

## **Executive summary:**

Production of fuel ethanol from lignocellulose has been shown to have a significantly more positive net energy and CO<sub>2</sub> balance than ethanol produced from grain or sugar beet. Especially, lignocellulosic waste raw materials offer abundant alternatives not competing with food and feed production. For converting lignocellulosic biomass into sugars and ethanol, novel technologies are, however, needed.

The overall aim of the HYPE project was to accelerate the implementation of new second generation biofuels from lignocellulosic raw materials by focusing on the identified key bottlenecks presently hindering commercialization. The goal of the project was to develop a novel consolidated bioprocess concept for the hydrolysis and fermentation of lignocellulosic feedstocks by achieving improvements in critical bottlenecking process stages. Wheat straw was chosen as the main raw material for bioethanol production. Other raw materials studied included corn stover, reed canary grass, sweet sorghum and willow. The previously developed IBUS technology served as basis for further development of the pretreatment. High consistency operation throughout the process was achieved, allowing high product concentration and potentially decreasing the ethanol production costs.

The enzymatic conversion of the pretreated substrate into sugars and further to ethanol was a key target for improvement. The high cost of enzymes is still considered one of the main barriers to economic production of cellulosic ethanol. Thermostable enzymes formed the basis to decrease the conversion costs. Optimized preparations of thermophilic enzymes were designed for rapid liquefaction stage and saccharification stages, and resulted in higher conversion compared to the reference enzymes. Increased knowledge on enzyme mechanisms and addition of novel components improved the hydrolysis further. The enzyme dosage could be decreased by at least 25% without compromising the performance, when compared to the reference enzymes. Additional cost reductions could be obtained by introduction of a diabatic vacuum stripping system to recover ethanol during fermentation, also allowing recovery of thermostable enzymes for recycling. The options for recovery and reuse of enzymes were further developed by designing novel enzymes and hydrolysis techniques.

The amount and cost of added enzymes could be also reduced by exploiting the capability of the fermenting organism (*Fusarium oxysporum*) to produce useful enzymes. The ethanol yield could be increased by improved fermentation of carbohydrates by two organisms, a xylose fermenting yeast and *F. oxysporum*. Pilot-scale testing and evaluation was accomplished at the Inbicon pilot plant, Skærbæk, Denmark. These tests included hydrolysis at very high dry matter both with thermostable enzymes and commercial enzymes. The outcomes of the pilot-scale testing were technical optimisation of process parameters and set of technical parameters utilized in the feasibility evaluation. Main conclusion of the feasibility study was that the consolidated process showed improved feasibility as compared to the base case if C5-fermentation at high consistency could be achieved. Main sensitivity parameters in terms of feasibility in full scale plants were biomass cost, ethanol price and enzyme cost.

The HYPE project tasks were accomplished by the 9 project partners representing different fields of science and industry. The whole value chain was covered from raw material supply and pretreatment, industrial

enzyme development and production to large scale saccharification, fermentation and ethanol recovery systems, supported by strong expertise of academic partners.

## **Project Context and Objectives:**

Fuels from lignocellulose biomass have a high potential to reduce GHG emissions, and hence are an important means to fulfil road transport CO<sub>2</sub> emissions targets. They can be a reliable fuel source, which can gradually reduce the dependence on oil imports, and can constitute part of a strategic reserve. In order to reach the goals set by the European Directives and the envisioned growth of biofuels during the next decades, it is essential to efficiently utilize the available agricultural and forest residues, as well as to extend the raw material sources to novel dedicated crops. Advanced conversion technologies are needed to produce ethanol and ethanol derivatives from a wider range of resources, including lignocellulosic biomass.

During the last years, major improvements in bioconversion technologies have been achieved. This is mainly due to advances in improved pretreatment technologies and biosciences, which have led to decreased costs of the enzymatic conversion step and to more efficient organisms for fermentation. Of the various pretreatment options, hydrothermal pretreatments (incl. steam pre-treatment) have been most extensively studied. Pretreatments disrupt the plant cell wall and improve enzymatic access to the polysaccharides. Hemicelluloses form a physical barrier around the cellulose, and several studies have shown that the maximum digestibility usually coincides with complete hemicellulose removal. At high severity conditions, however, hemicellulose is hydrolysed and further degraded to compounds toxic to the biological systems. Therefore, there is a tendency to restrict the severity of the pre-treatment methods. Consequently, more enzymes hydrolysing hemicelluloses have to be supplied in the enzyme mixtures. As pre-treatment method, delignification is generally not considered economically feasible although complete delignification improves the enzymatic digestibility. Partial delignification may, however, impede the action of enzymes because of readsorption or delocalization of lignin. The capital and operating costs for lignocellulose pretreatment may be as high as 20% of the total production costs of ethanol.

Along with the raw material costs, cellulases make up a significant cost factor in the production of cellulosic ethanol. Despite recent reductions in enzyme costs there is an obvious need for further development on a wider basis. Several approaches have been applied to improve cellulase performance and decrease the amount of enzymes needed to saccharify lignocellulosic substrates; improving individual cellulase components or complementing or replacing the set of cellulases by novel proteins. According to scientific and patent literature, improvements in the enzyme pattern have been introduced by both approaches. Lignin has been shown to bind cellulases and reduce their service life (1,2) and therefore, reducing their non-specific binding to lignin is a further challenge.

Presently, most commercial enzymes are only able to act at a temperature range close to 40-50. Thermostable enzymes offer potential benefits in the hydrolysis of lignocellulosic substrates; higher specific activity decreasing the amount of enzymes needed, enhanced stability allowing improved hydrolysis performance, better inhibitor tolerance and increased flexibility with respect to process configurations, all leading to improvement of the overall economy of the process. In the FWP5 TIME project, new thermostable fungal cellulases have been screened, cloned and produced, tested in hydrolysis experiments and used to design new process concepts (3). Three thermostable cellulases, identified as most

promising enzymes in their categories (CBH, EG and  $\beta$ -glucosidase) were cloned, efficiently produced in *Trichoderma reesei* and preliminarily mixed to compose a novel mixture of thermophilic cellulases (4). The thermostable enzyme mixture was evaluated in high temperature hydrolysis experiments on technical substrates (5). These thermostable enzymes were the basis for further development of enzymes and their application techniques.

A further approach to improve the performance of enzymes is to add enzymes active on other polymers in the matrix, such as hemicellulases, or to add other rate-limiting enzymes enhancing the hydrolysis. Thus, although the residual amount of hemicellulose in the pretreated raw material may be low, removal of the restricting layers of hemicellulose have been shown to improve the hydrolysis yield of cellulose, obviously by exposing and making available new cellulose layers to the action of cellulases for hydrolysis. The impact of removal of xylan by xylanase enzymes has been shown to increase the hydrolytic efficiency.

Obviously, one of the single most important process parameters with regard to process economy and efficiency is the overall substrate consistency level. Low content of biomass in the process not only increases the capital cost due to equipment size but also results in excessive energy requirement with regard to heating, cooling and distillation. Until recently, a dry matter content of 10% (w/w) or less has been the standard applied. When aiming at an ethanol concentration above 5% (w/w), the dry matter consistency has to be over 20% (w/w). In the FWP5 IBUS project, a new approach based on gravity mixing of biomass and enzymes was developed (6). Gravity mixing enables efficient hydrolysis and processing of biomass up to a dry matter level of 40% (w/w), and at the same time the efficient mixing improves the enzyme efficiency.

Lignocellulose substrates contain both hexose (C6) and pentose (C5) sugars. The traditional ethanol producing organism, yeast *Saccharomyces cerevisiae*, is not able to produce ethanol from pentose sugars. Lately, significant development has been achieved in increasing the ethanol yield by the fermentation of pentose sugars to ethanol using the tools of metabolic engineering. The traditional yeast, *S. cerevisiae*, is considered most competitive due to its long standing industrial tradition, good productivity and tolerance for high ethanol concentration. Stable xylose-fermenting *S. cerevisiae* strains have been obtained by two major ways; by modifying the oxidoreductive steps of xylose metabolism to xylulose or by inserting xylose isomerase genes. The present published yields and rates are, however, still lower than with traditional glucose fermenting yeasts. The theoretical improvement of ethanol yield through fermentation of all available sugars may be up to 30% (7).

The most thoroughly studied process configuration for the fermentation is the simultaneous saccharification and fermentation (SSF) process, where the hydrolysis and fermentation take place in the same stage. The method suffers, however, from the combination of the two process stages; i.e. hydrolysis and fermentation, which cannot be run at their optimum conditions. One of the major advantages of the SSF concept is the continuous removal of the sugars by fermentation, thus minimizing the severe end-product inhibition of cellulase enzymes. In an SSF process utilizing yeast, the enzymes usually act throughout the hydrolysis at suboptimal temperatures around 35°C. Consolidated bioprocessing (CBP)

refers to a conversion processes where the production of enzymes, hydrolysis of cellulose (and hemicellulose) and fermentation is carried out basically in one step. According to recent estimations, the projected bioethanol production costs by CBP could be essentially lower than with traditional methods (8). In spite of this great promise, no micro-organisms with optimal properties (rapid substrate hydrolysis and product formation) are currently available. An interesting organism for the consolidated process is *Fusarium oxysporum*, known to produce several enzymes acting on cellulose and hemicelluloses (9), and to be able to ferment sugars (glucose and xylose) into ethanol with reasonably good yield. This organism, however, has some limitations, such as relatively slow growth, ethanol and enzyme production rate under the micro-aerophilic conditions optimal for ethanol production. *F. oxysporum* has been shown to produce ethanol with a yield of about 1.8 mole ethanol/mole of glucose and 1 mole ethanol/mole of xylose (10).

The novel consolidated process concept, introduced in the HYPE project, offers several superior characteristics leading to significant cost reductions. The well pretreated raw material with a high dry matter content and low amount of toxic compounds, is liquefied (prehydrolyzed) with the novel thermostable enzymes, efficiently produced in the industrial fungal production strain (*Trichoderma reesei*), and fermented to ethanol with the enzyme producing organism *F. oxysporum* at high consistency. Enzymes produced by the fermenting organism would reduce the need of added, externally produced enzymes. Removal of ethanol during the fermentation would keep the ethanol concentration low and avoid end product inhibition.

In this project, the IBUS pre-treatment technology developed under FWP5 was the main reference pretreatment method used for the raw materials. In addition, other thermochemical methods were studied. The IBUS technology is based on hydrothermal treatment with hot water (160-195 C), resulting in high yield and excellent enzymatic digestibility of all carbohydrates. The method can be run in pilot and demonstration scales. The abundantly available feedstock, wheat straw, a side stream from agriculture, not competing with food production, was used as the main raw material. Other agricultural feedstocks studied included corn stover, reed canary grass, sweet sorghum and willow.

A special target in this project was to improve the performance of individual key cellulases by further improving their performance and by reducing their non-specific binding to lignin. Targeted modifications in the cellulose binding domains of cellulases were expected to reduce this undesirable phenomenon. The cellulose binding module (CBM) plays obviously a key role both in specific and unspecific binding, and was the target for improvement in this project. A deeper understanding was aimed at concerning the mechanism of enzymatic hydrolysis of cellulose by detailed biochemical and kinetic studies. Furthermore, potential new enzymes overcoming the bottlenecks of the hydrolysis were searched.

Using the thermostable enzymes, the hydrolysis temperature could be increased, as compared to present commercial enzymes. Clearly more efficient hydrolysis per enzyme activity unit or protein amount used could thus be obtained. Enhancing the thermal stability was expected to result in clearly improved specific activity, essentially decreasing the protein dosage required for efficient hydrolysis of lignocellulosic substrates. In addition, improved thermal stability was expected to lead also to potentially higher stability towards substrate derived inhibitors

and higher recovery in low temperature distillation conditions. The efficient hydrolysis of all carbohydrates present in the raw materials requires a complete set of lignocellulolytic enzymes. In this project, the synergistic importance of other accessory enzymes, especially those produced by the fermenting organism, *F. oxysporum*, were explored. Some of the limitations of this organism were studied by genetic and process technological improvements. *F. oxysporum* serves as an interesting new model organism for the consolidated bioprocessing, and allows estimation of the potential enzyme and ethanol production cost reductions. In this project, a xylose fermenting yeast was used as the state-of-the-art reference.

The technology development for a high consistency process (at 20% w/w or higher dry matter content) includes several factors, such as product inhibition, transport phenomena and water activity. The principle of gravity mixing was used for development of the hydrolysis process. The prehydrolysis (preferably at high temperature) allows to reduce rapidly the viscosity (or flowability) of the high solid content substrate and enables better mixing for the inoculation of the fermenting organism. The liquefaction and simultaneous saccharification and fermentation were tested in pilot scale combined with externally added liquefying and saccharifying enzymes. The possibility of stripping a substantial part of ethanol was investigated with the aim of extending the service life of the enzymes. The project thus aimed at providing solutions for an entire new process concept. The improvements achieved were estimated in feasibility studies.

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## **Project Results:**

A strong and multidisciplinary consortium was gathered to reach the objectives of the HYPE project; i.e. to accelerate the implementation of new second generation biofuels from lignocellulosic raw materials. The project tasks were carried out by the 9 project partners representing different fields of science and industry. The partners had expertise and complementary knowledge on different areas of lignocellulosic biomass conversion processes, including pretreatment of the feedstock (Inbicon, Biogold), development of novel processes and equipment for lignocellulose conversion and ethanol recovery (Inbicon, Biogold, Holm) and industrial production of enzymes (ROAL). The academic groups had long-term expertise on various aspects of lignocellulosic enzymes; screening, molecular biology, protein production, enzymology (VTT, UH, KU, UT) as well as in lignocellulose hydrolysis techniques (VTT, UH, KU, NTUA) as well as in microbial metabolism and fermentation techniques (VTT, NTUA). The whole value chain was thus covered; raw material supply and pretreatment, industrial enzyme development and production, and large scale saccharification, fermentation and ethanol recovery systems. Each partner has its clear tasks based on previous expertise. The contribution of each partner was necessary for successful execution of the project.

The project was divided into six technical workpackages covering the supply of selected raw materials, their pretreatment and analysis (WP1), development of new enzymes (thermostable, optimally binding, and additional, hydrolysis-enhancing enzymes; WP2), development of integrated and consolidated hydrolysis technology (WP3), improvement of the ethanol producing organism (WP4), optimization of the consolidated bioprocessing (WP5) and demonstration and evaluation of the feasibility of the techniques developed (WP6). In addition, one WP is devoted to project management, dissemination and exploitation (WP7). The project approach is summarized with responsible partners in each work package.

## **Raw material pre-treatment and supply**

Straw is presently one of the most relevant raw materials, not competing with food production. Wheat straw was chosen as the first reference biomass for bioethanol production in the HYPE project. Other raw materials studied include corn stover, reed canary grass, sweet sorghum and willow. Inbicon provided the partners of the project with well pretreated wheat straw and corn stover and Biogold with reed canary grass and willow, in as large quantities as needed for the different project tasks. In addition, NTUA pretreated the whole sweet sorghum crop as well as its bagasse. The raw materials were characterised before and after the steam pretreatment for the chemical composition and yields. The work in the HYPE project was benchmarked against the previously developed efficient hydrothermal Inbicon pretreatment technology. The pretreatment technology has several advantages as it only use steam, produces a very low amount of degradation products and provides a material for the enzymatic hydrolysis step with a highly available carbohydrate matrix. Pretreatment of the main feedstocks; wheat straw and corn stover, was performed in the Inbicon pilot plant which has the capacity to continuously pretreat 100 kg biomass/h. The biomass was first shredded to approx. 5 cm pieces prior to being hydrated to approx. 35-50% dry matter before being loaded into the pretreatment system where the biomass was pretreated through addition of steam. The pretreated material was unloaded from the pretreatment as a slurry which could be separated into a fibre fraction and a liquid fraction.

The fibre fraction contained more than 90% of the cellulose from the biomass, the majority of the lignin and some of the hemicellulose and other compounds from the biomass. The liquid fraction contained salts and dissolved hemicellulose from the biomass.

Reed canary grass and willow were pretreated by steam explosion or hot water extraction in the lab scale pretreatment system of Biogold. The biomasses were chosen as they are abundant in Eastern Europe and could be utilized to obtain a sustainable fuel production in this area with a very low carbon footprint. According to the results, reed canary grass showed similar solubilization and conversion properties as the side streams of the annual plants, wheat straw and corn stover, whereas willow, being a woody raw material, was very hard to solubilize and a good conversion of the carbohydrates in the pre-treated material could not be obtained with the applied pretreatment methods. The hydrothermal pretreatment of sweet sorghum bagasse was optimized to lead to an efficient conversion of the raw material.

Wheat straw and corn stover had very similar compositions; corn stover contained slightly less glucan, xylan, arabinan and lignin than wheat straw. Reed canary grass resembled wheat straw, although it contained slightly more glucan and slightly less xylan. Willow contained significantly less xylan and arabinan and significantly more lignin than wheat straw. The high lignin content might explain why the carbohydrates in pretreated willow were less converted to sugars in hydrolysis than those from the less lignin-containing biomasses. Hydrothermal pretreatment only relocates lignin on the cellulose chains in contrast with other pretreatment processes where lignin is more efficiently solubilized and extracted from the carbohydrates. On the other hand, sweet sorghum stalks contained a high amount of free sugars (sucrose, glucose and fructose) but only a low amount of lignin

### **Enzymatic hydrolysis of lignocellulose**

One of the major obstacles to cost-effective production of ethanol from lignocellulose is the high enzyme costs. This is caused mainly by two factors: high amount of enzymes needed in the processes and high production costs of enzymes. The major effort in the HYPE project was directed to decrease the amount of enzymes needed, but also possibilities to improve the cost-efficiency of enzyme production by finding better options for the raw materials were searched.

The HYPE hydrolysis process starting from fibrous material in high; 20-30% (w/w) dry matter content suspension occurs in two stages: liquefaction, transforming the fibrous material to free-flowing slurry and saccharification, producing the sugars for ethanol fermentation. Enzymes suitable for these stages were consequently studied both separately and in combination. The project focused on thermostable enzymes which, in addition to their general better stability, allow more flexibility and possibilities for improved performance of processing. The targets of the enzyme development were thus to improve their performance by optimization of the mixtures for liquefaction and hydrolysis and by screening and identifying single components of enzyme mixtures for improved total performance.

Moreover, one of the aims of the project was to study consolidated process for ethanol production, i.e. to utilize an organism in ethanol

production which also can contribute to hydrolysis by producing part of the required enzymes. The consolidated organism in the HYPE project was *Fusarium oxysporium*. Therefore, the complementation of the externally added enzymes with saccharifying enzymes of *Fusarium* was also studied in the process of combined hydrolysis and ethanol fermentation, simultaneous saccharification and fermentation (SSF). The experimental research enzymes used were produced in genetically modified fungal organisms as monocomponent preparations in laboratory or pilot scale and purified further when necessary also from other production organisms.

Since lignocellulose is a complex material, containing the carbohydrate polymers cellulose and hemicellulose and the aromatic lignin as major components, multicomponent enzyme mixtures are required for an efficient degradation to the monomeric sugars. Sugars can be produced by enzymes from cellulose - yielding glucose - and from hemicellulose - yielding several sugars such as xylose, mannose, galactose, arabinose, etc. All these sugars can be converted to ethanol if using an efficient fermenting organism, usually improved genetically (yeast, bacteria, fungi). Lignin is a polymer composed mainly aromatic units, and cannot be the source of sugars. However, lignin affects the hydrolysis by interfering with the action of enzymes, especially by preventing them to act on cellulose or hemicellulose, and adsorbing enzymes, thus binding them and making inaccessible to the actual polymers they should act on.

Natural lignocellulose is very resistant to microbial and enzymatic break-down, and therefore different pretreatments prior to enzyme hydrolysis are required in industrial processes. The composition and three-dimensional structure of the material also reflects this recalcitrance. As a consequence, in addition to an optimal cocktail of efficient enzymes acting on cellulose, a multitude of other enzymes is required to remove the surrounding and internal hemicellulose structures in biomass, on the other hand to produce efficiently sugars from hemicellulose and on the other hand to expose cellulose freely accessible to cellulolytic enzymes.

Enzymes hydrolysing cellulose to glucose are cellulases. They are schematically divided in three groups with fundamentally different functionalities: cellobiohydrolases (CBH's), endoglucanases (EG's) and  $\beta$ -glucosidases. The mechanism of cellulose hydrolysis is dictated by the fact that cellulose is insoluble, partially crystalline material. As a consequence, in order to break cellulose, the water soluble enzymes must first adsorb to the solid surface. After that they can perform the hydrolysis reaction: adding water molecules to the chemical bond they act on, cutting the polymer chain and releasing the hydrolysis products. Finally, enzymes need to move away from their location and find a new site suitable for their action. Cellobiohydrolases act on the ends of the cellulose polymer chains, releasing cellobiose. They typically act processively, i.e. proceeding along the chain and releasing successively cellobiose units from the same chain they are attached on. Endoglucanases typically act on the less crystalline areas of cellulose, cutting the chain and thus creating new chain ends for the action of CBH's. Cellobiose produced by CBH enzymes is released in the solution in which it is further hydrolysed to glucose by  $\beta$ -glucosidase enzymes. In addition to cellulolytic enzymes, there are other mechanisms to intensify cellulose break-down in nature. These mechanisms may be oxidative reactions depolymerizing cellulose. Recently specific enzymes for these reactions have also been discovered and their increasing effect in cellulose hydrolysis shown.

Enzymes produced by various micro-organisms in nature differ in their characteristics, and some enzymes are more suited to industrial processes than others, the pre-requisite being that they work optimally in the required conditions, such as pH and temperature. In the HYPE project especially thermostable enzymes, able to work in high temperature process conditions were studied. The suitability of the enzymes for industrial processing is analysed by different biochemical methods. Most important measure of the efficiency is the rate of conversion, which depends on the structure of the enzyme molecule and on the other hand on inhibition due to the chemical components present in the process. Since nature needs mechanisms to control these reactions, enzymes are often inhibited by the end products of the reaction, and thus cellobiohydrolases are inhibited by cellobiose and  $\beta$ -glucosidases by glucose. This is of course undesired in industrial use aiming at high sugar concentration, and thus enzymes with a high conversion rate at high sugar concentration are searched for.

Enzymes for hydrolysis of cellulose and hemicellulose for various industrial uses have been commercially available already for many decades and their production technology is advanced. Enzyme companies are looking for more active single proteins, operating also in concert with other enzyme components. The present production strains and methods are highly optimized, and actually what is desired as a result of screening is a gene coding for an efficient enzyme to be introduced in the production system. In case of thermostable enzyme mixture, these features bring a challenge: all the introduced enzymes should, in addition to higher efficiency, also have higher thermostability than the traditional components. This means that all key enzyme components in a mixture should be equally thermostable. In the HYPE project these components were produced separately as research enzymes and evaluated both separately and in designed mixtures with other thermostable enzymes.

### **Enzymes studied**

Thermostable enzymes were cloned and produced in fungal production hosts by Roal for both laboratory and pilot studies. When necessary the enzyme preparations were purified by optimized heat treatments or chromatography for detailed biochemical characterization. The primarily studied enzymes included several thermostable cellobiohydrolases, endoglucanases, xylanases and  $\beta$ -glucosidases. A glycoside hydrolase family 61 protein from NTUA was also evaluated in hydrolysis. Purified enzymes of *Trichoderma reesei* from VTT and of *Phanerochaete chrysosporium* at UT were used as reference in some of the studies. The reference enzymes were a commercial cellulase mixture, Celluclast, based on enzymes from *Trichoderma reesei*, supplemented with  $\beta$ -glucosidase, Novozymes 188 (Novozymes).

### **Characterization and optimization of the set of thermostable enzymes**

The enzyme mixtures were characterized both for the liquefaction of high dry matter biomass suspensions and for the total hydrolysis. In addition, the combination of thermostable enzymes and enzymes from *F. oxysporum* were studied. The role of different enzymes in liquefaction was studied at HU using a new methodology to follow continuously the change of apparent viscosity in high-consistency biomass suspensions up to 15% (w/w). Potential enzymes identified were studied for their ability to liquefy pretreated wheat straw. The results revealed that endoglucanase EGII/Cel5A was a key enzyme for liquefaction, most likely because of

breaking the water retaining fibre structures (11). It was evident that sufficient liquefaction effect was rather a consequence of cleavage at a proper site than the frequency of enzymatic cleavages. In contrast to liquefaction, improved saccharification required the synergistic action of various enzymes with different sites of attack and modes of action.

Synergistic action of CBH and EG enzymes was clearly seen in hydrolysis experiments at VTT. Even a low amount of 5% of Cel5A endoglucanase in the mixture gave near to optimal synergy effect at 55°C. In general, the highest synergy was observed using CBH:EG enzymes in ratios from 80:20 to 95:5. Furthermore, the optimization of the full enzyme mixture for hydrolysis of hydrothermally pre-treated wheat straw from Inbicon was also carried out using statistical design. Obtaining high degree of hydrolysis was not very sensitive to small changes in the ratios of the key enzymes. Thermoenzymes showed to be clearly more efficient also at 45 °C in optimized mixtures than their *Trichoderma*-derived counterparts used as the state-of art enzymes during the project. The optimization gave also the basis of the mixture design to be used in the pilot scale experiments at Inbicon.

Clear synergism was detected at UH and NTUA between *Fusarium* enzymes and the saccharifying enzymes. This indicated the potential benefit of introducing to the hydrolysis process enzymes from *F. oxysporum*, the organisms suggested for consolidated processing.

### **Screening of new enzymes**

Culture filtrates of 31 fungal strains were screened at VTT for synergistic effects with *Trichoderma* enzymes or the optimized mixture of thermostable enzymes. Several fungal culture supernatants enhanced synergistically the performance of the enzymes in total hydrolysis. An enzyme characterized as being of CBHI type was isolated from a fungal source and evaluated in hydrolysis. This enzyme had a clear improving effect on *Trichoderma* mixture when replacing 10 % of the total protein, as well on the mixture of thermostable enzymes.

In addition, several thermostable cellulases were screened at VTT from metagenomic libraries and characterized. They showed clear endoglucanase characteristics but low synergy in the enzyme mixtures for total hydrolysis. Even though one of them had highest endoglucanase activity at 90°C they could not be used as efficient components of thermostable mixtures because they seemed not to be compatible with fungal CBH enzymes.

### **Biochemical characterization of hydrolytic enzymes**

Kinetic constants and hydrolysis mechanisms of the key cellulases were studied in different hydrolysis conditions at UT. Several targeted methods were developed, e.g. for the measurement of the turnover numbers for cellobiohydrolase (CBH) catalyzed cellulose hydrolysis and a method for quantitative determination of apparent processivity of CBHs acting on cellulose under single-hit conditions. Based on the results, new aspects related to cellulose hydrolysis were discovered and novel interpretation for the processivity and synergy of cellulases were published by the scientists in UT (12, 13).

The thermostable enzymes were extensively studied and characterised with respect to kinetics and inhibition at UT. It was found that the cellobiohydrolases and endoglucanase used in the thermostable mix had

slightly higher  $k_{cat}$  compared to corresponding enzymes in the reference enzyme. Inhibition studies showed that all Cel7 CBH's were severely inhibited by cellobiose, but no significant difference was observed between enzymes. The Cel6 CBH's were much less inhibited by cellobiose but glucose had a similar inhibitory effect on the activities ( $IC_{50}$  for glucose 45 g/l). For comparison, the endoglucanases TrCel5A and TaCel5A had  $IC_{50}$  around 35 g/l of cellobiose and were not inhibited by 90 g/l of glucose. Comparison of thermostable  $\beta$ -glucosidases (AtBG3, TaBG3 and CtBG3) with the commercial  $\beta$ -glucosidase purified from Novozym 188 revealed that CtBG3 did not suffer from substrate inhibition and had high catalytic efficiency,  $k_{cat}$ . Thus, it is possible to obtain enzymes, better suited for high consistency hydrolysis.

Based on the measurement and kinetic equations, a model for hydrolysis in which CBH action is limited by the length of obstacle-free way on cellulose chain was proposed. This means that the length of the obstacle-free path available for a processive run on cellulose chain is limiting the processivity of CBHs on cellulose. In the model, the formed productive complex of CBHI and cellulose molecule was calculated to proceed with a rate of about one cellobiose unit per second. After encountering an obstacle, CBHs would "get stuck" onto the substrate. Consequently, the rate of further cellulose hydrolysis approaches the value determined by dissociation rate constant which is apparently low for processive CBHs. The consequence from this theory is that any component that aids to remove or displace obstacles should work synergistically with CBH and lead to the higher extent of total degradation. Synergistic hydrolysis of bacterial cellulose by CBHI and EG suggested the presence of another mechanism of synergy that operates in parallel with conventional endo-exo synergism and thus a mechanistic interpretation whereby EG accelerates the recruitment of stalled CBHs was proposed<sup>13</sup>.

Three thermostable  $\beta$ -glucosidases were also characterized at UT in terms of kinetic parameters of cellobiose hydrolysis and glucose inhibition and two xylanases at UH (14). They were also evaluated in hydrolysis experiments and the optimal ones were selected for more detailed studies and for optimizing enzyme mixtures for pilot scale trials.

### **Improvement of enzyme production**

In order to achieve cost reductions, a variety of novel raw materials were screened for an industrial process by Roal. A raw material from cereal processing industry provided better yields as compared to the reference medium. The cereal based raw material was also competitive and feasible to be used in the process. Industrial scale fermentations at an enzyme plant using the newly optimized raw material composition were started already during the HYPE project.

### **Development of hydrolysis technology**

Traditionally, hydrolysis and saccharification of lignocellulosic substrates has been performed using substrate concentrations in rather dilute systems in the range of 2-10% (w/w) DM (or water insoluble solids WIS), which is not economically viable because of e.g. higher costs of investments and distillation. The reason for not operating at higher solids concentrations have been the technical difficulties to mix and process the very viscous biomass, end-product inhibition of the enzymes and inhibition of the microorganisms by degradation products formed

during the pretreatments (15) . However, new reactors systems have been developed and scaled up, e.g. by Inbicon, and it has been already shown that it is technically possible to operate up to 25-30% (w/w) solids concentration (16, 17, 18). However, new enzymes that can ensure fast and efficient liquefaction and have better properties with respect to specific activity, stability and end product inhibition are needed. In this respect thermostable enzymes might offer an advantage over traditional commercial enzymes from e.g. *T. reesei* or *Aspergillus*<sup>4</sup>. In addition the process configuration has to be optimised to benefit the properties of these enzymes.

Development of integrated high temperature (HT) liquefaction, hydrolysis and saccharification technology for conversion of lignocellulosic biomass at high solids concentrations, above 20% (w/w) DM, has involved optimization of process conditions (temperature, residence time, solids concentration, enzyme composition etc.) of hydrolysis and saccharification, as well as the overall process configuration. Optimising the process conditions and process configuration are crucial to improve conversion yields at high solids hydrolysis and at the same time to minimise the cost of enzymes. Part of this work has been an extensive study of several of the factors affecting the enzyme performance and ability to recover enzymes when operating at high solids. Factors limiting the hydrolysis have also been identified and used for optimization of the hydrolysis. Furthermore, the amount of externally added enzymes by optimizing the hydrolysis conditions for various raw materials has been minimized.

### **Improving liquefaction at high solids concentration**

Lignocellulosic materials are characterised by the long and entangling fibers, which can swell and hold large amounts of water (19). The exact properties depend on the pretreatment conditions and severity, but all raw materials from the HYPE project shared these characteristics. From a processing point of view, this resulted in materials where no free water was present at solids concentrations above 20% (w/w) DM and which had very high initial viscosity. The materials were therefore very difficult to mix in traditional reactors and it was desirable to achieve a fast liquefaction (apparent viscosity reduction) potentially improving mass transfer and minimising the power required for mixing (17, 11). The thermostable endoglucanase TaEGII was selected as it proved to liquefy the material efficiently and to act at temperatures up to 75°C. The technique revealed that with TaEGII sufficient liquefaction could be achieved already at a low enzyme dosage during a fairly short reaction time (11, 20). The results also revealed, however, that it was most advantageous to carry out the liquefying step together with other synergistically acting cellulolytic enzymes.

Further studies of liquefaction were carried out at KU at solids concentrations up to 25% (w/w), which has been used at Inbicon in large scale. For studies at 25% (w/w) DM it was not possible to get reliable measurements of viscosity within the first 6 hours, due to the very high initial viscosity. In general it is challenging to perform rheological measurements due to the fibrous structure of the material (19). The results showed that the system followed pseudoplastic shear thinning (viscosity decreases with shear rate) and was thixotropic (viscosity decreases with time). In accordance with previous work, the data could well be described using the power law equation and the viscosity of different enzyme systems and over time could be compared using the

consistency index Kpl (21). The results showed that although Ta EGII was very efficient in reducing the fibre length, the viscosity was higher than in the system with a full enzyme mixture. Likely also factors, such as other cellulases and hemicellulases obviously play a role in the viscosity reduction and hydrolysis. From a process point of view, it was thus desirable to use a tailored cocktail already in the first hydrolytic stage because of the significantly better conversions obtained with combination of enzymes due to their synergistic properties. The most pronounced reduction in viscosity and fibre length took place during the first 6-8 hours. Efficient liquefaction of sweet sorghum at high consistency could be also obtained at NTUA.

### **Development of process configuration for liquefaction and saccharification**

After liquefaction the process can either continue as a hydrolysis (saccharification) at high temperature or the temperature can be lowered and the fermentation started. In the first option the process will benefit from the higher hydrolysis rate of the enzymes at the higher temperature but, on the other hand, end-product inhibition may be a serious issue especially when operating at high solids concentrations. It was therefore investigated how to combine the liquefaction and saccharification processes thereby optimising the overall efficiency of the hydrolysis and minimising the need for externally added enzymes by 1) combining (high temperature) liquefaction and saccharification, 2) by testing mixtures of enzymes (thermophilic, mesophilic and from *Fusarium*) and 3) and by choosing the best process configuration.

Liquefaction and saccharification was studied in a model system simulating the conditions for liquefaction and SSF by first carrying out high temperature liquefaction (45-55°C) followed by a second stage at 35°C, which is the temperature for fermentation, first at low solids concentrations (2%) and later at 30% (w/w) DM. Testing in small scale confirmed that 6 h of liquefaction was optimum and the best conversion was obtained when applying the full enzyme loading from the start. In a two-step configuration using thermostable enzymes in combination with traditional enzymes, it was possible to replace more than 30% of the commercial reference enzyme (Celluclast-Novozym 188) by a *Fusarium* preparation without affecting the final yield. More importantly, a synergistic effect of combining *Fusarium* enzymes with Celluclast was observed, most likely due to the presence of essential auxiliary enzyme activities in the *Fusarium* preparation. In a consolidated process the enzymes produced by *Fusarium* could therefore play a significant role in reducing the costs for external enzymes.

Further work on optimising liquefaction and saccharification was carried out at high consistencies; 20 to 30% (w/w) DM at KU. The work confirmed previous results that when increasing the initial solids concentration, the conversion decreases almost linearly (18, 22). This was independent of the substrate and enzymes used (i.e. the reference or thermostable mixture). The linear relationship between conversion and solids concentration could also be used to predict rather well the enzyme loading needed to keep unchanged conversion when changing the solids concentration.

Previously, it has been found that polyethylene glycol (PEG) has a positive effect on enzyme performance, likely due to reduced binding of enzymes onto lignin and improved stability (23, 24, 25). Experiments

confirmed that PEG had a positive effect on the performance of both commercial reference enzymes and the thermostable enzymes and improved the conversion of all HYPE raw materials (wheat straw, corn stover and reed canary grass). In hydrolysis experiments PEG could improve the conversion by up to 40% and in SSF experiments by 15%. This is well consistent with previous results. On pretreated spruce up to 85% improvement in hydrolysis has been reported (26), whereas 20% improvement has been reported on hydrothermally pretreated wheat straw (25).

The results on the liquefaction and saccharification studies revealed that the thermostable enzyme mixture, when dosed on equal enzyme basis, performed up to 30% better compared to the commercial reference on all raw materials studied. The data was also reconfirmed by results on SSF studied by VTT, where similar ethanol yields were obtained when using a 25-30% lower dosage of thermostable enzymes as compared to the reference (on enzyme protein basis).

A final validation of the process concept was carried out in liquefaction and simultaneous saccharification and fermentation studies (SSF) to eliminate the effect of end-product inhibition at higher solids concentration. A miniaturised version of the Inbicon reactor system was developed at KU. The results confirmed that at higher solids concentrations product inhibition was critical and therefore short high temperature liquefaction was preferable. In addition, these experiments were also used to investigate the stability of the enzymes throughout the whole process to evaluate the potential for enzyme recovery after ethanol separation. The results revealed that to maximise the recovery of enzyme activity, a short high temperature liquefaction (6 h) followed by a low temperature SSF gave the highest recovery. After 168 h hydrolysis and fermentation, more than 80% of the cellulase and hemicellulase activity was still present in the slurry when performing short liquefaction of 6 h. Addition of PEG was found to be very important for high recovery of enzyme activity. Again this is likely due to reduced binding and inactivation on lignin and maybe also improved stability in general.

In summary, the conditions for liquefaction and saccharification (as well as SSF) were investigated and optimised. This resulted in several possibilities to substantially reduce the enzyme requirements: up to 30% of the externally added enzyme could be replaced by enzymes from *Fusarium* inoculum, 25-30% less enzymes on a protein basis were needed when using the thermostable enzyme mixture, as compared to the reference commercial enzymes, addition of PEG could improve the enzyme performance by 15-40% depending on process configuration and enabled the recovery of 80% of the enzyme activity after fermentation.

### **Analysis of bottle necks for efficient processing of biomass at high solids concentrations**

Bottlenecks restricting the hydrolysis were identified in order to design optimal enzyme mixtures and hydrolysis methods and to improve the efficiency of the hydrolysis. Approaches to overcome the different structural limitations were studied by UH, UT and VTT. Several factors have been recognized to restrict the enzymatic hydrolysis of lignocellulosic substrates in general and at high solids concentrations in particular. Among these factors are problems associated with the function of cellulases; e.g. product inhibition, inhibition by other components, binding to cellulose and slow dissociation, need for

auxiliary enzymes, such as xylanases and binding of enzymes onto lignin(27, 28, 29, 30, 16). Kinetic characterization of the key enzymes clearly emphasised the bottleneck of the CBH's (especially Cel7) in high dry matter hydrolysis without simultaneous removal of sugars in fermentation, which results in high concentrations of glucose and cellobiose. It also highlighted the importance of sufficient addition of an efficient  $\beta$ -glucosidase to alleviate the cellobiose inhibition.

During the pretreatment a number of different degradation products are formed and in addition the hemicellulose is partly degraded and solubilized. These products might all affect the enzyme performance (31, 32, 33). Analysis of the pretreatment liquid revealed that severe inhibitors were formed as degradation products of the lignocellulosic carbohydrates. Mass-spectrometry was used to identify these compounds. These compounds were found to be about 100 times more inhibitory to cellobiohydrolase TrCel7A compared to cellobiose, one of the most potent inhibitor of TrCel7A. Inhibition of EGs was, however, relatively weak and the  $\beta$ -glucosidase (from Novozym 188) was not inhibited by the compounds. All studied enzymes were also able to slowly degrade the inhibitors. The most efficient in relieving the inhibitory effect was the thermostable enzyme mix followed by the reference enzyme mixture.

The action of cellulases can also be limited due to hemicelluloses covering the cellulose thereby restricting the access to cellulose or creating obstacles that block further action of the enzymes (34, 35, 36). Addition of xylanases, especially the thermostable xylanase TaXyn10A, clearly improved cellulose conversion by removing hemicellulose. Acetyl xylan esterases could further improve the conversion, whereas arabinofuranosidases did not improve the cellulose conversion.

Another structural barrier is lignin and several enzymes have been shown to bind irreversibly to lignin (30, 24, 1). The binding of cellulases reduces their activity and recyclability and therefore, reducing the non-specific binding on lignin is important. Various possibilities to overcome the binding to lignin were tested. PEG is believed to reduce the binding of enzyme to lignin and addition of PEG indeed both improved the conversion and the recovery of enzyme. To clarify the mechanisms of cellulase binding, the interactions of enzyme adsorption on lignin were studied in detail with the endoglucanase (Cel45A) catalytic core from *Melanocarpus albomyces* fused to carbohydrate binding modules (CBMs) carrying amino acid mutations in the aromatic tyrosine residues responsible for binding on crystalline cellulose. The results confirmed that the CBM was involved in binding on lignin and that in future, enzymes binding less on lignin could be developed by designing targeted modifications to the CBM. Binding was in general less strong on wheat straw lignin as compared to spruce lignin.

Generally it is recognised that CBM plays an important role in docking and concentrating cellulases, especially cellobiohydrolases, onto the surface of cellulose (37). However, it has also been observed that the lack of a CBM can be compensated by increasing the dry matter consistency in the hydrolysis. Since the CBM is involved in unspecific binding, an interesting hypothesis has been presented on the improved recovery of enzymes at high solids concentration by using cellulases without CBM (38). The results clearly showed that also using the thermostable enzymes the yields with CBM+ and CBM- enzymes became very close at high consistencies, above 20% (w/w) on the model cellulose Avicel and on PWS. Equal hydrolysis yields with and without the CBM were obtained already at

10% (w/w) consistency on pretreated wheat straw and spruce. Interestingly, about 60-80% of the CBM- enzymes could be recovered on the lignin containing substrates, whereas only 10-20% of the CBM+ enzymes could be recovered on lignin containing substrate without PEG. Thus, it can be concluded that same or better hydrolysis yields could be obtained with the CBM- enzymes as compared to the CBM+ enzymes, and simultaneously, a significantly higher proportion of the CBM- enzymes could be recovered for recycling.

The work on analysis of bottle necks has led to a number of important observations and findings: 1) some of the thermostable enzymes applied in the project have characteristics superior to the reference enzymes in high consistency hydrolysis, 2) hemicelluloses do act as barrier creating obstacles for the cellulases but do also produce inhibitors to the solution, 3) thermostable xylanases were shown to be superior as compared with the commercial reference enzymes with respect to removing these hindering structures, 4) the type and nature of lignin and the pretreatment or isolation procedure has a major impact on the adsorption of enzymes, 5) there is great potential in modifying the enzymes in order to reduce the non-specific adsorption on lignin.

### **Evaluation of high temperature process concepts and enzymes**

The optimum concept for high temperature and high solids enzymatic hydrolysis was based on the use of the thermostable enzymes in the liquefaction and hydrolysis at lower temperatures eventually by taking advantage of the enzymes produced by *Fusarium*, added together with the inoculum. The preferred operation involved short, high temperature liquefaction (50-55°C) for 6 h followed by SSF at 30-35°C. Ideally the whole enzyme loading was added initially, as the synergistic action of all enzymes was crucial for a fast and efficient liquefaction as well as for the overall hydrolysis. The thermostable enzymes were clearly more active at elevated temperatures and remained stable throughout the process. Addition of PEG was found to improve the conversion by more than 15% and in addition improved significantly the recovery of enzymes after fermentation. The improved recyclability of enzymes could also be benefited in the low temperature recovery systems of ethanol.

### **Ethanol production by the consolidated organism**

Along with high enzyme costs, one of the major obstacles to cost-effective production of ethanol from lignocellulose is the reduced ethanol yield due to the limited ability of most natural organisms to utilize the C5 sugars for ethanol production and the suboptimal performance of the microbial strains, especially in high dry matter fermentations. In the HYPE project, the fermentation performance of the naturally xylose-fermenting filamentous fungus *F. oxysporum* and a genetically improved yeast strain of *Saccharomyces cerevisiae* utilizing xylose were compared for their fermentation efficiency in high dry matter (= 15 %) conditions.

The ability of *F. oxysporum* to produce lignocellulolytic enzymes has been investigated in detail. The growth and enzyme induction of *F. oxysporum* on different pretreated raw materials in laboratory scale was studied. It was found that the growth of *F. oxysporum* was enhanced using spent grain and corn cobs as the carbon source (39). The activity profile of cellulolytic activities in the concentrated extract was fairly similar to the cellulolytic profile of the reference commercial preparation

(Celluclast 1.5 L and Novozyme 188). Only the  $\beta$ -glucosidase activity in *F. oxysporum* crude enzyme solution was, however, about two to three times higher.

The crude multienzyme preparation produced by *F. oxysporum* was evaluated with regard to saccharification of the solid fraction of the hydrothermally treated wheat straw (40). A linear inverse correlation was observed for the initial dry matter concentration versus the bioconversion yield. A bioconversion yield of about 64% (of theoretical) was obtained after on a low substrate concentration, while the yield was only 42% when the initial dry matter content of 18% (w/w) was used. Addition of surface active agent resulted in an increased bioconversion. Moreover, the removal of the accumulated sugars present in the reaction mixture after 25 h of incubation increased the overall hydrolysis yield to 80% of the theoretical.

The microorganism was also evaluated for its ability to be used as a fermentative organism in simultaneous saccharification and fermentation (SSF). Maximum cell concentration was found to decrease with increasing initial ethanol concentration. The fermentation parameters, such as the ethanol yield and substrate (glucose) consumed, were practically constant showing little variation at initial ethanol concentrations of 0-4% (w/w). Measurement of inhibition of growth and fermentation activity has been considered as the best index for determination of ethanol tolerance. It was found that the amount of ethanol required to reduce the growth of *F. oxysporum* by 50% was 3.7% (w/w). Similarly, the amount of ethanol required to reduce the production of ethanol by 50% was 2.3% (w/w). The *F. oxysporum* strain demonstrated good tolerance against known inhibitors formed during pretreatment of lignocellulosic materials and was able to grow in the presence of significant concentrations of individual inhibitory compounds such as furan derivatives, phenolic compounds and weak acids (41). Furthermore, the hydrolytic activities of the fungus were proved to be robust enough towards the inhibitors tested.

#### **Comparison of the two C5-utilizing microorganisms *F.oxysporum* and *S.cerevisiae***

The two C5-utilizing microorganisms; *F.oxysporum* and genetically improved VTT in-house *S.cerevisiae*, were compared in fermentation experiments in laboratory scale on two unwashed pre-treated wheat straw raw materials from Inbicon. These materials were used in simultaneous saccharification and fermentation (SSF) experiments to provide data for comparison of the VTT *S. cerevisiae* strain and the NTUA *F. oxysporum* strain. In these experiments, the commercial enzyme mixture and the thermostable enzyme mixture developed in HYPE were compared. The results showed that the ethanol yield obtained by *S. cerevisiae* increased linearly with increasing enzyme concentration up to 10 mg/g dry mass, and non-linearly with higher dosages. In bioreactor scale, the highest ethanol yield by *S. cerevisiae* relative to dry mass loading was obtained at 10% and 15% dry mass conditions but decreased when the dry matter concentration was increased to 20%. Increasing the enzyme dosage did not significantly improve the yields as compared to low dosages.

The ethanol concentration and yield were inversely related when the effect of PWS concentration on ethanol production by *F. oxysporum* was examined. Thus, at a low substrate concentration, 10 % (w/w) of the PWS pretreated at low severity, the ethanol yield was 92.6 % of the theoretical yield based on hydrolysed glucose or 59 % based on the total

glucose and xylose in the PWS. On the other hand, at the higher substrate concentration of 15 % (w/w), the ethanol yield was 79.3 % of the theoretical yield based on glucose or 50.7 % based on the total glucose and xylose. Fermentation by-products, acetate and glycerol, were accumulated at low concentrations. The ethanol production by *F. oxysporum* was based on both cellulose and hemicellulose consumption. Thus, at low substrate concentrations (10-15 % DM) *F. oxysporum* acted as an efficient consolidated fermentative microorganism, producing ethanol from both the cellulosic and hemicellulosic fractions and contributing also to the supply of extra enzymes.

When ethanol production with *F. oxysporum* was compared to the xylose utilizing yeast strain, it appeared that during the first 48 hours ethanol production was significantly faster with *S. cerevisiae*, but *F. oxysporum* reached somewhat higher final ethanol concentration after a long fermentation, when using the low severity PWS at 10-15% (w/w) DM. The multienzyme system of *F. oxysporum* has been reported to contain significant amounts of xylanase, feruloyl esterase, acetyl esterase,  $\beta$ -xylosidase and  $\alpha$ -L-arabinofuranosidase activities and this contributes to the efficient hydrolysis of PWS (42).

The advantages of *S. cerevisiae*, as compared to *F. oxysporum* include faster substrate conversion and better ethanol tolerance (above 5 % ethanol by *S. cerevisiae*, about 3.5 % by *F. oxysporum*). The potential production of hydrolytic enzymes is a benefit of *F. oxysporum*. Both organisms have good inhibitor tolerance. Depending on the process outline and requirements set for the fermenting strain various options can be considered. The optimal conditions for producing the *F. oxysporum* enzymes should be further optimized (before or during the ethanol production stage), as well as the requirements for aseptic operation during the ethanol production. Due to the lack of aseptic fermenters and the limited ethanol tolerance, the *F. oxysporum* was not used in the high DM pilot experiments.

### **Improvement of the *F. oxysporum* strain**

Phosphoglucomutase and transaldolase are metabolic bottlenecks in the glycolysis and pentose phosphate pathway of the *F. oxysporum* metabolism. Both enzymes were homologously overexpressed in the *F. oxysporum* strain (43). During growth on glucose the recombinant strain exhibited higher maximum specific growth rate, as compared to the wild type strain, while cell yields of both strains were similar. The increase of the relative concentrations of glutamate and its derivatives when grown on glucose indicated that a more efficient nitrogen assimilation mechanism was taking place. The recombinant strain was more efficient in the production of ethanol from glucose and produced lower amounts of acetic acid. During growth on xylose the recombinant strain produced much higher cell yield compared to the wild strain and exhibited slightly higher maximum specific growth rate. The final cell concentration was doubled by the recombinant strain. Also higher concentrations of intracellular amino-n-butyric acid in the transformant strain were detected, which could be attributed to the increased activity of phosphoglucomutase which affect channeling glucose towards cell wall biosynthesis, glycolysis pathway and ethanol production. The transformed strain produced elevated rates of acetate and improved levels of ethanol.

### **Ethanol recovery in the consolidated process**

One main reason for the lack of commercial production of 2G bioethanol is the high production cost which is still significantly higher than the production cost of 1G ethanol based on sugar cane, corn and wheat, on which the production has expanded rapidly. As explained before, one of the major reasons for the higher conversion costs are due to the amount and high cost of enzymes required to produce fermentable sugars from lignocellulosic raw materials. The amount of enzymes needed in cellulose hydrolysis may be up to 100 times higher than the amount needed in the hydrolysis of starch.

The last part of the enzymatic release of fermentable sugars (the saccharification) normally takes place during fermentation where the fermenting organism (yeast) converts the sugars to ethanol simultaneously with the hydrolysis; i.e. in the simultaneous saccharification and fermentation process (SSF). The enzymes and not all fermenting organisms, unfortunately, do not stand high concentrations of ethanol, which means that when the ethanol concentration in the fermentation broth reaches 4-5% (w/v) (similar to normal beer), the release of sugars and production of ethanol slows down.

The anaerobic conversion of sugars to ethanol is an extremely energy efficient process, by which about 93% of the energy in the sugar ends up in ethanol. The drawback is that a substantial amount of energy is consumed to recover the ethanol. When the ethanol concentration in the broth at the end of fermentation is above 10% (w/v), typical for 1G bioethanol, the distillation costs are still reasonable. The cost of distillation starting at ethanol concentration from 4-5% (w/v), which is typical for 2G bioethanol, is, however, much higher. The traditionally low dry matter (DM) content of the 2G ethanol broth means that the dry matter content of the remaining solids after distillation (the stillage) will also be relatively low, resulting in high cost for concentration by evaporation to obtain marketable products.

The strategy of the project was to integrate the three processes: liquefaction (prehydrolysis), fermentation (SSF) and product recovery. The central element in this integration was introduction of a novel diabatic distillation and evaporation process driven by mechanical vapour recompression (MVR). The starting point for the previous development of late Danish innovator Erik Jensen. The crucial limitation inherent by this solution was that the capacity of the described apparatus was far too small to cope with the typical demand of 10-20 m<sup>3</sup>/h of ethanol in the fuel ethanol industry. To overcome this problem, it was decided at Holm to develop a modular system, where the individual modules could be delivered in standard 20 or 40 feet container frames as plug-and-play modules.

The next challenge at Holm was to optimize the capacity of the individual modules. This required development of a calculation model for approximation of the diabatic distillation processes. Models were available for optimal design of conventional distillation processes, but they could not be used for the horizontal diabatic process. Based on the novel calculation model, a significant increase in the capacity of the Erik Jensen apparatus of the same size was succeeded and at the same time, the ratio of investment to capacity was reduced. Furthermore, the specific energy consumption was reported to be reduced by about 50% compared with the traditional solution, through introduction of sequential multistage recompression of the vapour in the condensation/rectifier chamber, which in this case, was to be divided in

pressure-tight sections. Patent applications protecting the technology were filed.

The reduction of primary energy consumption when the three-stage MVR is exploited was 75-85% compared with the conventional adiabatic process depending on the consumption of live steam to trim the MVR process. The cost reduction depends on the ratio between the local electricity and steam prices.

The most important contribution of the novel distillation system to the reduction of production cost of 2G bioethanol is due to the ability of the novel distillation system to remove the ethanol from the fermentation broth in the stripper without any deactivation of enzymes, especially when the enzymes are characteristically thermally stable, as the enzymes of the Hype project. High recovery of enzymes when mimicking the stripping was demonstrated by Holm together with KU on an ethanol broth from Inbicon using both the mixture of thermostable enzymes from the Hype project and the commercial reference enzymes. The critical parameter was the surface temperature of the heat transmission wall on the stripper side. Ethanol concentration in the broth was also shown to affect the stability of the enzymes. Both enzyme preparations were less stable at higher ethanol concentrations, but the effect was less severe with the thermostable enzymes. There was no loss of activity of the thermostable enzymes at 60°C within 15 min., whereas a loss of 50% of the commercial reference enzyme activity was observed after 15 min. Alternatively, if the residence time is shorter in the stripper, the thermostable enzymes can tolerate a higher temperature, which will improve the efficiency of the stripper. The trials were conducted at close-to-industrial conditions where the residence time in the stripper would be less than 15 min. The thermostable enzyme mixture revealed to be superior to the reference enzyme by being far more stable at industrial relevant conditions.

Combined with results obtained at KU showing that up to 80% of the initial enzyme activity remained after the SSF and assuming that the enzymes can be used twice, the theoretical savings of external enzymes could be up to 40-45%. If the enzymes can be used more than twice, the savings can be even more significant. The simultaneous development of the thermotolerant cellulolytic enzymes and the novel energy efficient diabatic distillation system with multistage MVR have been shown to be very successful and can provide a valuable push for the commercialization of 2G bioethanol.

#### **Pilot and demonstration units of Inbicon**

Based on the IBUS process (developed under the EU 5th Framework Programme), Inbicon has built an advanced biorefinery at the port of Kalundborg in Denmark. In this biorefinery, Danish wheat straw is converted to second generation (2G) ethanol, lignin pellets and C5 molasses. The Danish Energy Agency has granted the design and construction phase and the European Commission's Seventh Framework Programme for Energy Research (FP7) has granted the commissioning and first three years of operation.

By the end of 2010 the demonstration plant has been totally commissioned and has gone into production phase. The first 2G ethanol has been sold to Statoil and is now distributed in 100 filling stations all over Denmark as Bio95 2G gasoline. Lignin pellets are sold to DONG Energy and used as high-quality solid biofuel in power plants. The C5 molasses is sold as

biogas booster in local biogas plants. The demonstration plant has proved continuous operation from straw bales to fermentation and the expected yield of 2G ethanol (greater than 198 dm<sup>3</sup> t<sup>-1</sup> dry straw). The process is developed, the products are on the market, lignocellulosic ethanol is reality, but as with almost any other new energy technologies, further policy and market incentives are still needed before investors will construct first full scale commercial plants.

In parallel to building and operating the demonstration plant, Inbicon has participated in the HYPE project. The Inbicon process proven in demonstration scale has in the HYPE project been used as a base case for developing a novel continuous consolidated bioprocess concept. In the ultimate consolidated bioprocess one organism produces the needed enzymes to break down cellulose and hemicellulose to monomer sugars and the same organism would also be able to ferment both C6 sugars from cellulose and C5 sugars from hemicellulose to ethanol.

In the HYPE project, Inbicon developed a steam pretreatment working at very high dry matter which produces lignocellulose slurry with high recovery of both C6 and C5 sugars. This was the basis of a consolidated bioprocess. The tested organism *F. oxysporium* was not yet able to act as an efficient enough consolidated organism producing high titers of enzymes or converting especially the C5 sugars with a high yield, to be interesting in commercial scale. The best consolidated process is presently found to be a combination of commercially available enzymes and yeast that can convert both C6 and C5 sugars to ethanol with a satisfying yield. Such a consolidated process is being implemented at Inbicon's demonstration plant in 2012-13. It is expected that the ethanol production will increase with 50% due to the upgrade.

### **Pilot-scale testing and feasibility**

**Pilot-scale testing and evaluation has been done in the Inbicon pilot plant, Skærbæk, Denmark.**

The testing included development of a pretreatment stage functioning at up to 50% (w/w) DM which is important to reduce the cost of pretreatment. Furthermore the pretreatment unit was rebuilt and optimized for producing a combined slurry of C6 and C5 ready for a consolidated bioprocess. The pilot testing also included hydrolysis at very high dry matter both with thermostable HYPE enzymes and commercial enzymes. The outcome of the pilot-scale testing was both a technical optimisation of process parameters and a set of technical parameters provided for the feasibility evaluation.

Based on the outcome of pilot testing and data gathered from all tasks of the project, feasibility studies have been conducted. A commercial Inbicon facility has been used as base case and comparator to the developed consolidated bioprocess. The base case facility converts 150,000 t (129,000 t DM) biomass/year corresponding to 20 t biomass (17,2 t DM)/h. The process is based on the configuration of Inbicon's demonstration plant in Kalundborg, and the process is modelled in an in-house developed model.

Main conclusions of the feasibility studies were that the feasibility of a consolidated process versus the base case showed improved feasibility if C5-fermentation at high consistency can be achieved. The main

sensitivity parameters in full scale plants are biomass cost, ethanol price and enzyme costs.

### **List of abbreviations**

At *Accremonium thermophilum*  
BG3  $\beta$ -glucosidase (of the glucohydrolase family 3)  
C5 sugars, pentose sugars (xylose, arabinose)  
C6 sugars, hexose sugars (glucose, mannose, galactose)  
CBH cellobiohydrolase  
CBM Carbohydrate binding module  
CBM+ Enzyme with a carbohydrate binding module  
CBM- Enzyme without a carbohydrate binding module  
CBP consolidated bioprocessing  
Cel7 cellulase of the glucohydrolase family 7  
Celluclast-Novozym 188 commercial reference cellulase preparation  
Ct *Chaetomium thermophilum*  
DM dry matter  
EG endoglucanase  
EGII/Cel5A endoglucanase II, cellulase of the glucohydrolase family 5A  
FPU filter paper activity  
1G ethanol, presently produced from raw materials suitable for food production  
2G ethanol, produced from non-edible lignocellulose raw materials  
IC50 Kinetic constant for enzyme inhibition  
IU International unit for enzyme activity ( $\mu\text{mol}/\text{min}$ )  
kcat katalytic coefficient describing the efficiency of an enzyme  
KU University of Copenhagen  
Mtoe million tons of oil equivalent  
MVR mechanical vapour recompression  
NTUA National Technical University of Athens  
PEG polyethylene glycol  
PWS pretreated wheat straw  
SHF separate hydrolysis and fermentation  
SSF simultaneous saccharification and fermentation  
Ta *Thermoascus aurantiacus*  
Thermomix is the thermostable enzyme preparation composed and used in the HYPE project  
Tr *Trichoderma reesei*  
UH University of Helsinki  
UT University of Tartu  
VTT Technical Research Centre of Finland  
WIS water insoluble solids  
Xyn11A xylanase of the glucohydrolase family 11A

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## **Potential Impact:**

### **4.1.4.1 The socio-economic impact and the wider societal implications of the project**

For 2020-30, the gasoline demand in Europe has been forecasted to be about 140 million tons. If the biofuels have to provide one fourth of the demand, the estimated production of biofuels should be around 35 Mtoe. For 2010, the corresponding policy target is 18 Mtoe for the transport sector. The European Environmental Agency has estimated the biomass potentials in the EU25 from 2010 to 2030. The biomass resource potential till 2010 is estimated at approximately 180 Mtoe. More than half is expected to derive from waste and residues from both agriculture and forestry and about 25% is expected from agricultural energy crops. The figures for 2020 and 2030 reach up to 239 and 316 Mtoe, respectively (44). It is estimated that between 4 and 13% of the total agricultural land in the EU would be needed to produce the amount of biofuels to reach the level of liquid fossil fuel replacement required for the transport sector in the Directive 2003/30/EC. Thus, the agricultural waste products (straw) and dedicated energy crops (sweet sorghum, reed canary grass) represent a considerable biomass potential and a raw material target for bioconversion processes, providing also jobs in the new agro-biorefinery sectors.

The development of biofuels in the transport sector has a strategic impact on key environmental issues, such as climate change and global warming and on local pollution in compliance with the Kyoto commitment. It will also enhance European security of energy supply thus reducing oil dependency and help sustainable rural economic development. Europe has a leading position in the production of biodiesel whereas the production of bioethanol is still low compared to North America and Brazil.

The role of ethanol may be as an intermediate term alternative fuel, but a higher share of liquid biofuels in gasoline will depend on the development and commercialisation of new 2nd generation biofuels. Ethanol is especially interesting also in short to medium term because it can be used as a blend with gasoline, either directly or as ETBE, in the existing vehicles and distribution systems. It can also be used as E85 in FFV vehicles and as neat fuel in city buses to improve the quality of life in urban areas. This project aimed at accelerating the commercialization of second generation ethanol from agricultural residues by designing and realizing a radically new process concept. Development of lignocellulosic ethanol is necessary for the EU to achieve the goals of the use of biofuels for transport. The possibility of producing low cost ethanol would have an economic impact and would strengthen the competitiveness of the European biofuel industry. A leading position in lignocellulosic ethanol would also favour European industry to deploy this technology to third countries using local low-cost raw materials, such as sugar cane bagasse.

The project contributed to the above mentioned aspects by providing the following major outcomes:

- Data on the use and pretreatment of various 2G raw materials for ethanol production
- Chemical data on the selected native and pretreated biomasses
- Optimization of novel pretreatment techniques at high solid concentrations

- Knowledge on new thermostable enzymes and discovery of enzymes enhancing the hydrolysis
- Understanding on the action mechanism of especially cellulases and accessory enzymes
- Novel concepts for reusing enzymes
- Optimal conditions for hydrolysis processes by novel enzymes
- Evaluation of consolidated processes
- New distillation techniques
- Pilot scale data on the performance of novel cellulases in consolidated processes
- Feasibility study on the novel process methods

The project supported especially European industry in the biorefinery area. The biotechnical industry is also highly important in Europe. About 90% of the industrial enzymes in the world market are produced by European companies. Biotechnology is also regarded as a potential growth sector. Thus, the development of novel bio-based processes to the traditional energy sector would improve the competitiveness and open up new large volume enzyme applications for the enzyme industry. This project contributed to strengthening the competitiveness of bio-energy related industry in Europe. New options for technology transfer between EU and countries interested in second generation biofuels production, such as Brazil and China will expectedly emerge. It is also essential to introduce and support new companies, especially small and medium-sized enterprises (SME)s like Holm and Biogold, as well as 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 industry are needed for developing future biorefineries for economical and sustainable production of fuels.

#### **4.1.4.2 The main dissemination activities**

The dissemination of the scientific results obtained in the project was accomplished through seminars, scientific and technical meetings and conferences, as well as through numerous publications of the research groups. So far the group has published 15 papers, submitted 9 further manuscripts in acknowledged scientific journals, and already filed 2 patents. Protection of results by filing of potential patent applications was always considered before publication and agreed by the PMC. Dissemination to wider public was through the website (Blog), non-scientific publications and presentations. The new Blog-type pages gained a significant number of positive comments also from readers outside the research community. Presentations (20) were given in international conferences and workshops, and disseminated also nationally. The project had also educational and training aspects and resulted in PhD and MSc degrees (total 6) and Post Doc visits. Thus, the project fulfilled both the scientific, technical and educational goals.

In the project technology-oriented and research-oriented partners from different countries were brought together. Without the successful collaboration, shared knowledge, resources and facilities the goals would not have been possible to obtain.

#### **4.1.4.3 Exploitation of results**

The exploitation issues within the project have been constantly reviewed and discussed in every project meeting, twice a year. In order to ensure efficient exploitation of the results, Jan Larssen from Inbicon was nominated as the exploitation manager for the HYPE project. The companies Inbicon, Biogold, Holm and ROAL are in first hand responsible for the exploitation of the obtained results and discoveries. At all stages of the implementation, the consortium has made best use of the exploitable results of the project, in particular those with a commercial potential, primarily through its own resources, and later, eventually by other external services. The developed technologies are expected to be exploitable in short or medium term. The consortium has already filed patents or is in the process of patenting the relevant technical and product innovations developed during the course of the project. The project has generated a significant amount of data, methods and enzymes which will be exploited by partners after the HYPE project has ended.

The companies will exploit the results in various ways. ROAL intends to exploit the results in the biomass business evaluations and benefits also the networking contacts. INBICON aims at developing technically and economically feasible processes for biomass processing and plans commercial exploitation of R&D results to improve the speed of commercialisation of lignocellulosic ethanol. HOLM intends to use the results in further development and commercialization of the multistage MVR distillation and evaporation system and diabatic stripper potentially combined with recycling of enzymes based on the IPR generated. BIOGOLD will exploit the new knowledge generated in the area of hydrothermal pre-treatment of various types of biomass: wheat straw, reed canary grass and hybrid willow in its further businesses. These exploitations have started and will continue in 2013. In addition, the research partners disseminate and exploit the results in their further research on the novel enzymes and organisms, as well as on hydrolysis and fermentation methods and techniques.

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List of Websites:

Website: <http://blogs.helsinki.fi/hype-project/>

**List of beneficiaries with the corresponding contact names:**

University of Helsinki: Liisa Viikari (liisa.viikari (at) helsinki.fi)

VTT: Matti Siika-aho (matti.siika-aho (at) vtt.fi)

ROAL Oy: Terhi Puranen (terhi.puranen (at) roal.fi)

University of Copenhagen: Henning Jorgensen (hnj (at) life.ku.dk)

Holm Christensen Biosystemer APS: Børge Holm Christensen (biosystemer (at) mail.dk)

National Technical University of Athens: Paul Christakopoulos (hristako (at) orfeas.chemeng.ntua.gr)

University of Tartu: Priit Väljamäe (priit.valjamae (at) ut.ee)

BioGold Oy: Priit Kotli (priit (at) biogold.ee)

Inbicon A/S: Jan Larsen (janla (at) dongenergy.dk)