combined different analytical methods such as HPAEC-PAD and MALDI-TOF MS to specific enzymatic hydrolysis. For example methyl glucuronic acids cannot be quantified using traditional carbohydrate analysis via acid hydrolysis. Instead acid methanolysis was needed for the quantification of individual uronic acids. UH has established a method to quantify methyl glucuronic acids from lignocellulosic materials utilising a standard curve of glucuronic acid and a correction factor for methyl glucuronic acid content, based on the response ratio of methyl glucuronic acid versus glucuronic in gas chromatography after acid methanolysis (Chong et al. 2012 In press). Reflective to the hemicellulose complexity was also the novel disaccharide side-chain structure discovered by UH (Pastel et al. 2009,

Figure 5). This type of hemicellulose structure was found in greater amounts in corn cob arabinoxylan and in lesser quantities in wheat straw arabinoxylan (Pastel et al. 2009).

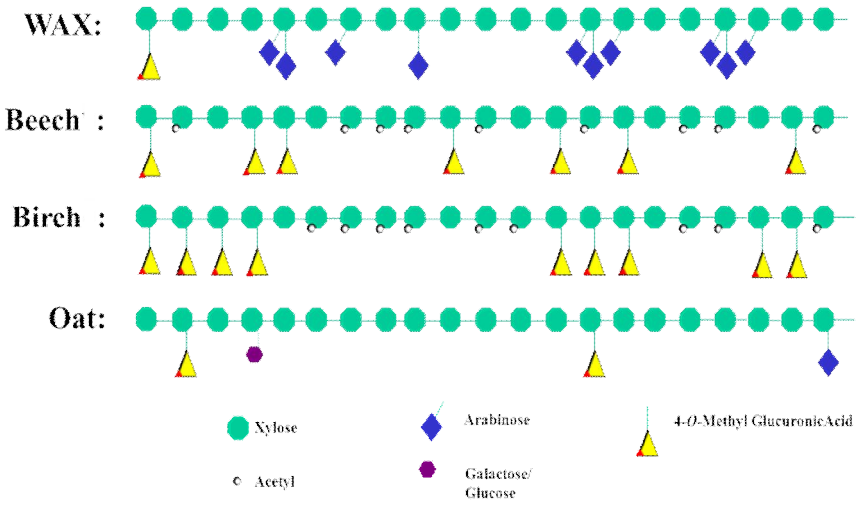

Figure 4 Schematic overview of the xylans in different plant biomasses. WAX; wheat arabinoxylan.

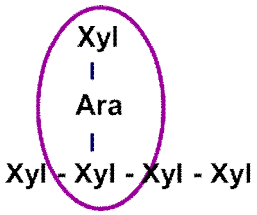

Figure 5 Novel hemicellulose structure found in in corn cob arabinoxylan and barley husk arabinoxylan, and in lesser quantities in oat spelt arabinoxylan and wheat straw arabinoxylan (Pastel et al. 2009). Xyl= xylose, Ara= arabinose.

The third major component in the lignocellulosic biomasses is lignin. Both amount and structure of lignin varied between different biomasses. In spruce wood, lignin is mainly composed of coniferyl alcohol, while grasses lignin is derived from polymerisation of coniferyl, sinapyl and *p*-coumaryl alcohols with significant contribution of cinnamic acids such as ferulic and *p*-coumaric acids. In wood lignin content is typically high (ca 30%) whereas in only ca 5% lignin was quantified from wheat bran. Isolated lignins are typically used in lignin chemical and structural analysis and studies related enzyme-lignin interactions. Lignin isolation methods were optimised for steam pretreated spruce by VTT (Rahikainen et al. 2011). The isolation procedure consisted of extensive enzymatic hydrolysis followed by protease treatment for removal of potentially lignin bound cellulases and hemicellulases. The resulting lignin rich hydrolysis residues contained less than 10 % (DM) carbohydrates and minor protein contamination (**Table 3**). Comparison of microscopic structure of steam pretreated spruce and residual lignins revealed the partial degradation of the cell wall structure in isolation process leaving mainly lignin-rich ‘sceletons’ left (**Figure 6**). The lignin rich hydrolysis residues were preferred in the project in elucidation of enzyme-lignin interactions due to their similarity to residual lignins present after completion of the hydrolysis and fermentation.

Table 3 Carbohydrate content, nitrogen content and BET surface area of steam pretreated spruce (SPS), enzymatic hydrolysis residue (EnzHR), acid hydrolysis residues (AcidHR) and microcrystalline cellulose (Avicel). nd = not determined (Rahikainen et al. 2011).

Sample	Glucose w-%	Xylose w-%	Mannose w-%	Total polysaccharides w-%	Nitrogen w-%	BET Surface area m^2/g
SPS	57.2	0.28	0.1	52	<0.05	12
EnzHR	8.7	<0.1	0.3	9	0.3[1]	2.5
AcidHR	0.2	<0.1	<0.1	<1	<0.05	80
Avicel[2]	97.6	1.3	1.3	90	nd	1.5

[1]The nitrogen content of the enzymatic hydrolysis residue before protease treatment was 1.2 w-%.

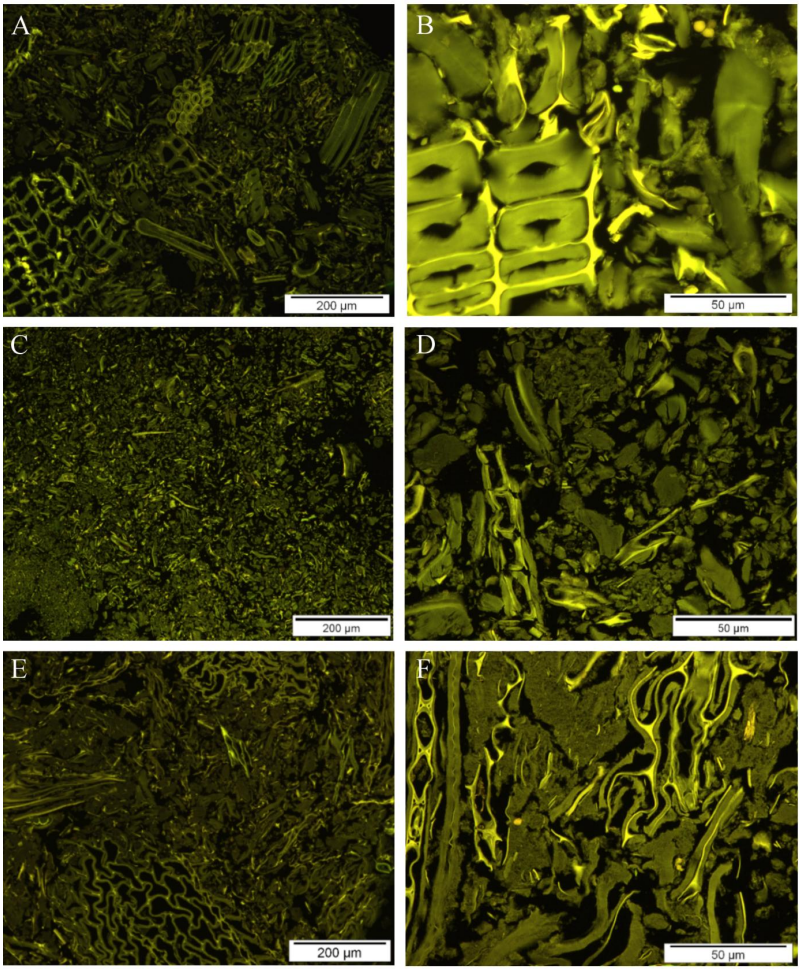

Figure 6 Fluorescent microscopic pictures of Steam pretreated spruce (SPS) A-B; Enzymatic hydrolysis residue (EnzHR) C-D; Acid hydrolysis residue (AcidHR) E-F. Lignin is visualized due to its autofluorescence (Rahikainen et al. 2011).

Enzymatic digestibility of pretreated lignocelluloses and analysis recalcitrant structures

The solids of the lignocelluloses remaining after pretreatment were enzymatically hydrolysed (Celluclast 1.5L and Novozym 188 (Novozymes, Denmark) and the hydrolysis residues were chemically and microscopically analysed. For example, sugar yields from the enzymatic hydrolysis of solid fractions of wheat straw pretreated at 190°C and 200°C are shown in Figure 7. The residual carbohydrates present in the

recalcitrant residues from wheat straw consisted mainly of arabinoylans and glucose. The scanning electron microscopy (SEM) (Figure 8) and atomic force microscopy (AFM) microscopy images of the untreated wheat straw revealed a smooth surface with ordered fibrils present within the vasculature. After the sequential extraction the structural organization appeared to be weakening. In the pre-treated sample the microfibrils were still visible however appeared to dissociating from the main connective tissue and following sequential fractionation these had dissociated and individualised. In the hydrolysis residues of wheat straw pre-treated at 190^{o}C the microfibrils were fragmented and after sequential extraction they appeared to be agglomerated. In the hydrolysis residues of the wheat straw pretreated at 200^{o}C there appeared to be much less material present and this consisted mostly of undigested vascular tissues. After the sequential extraction the residue exhibited further breaking down of material and appeared to contain mostly fragments of lignified material and microtubules with very little structure. These changes in the structural organisation of the supramolecules and the reduction in particle size were probably caused by the removal of cellulose, hemicelluloses, phenolics and lignin the assumption was supported by the biochemical analyses of the residues.

Pretreatment and enzyme digestion of lignocellulosic materials leaves a recalcitrant material which, although undigested, contains many degraded polymers and oligomers of hemicellulosic arabinoxylans and cellulose. Hence, recalcitrant material doesn't contain undegraded material, it contains degraded but insoluble material. These insoluble, but degradable polymers may be released in alkali after which they remain soluble, and are shown by HPSEC to be lower molecular weight than in the fresh control material. The insolubility is probably due to other cross-linking agents particularly residual lignin and possibly phenolic cross links. This effect is masked by the presence of small quantities of starch (bran) and glucan (corn) in the other substrates. Glucan being released which is held by hemicellulose however once solubilised remains solubilised. Combination of Klason lignin and phenolics retain glucose in the matrix. Due to the heterogeneous nature of the lignin matrix, techniques such as FT-IR do not give consistent results and as such cannot be used for identifying lignin.

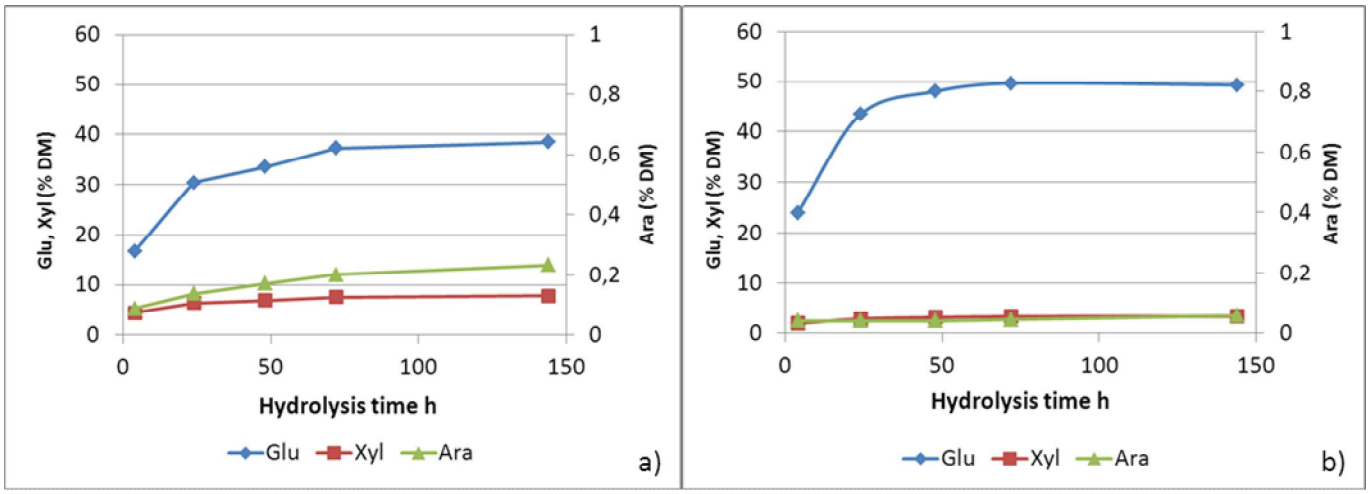

Figure 7. Yields (% dry matter, DM) of main sugars in enzymatic hydrolysis of solid fraction of wheat straw pretreated at a) 190°C and b) 200°C. Glu, glucose; Xyl, xylose; Ara, arabinose. Enzyme mixture used in hydrolysis: Celluclast 1.5L and Novozym 188.

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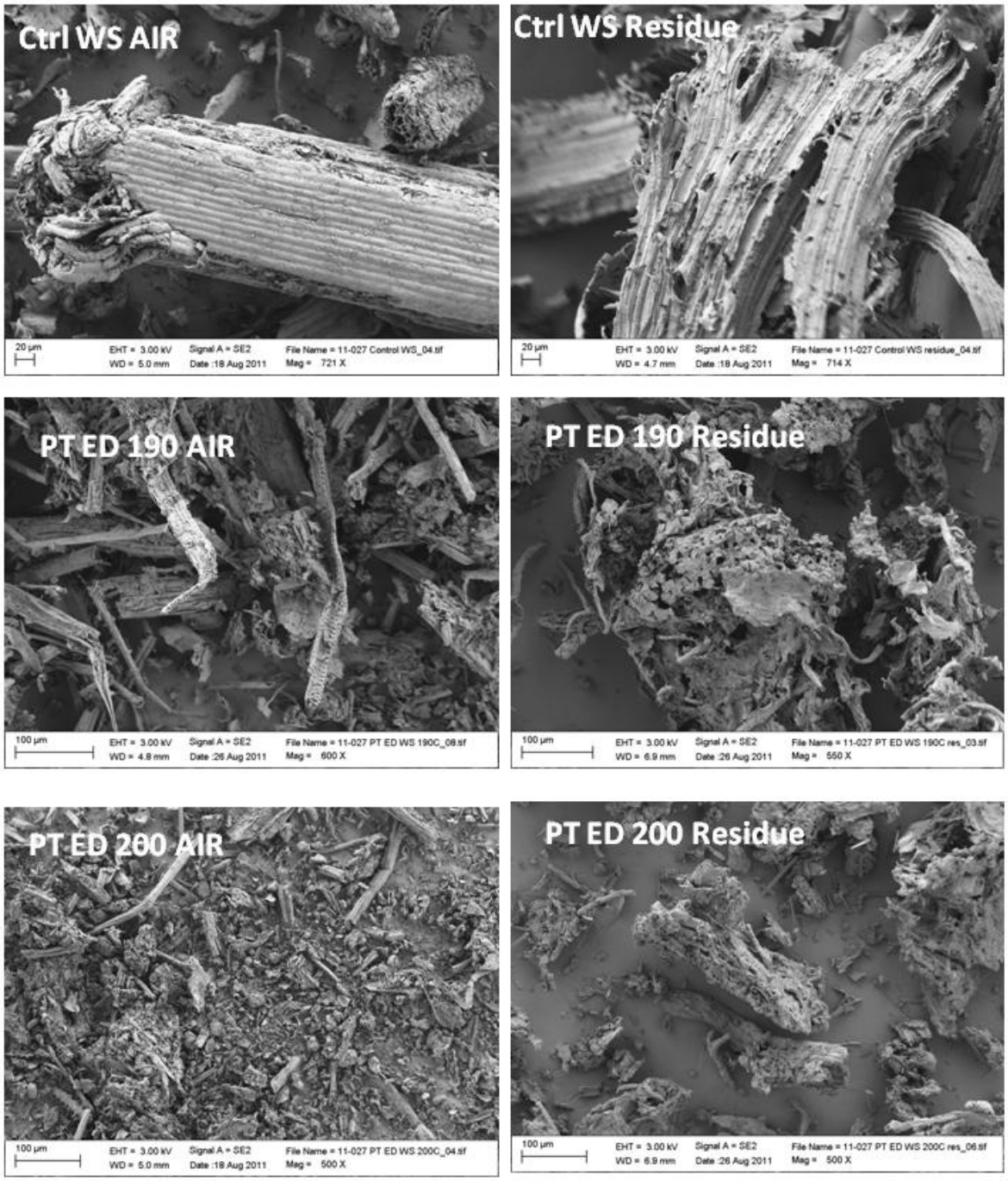

Figure 8. SEM photomicrographs of natural wheat straw (control), and enzymatic hydrolysis residues of wheat straw pretreated at 190°C (PT ED 190) and 200°C (PT ED 200) before fractionation (AIRs, left) and the recalcitrant residues after sequential extraction in alkali (Residues, left) (IFR).

Enzyme screening

Various (lingo)cellulolytic microbes are responsible for decomposing of plant biomass in nature. These microbes provide a vast source of novel cellulases and hemicellulases for more efficient conversion of the plant biomass intofermentable sugars. In the DISCO project, this source was approached using three different main strategies: functional screening for fungal cultures, functional screening for metagenomic libraries and *in silico* screening for fungal genomes.

The main provider of fungal cultures in DISCO project was Budapest University of Technology and Economics (BUTE), which holds a large culture and soil sample collection. About 700 fungal strains, of which 70% were new soil isolates, were initially screened for (hemi)cellulolytic activity using a two-phase screening strategy. In the first stage, the fungi or soil isolates were cultivated on agar plates containing cellulose or lignocellulose as a sole carbon source to select isolates presumably rich on (hemi)cellulase production (**Figure9**). In the second stage, the selected fungi were cultivated in semi-liquid media again containing cellulose or lignocellulose as the carbon source. The enzyme mixture produced by the fungi were analysed for various hemicellulase and cellulase activities. Using this two phase screening, tens of promising cellulolytic and/or hemicellulolytic fungal strains were discovered.

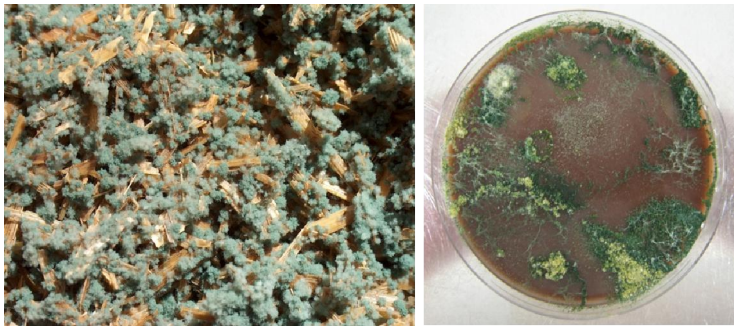

Figure 9 Lignocellulolytic fungal strains.

The enzyme mixtures produced by fungal cultures are typically complex containing many synergistically acting activities. This makes evaluation of different enzyme components in the mixture challenging. The cellulolytic and hemicellulolytic fungal strains discovered at BUTE were further evaluated using two types of approaches: sophisticated analysis of hydrolysis products from specific defined hemicellulose substrates with crude enzyme mixtures and fractionation and testing of partially purified enzymes. The analysis method that the University of Wageningen developed for screening for hemicellulases in the crude fungal enzyme mixture was based on the selection of structurally defined hemicellulose substrates e.g. wheat arabinoxylan and Eucalyptus xylan. When these hemicellulose substrates were hydrolysed with the fungal crude enzymes and the hydrolysis products were analysed using HPAEC-PAD (**Figure 10**) and MALDI-TOF MS (**Figure 11**) methods, a finger printing of the hemicellulolytic activities present in the crude enzyme was obtained (Van Gool et al. 2011). In addition to the soluble hemicelluloses, the similar type of screening approach was carried out for insoluble xylan rich substrates such as wheat straw (VanGool et al. 2012). Altogether ca 250 fungal crude enzymes were analysed with this method to identify fungal strains producing promising hemicellulases and to understand the effects of carbon source on the produced enzyme pattern. Based on the screening, nineteen promising hemicellulase producing strains were selected for further analysis and gene cloning/genome sequencing.

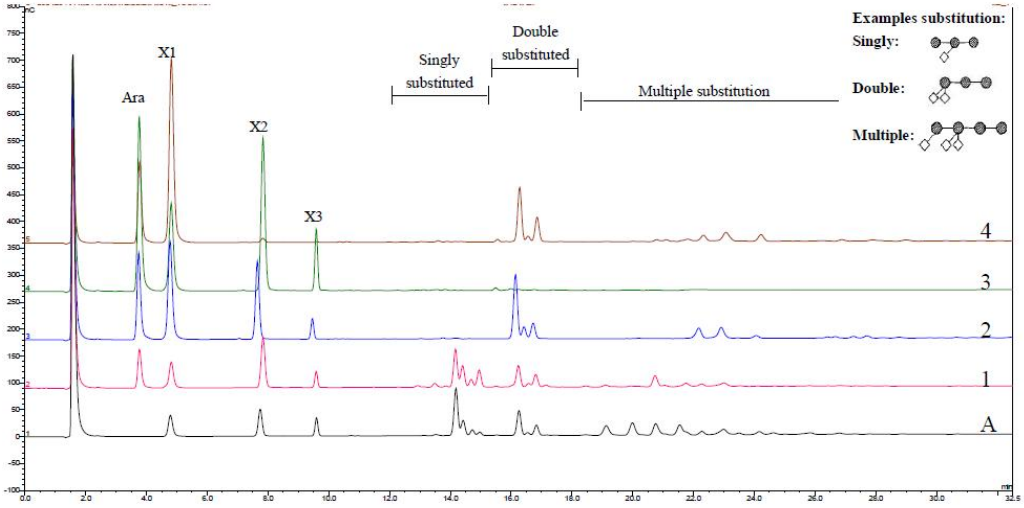

Figure10 Examples of typical hydrolysis product pattern (analysed with HPAEC-PAD) derived from incubation of wheat arabinoxylan (WAX) with the hemicellulolytic fungal cultures for 24h at 35ºC and pH 5. A=endoxylanase pattern; 1=endoxylanase look-a-like degradation pattern, 2=much arabinose and double substituted xylo-oligomers present -> arabonoxylan hydrolases acting on monosubstituted xylan present; 3=Monomers and linear xylans -> endo-xylanases (possibly very tolerant towards arabinose substitution) and arabino-xylan hydrolase present; 4=Much xylose present-> effectice β-xylosidase present. (Van Gool et al. 2011)

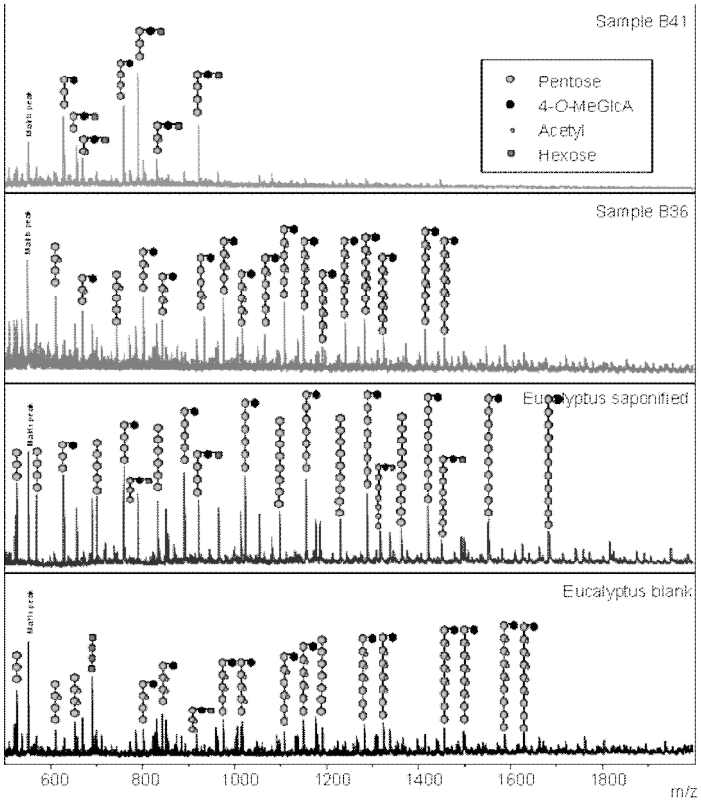

Figure 11 Hypothetical structures in Maldi-TOF mass spectra of Eucalyptus hemicellulose samples after different treatments: Blank unmodified eucalyptus xylan hydrolysate, saponified eucalyptus xylan hydrolysate, enzyme sample with strong in hexose release (B36), enzyme sample strong in acetyl and xylose release (B41). Positions of substituents are only indicative. (Van Gool et al. 2011)

Similar approach was performed by the University of Helsinki, which was screening for novel hemicellulase (β-xylosidase) activity acting on the specific 1→3-liked 2-*O*-β-D-xylopyranosyl-α-L-arabinofuranosyl side chains (**Figure 13**). This type of structure is not hydrolysed by any of the generic β-xylosidases or α-arabinosidases but the related hydrolysing activity was identified in a number of hemicellulolytic fungal strains discovered in the screening at BUTE.

The fractionation type screening of the fungal cultures was carried out at VTT Technical University of Finland and Institute of Biotechnology (INBI, Russia). At VTT, cellobiohydrolase type- cellulases were screened from the interesting fungal cultures using cellobiohydrolase (CBH) affinity columns described in van Tilbeurgh et al. (1984) (**Figure 12**). The CBH pools obtained in the affinity purification were tested in the hydrolysis of cellulose and lignocellulose and for adsorption to lignin. At INBI, novel efficient β-xylosidases were screened from fungal cultures using a combination of peptide mass fingerprinting, PCR-cloning, FPLC-fingerprinting and related enzyme assays.

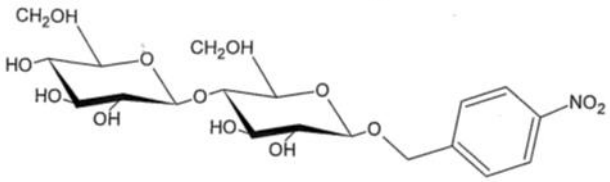

Figure 12-nitrobentzyl 1-thio-β-cellobioside-ligand in the cellobiohydrolases affinity columns. Coupling of the ligand occurs via the primary amine in the lignad with the cyanate ester/cyclic imidocarbonate in the CNBr activated Sepharose matrix.

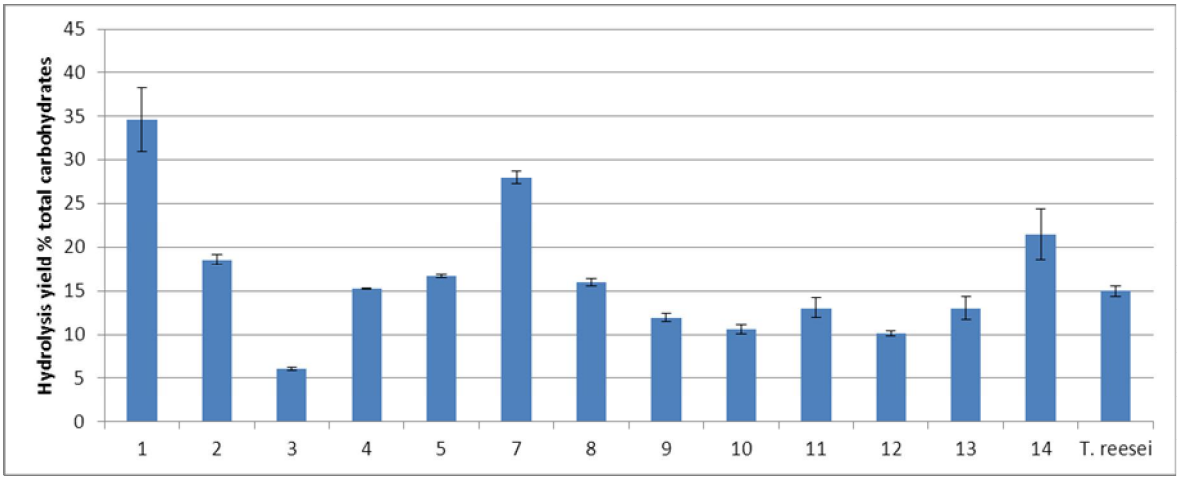

Figure 13. Testing of fungal CBH affinity pools in hydrolysis of crystalline cellulose (1% Avicel) in defined mixtures with fungal endoglucanases and β-glucosidase. Each number presents CBH pool from different strain. T. reesei= CBH affinity pool from *Trichoderma reesei*, reference.

The advantage of the screening approach utilizing metagenomic libraries is that enzymes produced by microbes which cannot be cultivated in normal laboratory conditions can be obtained. It has been approximated that only 1 % of the total species in one habitat can be culticated. Metagenome is the total DNA of all micro-organisms living in a defined habitat for example forest soil, compost etc.

In construction of metagenomic libraries, this total DNA is isolated and transferred in pieces to artificial host microbial organism. The result is a collection of microbial cells which contain pieces of foreign metagenomic DNA, a metagenomic library. These libraries can be used for activity based screening for desired enzyme activities or particular DNA sequences. At VTT, five metagenomic libraries were screened for cellulases and hemicellulases in collaboration with nationally funded research project Metageno and EU funded projects EU-DISCO and NEMO (**Figures 14 and 15).** The screening was done using a two phase method: 1) agar-plate based screening of cellulase and xylanase positive clones and 2) qualitative analysis of cellulase and xylanase activities in the host cell lysates. Altogether 80 cellulase or xylanase positive clones were identified and analysed in the projects.

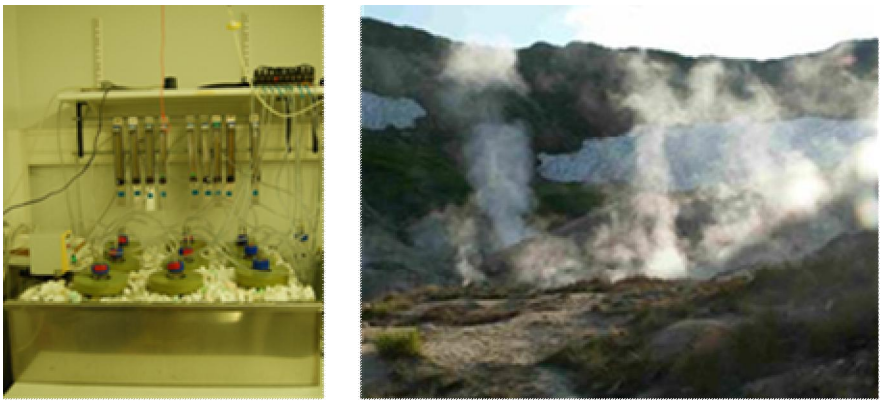

Figure 14 Different habitats for construction of metagenomic libraries a) controlled composts b) hot springs.

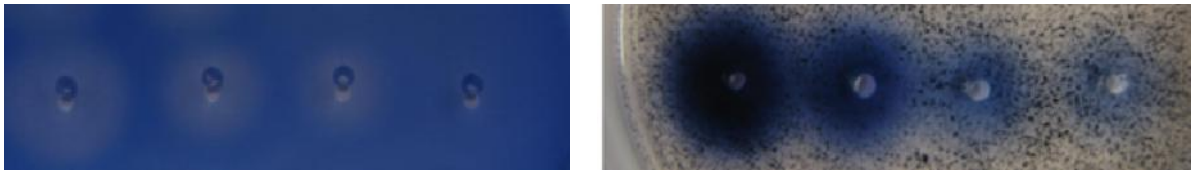

Figure 15 Detection of cellulase and xylanase activities in the functional screening of metagnomic libraries using Azo-dyed cellulose (left) and Azurine cross-linked xylan (right).

In silico screening approach of fungal genomes was also utilized in the DISCO project. This approach is based on gene sequence similarity of enzymes with similar functions and reaches e.g. enzymes which wild type fungi do not produce detectable quantities in laboratory condition. Genome mining at from the *Myceliophthora thermophila* C1 genome and some other fungal genomes by Dyadic resulted in 21 novel genes representing cellulases and hemicellulases. The genes were transferred to Dyadic's C1 white mutant production host and produced for characterization and testing.

Gene cloning, heterologous production and genome sequencing

Production of the novel enzymes in suitable host organism is a must for enzyme characterization when metagenome screening or genome mining are utilized. From fungal cultures enzymes can often be purified to some amounts, but for scaling up the cloning of the gene and overproduction of the enzyme in a suitable host is usually necessary. **Table 4** summarizes gene cloning and enzyme production approaches done in the DISCO project. Most of the cloned produced enzymes come from the genome mining approach and metagenomic screening, but also number of enzyme genes were cloned and produced from the fungal strains discovered in the activity based screening of fungal

cultures. Dyadic's gene expression system in C1 white mutant fungal strain was very efficient in production of novel enzymes with over 20 enzymes overproduced during the project using this system.

Table 4 Summary of gene cloning and enzyme production

Source	Number of genes	Enzyme type	Production host	Responsible partner
Fungal cultures	4	cellobiohydrolases, β-xylosidase and accessory enzymes	*Trichoderma reesei*, C1	Dyadic, VTT
Metagenomes	10	endoglucanases and xylanases	*Escherichia coli*	VTT
Genome mining	21	endoglucanases, xylanases, β-xylosidases, mannanases and accessory enzymes	C1	Dyadic

In addition to cloning of individual genes, the genomes of two fungal strains discovered in the project were sequenced at Institute of Biotechnology at University of Helsinki using the 454 Titanium sequencer. These novel fungal genome sequences are used in future genome mining approaches in search for novel enzymes.

Discovered enzymes and reaction mechanisms

Various different (hemi)cellulolytic activities were discovered within the DISCO project. These acticities include enzyme types needed for the hydrolysis of the plant lignocellulosic biomasses studied in the project (**Table 5**). Fifteen of the enzymes act on cellulose while 24 of them hydrolyse either backbone or substituents of xylan or mannan type hemicelluloses.

Table 5 Summary of DISCO enzymes acting on the DISCO raw materials.

Enzyme	Screen	Activity	Number	Potential raw material substrate
Cellobiohydrolases	Screening of fungal cultures	Hydrolysis of cellulose	3	**Wheat straw, wheat bran, corn cob, spruce**
β-xylosidase	Screening of fungal cultures, genome mining	Hydrolysis of xylan backbone	4	**Wheat straw, wheat bran, corn cob**
α-glucuronidase	Screening of fungal cultures	Hydrolysis of xylan substituents	3	**Wheat straw, wheat bran, corn cob**
β-D-Xylp-(1->2)-α-L-Araf-(1->3) hydrolysing xylosidase	Screening of fungal cultures	Hydrolysis of β-D-Xylp-(1->2)-α-L-Araf-(1->3)	2	**Wheat straw, wheat bran, corn cob**
Xylanase	Metagenomiic libraries, genome mining	Hydrolysis of xylan backbone	10	**Wheat straw, wheat bran, corn cob**
Acetyl esterase	genome mining	Hydrolysis of xylan and mannan substituents	1	**Wheat straw, wheat bran, corn cob**
α-galactosidase	genome mining	Hydrolysis of xylan and mannan substituents	2	**Wheat straw, wheat bran, corn cob, spruce**
Mannanase	Genome mining	Hydrolysis of mannan backbone	2	**Spruce**
Endoglucanases	metagenomes, genome mining	Hydrolysis of cellulose	9	**Wheat straw, wheat bran, corn cob, spruce**
β-glucosidase/β-xylosidase	**genome mining**	**Hydrolysis of cellulose (cellobiose) and xylan**	**3**	**Wheat straw, wheat bran, corn cob, spruce**

Biochemical properties such as pH and temperature optimum and stability and substrate specificity and inhibition were characterized for the discovered novel enzymes. In addition, mechanistic studies and a mode of action of the enzymes in the hydrolysis of cell walls (e.g. synergy between cellulases and xylanases) and complex xylans (both isolated substrates and soluble fraction from pretreated lignocelluloses, **Figure 16**) were studied by WU and UH. Furthermore, mechanism in enzyme-lignin interaction were elucidated for a number of enzyme mixtures and isolated enzymes by VTT and INBI. Highlights from the published results are described in the following chapters.

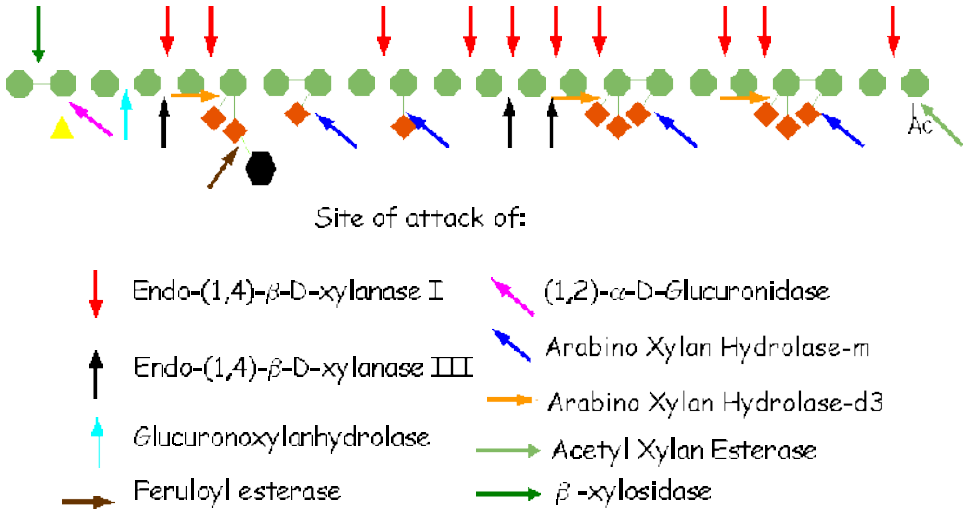

Figure16 Sites of attack of enzymes active on complex xylans

Properties of two glycosidic hydrolase family 10 xylanases Xyl 1 and Xyl3 discovered in genome mining of *Myceliophthora thermophila* C1 are described in detail in the recent publication by VanGool et al. 2012 (WU,DNL, INBI). Both enzymes contain carbohydrate binding modules. The enzyme variants lacking the CBM were also obtained via partial proteolysis and characterized. The performance of the studied xylanases on different types of xylanas or xylan rich substrates are shown in **Table6** The studied xylanases act differently towards high and low substituted xylan, also the distribution pattern of the substituents e.g. glucuronic acids was shown to be important. Alkali-labile interactions of xylan with other cell wall polymers, as well as a presence of cellulose hindered activity of xylanases. Contrary to earlier observations, the presence of a CBM did not increase the xylanases activity towards the studied substrates.

Table 6 Performance of the GH10 xylanases for various substrate characteristics (Van Gool et al. 2012)

	Lower degree of substitution	Randomisation of glucuronic acid substituents	Alkaline treatment of wheat straw	Removal of cellulose	Prevention of xylan self-association
Xyl1dCBM	0	+	++	++	++
Xyl1	-	+	++	-	++
Xyl3dCBM	++	+	++	-	++
Xyl3	+	+	++	++	+

-: Lower efficiency
0: No effect
+: Higher efficiency
++: Very high efficiency

GH family 3 glycosidic hydrolase Bxl5 discovered in the genome mining of *Myceliophthora thermophila* C1 was characterized (In Dotsenko et al (2012) partners INBI and DNL). The Bxl5 had high thermal stability and broad substrate specificity. The exo-acting enzyme is efficient in β-glucan hydrolysis providing almost 100 % conversion of laminarin and barley β-glucan to glucose. In addition, Bxl5 was effective in the xylan hydrolysis in combination with endoxylanases (**Figure 17**).

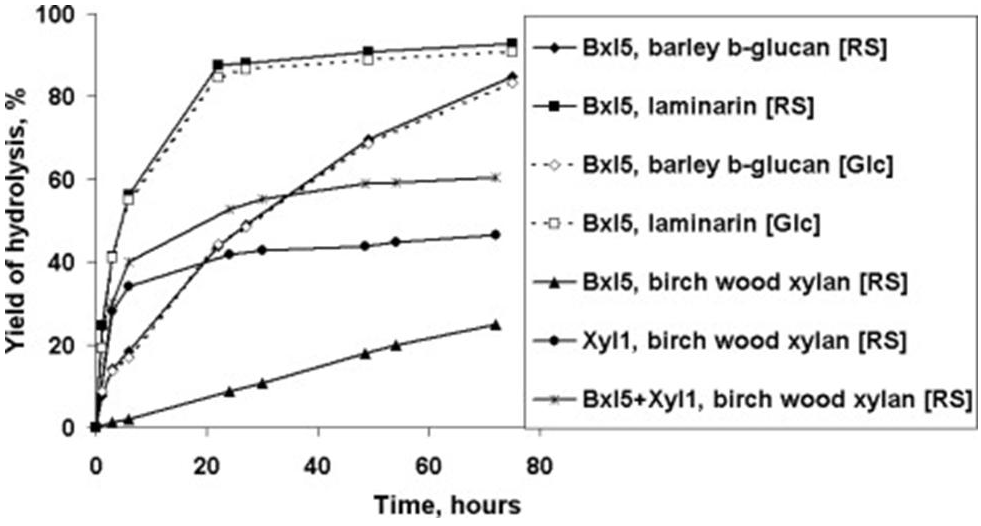

Figure 17 Release of glucose and reducing sugars during 72-h hydrolysis of laminarin and barley β-glucan by Bxl5 from C1 and birch wood xylan by Bxl5 and Xyl1 from C1 (pH5.0, 50°C) (Dotsenko et al. 2012).

Activity of *Myceliophthora thermophila* C1 and *Trichoderma reesei* mannanases were analysed and compared in the experiments combining statistical modelling and hydrolysis experiments with different substrates (Klyosov et al. 2012). The results indicated that the enzymes differ in substrate specificity: *T. reesei* enzyme required minimum four unsubstituted residues for the hydrolysis while

the *Myceliophthora thermophila* mannanase requireed at least five unsubstituted mannose residues for hydrolysis (**Figure 18**).

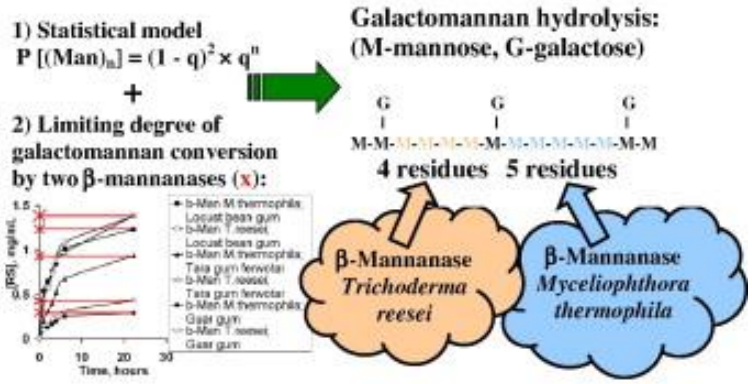

Figure 18 Statistical and experimental modelling of action of *Myceliophora thermophile* and *Trichoderma reesei* mannanases on mannan substrates (Klyosov et al. 2012).

Lignin hinders hydrolysis of cell wall carbohydrates by acting as a barrier and by non-productive binding of the hydrolysing enzymes. In the DISCO project this topic was approached by screening of enzymes with low-adsorption to lignin as well as analysing the mechanisms in lignin-induced inhibition. The mechanisms in lignin-induced inhibition was initially studied with *Trichoderma reesei* cellulase mixture and using lignin rich hydrolysis residues of pretreated spruce obtained via extensive enzymatic hydrolysis combined with a protease treatment or via acid hydrolysis of cell wall carbohydrates (Rahikainen et al. 2011, VTT). It was observed that the lignin-caused enzyme inactivation was due to temperature driven denaturation of the cellulases on the lignin surfaces (**Figure 19**, Rahikainen et al. 2011). In the screening of the low-lignin binding cellulases and xylanases it was seen that adsorption alone does not predict inhibitory effect of lignins on the enzyme activity but that the degree of inhibition is also affected by e.g. the enzyme resistance to surface denaturation in the hydrolysis temperature and sensitivity to lignin derived soluble compounds (unpublished data).

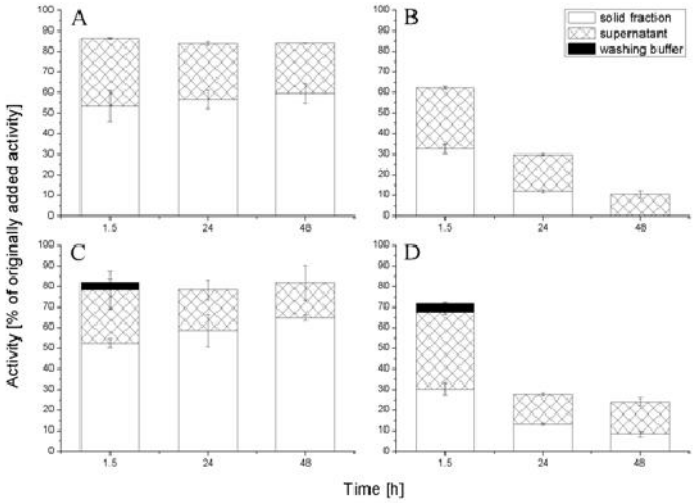

Figure 19 Effect of temperature on the distribution of endoglucanase and exoglucanase (Cel7A) activity during adsorption to enzymatic residual lignin (EnzHR). Endoglucanase activity in A) + 4°C B) + 45°C. Exoglucanase activity (Cel7A) in C) + 4°C D) + 45°C (Rahikainen et al. 2011).

DISCO enzymes in the hydrolysis and fermentation of plant lignocellulosic biomass

Efficient total hydrolysis of plant lignocellulosic biomass requires a complex mixture of different enzymes acting synergistically, therefore the hydrolysis of lignocellulose with a single enzyme component does not reveal much of the enzyme quality. As such, benefits of the novel cellulases and xylanases in the hydrolysis of lignocelluloses was tested by adding the novel components into commercial enzyme mixture Celluclast 1.5L fortified with Novozym 188 (Novozymes, Denmark) and into Dyadic's non-commercial enzyme product, which is based on the *Myceliophthora thermophila* C1 enzymes (INBI, VTT and UH). Lignocellulosic substrates used in testing were wheat straw and corn cobs both of which are abundant agricultural residues and chosen as the project substrates. The wheat straw and corn cobs were pretreated using the process based on pressurized hot water at Biogold and steam explosion with dilute acid catalysis at SEKAB E-Technology, respectively. The effects of the novel enzymes were dependent on both the enzyme mixture and substrate as well as hydrolysis temperature.

The enzymes showing most promising in the hydrolysis experiments were further tested in the hydrolysis combined to ethanol fermentation (SSF) with wheat straw, wheat bran and corn cob substrates at VTT, IFR and Biogold. Again, the effects of the novel enzymes varied depending on the enzyme mixture to which they were added and the used substrate. In general the best effects in terms of improved ethanol production were observed when the novel efficient xylanase was added to enzyme mixture, even though the yeast used in the project was traditional glucose fermenting strains not able to utilize xylose. This reflects the role of hemicelluloses in restricting cellulose conversion.

Two types of process designs were tested in the project. In simple SSF, the temperature was kept constantly relatively low (35°C) and the yeast was added to the substrate simultaneously with the enzymes. In another approach, the enzymes were added first and incubated with the substrate for 24h at elevated temperature, after which the temperature was decreased to 35°C and the yeast was added to the mixture. In the latter type of process design, the initial free glucose concentration at the time of yeast addition was high compared to a simple SSF allowing faster beginning for the fermentation. In addition, this type of 'pre-hydrolysis' can utilize heat generated in the pretreatment step, pre-

hydrolysis also allows high dry matter consistency in the process. The large scale pilot experiments using these two kinds of process designs were carried out with corn cob substrate at SEKAB E-Technology pilot plant at Örnskoldsvik, Sweden (**Figure 20**).

Figure 20 SEKAB E-Technology pilot plant located at Örnskoldsvik, Sweden.

The project aimed at lowering the cost of ethanol by the aid of novel more efficient enzymes. Cost calculation were carried out for the SFF process with corn cobs substrate as described in Humbird et al. 2011 using price estimates 4,24 $/kg for protein and 2,83 $/gallon for ethanol. The cost of ethanol was dependent on which enzyme mixture was used in which process designs. Comparison of the enzyme costs of mixtures containing novel enzymes showed that lowest cost for ethanol was obtained in both process designs with mixtures supplemented with novel xylanase from *Myceliophthora themophila* C1.

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4.1.4 Potential Impact

Sosio-economic impact

This project contributed towards the overall objectives defined in the European Technology Platform for Sustainable Chemistry and the 7th FP Work Programme 2007 by developing sustainable solutions for energy and chemical production, utilization of renewable resources in a sustainable manner, and exploiting agricultural and industrial wastes. Furthermore Theme 2: Food, Agriculture and Fisheries, and Biotechnology building a European Knowledge Based Bio-Economy was well addressed in the project. The DISCO project brought together scientific groups with the industry, especially SMEs, to exploit new and emerging research opportunities that address environmental, social, and economic challenges, more specifically the growing demand for sustainable use and production of renewable bio-resources. Particularly the project focused on the topic "KBBE-2007-3-2-01:"LIGNOCELLULOSIC ENZYMES - Development of cellulases for lignocellulosic biomass pre-treatment" with the special target on developing "efficient enzyme mixtures of cellulases and other necessary enzymes for the hydrolyzation of lignocellulosic biomass into fermentable sugars". The project contributed to the above mentioned goals by providing the following major outcomes:

- development of various analytical tools to biomass analysis as well as analytics related to (hemi)cellulases
- structural and biochemical data on the selected biomasses (wheat straw, wheat bran, corn cobs and spruce)
- optimized pretreatment procedures for the selected biomasses
- discovery of various novel enzymes for the hydrolysis of lignocellulosics
- genome data on the selected (hemi)cellulolytic fungi
- novel mechanistical data on the action of especially hemicelulases and accessory enzymes and their synergy to cellulases
- elucidation of the role of lignin in the hydrolysis of plant biomass, the inhibitory role of lignin
- pilot scale data on the performance of the cellulase mixtures for technical and economical evaluation

The project supported especially European industrial enzyme producers. Europe has traditionally been very strong in industrial enzyme production. The two largest companies Novozymes and Danisco/Genecor share currently about 70% of the total market. Total hydrolysis of lignocellulosic biomass opens up new large volume enzyme applications for the enzyme industry. It is also essential to introduce and support new companies, especially SMEs like DNL to the area. Furthermore it is important to support the new member states and their industries. The Estonian SME Biogold greatly contributed to the outcomes of the project and established an important European network valuable also in future.

Combined efforts and European level collaboration with scientists and the industry are needed for developing future biorefineries for economical and sustainable production of fuels and chemicals. The project aimed at developing enzymatic tools for efficient and economical lignocellulosic biomass total hydrolysis. This aim was well achieved by bringing together research-oriented and technology-oriented partners from different countries. The project successfully combined various expertises including classical and more modern enzyme screening technologies, enzymology, applied enzymology, fermentation technology, expertise on raw materials, in their characterization and also large scale production, enzyme production and commercial exploitation, technologies for saccharification and fermentation also in pilot scale and techno-economical process evaluation.

Collaboration among the partners was surprisingly good. This can be seen by various co-publications and visits between the members. Without the shared knowledge, resources and facilities the goals would not have been possible to obtain.

Education and training of PhD students and other academic personnel was also carried out within the project. Due to the multidisciplinary nature of the project, the project offered great opportunities for the students. Altogether 5 Master's theses and one PhD thesis was completed in the Disco project. In addition, two bachelor theses were prepared and two Ph.D thesis, (partially) based on the work carried out in Disco are estimated to be completed at year 2013. Project and project results have been disseminated in many scientific meetings. In addition, two PhD courses have been organized by UH.

Dissemination activities

www-Pages for the Disco project can be found at http://www.disco-project.eu/. The pages contain both a public and restricted site, available only for the DISCO members. The first leaflet for the DISCO project, totally 650 copies have been ordered and distributed by the partners. Altogether three press releases have been published.

Altogether nine peer reviewed scientific papers have been published so far in the DISCO project. In addition, several manuscripts have been submitted to journals and other ones are in preparation by partners. The results from the project have been disseminated in various scientific meetings.

Exploitation of the results

The expoitation issues within the project have been constantly reviewed and discussed in every project meeting, twice a year. Professor Maija Tenkanen (UH) was nominated as the expoitation manager for the Disco project. The companies DNL, Biogold, Afrifirm and GRW are in first hand responsible for the exploitation of the obtained results and discoveries. The project has generated lot of data, methods and enzymes which will be exploited after the Disco project will be ended by partners. The major achievements in DISCO can be summarized as:

- development of various analytical tools,
- structural and biochemical data on the selected biomasses (wheat straw, wheat bran, corn cobs and spruce)
- optimized pretreatment procedures for the selected biomasses
- discovery of novel enzymes for the hydrolysis of lignocellulosics
- genome data on the selected (hemi)cellulolytic fungi
- mechanistical data on the action of especially hemicelulases and accessory enzymes and their synergy to cellulases
- elucidation of the role of lignin in the hydrolysis of plant biomass, the inhibitory role
- pilot scale data on the performance of the cellulase mixtures for technical and economical evaluation.

The address of the project public website

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- The DISCO project website can be viewed at http://www.disco-project.eu.