INSIGHT INTO THE BIOMASS TO ETHANOL PROCESS WITH EMPHASIS ON CELLULASE PRODUCTION BY TRICHODERMA REESEI

PhD thesis
Miklós Gyalai-Korpos

2012
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TRICHODERMA REESEI

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Miklós Gyalai-Korpos

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Department of Applied Biotechnology and Food Science
Non-Food Research Group

2012
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- Ford Focus Flexi Fuel, © Miklós Gyalai-Korpos
- Bagasse of sweet sorghum variety 'Monori Édes', © Miklós Gyalai-Korpos
- *Trichoderma reesei* Δenv1 hyphae, courtesy of Monika Schmoll © Verena Seidl-Seiboth

Cover design by Lounge Design.
I’m really grateful to everybody (in Budapest, Vienna, Madrid etc., in the university and outside of it) who helped me in any way during my PhD years and after: finally the result is here, in your hands.

I beg your pardon for these short acknowledgments but as you may know, this thesis got prepared in a heavy schedule – and always the most important things remain last. So I rather missed out everybody than forgetting somebody…
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>4-O-Me-α-D-GlcPA</td>
<td>α-D-4-O-methylglucopyranosic acid</td>
</tr>
<tr>
<td>α-L-Araf</td>
<td>α-L-arabinofuranose</td>
</tr>
<tr>
<td>α-L-Fucp</td>
<td>α-L-fucopyranose</td>
</tr>
<tr>
<td>AMG</td>
<td>amylglucosidase</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>β-D-Galp</td>
<td>β-D-galactopyranose</td>
</tr>
<tr>
<td>β-D-Glcp</td>
<td>β-D-glucopyranose</td>
</tr>
<tr>
<td>β-D-Manp</td>
<td>β-D-mannopyranose</td>
</tr>
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<td>β-D-Xylp</td>
<td>β-D-xylopyranose</td>
</tr>
<tr>
<td>β-L-Arap</td>
<td>β-L-arabinopyranose</td>
</tr>
<tr>
<td>BGA</td>
<td>beta-glucosidase activity</td>
</tr>
<tr>
<td>BLR1/blr1</td>
<td>blue light regulator 1 protein/gene (of <em>Trichoderma</em>)</td>
</tr>
<tr>
<td>BLR2/blr2</td>
<td>blue light regulator 2 protein/gene (of <em>Trichoderma</em>)</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBH1</td>
<td>cellobiohydrolase 1</td>
</tr>
<tr>
<td>CIEMAT</td>
<td>Centro de Investigaciones Energéticas Medioambientales y Tecnológicas (energy research centre in Madrid, Spain)</td>
</tr>
<tr>
<td>DM</td>
<td>dry mass</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNS</td>
<td>dinitrosalicylic acid</td>
</tr>
<tr>
<td>EC</td>
<td>Enzyme Commission (number)</td>
</tr>
<tr>
<td>EGA</td>
<td>endo-glucanase activity</td>
</tr>
<tr>
<td>FAOSTAT</td>
<td>Statistical Database of Food and Agriculture Organization of the United Nations</td>
</tr>
<tr>
<td>FPA</td>
<td>filter paper activity</td>
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<tr>
<td>FPU</td>
<td>filter paper unit</td>
</tr>
<tr>
<td>GH</td>
<td>glycoside hydrolase</td>
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<tr>
<td>Glc</td>
<td>glucose</td>
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<tr>
<td>Glc2</td>
<td>cellobiose</td>
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<tr>
<td>GlcA</td>
<td>glucosamine</td>
</tr>
<tr>
<td>ha</td>
<td>hectare</td>
</tr>
<tr>
<td>HMF</td>
<td>hydroxymethylfurfural</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IEA</td>
<td>International Energy Agency</td>
</tr>
<tr>
<td>IU</td>
<td>international unit of enzyme activity</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>KSH</td>
<td>Központi Statisztikai Hivatal (Hungarian Central Statistical Office)</td>
</tr>
<tr>
<td>LHW</td>
<td>liquid hot water pretreatment</td>
</tr>
<tr>
<td>LIQ</td>
<td>liquid fraction of steam pretreated wheat straw</td>
</tr>
<tr>
<td>MFC</td>
<td>microbial fuel cell</td>
</tr>
<tr>
<td>NREL</td>
<td>National Renewable Energy Laboratory (Golden, CO, USA)</td>
</tr>
<tr>
<td>R&amp;D</td>
<td>Research and Development</td>
</tr>
<tr>
<td>REF</td>
<td>reference (sample/run)</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RS</td>
<td>reducing sugar</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SF</td>
<td>Solka Floc 200 FFC</td>
</tr>
<tr>
<td>SSF</td>
<td>simultaneous saccharification and fermentation</td>
</tr>
<tr>
<td>TRIS</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet light</td>
</tr>
<tr>
<td>WC-1/wc-1</td>
<td>white Collar 1 protein/gene (of Neurospora)</td>
</tr>
<tr>
<td>WC-2/wc-2</td>
<td>white Collar 2 protein/gene (of Neurospora)</td>
</tr>
<tr>
<td>WG</td>
<td>wheat grain</td>
</tr>
<tr>
<td>WIS</td>
<td>water insoluble solid</td>
</tr>
<tr>
<td>Xyl</td>
<td>xylose</td>
</tr>
<tr>
<td>XYLA</td>
<td>xylanase activity</td>
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</tbody>
</table>
ABSTRACT

Lignocellulosic materials, in the form of agricultural and forestry residues, are produced in abundant amounts worldwide. Furthermore, the cultivation of dedicated energy crops enables the possibility to expand production potentials. Thus, biomass could contribute to ease the ever growing energy demand of mankind, especially in the transport sector. However, in order to utilize the carbohydrate content of biomass for fuel ethanol production, a multi-steps process, consisting of pretreatment, hydrolysis and fermentation, must be applied. The scheme is well established but the process is not proven yet on an industrial scale, only a few demo and pilot plants exist. One of the barriers of the industrial-scale spread is the high price of cellulase enzymes needed for the hydrolysis.

In my PhD thesis I investigated the following aspects of the biomass-to-ethanol process: device development, feedstock suitability and cellulase production. Together with a company I constructed and statistically evaluated a device designed to track ethanol fermentation. By displaying gas volume formation on eight parallel channels simultaneously in real time, this device makes it possible to observe the effect of different experimental conditions and to study the kinetics of gas formation, in particular ethanol fermentation.

Another goal of my work was to prove the feasibility of ethanol production based on Hungarian sweet sorghum varieties. After different alkali pretreatments sweet sorghum bagasse could be effectively fermented to ethanol (69.9% conversion value after pretreatment with 2% NaOH at 25°C). Taking into account the ethanol potential of the juice too, contributing in 45%, an 8 300 L/ha yield could be calculated based on the most successful pretreatment.

One possibility for reducing enzyme cost is the use of inexpensive and process related carbon sources. Therefore, I aimed to introduce onsite available substrates for cellulase production by T. reesei. Side stream of pretreatment is the liquid fraction arising after the separation of the pretreated biomass slurry. It is composed of valuable carbohydrates along with compounds that are potentially toxic to microbes (mainly furfural, acetic acid and formic acid). After detoxification and dilution the liquid fraction of steam pretreated wheat straw was found to be a proper substrate for cellulase production resulting in a secreted enzyme mixture with large amount of xylanases. Moreover, supplementing this medium with ground wheat grain also carries apparent advantages by optimizing the enzyme mixture (BGA/FPA ratio close to 1) and...
provides the possibility for integration of second generation ethanol technologies with on-site cellulase production into wheat grain processing (first generation) ethanol factories.

I applied another approach to the liquid fraction of steam pretreated sweet sorghum bagasse; *T. reesei* was adapted to this carbon source. Pre-adaptation was carried out on agar plates containing different dilutions of liquid fraction. The inhibitory nature of liquid fraction and the adaptation ability could be demonstrated well by measuring the diameters of the colonies. The gained adaptation ability of the strains could be transferred into submerged media where these strains proved to be successful by means of reducing the prolonged lag phase caused by inhibitors as well as by higher final enzyme activity produced.

The light dependent regulatory manner of different pathways in *Trichoderma* reflects the different physiological requirements of growth on the surface or within the substrate. Since cellulase production is also affected by light, I aimed to exploit this possibility in up-scaled fermentation (30 liters). Some of the deletion mutants of *T. reesei* QM9414 modified in light signaling pathways secreted higher levels of activity than the wild type. Detailed results showed that there are different reasons behind enhanced activities. Deletion of *env1* resulted in the secretion of a more effective enzyme mixture, while deletion of *blr2* led to enhanced total protein secretion. Based on these findings it was assumed that the major components of the light response pathway (BLR1, BLR2 and ENV1) could be crucial regulators or checkpoints in (light dependent) production of extracellular plant cell wall degrading enzymes, even in the predominantly dark conditions of a steel fermenter. Although cellulase production by *T. reesei* is a well-studied process, the possibility laying in signaling pathway alteration to enhance process efficiency and strain improvement is only in its beginnings at best.
LIST OF PUBLICATIONS

This thesis is based on the following scientific papers, which are referred to by their roman numerals. The papers are appended at the end of the thesis. All the papers have been reprinted with the permission of the copyright holder.


Oral presentations


Gyalai-Korpos, M., Réczey K. Role of di- and polysaccharides in the bioethanol potential of sweet sorghum. (Di- és poliszacharidok szerepe a cukorcirok etanol potenciáljában.) *Workshop of the Polysaccharide-chemistry committee (MTA)* Budapest, Hungary November 7, 2007

Gyalai-Korpos, M., Kádár, Zs., Geier, J., Réczey, K. Possibilities to monitor ethanol fermentation in laboratory scale – introducing an online system. (Etanol fermentáció nyomon követésének lehetőségei laboratóriumi körülmények között – Fermentációs online monitoring rendszer bemutatása.) *Conference of Chemical Engineering ’07* Veszprém, Hungary April 25-27, 2007
Poster presentations


Gyalai-Korpos, M., Geier, J., Réczey, K. Tracking ethanol fermentation in laboratory scale. *Intensive Program in Biorenewables* Iowa State University, Ames, IA, USA June 3-15, 2009


**Conference proceedings**


**Other articles in Hungarian language**


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1. Introduction

Recent developments in the world oil market have demonstrated clearly that security of supply risks, the growing need for energy in developing countries and price volatility constitute a constantly increasing problem for the energy supply of transport. In the year 2009 93.5% of the energy used in transport was delivered by oil based products (IEA, 2011). Many prominent technologies including bioethanol production have been introduced in order to substitute oil at least partially and thus decrease the associated risks. However, many of these technologies are currently not mature for the market, mainly due to high production costs creating technical challenges in order to decrease them. Hence, more effort should be put into research and development of these technologies.

Bioethanol, when taking into account sustainability criteria, can be an outstanding energy carrier instead of petrol. Due to biofuel mandates and tax allowances, first generation bioethanol has gained some market penetration but also raised some serious debates related to land use and competition with food and feed. Contrarily to first generation ethanol based on starch (e.g. corn or wheat) or sucrose (e.g. sugar cane juice) containing feedstocks, second generation ethanol production is based on lignocellulosic residues. Lignocellulosic materials, in the form of agricultural and forestry residues, are produced in abundant amounts worldwide. Moreover, the amount can be further expanded by cultivating dedicated energy crops on marginal lands. However, because of the more resistant structure of lignocellulose, enzymatic hydrolysis must be preceded by pretreatment. The aim of the pretreatment is to set cellulose chains free of the lignin matrix to be available for cellulase enzymes and eliminate the other component of lignocelluloses, namely hemicelluloses (composed mostly of pentoses) in a soluble form (Alvira et al., 2010). Many process designs have been proposed for the biomass-to-ethanol process (Figure 1.). However, because of these multi-steps designs the related costs are too expensive for market introduction. Recently, good progress has been made to mainstream the technology by building pilot scale facilities (Winters, 2011).

A 2010 study of the Boston Consulting Group revealed three areas in which further efforts need to be done to reach cost competitiveness of lignocellulosic ethanol; these are reduction of enzyme cost, improvements in feedstock yield and quality, and the usage of the advantages of up-scaling (Balagopal et al., 2010). These priorities are in line with the recommendations drawn up by the IEA (Jagger, 2011).
This thesis concentrates on the utilization of whole sweet sorghum plant for bioethanol production and cellulase fermentation on diverse substrates by various mutant strains of filamentous fungus \textit{Trichoderma reesei}.

![Diagram of biomass-to-ethanol process](image)

**Figure 1. – Process design of the biomass-to-ethanol process (Gírio et al., 2010)**

- SHF: separate hydrolysis (of cellulose) and fermentation (of glucose)
- SHCF: separate hydrolysis and co-fermentation (of pentoses and hexoses)
- SSF: simultaneous saccharification and fermentation (of glucose)
- SSCF: simultaneous saccharification and co-fermentation (of pentoses and hexoses)
- CBP: consolidated bioprocessing (enzyme production, hydrolysis and fermentation of all sugars in a single step)

**Aims and outline of the thesis**

The thesis aims to investigate the following aspects of bioethanol production as earlier identified crucial points of commercialization:

1. I aimed the construction and evaluation of a new device that is capable to monitor ethanol fermentation on parallel channels in real time.

2. The goal of the work was to prove the feasibility of ethanol production based on Hungarian sweet sorghum varieties.

3. Related to cellulase enzyme production the work aimed to introduce onsite available substrates for cellulase production for \textit{T. reesei}.

4. A novel strain development method in order to enhance cellulase secretion is arising with the understanding of signaling pathways. The purpose was to upscale this promising approach by means of testing mutants of \textit{T. reesei} in laboratory fermenter.
2. Background

This section intends to give an insight into the current state of lignocellulose based ethanol production with main emphasis on feedstocks, pretreatments and enzyme production by *T. reesei*.

2.1 Overview of ethanol production

The basis of ethanol production is the fermentation of glucose and fructose to ethanol usually by baker’s yeast (*Saccharomyces cerevisiae*), in a process well-known by humankind for many thousand of years. Glucose can be obtained from a variety of raw materials: sugar feedstocks (e.g. sugar cane, sweet sorghum, sugar beet) contain it in the form of sucrose (a dimer of glucose and fructose), grains (e.g. corn, wheat) store it in the form of starch – an energy deposit to facilitate germination of seedlings, and biomass in general has it in the form of cellulose and to a lesser extent of hemicellulose – the structural polymers of plant cell wall. However, hemicellulose contains other types of sugars too that cannot be converted to ethanol by baker’s yeast but rather by other microbes (Gomez et al., 2008). Dependent on the raw materials, different processes are used to liberate glucose and produce ethanol. In case of sugar and starch feedstocks the technology, known as first generation is well established, while the process based on lignocellulosic biomass, called as second generation is currently on a pilot scale. As a consequence of the conscious ethanol policy in Brazil that was adopted in the 1970s, an expansion of sugar cane based ethanol production occurred leading to other economy wide benefits: by 2004 this ethanol became fully competitive with gasoline without government intervention (Goldemberg, 2007) and in 2009 more than 80% of Brazil automobile production was flexi-fuel vehicle (that can run on any blend of gasoline-ethanol) (Balat and Balat, 2009).

Nevertheless, because of the different boundaries, aspects and methodologies it is very difficult to provide an overall and objective comparison of the ethanol producing technologies. One should also notice that competitiveness of biofuels cannot be judged without taking into account the different subsidy schemes, the non-existing industrial scale experiences with lignocellulosics, as well as the utilization of process by-products (e.g. distillation heat provided by burning of bagasse). Therefore, comparisons must be handled with caution and with the minimal understanding of methodologies which is beyond the scope of this thesis.
However, one possible indicator that avoids the discussion of prices, is the EROI (Energy Return on Energy Invested) approach expressing the ratio of the usable acquired energy to the energy expended; one report suggests values of 0.8-10 and 0.8-1.6 for sugar cane based and corn based ethanol respectively (Murphy and Hall, 2010). But, these are also significantly lower than those of world oil production (EROI = 35) which has been, however, decreasing as resources deplete (Murphy and Hall, 2010).

After fermentation the usual ethanol concentration obtained in corn based industrial processes is in the range of 10-12% that must be further enhanced by downstream processing (distillation and dehydration) (Bothast and Schlicher, 2005). This concentration is influenced by the ethanol/sugar tolerance of the yeast strain applied: normal bakers’ yeast has a high tolerance up to 15% ethanol (Vertés et al., 2008). An early report indicates that above approximately 180 g/L glucose concentration the degree of utilization significantly drops (Gray, 1945). Modification of microbes (genetic engineering, new strains) or process based technologies, such as fed-batch and very high gravity (VHG) fermentation make it possible to obtain a higher ethanol concentration and thus to decrease water and energy demand of the process (Puligundla et al., 2011). In a recent review for different VHG fermentation experiments with osmotolerant strains and/or additives decreasing stress (osmoprotectants and nutrients), ethanol concentrations of 14-24 v/v% and 14-19 v/v% were reported for starchy and sugar substrates, respectively (Puligundla et al., 2011). When using lignocellulosic substrates it is not the yeast ethanol tolerance that limits but the possible concentration of the lignocellulosic substrate (viscosity, mass transfer problems). Nevertheless, from a techno-economic aspect the lower limit for ethanol concentration is 4%; below this there is a significant increase in energy demand, hence lignocellulosic biomass based process designs aim to reach this limit (Erdei et al., 2010).

### 2.1.1 Structure of lignocellulosic biomass

The real potential in second generation is that a wide range of abundant raw materials can be used from dedicated energy plants (Somerville et al., 2010) to residues of agriculture (Kim and Dale, 2004). Lignocellulose is composed of three polymers, namely cellulose, hemicellulose and lignin. Cellulose chains through hydrogen bonds form microfibrils giving the resistance of crystalline cellulose and also the structural base for plant cell wall. The microfibrils are linked by hydrogen bonds and are embedded into the hemicellulose matrix that maintains flexibility. With aging of the plant this whole structure is getting
coated by lignin in order to be waterproof, rigid and resistant to microbes and chemicals (Gomez et al., 2008). These microfibrils are further organized to form macrofibrils (Figure 2.). The ratio of these ingredients varies from species to species, and even with age of the plant as cell wall types change; in general cellulose fraction is the most dominant one with 40.6-51.2% of the wall material, hemicelluloses represent 28.5-37.2% followed by lignin with 13.6-28.1% (Pauly and Keegstra, 2008). However, in some cases hemicelluloses can even account up to 50% of the cell wall (Gírio et al., 2010).

Figure 2. – Structure of lignocellulose (Rubin, 2008)

Hemicellulose and cellulose are carbohydrate polymers and thus possible subjects of ethanol production. Cellulose is a linear chain homopolymer of gluoses with β-1,4 bonds where each glucose is inverted compared to its neighbor (Gomez et al., 2008). Hence, the real repeating unit of cellulose is cellobiose. Along the chain where the ordering of gluoses falters amorphous regions occur giving place for easier enzyme attack (Gomez et al., 2008).

In contrast to cellulose, hemicellulose is not a chemically well defined compound but rather a family of polysaccharides in highly branched structures (Rubin, 2008). Hemicellulose may contain pentoses (β-D-xylose, α-L-arabinose), hexoses (β-D-mannose, β-D-glucose, α-D-galactose, α-L-rhamnose,
α-L-fucose), and uronic acids (α-D-glucuronic, α-D-4-O-methylgalacturonic and α-D-galacturonic acids). Moreover, the hydroxyl groups of sugars can be partially substituted with acetyl groups (Gírio et al., 2010). Depending on the plant variety, these compounds form different polysaccharides of which xylan backbones with different chains are the most common ones (mainly in hardwoods and herbaceous plants) (Gírio et al., 2010) (Table 1). Side chains are usually composed of sugars and uronic acids, but in arabinoxylans the arabinosyl residues are often esterified with ferulic acid residues (Lindsay and Fry, 2008). Noteworthy, xylan homopolymers are present only in algae (Gírio et al., 2010). In softwoods, mannan based hemicelluloses (glucomannans and galactoglucomannans) are the dominant ones (Gírio et al., 2010).

**Table 1. – Main types of hemicellulose polymers, adapted from Gírio et al., 2010**

<table>
<thead>
<tr>
<th>Type</th>
<th>Plants (amount, % of DM)</th>
<th>Backbone</th>
<th>Side chains</th>
<th>Linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinogalactan (AG)</td>
<td>Softwoods (1-3%, in some species up to 35%)</td>
<td>β-D-Galp</td>
<td>β-D-Galp α-L-Araf β-L-Arap</td>
<td>β-1,6 α-1,3 β-1,3</td>
</tr>
<tr>
<td>Xyloglucan (XG)</td>
<td>Hardwoods, grasses (2-25%)</td>
<td>β-D-Glc (β-D-Xylp)</td>
<td>β-D-Xylp β-D-Galp α-L-Araf α-L-Fuc β-1,4 α-1,3 β-1,2 α-1,2 Acetyl</td>
<td></td>
</tr>
<tr>
<td>Glucomannan (GM)</td>
<td>Softwoods, hardwoods (2-5%)</td>
<td>β-D-Manp β-D-GlcP</td>
<td>β-D-Galp Acetyl</td>
<td>α-1,6</td>
</tr>
<tr>
<td>Galacto-GM (GGM)</td>
<td>Softwoods (10-25%)</td>
<td>β-D-Manp β-D-GlcP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucuronoxylan (GX)</td>
<td>Hardwoods (15-30%)</td>
<td>β-D-Xylp</td>
<td>4-O-Me-α-D-GlcP Acetyl</td>
<td>α-1,2</td>
</tr>
<tr>
<td>Arabino-GX (AGX)</td>
<td>Grasses, cereals, softwoods (5-10%)</td>
<td>β-D-Xylp</td>
<td>4-O-Me-α-D-GlcP β-L-Araf</td>
<td>α-1,2 α-1,3</td>
</tr>
<tr>
<td>Arabinoxylans (AX)</td>
<td>Cereals (up to 30%)</td>
<td>β-D-Xylp</td>
<td>α-L-Araf Feruloy</td>
<td>α-1,2 α-1,3</td>
</tr>
<tr>
<td>Glucuronoxylans (GAX)</td>
<td>Grasses and cereals (15-30%)</td>
<td>β-D-Xylp</td>
<td>4-O-Me-α-D-GlcP Acetyl</td>
<td>α-1,2 α-1,3</td>
</tr>
<tr>
<td>Homoxylans (X)</td>
<td>Algae</td>
<td>β-D-Xylp</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Lignin is a complex, non-sugar polymer coating the other two polymers of cell wall and thus delivering recalcitrance towards microbiological attacks (Gomez et al., 2008). The main building blocks of lignin are three different phenolic monomers (p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) which can form a wide variety of bondings such as β-aryl ethers, phenylcoumarans, resinols, biphenyls and biphenyl ethers (Ralph et al., 2004). Its structural qualities – aromaticity, structural heterogeneity and extensive carbon-carbon crosslinking – make it the most recalcitrant component of the plant cell wall (Chang, 2007).

Because of its non-sugar base lignin cannot be fermented to ethanol, but it can contribute to the energy balance of ethanol production or other related processes in another way. Pulping residues are typically burned to generate electricity and heat for running refineries (Chang, 2007).

2.1.2 Sweet sorghum – a candidate for sustainable bioethanol plant

Sweet sorghum is a sugar cane-like C4 plant, containing juice in the stem with large amount of sucrose (11-23% Brix, depending in variety and conditions (Almodares and Hadi, 2009; Byrt et al., 2011)) that can be effectively extracted by squeezing and thereafter readily fermented to ethanol by baker’s yeast. The leftover, built up of lignocellulose, is called bagasse similar to sugar cane processing. Traditional uses of the juice include production of syrup, alcoholic beverages, crystalline sugar and in some regions stalks are also consumed fresh (Saballos, 2008). Besides these uses another possibility is gaining growing attention: to cultivate it as energy crop for bioethanol production (Almodares and Hadi, 2009; Byrt et al., 2011, Ratnavathi et al., 2011). Ethanol yields of 2 100-8 000 L/ha have been reported with one harvest annually (Byrt et al., 2011) that significantly exceed the ethanol yields from starchy materials, such as corn and wheat grain (Balat and Balat, 2009). Furthermore, when comparing the energy performance of wheat and sweet sorghum monocultures a significantly higher net energy gain for sweet sorghum was demonstrated in Northern Italy (Monti and Venturi, 2003).

2.1.2.1 Origin and cultivation of sweet sorghum

Cultivated sorghum (Sorghum bicolor spp. bicolor (L.) Moench) is in the subgenus Sorghum and originates from semi-arid regions of Africa. However, due to its adaptive capacity, nowadays it is cultivated on a wide spectrum of climates on every continent except Antarctica because of their (Saballos, 2008). Domestication of sorghum species started already at least 5000 years ago
Sweet sorghum varieties belong to the above domesticated species together with grain, forage and broomcorn sorghums (Murray et al., 2009). These names describe well the diversity of phenotypes, as well as the aim and direction of selection – sweet sorghum has been selected for accumulation of high amount of sucrose in the stem. Nevertheless, this close relationship also means that there are no discrete objective criteria to differentiate sweet sorghums from grain sorghums: the most obvious phenotypic difference, juicy versus dry stem is controlled by a major gene (Murray et al., 2009). After genetic investigation of different sweet sorghum varieties Murray et al. classified sweet sorghums into the groups of syrup (historical and modern) types, modern sugar/energy types and amber types (Murray et al., 2009).

In contrast to sugar cane the cultivation of sweet sorghum is also possible in temperate climates, however, like an annual crop, with one harvest per year – frost terminates the growth of the plant. Tropical regions allow for a second harvest too (Saballos, 2008). In the former case, the cultivation period is between April and September-October when sugar content of the stem peaks. Cultivation of sweet sorghum possesses the following advantages arising from the physiology and biochemistry of C4 plants that usually can generate high biomass yield with minimal inputs (list based on Rooney et al., 2007; Saballos, 2008 and Byrt et al., 2011):

- high conversion efficiency of light into biomass (biomass yields competing with switchgrass and miscanthus) resulting in high sugar, and thus ethanol yields;
- high water use efficiency and thus low water requirement that is 25% of that needed for sugar cane and 50-66% needed for maize production (no irrigation);
- drought tolerance – even though drought leads to reduction of plant growth, enhanced accumulation of sucrose and starch was observed in drought-stressed stems and thus resulting in equal sugar yields (Massacci et al., 1996);
- reduced demand for fertilizer due to the high leaf nitrogen use efficiency and large fibrous root system;
- modest demand for soil quality (that are not appropriate for corn or wheat) – high tolerance towards salinity and water-logging;
- pest and disease management is less complex, usually good cultural practices prove to be enough.

These properties allow greater tolerance towards consequences of climate change (temperature extremities, droughts). For sorghum crops higher temperatures and higher atmospheric level of CO₂ may become beneficial by means of biomass yield (Prasad et al., 2009, Allen et al., 2011). On the other hand cultivation of sweet sorghum is possible also on marginal lands and therefore it can contribute to sustainable ways to produce bioethanol by, for instance, avoiding the food versus fuel debate often related to bioethanol production. Besides other C4 crops such as switchgrass and sugar cane, sweet sorghum is somehow overlooked, however, its untapped genetic diversity as presented by its viability in almost every climate condition, carries enormous potential for further breeding (Rooney et al., 2007; Saballos, 2008).

2.1.2.2 Ethanol production based on sweet sorghum

In Asia (China, India and the Philippines) and South America fermentation of sweet sorghum juice is carried out on a small to medium scale (Saballos, 2008). Contrarily, the conversion of sweet sorghum bagasse to ethanol is still in an experimental phase (Ballesteros et al., 2004; Sipos et al., 2009; Li et al., 2010; Yu et al., 2010; Shen et al., 2011; for review see Ratnavathi et al., 2011). Economic evaluation of a sweet sorghum biorefinery for ethanol production from bagasse has been studied under North Chinese circumstances (Gnansounou et al., 2005), while the economy of juice processing has been investigated more deeply, for example, in contexts of India (Prasad et al., 2007), upper Midwest of the USA (Bennett and Anex, 2009), Zimbabwe (Woods, 2001) and Inner Mongolia (Wang and Liu, 2009).

Under moderate climate the technological difficulty of sweet sorghum processing is the short harvest period making the juice available only for 1-2 months annually: the juice cannot be stored because the microbes including its natural microbial flora degrade the easily fermentable sugar content (Daeschel et al., 1981). Without any preservation up to 12-30% of the fermentable sugar content can be lost in 3 days and 40-50% in a week at room temperature (Wu et al., 2010). Many methods have been proposed to elongate the availability of the juice, for instance: refrigerating (Wu et al., 2010), evaporation (Hodúr et al., 2008), ensiling the whole stalks (Bennett and Anex, 2009), proper harvest and processing method (Lingle et al., 2012) and lowering the pH with the addition of different acids (Hodúr et al., 2008; Feczák personal communication).
However, one must note that storage may lead to elevated energy and/or chemicals needs also influencing process economy. It is worth to note that according to the findings of Bennett and Anex (2009) seasonal fermentation of sweet sorghum juice is competitive with corn based ethanol process, however, when considering the effect of storage by ensiling the whole stalks this benefit does not exist anymore. The papers of Bennet and Anex (2009) and Gnansounou et al. (2005) suggest that fermentation of sweet sorghum juice under moderate climate could only be a complementary process, for example, in a biorefinery concept. The utilization of bagasse and any other lignocellulosic residues could balance the annual short availability of the juice meaning also a great opportunity for integration of first and second generation technologies.

With a view to Hungary, different cultivars of sweet sorghum, especially the ones that have been bred in Hungary (such as Monori Édes, Berény, Róna) and adapted to local environmental conditions, can be sustainable solutions for rural fuel supply. In field trials juice yields of 25-50 t/ha with 10-20% sugar content have been presented even under non-favorable conditions (Antal et al., 2008; Blaskó et al., 2008; Feczák personal communication).

### 2.1.3 Wheat grain and straw

Wheat is one of on the largest area and in amount cultivated grains, in 2010 globally on 217 million ha yielded 651 million metric tons of grain (FAOSTAT website\(^1\)). Wheat grain is mostly produced for food purposes. In context of ethanol production, after liquefaction the starch content of the grain is digested with amylases to liberate glucose fermentable to ethanol by baker’s yeast. Based on literature survey an average net energy balance of 2.17 was calculated for wheat grain ethanol under European circumstances (Gnansounou and Duriat, 2005). However, lively debates have been going on about issues related to first generation ethanol production (i.e. land use change, water use, food supply) often with contradictory results (Dale, 2008; Pimentel et al., 2008; Dominguez-Faus et al., 2009). Consequently, serious concerns have been raised about the sustainability of first generation ethanol produced on food grains (Somerville, 2007; Zhang and Long, 2010).

This background urges us to explore ethanol production based on byproducts and to seek the transition and integration possibilities of first and second generation technologies with making use of already operating infrastructures

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\(^1\) Crops production data, Area harvested, Production – accessed February 20, 2012
Wheat straw, as a byproduct of wheat harvesting, is available in an abundant amount. Considering a 1.3:1 residue to wheat grain ratio on dry weight basis² (Milbrandt, 2005) and that approximately 30-40% of straw is left on the field to prevent erosion, near 450 million metric tons of wheat straw could be collected. In 2010 3 745 190 metric tons of wheat grain was harvested in Hungary (KSH website³) which – applying the above calculation – is equal to more than 2.5 million metric tons of processable wheat straw. In line with this enormous theoretical potential, some second generation ethanol demonstration plants have already started to process wheat straw including Iogen in Canada (Solomon et al., 2007) and Inbicon in Denmark (Inbicon website⁴).

2.2 Pretreatment and utilization of pretreated materials

Pretreatment is necessary to disrupt the resistant lignocellulose matrix consisting of cellulose, hemicellulose and lignin. The main aim of pretreatment is to make the cellulose surface accessible for cellulase enzymes during the subsequent hydrolysis by means of removing lignin coating and loosening cellulose fibrils (Alvira et al., 2010). In turn, cellulases and other accessory enzymes liberate monomeric sugars, mostly glucose further subjected to fermentation usually by baker’s yeast. Pretreatment technologies as a crucial step for biomass-to-ethanol process have been extensively studied and reviewed during the years (Mosier et al., 2005; Yang and Wyman, 2008; Hendriks and Zeeman, 2009; Nigam et al., 2009; Alvira et al., 2010; Agbor et al., 2011; Balat, 2011; Tomás-Pejó et al., 2011). As a result of this many biological, chemical, physical and mixed methods have been identified and further developed; some of them have reached the maturity to be applied in pilot plants (Table 2.). In every case pretreatment is preceded by mechanical comminution to reduce particle size and increase specific surface.

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² moisture content of wheat grain is 13.5% (Milbrandt, 2005)
⁴ Biomass refinery, Feedstocks – accessed February 20, 2012
Table 2. – Non-exhaustive summary of pretreatment methods, adapted from Tomás-Pejó et al., 2011.

<table>
<thead>
<tr>
<th>Method and principle</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milling</td>
<td>Reduces cellulose crystallinity</td>
<td>High energy consumption</td>
<td></td>
</tr>
<tr>
<td>Concentrated acid: soaking in acids (either organic and inorganic) on different temperatures and residence times</td>
<td>High glucose yield Ambient temperatures also effective</td>
<td>High cost of acid and its recovery Corrosion problem Formation of inhibitors</td>
<td>BlueFire Ethanol (USA) Weyland AS (Norway)</td>
</tr>
<tr>
<td>Diluted acid: soaking in acids (either organic and inorganic) on different temperatures and residence times</td>
<td>Less corrosion problem than with concentrated acid Less formation of inhibitors Hemicellulose solubilization</td>
<td>Generation of degradation products Low sugar concentration in exit stream</td>
<td>SEKAB (Sweden)</td>
</tr>
<tr>
<td>Alkaline: soaking in alkalines (mostly NaOH, KOH, Ca(OH)₂) on different temperatures and residence times</td>
<td>Effective lignin and hemicellulose solubilization Ambient temperatures</td>
<td>Alkali removal Efficiency depends on lignin content Possible loss of sugars and formation of inhibitors</td>
<td></td>
</tr>
<tr>
<td>Organosolv: using organic or aqueous solvents (such as ethanol, acetone, glycerol…) on elevated temperature</td>
<td>Lignin and hemicellulose hydrolysis Highly digestible cellulose Recovery of lignin possible</td>
<td>High cost Solvents need to be drained and recycled Significant amount of inhibitors</td>
<td>Lignol Energy Corporation (Canada)</td>
</tr>
<tr>
<td>Ozonolysis: applying ozone usually at atmospheric pressure and room temperature</td>
<td>Reduces lignin content No generation of toxic compounds No effect on cellulose</td>
<td>High cost of large amount of ozone</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. – Continued

<table>
<thead>
<tr>
<th>Method and principle</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet oxidation: an oxidative pretreatment applying oxygen or air as catalyst</td>
<td>Efficient removal of lignin</td>
<td>High cost of oxygen and alkaline catalyst</td>
<td>Risö (Denmark) (laboratory scale)</td>
</tr>
<tr>
<td></td>
<td>Solubilization of hemicellulose but low formation of inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Minimizes the energy demand (exothermic)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liquid Hot Water (LHW): uses water at high pressure to keep it in liquid state at elevated temperatures</td>
<td>Requires no catalyst and chemicals</td>
<td>High water demand</td>
<td>Inbicon (Denmark)</td>
</tr>
<tr>
<td></td>
<td>Low-cost reactor</td>
<td>High energy requirements</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemicellulose is solubilized</td>
<td>Low-solids processing during pretreatment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reduced concentration of degradation products</td>
<td>Lignin not affected significantly</td>
<td></td>
</tr>
<tr>
<td>Ammonia Fiber Explosion (AFEX): uses liquid anhydrous ammonia at moderate temperatures and high pressure with varying residence times</td>
<td>Increases accessible surface area</td>
<td>Not efficient for raw materials with high lignin content</td>
<td>DuPont Danisco Cellulosic Ethanol (USA)</td>
</tr>
<tr>
<td></td>
<td>Low formation of inhibitors</td>
<td>High cost of large amount of ammonia</td>
<td></td>
</tr>
<tr>
<td>Steam explosion</td>
<td>Lignin transformation and hemicellulose solubilization</td>
<td>Generation of toxic compounds</td>
<td>Abengoa Bioenergy (Spain) Iogen (Canada)</td>
</tr>
<tr>
<td></td>
<td>Cost-effective</td>
<td>Partial hemicellulose degradation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Higher yield of cellulose and hemicellulose in the two-step method</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. – Continued

<table>
<thead>
<tr>
<th>Method and principle</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supercritical fluids: mostly applying supercritical CO₂</td>
<td>Increases accessible surface area</td>
<td>Very high pressure requirements</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cost-effective</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No generation of toxic compounds</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lignin removal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ionic liquids: salts with low melting point and thus liquids in low temperatures (usually large organic cation with small inorganic anion)</td>
<td>Break down the hydrogen bonds causing solubilization and reducing cellulose crystallinity</td>
<td>Recycling is cost and energy intensive</td>
<td>Possible toxicity to enzymes and microbes</td>
</tr>
<tr>
<td></td>
<td>No toxic or explosive gases are formed</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Minimal formation of degradation products</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biological: employing microbes mainly brown, white and soft rot fungi</td>
<td>Degrades lignin and hemicellulose</td>
<td>Requires long incubation times</td>
<td>Requires careful control of growth conditions</td>
</tr>
<tr>
<td></td>
<td>Low energy consumption and capital cost</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No chemicals and mild conditions</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No inhibitor formation</td>
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<td></td>
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</tbody>
</table>

Nowadays, steam explosion is one of the most widely employed pretreatment technologies. Steam explosion is a hydrothermal physical-chemical process that applies severe pressure and temperature conditions for an exact time after that the pressure is rapidly expanded to atmospheric causing destruction in the lignocellulosic structure (Alvira et al., 2010). Because of the harsh conditions, some components are removed from the matrix and even further degraded. Partial hydrolysis and solubilization of the hemicellulose fraction occur (Jorgensen et al., 2007), and therefore, these substances remain in liquid
fraction, also referred as the hydrolyzate, after separation of the pretreated slurry. Lignin is also redistributed and removed to some extent (Tomás-Pejó et al., 2011). The breakage of useful sugar molecules and other bonds creates soluble compounds with inhibitory effect towards microbial growth and thus affecting the subsequent steps negatively (Palmqvist and Hahn-Hägerdal, 2000a; Palmqvist and Hahn-Hägerdal, 2000b; Zaldivar et al., 2001). Importantly, the levels of inhibitors depend on substrate composition and pretreatment conditions (Palmqvist and Hahn-Hägerdal, 2000a). After pretreatment the slurry is either used without separation or separated to a fiber fraction containing cellulose that is further exposed to enzymatic hydrolysis and a liquid fraction containing soluble sugars and inhibitory compounds.

2.2.1 Possible utilizations of the liquid fraction

Although the inhibitory property of the liquid fraction makes further utilization difficult, it is of interest because of its high sugar content – mostly oligomeric forms of pentoses – available onsite. Probably the most investigated alternative involves producing ethanol from the sugar content of the liquid fraction by naturally occurring yeasts (e.g., *Pichia stipitis*) or engineered bacteria or yeast strains (e.g., *Escherichia coli* or *S. cerevisiae*) which are able to convert both hexoses and pentoses simultaneously to ethanol (Olsson and Hahn-Hägerdal, 1996; van Maris et al., 2006). This makes it possible to enhance overall ethanol yield of the process. Hence, extensive work has been done on developing inhibitor tolerant and pentose utilizing yeast strains (Hahn-Hägerdal et al., 2007; Chandel et al., 2011a; Kuhad et al., 2011; Dumon et al., 2012). However, microbial pentose utilization is not limited to ethanol formation: xylitol, a sweetener, production by different yeast strains has also been examined using xylose rich hydrolyzates as substrate (Parajó et al., 1998; Sene et al., 2001; Silva and Roberto, 2001; Huang et al., 2011).

Biogas (Kaparaju et al., 2009a; Wang et al., 2009) and biohydrogen (Kaparaju et al., 2009b) productions have also been reported on hydrolyzate of hydrothermally treated wheat straw. The liquid fraction of steam-pretreated willow was investigated for β-glucosidase production as carbon source for different *Aspergillus* and *Penicillium* strains (Réczy et al., 1998).

Attempts have been made to employ this fraction for onsite cultivation of *T. reesei* in order to produce cellulase enzymes (Szengyel et al., 1997; Réczy et al., 1998; Hayward et al., 1999; Bigelow and Wyman, 2002; Lo et al., 2005), additionally, during enzyme production *T. reesei* is able to consume some
inhibitors in low concentration under certain conditions (Palmqvist et al., 1997; Larsson et al., 1999; Szengyel and Zacchi, 2000). However, these efforts with *Trichoderma* have mostly failed when the total concentration of the inhibitors exceeded the level of tolerance of *T. reesei*. Even so, application of the liquid fraction for enzyme production is advantageous in the biomass-to-ethanol process economy: based on techno-economic simulation it has already been concluded that utilization of the liquid fraction can be a more feasible option than purchased enzymes (Barta et al., 2010).

### 2.2.2 Properties of the liquid fraction

Effects and mechanisms of inhibition have been investigated in different, mostly ethanologenic yeasts and bacteria (Palmqvist and Hahn-Hägerdal, 2000b; Klinke et al., 2004; Almeida et al., 2007; Almeida et al., 2009; Taherzadeh and Karimi, 2011) but harmful effects have been reported for the filamentous fungus *T. reesei* too (Szengyel and Zacchi, 2000). In many cases the exerted inhibitory effect does not only depend on the individual concentration of each inhibitor but also on interactions among them (Mussatto and Roberto, 2004; Almeida et al., 2007; Tomás-Pejó et al., 2011). The inhibitors based on their chemical structure and origin can be divided into three groups (list based on the reviews of Taherzadeh and Karimi, 2011 and Tomás-Pejó et al., 2011):

1. **Furans**: Predominant degradation products present in the liquid fraction of steam explosion are derivates of furan, namely hydroxymethylfurfural (HMF) and furfural, derived from hexoses and pentoses, respectively, by dehydration in the presence of acids. Hence, they are unavoidable in those high temperature lignocellulose pretreatments where acids are present. Inhibitory effects of furfural depend on concentration and strain used, but in general concentration of 1 g/L and beyond influences many bacteria, yeasts and filamentous fungi negatively by, for instance, decreasing growth rate and enlarging lag phase. However, cells, including yeasts and *T. reesei* can tolerate and even decompose furan to furfuryl alcohol and/or furoic acid which are less toxic (Taherzadeh et al., 1999; Szengyel and Zacchi, 2000). HMF has been reported less severe inhibitor with mainly causing prolongation of lag phase but in high concentrations can completely abolish cell growth. Bioconversion of HMF can also occur but slower than that of furfural.
2. Carboxylic acids: Further breakdown of furan and HMF leads to formic and levulinic acid formation. Another aliphatic acid, acetic acid, originates from acetyl groups of hemicellulose. Usually acetic acid is present in the highest concentration followed by formic and levulinic acid. Besides this, the presence of other acids is also possible depending on pretreatment conditions. The inhibitory effects of these acids depend on their dissociation properties (pKa value) and thus on the pH of the medium. Undissociated carboxylic acids can diffuse through the cell membrane and inside of the cell, due to the higher inner pH, they dissociate. This leads to decreasing inner pH; hence the cell tries to get rid of protons by operating pumps using a lot of ATP that with overcoming pumping capacity can lead to cell death. With this surplus energy demand no other process can obtain sufficient energy, resulting in lower yields. However, if acids only in small concentration are present the phenomenon detailed above can act as a stimulant by increasing ATP production. Due to the lower pKa value formic and levulinic acids are more toxic than acetic acid. Moreover, the smaller molecular size of formic acid and the highly hydrophobic nature of levulinic acid make it easier to penetrate through cell membrane.

3. Phenolic compounds: A wide range of phenolic compounds (aldehydes, ketones and acids) derived from lignin degradation are usually also detected in liquid fraction in lower concentrations. However, some phenolic compounds may originate from extractives of plant cell wall. Main compounds are 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, vanillin, vanillic acid, syringaldehyde, syringic acid, cumaric acid, ferulic acid and cathecol but a lot of other phenolic degradation products remain still unidentified. The reason for toxicity is their influence on the integrity of biological membranes resulting in declining growth rate; however, it is hard to define an exact inhibition mechanism. Toxicity increases with decreasing molecular weight.

Based on many studies the following order of inhibition strength has been proposed: phenolic compounds, particularly vanillin, were found to be the inhibitoriest, followed by furans and acids (Chandel et al., 2011b). Because of the complex effect of pretreatment and the variable background matrix of raw material and liquid fraction many degradation products cannot be identified accurately (Tomás-Pejó et al., 2011). Therefore, besides these main inhibitors (Figure 3.) other toxic compounds may be present in the liquid fraction. Recently, the role of glycolaldehyde, the simplest sugar of two carbons, was
investigated. It was found that it may be the main substance in the liquid fraction causing inhibition since the concentration in LHW pretreated biomass (up to 24 mM) is much higher than that needed for severe inhibition in yeast (1-10 mM) (Jayakody et al., 2011).

Figure 3. – Inhibitors formation during pretreatment of biomass (Almeida et al., 2007)

In addition to microbes inhibitors have also potential negative effect on cellulase enzymes. The same inhibition strength was reported on commercial enzyme preparation as for microbes, in declining order: lignin derivates (i.e. phenolics), furans and least inhibitory effects were found for acids (Jing et al., 2009). In line with this finding, phenolic compounds were found to inhibit cellulase enzymes significantly in the hydrolysis step (Kim et al., 2011; Ximenes et al., 2011). Cantarella et al. (2004) investigated the effects of different inhibitors individually: acetic acid (2 g/L), small amounts of furfural, HMF, syringaldehyde, 4-hydroxybenzaldehyde, and vanillin (0.5 g/L) did not significantly affect cellulase activity, whereas high concentrations of formic acid (11.5 g/L) inactivated and of levulinic acid (29.0 g/L) partially affected cellulases.

2.2.3 Detoxification methods

A number of methods have been proposed in order to decrease the concentration of inhibitors. These methods can be classified into biological, chemical and
physical ones, often applied also in combination (Mussatto and Roberto, 2004; Chandel et al., 2011b). However, it is difficult to compare the effect of these methods due to differences of raw materials and pretreatment conditions, as well as the circumstances of subsequent utilization: type of microbes, their adaptation and tolerance. As a general result of detoxification, compared to non-detoxified hydrolyzates, kinetics of the microbial reaction will improve and thus, yield and productivity will increase (Mussatto and Roberto, 2004). However, because of the severity of some methods or due to the consumption by microbes some loss in sugar concentration also occurs. A short summary is given in Table 3, concentrating on removal of compounds as determined by the principles of each method (based on the reviews of Mussatto and Roberto, 2004; Chandel et al., 2011b).

<table>
<thead>
<tr>
<th>Method</th>
<th>Removed compounds</th>
<th>Main properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic</td>
<td>Phenolics</td>
<td>Addition of laccase and peroxydase enzymes from white rot fungi</td>
</tr>
<tr>
<td>In-situ microbial detoxification</td>
<td>Furfural, HMF, acids, phenolics</td>
<td>Utilization of different microbes to metabolize inhibitors</td>
</tr>
<tr>
<td>Vacuum evaporation</td>
<td>Volatile compounds (acetic acid, furfural, vanillin)</td>
<td>Volatile compounds are stripped out but because of volume loss non-volatile ones are concentrated</td>
</tr>
<tr>
<td>Membrane separations</td>
<td>Acetic acid</td>
<td>Liquid fraction is pumped through an adsorptive membrane</td>
</tr>
<tr>
<td>Overliming</td>
<td>Furans and phenolics compound</td>
<td>Addition of Ca(OH)₂ to adjust the pH to 10 causing precipitation of gypsum</td>
</tr>
<tr>
<td>Ion exchange resins</td>
<td>Phenolics, acids, furfural</td>
<td>Treatment with anion exchange resin.</td>
</tr>
<tr>
<td>Activated charcoal</td>
<td>Phenolics</td>
<td>High specific surface of charcoal adsorbs a lot of toxic compounds.</td>
</tr>
</tbody>
</table>

*usually the removal of compounds is only partial – concentration of the above given substances is affected the most

Besides these classical detoxification methods a novel approach was presented by Borole et al. (2009). In their work microbial fuel cell (MFC), an electrical device treating water and converting soluble organic matter into electricity and hydrogen, was applied to the liquid fraction. MFC was demonstrated to reduce the concentration of inhibitors including sugar-degradation products, furfural and HMF, and lignin degradation products. This way MFC can contribute to
higher ethanol yield combined with water recycling and electricity supply (Borole et al., 2009)

### 2.2.4 Microbial strategies to overcome inhibition

Other strategy to overcome inhibition of the substances in liquid fraction is to improve the microbes processing it. Basically there are three ways for this approach (Almeida et al., 2009; Taherzadeh and Karimi, 2011). One is to acclimatize the microbes to toxic compounds usually by sequentially transferring the growing cells into media containing increasing inhibitor concentrations or liquid fraction ratios. With adaptation the long lag-phase caused by the presence of inhibitors can be significantly shortened, as well as the ethanol yield increased (Parawira and Tekere, 2011). This has been demonstrated many times as a successful tactics in case of ethanol fermenting yeasts (Amartey and Jeffries, 1996; Sene et al., 2001; Silva and Roberto, 2001; Huang et al., 2009; for review see Parawira and Tekere, 2011). In contrast the possible adaptation of *T. reesei* has rarely been studied. Bigelow and Wyman (2002) reported that adaptation is an encouraging option but because of the difficulty in maintaining the adapted strains a detailed study including enzyme activity measurements was not carried out. Hayward et al. (1999) found that despite increased growth as a consequence of adaptation, strains with increased enzyme production could not be obtained.

The second option is to understand the cellular mechanism of inhibition and inhibitor decomposition, and thus manipulate the genome in order to enhance tolerance and metabolism with tools of genetic engineering (Almeida et al., 2009). Many yeast strains overexpressing genes encoding inhibitor converting enzymes (e.g. laccase, furfural reductase) have been created this way (Parawira and Tekere, 2011). Third, the application and improvement of different fermentation techniques, including fed-batch operation, high cell density and encapsulation of cells, can also be successful (Taherzadeh and Karimi, 2011).

### 2.3 Enzymes for biomass conversion

Lignocellulosic materials in the form of decaying plant materials are an abundant source of energy and carbon in natural habitats. Therefore many organisms, mostly microbes are able to decompose the structural plant cell wall carbohydrates in order to utilize the monomers as carbon and energy source. Many genera of anaerobic and aerobic bacteria as well as fungi show cellulolytic activity. Anaerobe microbes mostly degrade cellulose by complexed cellulase systems, referred to as cellulosomes, which are often present only on
the surface of the cell. Aerobes usually secrete a large amount of distinct enzymes into the surrounding environment (Lynd et al., 2002). From a practical point of view these enzymes can be easily recovered and utilized.

Among fungi, even the most primitive group, the anaerobic *Chytridomycetes* living in gastrointestinal tracts of ruminant animals and the more evolved *Ascomycetes*, *Basidiomycetes*, and *Deuteromycetes* contain high numbers of species exhibiting cellulose degrading ability (Lynd et al., 2002). Filamentous fungi from different genera, such as the ascomyceta *Trichoderma*, *Penicillium* and *Fusarium*, and the basidiomyceta *Humicola* and *Phanerochaete* are the most applied ones in cellulase production (Singhania, 2011). Different accessory, mostly hemicellulolytic enzymes for complete plant cell wall degradation produced by *Aspergilli* also possess industrial importance (de Vries and Visser, 2001). Nevertheless, one of the most researched fungi, with more than 50 years of history, is *T. reesei*, while, a number of other fungal strains (*Penicillium*, *Acremonium* and *Myceliophthora*) are getting more and more attention in the biofuel industries (Gusakov, 2011).

Cellulases accounted for 20% of the expanding multibillion dollars value global enzyme market between 2005 and 2010 (Singhania, 2011). The cellulases are used in several processes including ones of the textile, food, feed, pulp and paper industries (Tolan and Foody, 1999; Bhat, 2000; Singhania, 2011). Moreover, a new market potential can arise with the commercialization of cellulosic ethanol plants for what currently a main bottleneck is the price of enzyme production (Singhania, 2011). As already indicated in an early review, cellulase enzymes were found to be responsible for 43.4% of the cost of ethanol in 1980 (Ryu and Mandels, 1980). During the time cost of cellulosases have been decreased considerably, but still is one of the barriers of industrial-scale spread (Geddes et al., 2011).

### 2.3.1 A prolific enzyme producer: *T. reesei*

The ascomycete *T. reesei*, anamorph of *Hypocrea jecorina* is one of the most powerful cellulase producing microorganisms that is applied on industrial scale as well. The two major players on the market of cellulase enzymes developed for biomass conversion are Genencor and Novozymes, both apply different *T. reesei* mutants for enzyme production (Singhania, 2011).

Today numerous hypercellulolytic *T. reesei* strains are available that have been developed from QM6a isolated on the Solomon Island during the Second World War and originally identified as *T. viride* (Seidl and Seiboth, 2010; Peterson and
Nevalainen, 2012). QM6a has been recognized as a good cellulase producer (Mandels and Reese, 1957) which had turned to be a benefit from the instant problem of decomposing cotton tents leading to its discovery. Later QM6a was found to be distinct from *T. viride* and classified under a new species name of *T. reesei* in honor of Natick laboratory researcher Elwyn T. Reese who has isolated it (Bayer, 2009).

During the years, various mutation lines have been developed from this isolate and from its ancestors through the classical way, i.e. using mutagen agents: various chemical compounds (Durand et al., 1988; Kawamori et al., 1985; Nevalainen et al., 1980), combined with UV irradiation (Gadgil et al., 1995; Jun et al., 2009; Montenecourt and Eveleigh, 1977) or high voltage electrons (Mandels et al., 1971) all with subsequent screening on special media. The initial aim of strain selection was to improve cellulase yields and create catabolite derepressive mutants. The mostly and still used catabolite derepressive RUT C30 had been isolated as a result of a three round mutagenesis process combining the methods above (Eveleigh, 1982). The strain development work running parallel in different institutions also resulted in other hyperproductive strains (Montenecourt, 1983) such as the still catabolite repressive QM9414 (Montenecourt and Eveleigh, 1977) and the publicly not available CL847 with outstanding performance (Warzywoda et al., 1983; Seidl and Seiboth, 2010) (Figure 4.). As this strain selection was based on screening for favorable phenotypic alteration, the proper genetic reasons for enhancement in cellulase production are not known exactly. Recently, a lot of efforts have been made in order to uncover the genetic roots of cellulase hyperproduction of RUT C30 revealing large genomic deletions and more other mutations affecting the function of many different genes compared to QM6a (Le Crom et al., 2009; Seidl et al., 2008).
The reason for carbon catabolite derepression was also found. In RUT C30 the crel gene, a key transcription factor for carbon catabolite repression is present in a non-functional truncated form (Ilmén et al., 1996). However, the changes affecting the whole genome have made it clear that many mutations in different places and with different cellular functions not closely related to cellulase expression may be necessary to cause hyperproduction (Seidl and Seiboth, 2010).

Naturally, the above mutations also had an effect on the phenotype for example by means of morphology (Seidl and Seiboth, 2010) and carbon source assimilation (Le Crom et al., 2009; Seidl et al., 2008). Probably the most obvious phenotypic difference from QM6a and even from QM9414 is the lack of yellow pigment formation in RUT C30 both on agar plates and in submerged cultures (Peterson and Nevalainen, 2012, Seidl et al., 2008).

2.3.2 Cellulase enzyme system of T. reesei

With respect to the very recalcitrant structure of lignocellulose it is no surprise that cellulases and hemicellulases designated to decompose cellulose and hemicellulose, respectively, are not able to penetrate on the intact surface of the plant cell wall. Pretreatment, loosening the plant cell wall structures is necessary to provide surface for the enzymes to attack. The enzymatic decomposition of cellulose, once it is accessible for the enzymes, requires the synergistic action of at least 3 major enzyme classes, members of the glycoside hydrolases (GH, EC 3.2.1., classification of GHs: www.cazy.org): endoglucanases (EC 3.2.1.4), exoglucanases, also known as cellobiohydrolases
Endoglucanases attack randomly accessible $\beta$-1-4-bonds among the cellulose chain in order to create oligomers and free termini for exoglucanases that progress onward the chain while cleaving cellobiose from the ends. There are two types of exoglucanases depending on whether they can attack on the reducing or the non-reducing end of cellulose chains (Figure 5.). These enzymatic attacks take place on the surface of the solid substrate and yield water soluble oligomers and cellobiose. In order to complete the hydrolysis $\beta$-glucosidases are necessary to split cellobiose into glucoses (Serpa and Polikarpov, 2011).

All of the three cellulases are secreted by *T. reesei* into culture medium, however, in very different amounts: while exoglucanases account for 80% of the total cellulolytic proteins produced (Serpa and Polikarpov, 2011), $\beta$-glucosidases make up less than 1% of the enzyme cocktail (Lynd et al., 2002). This defect of *T. reesei* possesses crucial importance, since optimal activity of $\beta$-glucosidases is necessary for complete hydrolysis of pretreated biomass to glucose and thus to prevent cellobiose inhibition of exoglucanases and endoglucanases. However, $\beta$-glucosidases are inhibited by glucose; its consumption can be facilitated by process based strategies (i.e. SSF).
Besides GHs, two secreted proteins missing the catalytic core were found only with carbohydrate-binding domain and expression of swollenin was also reported (Foreman et al., 2003). Swollenin consists of a cellulose-binding domain and an expansin-like domain (expansins are plant proteins with cell wall loosening action) showing its role in loosening the plant cell wall (Saloheimo et al., 2002).

Because of the more diverse structure of hemicelluloses, the types of enzymes with affinity towards them are also numerous (Manju and Singh Chadha, 2011; Dumon et al., 2012). The most important ones, the xylanases liberate xylose from xylan backbones working in exo-endo synergy (endo-1,4-β-xylanases, EC 3.2.1.18 and exo-1,4-β-xylosidase, EC 3.2.1.37) similar to cellulases with the exception that exo-1,4-β-xylosidase can act only on non-reducing ends (Manju and Singh Chadha, 2011). However, since polysaccharides of hemicelluloses are highly branched many additional enzymes are needed for de-branching: α-L-arabinofuranosidase (EC 3.2.1.55), α-D-glucuronidase (EC 3.2.1.139), acetylxylan esterase (EC 3.1.1.72), α-galactosidases (EC 3.2.1.22), and β-mannosidases (EC 3.2.1.25), acetyl-xylan-esterases (EC 3.1.1.72), ferulic acid esterases (EC 3.1.1.73), and p-coumaric acid esterases (EC 3.1.12) (Shallom and Shoham, 2003).

2.3.3 Cultivation conditions of T. reesei

Cellulase enzyme production by T. reesei can take place in solid state, as well as in submerged fermentation with the latter being the more used option (Singhania, 2011). The medium composition, such as carbon source and nutrients (Gupta et al., 1972; Knapp and Legg, 1986; Cochet, 1991; Esterbauer et al., 1991; Ahamed and Vermette, 2008) and operational parameters, such as pH (Knapp and Legg, 1986; Cochet 1991; Chahal et al., 1992; Hayward et al., 2000; Juhász et al., 2004; Al-Taweil et al., 2009; Adav et al., 2011) have significant effect on cellulase secretion. Therefore, with optimizing fermentation conditions a significant gain in cellulase secretion can be obtained. Recently it has been showed that light also influences cellulase enzyme production that may have to be taken into account when designing fermentation experiments (Schmoll et al., 2010).

Regarding carbon sources a wide range of substrates has been applied beginning with simple soluble sugars such as glucose, lactose or xylose (Mohagheghi et al., 1988; Warzywoda et al., 1992; Chaudhuri and Sahai, 1993; Domingues et al., 2001) or different cellulose preparations (Hayward et al., 2000). In an
industrial point of view products related to biomass processing, just as pretreated biomass, have also been applied widely (Acebal et al., 1986; Doppelbauer et al., 1987; Shin et al., 2000; Sukumaran et al., 2009). \textit{T. reesei} fits the secreted enzyme mixture to the characteristics of the used substrate, therefore applying the same substrate both in enzyme production and subsequent hydrolysis may be beneficial (Sipos et al., 2010).

Cellulase secretion in \textit{Trichoderma} is an inducible phenomenon, but the mechanism of cellulose sensing is still unclear, mostly due to its water insolubility. Hence, the “real inducer” by means of transmitting the signal about the presence of cellulose into the cell is still not known exactly. A proposed route for induction is that cellulase enzymes on the surface of conidia attack the substrate in order to have carbon source for germinating. In turn cellulase expression will be facilitated in the growing cells by consumed cellulose derived products with inducing property (Kubicek et al., 1993; Suto and Tomita, 2001). This scheme is also supported by the observation that during conidia formation the genes encoding cellulase and hemicellulase enzymes are significantly upregulated (Metz et al., 2011) explaining well that these enzymes are present in large amount on conidia (Kubicek et al., 1988).

Several small compounds, like sophorose (Mandels et al., 1962; Sternberg and Mandels, 1979) and sorbose (Nanda et al., 1986; Nogawa et al., 2001), possess the capability to induce cellulase secretion, but an industrial application of these compounds, as carbon sources, is unfeasible because they are expensive to purchase. Lactose, another inducer, could be a preferable choice for industry because whey, a byproduct of cheese processing, contains lactose. However, whey is not readily available onsite as it is not biomass related (Chen and Wayman, 1992; Warzywoda et al., 1992). The liquid fraction obtained after pretreatment of biomass contains both glucose and xylose oligomers which induce cellulase secretion in \textit{Trichoderma} (Wang et al., 1988); by its availability it can be a good substrate for onsite enzyme production.

Although starch is usually not recognized as inducer for cellulase production in \textit{Trichoderma} species, an inductive effect was reported for starch-derived compounds in \textit{T. reesei} RUT C30 (Chen and Wayman, 1992; Wayman and Chen, 1992). One possible explanation is that during acidic or enzymatic hydrolysis of starch, reversion products, such as sophorose, are also formed and these products can act as inducers on cellulase transcription (Chen and Wayman, 1992). However, the same positive effect was observed in case of
utilizing native starch as partial carbon source supplemented with cellulosic materials (Wayman and Chen, 1992; Taj-Aldeen, 1993).

2.3.4 Novel approaches for further development of T. reesei

With the improvement of laboratory methods and genetic engineering tools it had became possible to modify the genome and thus optimize cellulase secretion in site-directed mutagenesis. Prerequisite of this strategy was at least the partial understanding of transcriptional regulation of cellulase genes on which updated reviews have been published elucidating the possible roles of many transcription factors (Aro et al., 2005, Mach and Zeilinger, 2003, Schmoll and Kubicek, 2003).

The next step in strain improvement was to take advantages of genome sequence (Baker et al., 2008). Surprisingly, genome sequence of T. reesei QM6a revealed unexpectedly low numbers of genes coding cellulases, hemicellulases and pectinases (Martinez et al. 2008) despite the high efficiency of cellulases produced. Albeit these genes are clustered which helps a more effective control of gene expression, the key recognizing T. reesei as a superior cellulase producer may be its efficient signal transduction system (Schmoll, 2008). Therefore, besides improving the produced enzymes themselves or the efficiency of the promotors by which their expression is controlled, another strategy can be the exploitation of signal transduction processes (Kubicek et al., 2009).

Signaling mechanisms and pathways with their elements (receptors, trans-factors and other proteins) convey signals from nature into the nucleus and thus influencing gene expression by targeting different downstream gene elements. The well functioning of this machinery greatly contributes to successful adaptation and survival by receiving and interpreting numerous biotic and abiotic factors one of which is light. Light has both beneficial (energy, information) and harmful (UV radiation, DNA mutation) effects on living organisms, therefore the given reactions to light have crucial importance in survival. In contrast to plants, which utilize light as energy source, for fungi light is merely a source of information. As the many effects of light are common within the fungal kingdom, the pathways of light sensing and its elements often share significant homology (Idnum and Heitman, 2005; Corrochano, 2007).
2.3.4.1 Effect of light on cellulase expression in *Trichoderma*

Light, especially blue light affects or initiates a number of physiological processes in fungi in general and also in *Trichoderma*, e.g. growth, conidiation and metabolic pathways (Herrera-Esrella and Horwitz, 2007; Tisch and Schmoll, 2009; Rodriguez-Romero et al., 2010; Schmoll et al., 2010). These responses given to light reflect the changes in the surrounding ecological niche. *T. reesei* as a saprophyte mainly resides inside of decaying plant material, that is, in darkness. Presence of light means the open surface of the habitat where, for instance, the successful dissemination of conidia is expected or existence of possible mating partner is assumed. The first observation on photobiology of *T. reesei* was also made on light triggered conidiation; a brief light pulse applied on agar plates with growing mycelia leads to forming a ring of conidia on what had been the edge of the colony in the moment of the pulse (Gressel and Galun, 1967).

Cellulase expression in *T. reesei* has also been observed to be influenced by light: protein ENVOY modulates cellulase transcription in a light dependent manner (Schmoll et al., 2005). Although ENVOY, a putative photoreceptor is crucial in light tolerance, it has a more widespread cellular role even in darkness. However, overexpression of ENVOY in darkness is not enough to mimic light showing that additional components or post-translational modifications are involved (Schuster et al., 2007).

Recently, also an influence of the two photoreceptors BLR1 and BLR2 on cellulase expression has been shown suggesting that these regulators act positively on this process and corroborating the link between cellulase gene expression and light response (Castellanos et al., 2010). BLR1 and BLR2 are orthologs of the well studied White Collar proteins (WC-1 and WC-2), the major photoreceptors of the model organism *Neurospora crassa* that have homologs across the fungal kingdom with conserved encoding genes in almost all fungal genomes sequenced (Rodriguez-Romero et al., 2010). These proteins have been reported to be essential in almost all light responses; however, wc-independent light responses are also possible (Liu et al., 2003). The *T. reesei* blue light photoreceptor complex (BLR1 and BLR2) have similar structural domains as WC proteins and plays an important role in photoconidiation and gene regulation in response to light, but light-independent regulatory roles were also reported for these proteins (Castellanos et al., 2010).
Moreover, the G-protein signaling and cAMP pathways also influence cellulase gene expression in a light dependent manner with possible involvement of ENVOY and/or BLR proteins (Schmoll et al., 2009; Seibel et al., 2009; Tisch et al., 2011a). A genome wide analysis showed that in *T. reesei* QM9414 2.7% of the genes are at least two-fold differentially regulated in light and darkness. GH enzymes were either up- or downregulated showing that light cannot be considered as a negative or positive factor but rather a signal optimizing enzyme mixture (Tisch et al., 2011b).
3. Materials and methods

3.1 Raw materials

3.1.1 Sweet sorghum varieties

During the studies two sweet sorghum varieties were used: ‘Monori Édes’ for Paper II and ‘Berény’ for Paper III.

Sweet sorghum variety ‘Monori Édes’ developed by Agroszemek Ltd in Hungary can produce high green biomass yield, i.e. 80-100 t/ha. After harvest stems got crushed to extract the juice (40-50 t/ha, 14-20% Brix), residue of the process is the bagasse (40-50 t/ha, 50% dry weight). Harvest and squeezing were performed during the autumn of 2007 on the site of Agroszemek Ltd. near to Hódmezővásárhely, Hungary. The juice got conserved by acidification with nitric acid (pH = 3.0-3.5) and kept frozen until use.

Total sugar content of this juice was 163.8 g/L composed of mainly sucrose and its components (Table 4.). The reason for the partial hydrolysis of sucrose into fructose and glucose is the acidification and storage.

Table 4. – Composition of sweet sorghum juice ‘Monori Édes’

<table>
<thead>
<tr>
<th>Sweet sorghum juice g/L</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose</td>
<td>14.2±0.3</td>
</tr>
<tr>
<td>Glucose</td>
<td>16.5±1.6</td>
</tr>
<tr>
<td>Sucrose</td>
<td>133.1±5.8</td>
</tr>
</tbody>
</table>

Sweet sorghum variety ‘Berény’ was cultivated at Research Institute, Karcag, Hungary (Centre for Agricultural Sciences and Engineering, University of Debrecen, Hungary) in 2006. After squeezing the bagasse was collected, chopped and dried at 50°C to 85-90% dry matter content. For purposes of Paper III only the bagasse was used.

3.1.2 Wheat grain and straw

Wheat grain and straw were used in experiments in Paper IV. Wheat straw was collected in 2008 in Soria, Spain. It was milled using a laboratory hammer mill to obtain a chip size of 10 mm, and was stored at room temperature until use. Mazurka-type wheat grain with starch content of 65.9% was harvested in the fall of 2009 in Somodorpuszta, Hungary. Dried grain was ground, and the
following size distribution was obtained: 49.8% (related to total weight) was between 1.00 and 0.32 mm, while 82.2% was between 1.40 and 0.10 mm.

### 3.1.3 Other raw materials used

Wheat distiller’s grain was used as supplementary nitrogen source for enzyme production experiments. It is composed of 32.2% protein, 18.6% glucan and 14.6% xylan.

In some cases to cultivate *T. reesei* the widely used model substrate, Solka Floc 200 FCC commercial cellulose product (International Fiber Corporation, North Tonawanda, NY, USA) was applied as carbon source. Solka Floc 200 is prepared from milled pinewood by several extraction steps resulting in delignified product with high cellulose and xylan content.

### 3.2 Pretreatments

For Paper II chopped, dried and ground bagasse of ‘Monori Édes’ was pretreated on eight different ways with combining the following parameters: type of base (NaOH versus KOH), concentration of base (1% versus 2%) and temperature – time combination (25°C, 3 days versus 121°C, 1 hour). Ground bagasse (0.3 – 1.4 mm) at 10% dry weight content (40 g dry matter in 400 g total mass) was soaked into alkali solution in 1000 mL screw-capped bottles and left at room temperature for 3 days or autoclaved at 121 ºC for 1 hour. After pretreatments the mixtures were separated by vacuum-filtration and the solid fraction was washed with hot distilled water to remove solubles. Filter cake was dried at 50ºC, analyzed and used for enzymatic hydrolysis. Since the conditions of pretreatment were the subject of investigation, the composition of differently pretreated bagasse is presented in the chapter Results and Discussion.

For Paper III bagasse of ‘Berény’ was steam pretreated at the Department of Chemical Engineering, Lund University, Sweden. The material was steamed at atmospheric pressure for 1 hour in order to reach 50% moisture content, and then impregnated with 2% SO₂ (based on moisture content) in plastic bags for 30 min. Steam pretreatment was performed in a reactor with 10 L working volume (Palmqvist et al., 1996). Temperature was set and maintained by injection of saturated steam. After 10 min of residence time at 190°C the pressure was released. Slurry of steam pretreated sweet sorghum bagasse was collected from the cyclone and washed with triple amount of warm (60-70°C) distilled water to remove the majority of the water soluble substances, then the liquid and fiber fractions were separated by vacuum-filtration. Composition of
pretreated bagasse and liquid fraction after separation are presented in Table 5. and 6., respectively.

Table 5. – Composition of sweet sorghum bagasse prior and after pretreatment

<table>
<thead>
<tr>
<th>Sweet sorghum bagasse</th>
<th>Pretreated bagasse (separated, washed solid fraction) % of dry mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucan</td>
<td>36.3±1.4</td>
</tr>
<tr>
<td>Xylan</td>
<td>25.6±0.9</td>
</tr>
<tr>
<td>Arabinan</td>
<td>2.0±0.1</td>
</tr>
<tr>
<td>Lignin</td>
<td>18.6±0.3</td>
</tr>
<tr>
<td></td>
<td>58.3±1.2</td>
</tr>
<tr>
<td></td>
<td>12.8±0.6</td>
</tr>
<tr>
<td></td>
<td>2.1±0.1</td>
</tr>
<tr>
<td></td>
<td>24.6±0.5</td>
</tr>
</tbody>
</table>

Table 6. – Composition of liquid fraction of steam pretreated bagasse

<table>
<thead>
<tr>
<th>Liquid fraction g/L</th>
<th>Liquid fraction g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Monomers</em></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0.8±0.0</td>
</tr>
<tr>
<td>Xylose</td>
<td>3.2±0.3</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td><em>Oligomers</em></td>
<td></td>
</tr>
<tr>
<td>Cellobiose</td>
<td>0.3±0.0</td>
</tr>
<tr>
<td>Glucan</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>Xylan</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>Arabinan</td>
<td>0.1±0.0</td>
</tr>
<tr>
<td><em>Inhibitors</em></td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.7±0.0</td>
</tr>
<tr>
<td>Formic acid</td>
<td>0.8±0.0</td>
</tr>
<tr>
<td>Furfural</td>
<td>0.4±0.0</td>
</tr>
<tr>
<td>HMF</td>
<td>0.1±0.0</td>
</tr>
</tbody>
</table>

Steam explosion of wheat straw (Paper IV) was performed in a 10 L reactor at 210°C for 2.5 min using Masonite technology (Marchessault et al., 1983) in CIEMAT (Madrid, Spain). These conditions were established by Ballesteros et al. (2006) to reach on optimal sugar recovery and hydrolysis yields. Separation of the slurry was similar than above except a major difference: the solid fraction
was only washed after separation of liquid fraction, thus this liquid fraction did not contain any washing water. Composition of pretreated wheat straw after separation and washing is presented in Table 7, while composition of the related liquid fraction in Table 8.

**Table 7. Composition of wheat straw prior and after pretreatment**

<table>
<thead>
<tr>
<th></th>
<th>Wheat straw</th>
<th>Pretreated wheat straw (separated, washed solid fraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of dry mass</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucan</td>
<td>40.7±2.7</td>
<td>57.5±0.8</td>
</tr>
<tr>
<td>Xylan</td>
<td>27.6±2.4</td>
<td>6.0±0.1</td>
</tr>
<tr>
<td>Lignin</td>
<td>17.0±0.8</td>
<td>30.1±0.1</td>
</tr>
</tbody>
</table>

**Table 8. Composition of liquid fraction of steam pretreated wheat straw**

<table>
<thead>
<tr>
<th></th>
<th>Liquid fraction g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Monomers</strong></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>Xylose</td>
<td>5.5±0.2</td>
</tr>
<tr>
<td>Others(^a)</td>
<td>2.6±0.3</td>
</tr>
<tr>
<td><strong>Oligomers</strong></td>
<td></td>
</tr>
<tr>
<td>Glucan</td>
<td>6.6</td>
</tr>
<tr>
<td>Xylan</td>
<td>35.0</td>
</tr>
<tr>
<td>Others(^a)</td>
<td>1.9</td>
</tr>
<tr>
<td><strong>Inhibitors</strong></td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>8.8±0.8</td>
</tr>
<tr>
<td>Formic acid</td>
<td>7.2±0.9</td>
</tr>
<tr>
<td>Furfural</td>
<td>2.1±0.5</td>
</tr>
<tr>
<td>HMF</td>
<td>0.7±0.0</td>
</tr>
</tbody>
</table>

\(^a\) galactose, mannose and arabinose or related oligomers

### 3.3 Microbial strains

For enzyme production experiments in Paper III and IV *T. reesei* RUT C30 (ATCC56765) strain obtained from the American Type Culture Collection was
used. It was maintained at 30°C on malt agar slants composed of 20 g/L malt extract, 20 g/L agar, 5 g/L glucose, and 1 g/L peptone.

For the comparative enzyme production experiments in Paper V *T. reesei* QM9414 (ATCC26921) and the following descendents defective in light sensing pathways were used: *Δenv1, Δblr1* and *Δblr2*, missing the open reading frames of respective genes. Strains *Δblr1* and *Δblr2* were kindly provided by Alfredo Herrera-Estrella, while QM9414 and *Δenv1* by Monika Schmoll. Strains were maintained on malt agar plates (30 g/L malt extract, 1 g/L peptone, 20 g/L agar) in darkness at 30°C.

For fermentation experiments in Paper I and II, blocks of baker’s yeast (*S. cerevisiae*), product of Lesaffre Magyarország Élesztőgyártó és Kereskedelmi Kft., Budafok, Hungary were purchased in a local store.

### 3.4 Cellulase production

#### 3.4.1 Inoculum preparations

Conidia from 14-day-old slants were harvested with sterile distilled water. This suspension was used to inoculate Erlenmeyer flasks containing 200 mL of sterile modified Mandels’ basal medium (Mandels and Andreotti, 1978) to obtain a final concentration of 10⁸ conidia/mL. This medium contained 1.87 g/L (NH₄)₂SO₄, 2.67 g/L KH₂PO₄, 0.53 g/L CaCl₂.2H₂O, 0.81 g/L MgSO₄.7H₂O, 0.40 g/L urea, 5.0 mg/L FeSO₄.7H₂O, 1.7 mg/L MnSO₄.H₂O, 1.4 mg/L ZnSO₄.7H₂O, 2.0 mg/L CoCl₂.6H₂O; 1.00 g/L peptone, 0.33 g/L yeast extract and 10 g/L Solka Floc as carbon source. These components were suspended:

- in tap water (Paper IV);
- in 0.1 M citrate-phosphate buffer (pH 5.0) (Paper V);
- either in tap water or liquid fraction of pretreated bagasse or a mixture of liquid fraction-water in different ratios (1:1, 1:2, 1:3, 1:4) (Paper III).

Inoculated flasks were closed with cotton plugs and incubated at 30°C and 250-300 rpm in darkness on a rotary shaker for 3 (Paper V) or 4 days (Paper III and IV).

#### 3.4.2 Fermenter experiments

QM9414 and the modified strains in Paper V were analyzed in a 30 L double-walled stainless steel laboratory fermenter (Biostat C-DCU 3, B Braun Biotech, Germany) equipped with pO₂, pH, temperature, pressure and foam sensors in
20 L working volume. Agitation was provided by a top-drive agitator with 3 Rushton impellers each with 6 blades.

Medium components (10 g/L Solka Floc 200 and 5 g/L wheat distiller’s grain, 0.83 g/L KH₂PO₄ and 0.83 g/L (NH₄)₂SO₄) were suspended in tap water and sterilized in the fermenter. Medium was inoculated in 10% (v/v), i.e. with 2 L of inoculum resulting from the aseptic combination of ten flasks of inoculum into the sterile inoculating equipment.

During fermentation runs pH was continuously controlled and adjusted to 5.8 by addition of 10% (v/v) NH₄OH or 10% (v/v) H₃PO₄. pO₂ level was maintained on 30% by cascade control of air flow rate as first priority, varying between 5 and 12 L/min and velocity of agitation as secondary, shifting between 250 and 600 rpm. Beyond 30% of dissolved oxygen content air flow rate and agitation worked on the minimal values for both variables. Temperature was set to 28°C. Operation parameters (pH, volume of added base and acid, pO₂, stirring velocity and air flow rate) were recorded by MFCS/win 2.0 (B Braun Biotech) software. To control foaming, ionic antifoam emulsion (Sigma-Aldrich Antifoam A) was added automatically.

Fermentations were carried out in duplicate. Samples were withdrawn regularly, centrifuged (3400 g, 5 minutes) and whole broths were also saved for cell mass measurement.

### 3.4.3 Pre-adaptation of T. reesei to liquid fraction

In Paper III the adaptation possibility of T. reesei was investigated, firstly, by pre-adaptation on agar plates containing liquid fraction in different dilutions. The medium components for agar plates (5 g/L glucose, 1 g/L peptone, 20 g/L Bacto-agar) were suspended in either tap water or in liquid fraction or in the mixture of these (liquid fraction:water 1:1 or 1:3) and pH was set to 6.0 by adding solid NaOH. The medium was sterilized on 121°C for 20 min and after that plates were prepared.

Plates were inoculated by placing a piece of agar from a previously cultured plate in the middle of the new plate. The plates were grown at room temperature in the laboratory. The diameter of the cultures was measured daily in the same time. Rate of growth was expressed as the difference of the circle areas covered by the colony on two different days and divided by the numbers of days between the two readings (mm²/day).
3.4.4 Enzyme production on liquid fraction in shake flasks

The liquid and solid fractions of the pretreatments were used for enzyme production experiments (Paper III and IV). Same protocol was applied in Paper III and IV. The medium for cellulase production was composed of (NH₄)₂SO₄, KH₂PO₄, and carbon sources at different concentrations (Table 9.). Concentrations of the two salts were equal and were based on the amount of the carbon source. When the carbohydrate content was 15 g/L or lower, 0.83 g/L of both salts were used; what was doubled (1.66 g/L) when the carbohydrate content was above 15 g/L. Each medium contained an additional 5 g/L wheat distiller’s grain. To avoid pH changes solid substances were suspended in 0.1 M TRIS-maleic acid buffer (pH 5.8) prepared in either tap water or liquid fraction in dilutions indicated in Table 9. TRIS-maleic acid buffer can stabilize the pH during cultivation, and some positive effects on cellulase production were also observed (Juhász et al., 2004).

Table 9. – Composition of media used for shake flask experiments

<table>
<thead>
<tr>
<th>Medium</th>
<th>Carbon sources</th>
<th>Liquid fraction:water ratios</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation</td>
<td>10 g/L washed pretreated bagasse, liquid fraction</td>
<td>1:0; 1:1 (100%, 50%)</td>
<td>Paper III</td>
</tr>
<tr>
<td>Preliminary</td>
<td>Only liquid fraction</td>
<td>1:0; 1:1; 1:3 (100%, 50%, 25%)</td>
<td>Paper IV</td>
</tr>
<tr>
<td>SF</td>
<td>5 g/L Solka Floc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WG</td>
<td>Ground wheat grain in concentration of 12.5 g/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFWG</td>
<td>Ground wheat grain in concentration of 6.25 g/L plus 5 g/L Solka Floc</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>LIQ</td>
<td>Detoxified liquid fraction</td>
<td>1:3</td>
<td></td>
</tr>
<tr>
<td>LIQWG</td>
<td>Detoxified liquid fraction plus 12.5 g/L raw wheat grain</td>
<td>1:3</td>
<td></td>
</tr>
<tr>
<td>LIQWIS</td>
<td>Detoxified liquid fraction plus 14.3 g/L washed pretreated wheat straw</td>
<td>1:3</td>
<td></td>
</tr>
<tr>
<td>REF</td>
<td>14.3 g/L washed pretreated wheat straw</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

After sterilization the 150 mL aliquots in Erlenmeyer flasks, media were aseptically inoculated with 15 mL 4-day-old preculture at 10% (v/v), and...
cultures were propagated at 30°C and 300 rpm on a rotary shaker. Samples were withdrawn regularly and centrifuged (3,400g, 5 min) to separate supernatants for further analysis. Fermentations were terminated after 7 or 11 days, and in case of Paper IV whole fermentation broths were stored at -20°C to test some of them in hydrolysis assays.

3.5 Hydrolyses

Pre-produced enzymes and commercial enzyme preparations were used for hydrolysis experiments. Celluclast 1.5L (Novozymes A/S, Bagsvaerd, Denmark) is a complete cellulase mixture obtained from T. reesei and Novozym 188 (Novozymes A/S, Bagsvaerd, Denmark) is a β-glucosidase preparation of Aspergillus niger origin. Both preparations were kindly provided by Novozymes.

3.5.1 Hydrolysis of pretreated washed sweet sorghum bagasse

Bagasse samples were hydrolyzed at 50°C at 2% dry weight content in 0.05 M acetate buffer (pH 4.8) by Celluclast 1.5L and Novozym 188 at 20 and 40 IU/g dry matter content, respectively. The process was carried out in 250 mL screw-capped bottles containing 4 g dry pretreated or raw bagasse in 200 g total mass with magnetic stirring (250 rpm). 1.8 mL of samples was taken at the start of hydrolysis and after 1, 3, 4, 6, 24 and 48 hours. Samples were centrifuged in Eppendorf tubes at 15 000 rpm for 5 minutes, and subsequently reducing sugar determination was carried out from the supernatant to track hydrolysis. After 48 hours the hydrolyzates were cooled down to 30 ºC and moved to the fermentation step.

3.5.2 Hydrolysis of pretreated washed wheat straw

Hydrolysis experiments were carried out with the enzymes produced on the liquid fraction of pretreated wheat straw with different supplementations (Paper IV). Reaction volume was 5 mL in test tubes with magnetic stirring at 50°C. Dry WIS content was 2% suspended in 0.05 M acetate buffer (pH 4.8). The enzyme to substrate ratio was equivalent to 20 FPU/g glucan. As a control, the substrate was hydrolyzed by Celluclast 1.5L supplemented with Novozym 188 in 20 FPU/g glucan cellulase and 20 IU/g glucan total β-glucosidase dosages.

Hydrolysis was run for 72 hours with withdrawals at 0, 3, 6, 9, 24, 48, and 72 hour. At each time point, three tubes were taken (representing triplicates) and boiled for 5 min to inactivate the enzymes. They were then centrifuged, and the
supernatant was prepared for high performance liquid chromatography (HPLC) analysis to measure cellobiose, glucose, and xylose concentrations.

### 3.6 Ethanol fermentation

Batch fermentations of different samples were carried out in a volume of 200 mL at 30 ºC in 250 mL screw-capped bottles with magnetic stirring (250 rpm) connected to the monitoring device. Baker’s yeast suspension was added in order to obtain a cell concentration of 2 g dry weight per liter. Fermentation was tracked by measuring CO₂ production by a novel online fermentation module device developed by Nonfood group (at Budapest University of Technology and Economics) and Stereo Vision Ltd described in Paper I.

Fermented samples included sweet sorghum juice, pre-hydrolyzed pretreated sweet sorghum bagasse samples and for the statistical evaluation a synthetic medium. Synthetic medium contained glucose in different concentrations (10 g/L, 20 g/L, 30 g/L, 40 g/L, 60 g/L, 80 g/L) and the salts of 0.3 g/L MgSO₄, 2.0 g/L NH₄Cl, 1.0 g/L K₂HSO₄.

At the end of fermentation when CO₂ production ceased, flasks were sampled. Samples were centrifuged at 9 000 rpm for 5 min. Supernatant was analyzed for sugar and ethanol concentration by HPLC.

### 3.7 Other methods

#### 3.7.1 Detoxification

In the case of the experiments carried out for Paper IV liquid fraction of pretreated wheat straw was detoxified in a two step process. Firstly, liquid fraction was vacuum-evaporated at 50°C for 10 min, during which the starting volume of 250 mL decreased by 60 mL. This loss was replaced by distilled water to obtain the initial volume again. Secondly, this solution was overlimed at 35°C by adding Ca(OH)₂ powder to pH 10 with continuous stirring. Once a pH of 10 was achieved, the solution was stirred for an additional 1 h. To remove any precipitate, the overlimed liquid was centrifuged (3,400g, 5 min); the supernatant was stored at 4°C until use in cellulase production. The pH was acidified to value 5.8 during fermentation medium preparation.

#### 3.7.2 Western blotting

For exact comparison in Paper V the secreted amount of the protein CBH1 was quantified by Western blotting. Proteins from 0.5 mL fermentation supernatant were precipitated by adding 1 mL of 96% ethanol. Western blotting was
performed according to standard protocols (Sambrook et al., 1989). Briefly, after centrifugation the pellet was dissolved in 0.2 mL SDS buffer; 10 μL representing equal amounts of culture filtrate were loaded into a 7.5% SDS-PAGE gel and run at 15 mA. After separation samples were transferred to nitrocellulose membranes (Hybond-C Extra, Amersham Biosciences, UK).

3.8 Analyses

The different liquid fractions were analyzed for sugar and inhibitor content and the fiber fractions were analyzed for structural carbohydrates and lignin content. Before composition analyses, all solid samples were dried and ground into fine powder. Fermentation supernatants were analyzed for enzyme activities, residual reducing sugar and extracellular protein content. Fermentation broths from fermenter experiments were analyzed for cell mass concentration. All measurements were carried out at least in duplicate.

3.8.1 Composition analyses

3.8.1.1 Reducing sugar (RS)

For measurement of reducing sugars, 3 mL of the dinitrosalicylic acid reagent (DNS) was added to 1.5 mL of appropriately diluted sample, and the mixture was placed in boiling water for 5 min (Miller, 1959). After cooling and adding 16 mL of distilled water, absorbance was read at 550 nm in order to determine the concentration of RS. Glucose was used to prepare standard curve.

3.8.1.2 Extracellular protein content

Extracellular protein content was determined by Bradford method (Bradford, 1976) using Coomassie Blue G250 reagent. Calibration curve was prepared with Bovine Serum Albumin.

3.8.1.3 Cell mass determination

For cell mass determination 100 mL fermentation broth obtained from fermenter experiments was vacuum-filtered onto a dry and preweighed filter cloth and washed with distilled water. After drying at 105°C for 6 hours the filter cloth containing a filter cake was weighed again. For further analysis 0.5 g homogenous sample from each filter cake was subjected to a two step sulfuric acid hydrolysis to release N-acetyl-glucosamine and glucosamine from fungal cell wall (building units of chitin and chitosan): the sample was incubated with 60% (w/v) sulfuric acid at room temperature for 24 hours with subsequent dilution to 1 N and hydrolyzed at elevated temperature (121°C for 1 hour)
(Sakurai et al., 1977). This two-step hydrolysis results in complete deacetylation, thus N-acetyl-glucosamine was also converted to glucosamine (Zamani et al., 2008). The room temperature mixture was neutralized by addition of 1 N solution of NaOH and the total volume was measured. A portion was centrifuged at 3400 g for 5 minutes and supernatants were analyzed for glucosamine concentration by the method of Blix (Blix, 1948) as described by Bussari et al. (2008). Glucosamine content was calculated according to a calibration curve prepared with reagent grade D-glucosamine.HCl (Sigma-Aldrich). Cell mass is expressed as mg glucosamine/mL fermentation broth.

3.8.1.4 Determination of biomass composition

Glucan and xylan contents of raw materials before and after pretreatments were determined using a two-step sulfuric acid hydrolysis based on the method described by Hägglund (1951). A modified method was performed in which 0.5 g raw material was dispersed in 2.5 mL 72% sulfuric acid and maintained at room temperature for 2 h with occasional stirring. Next, 75 mL of distilled water was added and autoclaved for 1 h at 121°C. The reaction mixture was separated on preweighed, dried G4 glass filtering crucibles by applying vacuum. The filtrate was analyzed for carbohydrates by HPLC. The filter cake was washed with hot distilled water and dried at 105°C. This residue was defined as lignin content, and it was corrected for ash content, which was determined after incineration at 550°C for 6 h.

For starch content determination a slightly modified method of NREL (NREL, 1996) was applied. A dry mass of 0.5 g of ground wheat grain was suspended in a mixture of 25 mL distilled water and 10 mL 2 M NaOH. It was then incubated at 90°C for 20 min, followed by cooling to a temperature below 50°C. The sample was neutralized through the addition of 10 mL 2 M HCl. Then 10 mL of 0.1 M Na-acetate buffer (pH 4.2) and AMG 300L (Novozymes A/S, Bagsvaerd, Denmark) amylglucosidase of A. niger at 600 U/g solid were added for a 12-h hydrolysis at 40°C. This mixture was centrifuged at 3,400g for 5 min, and the supernatant was analyzed for glucose via HPLC. Measured glucose concentrations were reduced by the glucose content of AMG 300L.

Initial sugar content of sweet sorghum juice was determined by ‘Sucrose, D-fructose and D-glucose’ kit according to the attached description (Megazyme International Ltd., Ireland).
3.8.1.5 HPLC analyses

Samples for HPLC analysis were prepared by filtering through a regenerated cellulose syringe filter with 0.45 μm pore size (ProFill, Langerwehe, Germany). The glucose, cellobiose, xylose and ethanol contents of different samples were separated on an Aminex ion exclusion HPX-87H cation-exchange column (BioRad, Hercules, CA, USA) running at 65°C with 5 mM sulfuric acid as mobile phase at a flow rate of 0.5 mL/min. After separation, compounds were detected by a Shimadzu RID-I0A refractive index detector (Shimadzu, Kyoto, Japan) as presented on Figure 6. and in Table 10. (Paper I, Paper II, Paper III and hydrolysis samples in Paper IV). Based on many years of gathered laboratory experiences in the research group and also supported by Bonn and Bobleter (1984) this HPLC system with the respective parameters is convenient to measure sugars and ethanol simultaneously in fermentation samples. The inhibitor content of liquid fraction in Paper III was also measured by this system.

Figure 6. – Chromatogram of the calibration mixture by the HPLC system described above. Peaks from the left: cellobiose, glucose, xylose, arabinose, (acetonitrile) and ethanol.
Table 10. – Properties of compound identification by the HPLC system described above. Concentrations assumed to exceed the calibration range were measured after dilution with distilled water.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time min</th>
<th>Calibration range g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellobiose</td>
<td>9.0</td>
<td>0.2-5.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>11.0</td>
<td>0.3-10.0</td>
</tr>
<tr>
<td>Xylose</td>
<td>11.8</td>
<td>0.3-10.0</td>
</tr>
<tr>
<td>Arabinose</td>
<td>12.8</td>
<td>0.2-5.0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>26.2</td>
<td>0.2-7.0</td>
</tr>
</tbody>
</table>

Total sugars (including both monomers and oligomers) of the liquid fraction used in Paper IV were determined after mild acid hydrolysis (4% (v/v) H₂SO₄, 120°C and 30 min). Monomeric and total sugar concentrations in liquid fractions were measured by HPLC (Waters, Mildford, MA, USA) with a 2414 refractive index detector (Waters, Mildford, MA, USA). A Transgenomic CarboSep CHO-682 carbohydrate analysis column was employed for the separation, and operating at 80°C with deionized water as mobile phase (0.5 mL/min).

The inhibitor content of liquid fraction in Paper IV was analyzed as follows. Furfural, HMF, vanillin, 4-hydroxybenzaldehyde, coumaric acid, and ferulic acid were analyzed on an Aminex HPX-87H column at 65°C. For the mobile phase, 89% 5 mM H₂SO₄ and 11% acetonitrile, at a flow rate of 0.7 mL/min, were used. For detection, a 1050 photodiode-array detector (Agilent, Waldbronn, Germany) was employed. Acetic and formic acid were also quantified by HPLC (Waters, Milford, MA, USA) with a 2414 refractive index detector and an Aminex HPX-87H column maintained at 65°C with a 5 mM sulfuric acid mobile phase at a flow rate of 0.6 mL/min.

**3.8.2 Enzyme activity assays**

**3.8.2.1 Filter Paper Activity (FPA)**

FPA measurements were performed to describe the overall saccharification potential of produced enzymes. 0.5 mL of appropriately diluted supernatant liberating approximately 1 mg glucose equivalent under assay conditions was mixed with 1.0 mL of 0.05 M Na-acetate buffer (pH 4.8) and a 6×1 cm strip of Whatman grade No. 1 filter paper equal to 50 mg of cellulose was added. After incubation for 1 hour at 50°C, 3 mL of DNS reagent was added to stop the reaction. Liberated reducing sugar was measured as described above. The filter
paper unit (FPU) per mL was defined as the amount of glucose released given in μmol/min.

3.8.2.2 Xylanase activity (XYLA)
To measure xylanase activity, 0.1 mL properly diluted supernatant liberating approximately 0.2 μg xylose was added to the mixture of 0.4 mL 0.05 M citrate buffer (pH 5.3) and 0.5 ml of 1% (w/v) birchwood xylan (Sigma Aldrich) solution prepared in the same buffer. After incubation at 50°C for 10 min the reaction was terminated by adding 1.5 mL of DNS reagent and then the mixture was kept at 100°C for 5 min. Absorbance was read at 550 nm. A xylose calibration curve was used to calculate the activity defined as the amount of xylose released given in μmol/min.

3.8.2.3 β-glucosidase activity (BGA)
β-glucosidase activity was assayed according to the procedure of Berghem and Petterson (1974) using 5 mM 4-nitrophenyl-β-D-glucopyranoside (Sigma-Aldrich) as substrate in Na-acetate buffer (pH 4.8). 1 mL pre-incubated substrate was mixed with 0.1 mL sample. After the reaction (10 min at 50°C) was terminated with addition of 2 mL of 1 M Na₂CO₃ solution and diluted with 10 mL of distilled water the released 4-nitrophenol was measured at 400 nm against substrate blank.

3.8.2.4 Endo-glucanase activity (EGA)
For endo-glucanase activity measurement tenfold diluted supernatant was added to Azo-CM-cellulose solution (S-ACMC; Megazyme International Ltd., Ireland). The procedure was carried out according to the manufacturer’s instructions.

3.8.3 Calculations
Statistical analyses were performed with STATISTICA 9.0 software (StatSoft, Inc., Tulsa, OK, USA). The significance level for t-test for independent samples was set to a p-value of 0.05.

In Paper II ethanol conversion was calculated as follows:

\[
\text{conversion}^{\text{EtOH}}_{\text{final}}\left(\%\right) = 100 \cdot \frac{c^{\text{final}}_{\text{EtOH}}[g/L]}{0.51 \cdot c^{\text{initial}}_{\text{sugars}}[g/L]}
\]
In case of bagasse the initial sugar concentration was calculated based on the glucan content of the washed pretreated sample. Value of 0.51 is the stoichiometrical factor for ethanol conversion.

Ethanol yields for bagasse were calculated as follows:

\[
\text{yield}_{\text{EtOH}}[\text{L/ha}] = \frac{c_{\text{final}}^\text{EtOH}[\text{g/L}] \cdot V_{\text{sample}}[\text{L}]}{m_{\text{sample}}[\text{g}] \cdot 789.3[\text{g/L}] \cdot 45 \cdot 10^9[\text{g/ha}] \cdot 0.5 \cdot \frac{\text{glu}_{\text{raw}}[\%]}{\text{glu}[\%]}}
\]

Whereas,
- 789.3 is the density of ethanol
- 45 t/ha is a hypothesized green yield of bagasse with 50% dry mass content
- the last member of equation, the ratio of glucan contents (actual compared to the one of raw bagasse) is a correction factor to estimate mass loss during pretreatment (since \(m_{\text{sample}}\) is the mass of washed pretreated sample)

Ethanol yield for juice was calculated as follows:

\[
\text{yield}_{\text{EtOH}}[\text{L/ha}] = \frac{c_{\text{final}}^\text{EtOH}[\text{g/L}]}{789.3[\text{g/L}] \cdot 1050[\text{g/L}]} \cdot 45 \cdot 10^9[\text{g/ha}]
\]

Whereas,
- 1050 is a hypothesized density of sweet sorghum juice (based on sugar content)
- 45 t/ha is the hypothesized juice yield

Calculations related to the development of the online fermentation monitoring system are given the Results and Discussion session as those were part of the device and method development.
4. Results and discussion

In this chapter, I present and discuss the main results published in papers I-V. Paper I describes a method and device development for fermentation tracking that is crucial for laboratory practices and testing new approaches. Paper II and III contain experiments to support the viability of whole sweet sorghum plant utilization with regards to both ethanol fermentation and onsite cellulase enzyme production by *T. reesei*. Paper IV investigates another substrate, the liquid fraction of pretreated wheat straw for a similar aim of process integration by means of onsite enzyme production by *T. reesei*. Paper V possesses a different way for the enhancement of cellulase production: it aims to take advantages of the modern genetics to improve cellulase secretion in *T. reesei*.

4.1 A novel device for tracking ethanol fermentation (Paper I)

In order to evaluate and draw correct conclusions from experiments the existence of proper methods and dedicated devices is necessary. First, a device was developed that is capable to monitor ethanol fermentation in real time. With this feature and the eight parallel sample sites it is possible to see the differences between substrates, pretreatments and media as soon as fermented and not only after timely analyses.

4.1.1 Description of the system and principle of operation

The system is basically divided into two parts, the measuring device and the processing software. The main component part of the device is a U-tube (with 15 mm internal diameter and height of about 25 cm) filled with $10^{-2}$ M HCl solution to prevent CO$_2$ loss and equipped with two copper electrodes with different lengths. The end of the longer electrode is permanently in the acid solution while the shorter one only when the device is under pressure, i.e. effluent gas enters the tube. Between the two legs a transfer tube is placed with 3 mm internal diameter and connection points to the main legs at different heights (Figure 7.).
1. Gas introductory tube (from reactor)
2. Transfer tube between the two legs
3. Gas outlet
4. Shorter electrode (+4.4 V voltage)
5. Longer electrode (0 V voltage)

Figure 7. – Structure and parts of the U-tube applied in the online fermentation monitoring device

This structure of the U-tube and thus the operation principle are originally described by Veiga et al. (1990) in order to measure the volume of biogas in aerobic digestion. Compared to that design some minor differences were applied to the U-tube used in our setup. First, the size was decreased resulting in discharge events with volume around 12 mL instead of the 50 mL as described by Veiga et al. Second, the original design was supplied by a voltage of 220 V, while in our case it was only 4.4 V supplied through the USB port of a PC. Nevertheless, the major difference is the method of signal processing: with being connected to a PC, it became possible to follow the process in real time and obtain a view into the reaction kinetics. This real time tracking is possible on 8 channels simultaneously – to measure more samples at the same time is not mentioned by Veiga et al. Furthermore, we carried out a statistical evaluation and attempted to define the accuracy of our device specific for ethanol fermentation.

The operation principle is the following: the produced CO₂ enters in either leg of the tube and leaves from the other while the same volume of liquid as its own is moving through the legs. The gas leaves the tube in fixed increments through an inverted siphon (transfer tube) placed between the two legs of the U-tube. The different lengths of the electrodes and thus their changing contact with the solution depending on the level make it possible to detect changes in the liquid
surface level and thereby count the number of discharge events. These events get registered on a personal computer by a software, developed by Stereo Vision Ltd. As the measuring device was calibrated, the exact volume leaving the tube during a discharge event is known hence by cumulating the number of these events the volume can be calculated. As the main feature the software displays a cumulated CO₂ versus time plot in real time. The measured data and the plot can be exported to a Microsoft Excel file for further editing and studying.

Principle of calibration is also displacement using an empty bottle instead of fermentation sample connected to the device. The 8 channels were parallel calibrated with 500 mL, about 31°C (the temperature of fermentation) water running through the system and displacing the same volume of air leaving the bottle via the U-tube imitating displacing events. The event volume was calculated from the volume of the transferred water measured with a measuring cylinder divided with the number of displacement events (mL/event).

4.1.2 Evaluation of the results

As the event volume is of crucial importance for reliable results, the reliance and repetitiveness of the calibration method was investigated. For this reason calibration on each channel was repeated many times even at different days and testing whether the same units got obtained. Based on the repeated calibration and statistical evaluation the method of calibration was accepted, as well as the event volume was considered as reliable and accurate. In case of all channels the event volumes were about 12 mL that is equal to a resolution of 0.045 g of hexose substrate or 0.023 g of ethanol assuming a method with good sensitivity. These volumes were used through the tests to estimate the volume of gas produced.

Similar to calibration the fermentation runs were repeated many times on different days and also the same samples were run on different channels to test the compatibility of channels. Based on statistical evaluation it was shown that the repeated measurements of the same sample on different days and channels deliver results with a standard deviation of 116.2 mL (equal to 10 displacement events or 0.23 g ethanol). This means that the influence of the choice of channel and day for the measurement is inside the acceptable range; thus channels are compatible with the others and can reliably repeat measurements.

In order to determinate the concentration of ethanol based on the produced amount of CO₂ as measured with online fermentation monitoring system an
equation was developed. Main assumptions of this equation are the stoichiometrical equal amount of produced ethanol and CO$_2$ during fermentation and the ideal gas law describing the connection between the volume and molecular amount of gas:

$$c_{\text{EtOH}} [\text{g/L}] = \frac{(p_{\text{air}} [\text{Pa}] + 0.09 \cdot 1000 \cdot 9.81) \cdot V_{\text{CO}_2} [\text{mL}] \cdot 10^{-6}}{R \cdot T_{\text{air}} [\text{K}] \cdot \frac{46}{V_{\text{sample}} [\text{L}]}}$$

Whereas,
- $p_{\text{air}}$ is the average atmospheric pressure during measurement,
- 0.09 is the maximum deflection in the U-tube just before inversion [m],
- 1000 is the density of $10^{-2}$ M HCl which was applied to minimize CO$_2$ loss by solution [kg/m$^3$],
- 9.81 is the acceleration of gravity [m/s$^2$],
- $V_{\text{CO}_2}$ is the total volume of the produced gas during the measurement,
- $10^{-6}$ is a factor to convert mL to m$^3$,
- $R$ is the Regnault constant [8,314 J/mol K],
- $T_{\text{air}}$ is the average air temperature during the measurement,
- 46 is the molar weight of ethanol [g/mol],
- $V_{\text{sample}}$ is volume of the fermentation sample [L].

Simplification of this equation was carried out using standard conditions (25ºC, 100 000 Pa) that approximates well actual laboratory conditions:

$$c_{\text{EtOH}} [\text{g/L}] = \frac{0.0019 \cdot V_{\text{CO}_2} [\text{mL}]}{V_{\text{sample}} [\text{L}]}$$

In order to estimate the error due to this simplification error propagation hypothesis was tested with possible maximal variances from the standard conditions under the conditions of the experiments (15 hPa and 3ºC). The result of the test was ± 0.0052, which is minor so the simplification was valid and did not result in error propagation.

Results delivered this way were compared to results measured by HPLC. According to above equation a slope of 0.0019 is expected (1 mL of CO$_2$ is equal to 0.0019 g of ethanol) but based on measurements a value of 0.0016 was 48
obtained, however, the second member of the equation describing the slope was not zero leading to a constant bias. Furthermore, the more events were detected (the higher was the glucose/ethanol concentration), the higher was the difference of the measured (by HPLC) and calculated ethanol amount. This dependence of the bias on glucose concentration (i.e. on produced volume) was confirmed by residues plotting too.

Apart of this discrepancy, the device is suitable for ethanol fermentation tracking in real time with its obvious advantages. This visualization of fermentations makes it also possible to study the kinetics of product formation. The device has been used for the ethanol fermentation tracking of different raw materials including sweet sorghum juice and bagasse (Paper II), as well as test runs have been conducted to monitor biogas formation of swine manure.

4.2 Utilization of whole sweet sorghum plant ‘Monori Édes’ for ethanol production (Paper II)

A process design, based on sweet sorghum, has been proposed by Sipos at al. (2009) making it possible to integrate second generation technologies into first generation ones (Figure 8.). This design merges cellulase enzyme production into the process by utilizing the liquid fraction of pretreatment as onsite substrate (option 1). Based on this scheme both the juice and the pretreated bagasse were utilized for ethanol production (Paper II).

![Figure 8. – Scheme of integrated sweet sorghum processing (Sipos et al., 2009)](image-url)
4.2.1 Fermentation of the juice

The juice containing originally sucrose, glucose and fructose in a total concentration of 163.8±7.3 g/L was fermented by baker’s yeast in the device described above. As a result a final ethanol concentration of 68.7±2.1 g/L was reached that is equal to a conversion value of 78.9% based on total initial sugar content.

On basis of the obtained effluent gas volume curve the fermentation started already after approximately 20 min after inoculation. Reaching a constant velocity in 70 minutes for an 8 hour interval which was slowly declining and gas formation stopped after 24 hours (Figure 9.). In view of this plot, it was sampled to measure ethanol concentration considered as final.

![Figure 9. – Gas production curves of sweet sorghum juice fermentation recorded by the online fermentation monitoring system](image)

4.2.2 Different alkali pretreatments of bagasse

Contrary to the juice, which is readily fermentable, bagasse is needed to be pretreated and hydrolyzed previous to fermentation. Pretreatment was carried out in eight different ways based on combination of 3 parameters on two levels. After pretreatment the slurry was separated and washed. Composition of the washed pretreated solids was determined in order to evaluate the effectiveness of pretreatments (Table 11.).

Pretreatments resulted in an enhanced glucan (from 41% to 45-67%) and xylan (from 17% to 23-29%) content. With increasing alkali concentration the glucan
content increased, this effect was especially significant at 121°C. Consequently, the largest increase was caused by harsher conditions, i.e. 2% concentration at 121°C. Increasing the concentration of alkali did not lead to enhanced xylan content at room temperature, whereas at 121°C a slight increase was observed.

Concerning the type of alkali, NaOH proved to be a more efficient pretreating agent than KOH in both concentrations and at both temperatures by means of increasing glucan content (Table 11.). Reason for this is that NaOH causes disruption of hydrogen-bonds in cellulose and hemicellulose, breakage of ester linkages between lignin and xylan, and deprotonation of phenolic groups; these lead to swelling of cellulose and solubilization of lignin (Chen et al., 2007).

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Glucan %</th>
<th>Xylan %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw material</td>
<td>41.30±0.07</td>
<td>17.41±0.15</td>
</tr>
<tr>
<td>1% NaOH 25°C</td>
<td>48.24±0.57</td>
<td>25.16±0.35</td>
</tr>
<tr>
<td>2% NaOH 25°C</td>
<td>50.02±0.52</td>
<td>23.34±0.13</td>
</tr>
<tr>
<td>1% KOH 25°C</td>
<td>44.59±1.19</td>
<td>24.25±0.29</td>
</tr>
<tr>
<td>2% KOH 25°C</td>
<td>48.63±0.68</td>
<td>24.44±0.30</td>
</tr>
<tr>
<td>1% NaOH 121°C</td>
<td>49.57±0.67</td>
<td>25.75±0.46</td>
</tr>
<tr>
<td>2% NaOH 121°C</td>
<td>66.86±0.39</td>
<td>28.76±0.35</td>
</tr>
<tr>
<td>1% KOH 121°C</td>
<td>45.78±0.59</td>
<td>24.56±0.74</td>
</tr>
<tr>
<td>2% KOH 121°C</td>
<td>64.85±0.36</td>
<td>29.37±0.16</td>
</tr>
</tbody>
</table>

### 4.2.3 Enzymatic hydrolysis and fermentation of pretreated bagasse samples

Subsequent hydrolysis was tracked by regular sampling and measurement of liberated RS describing well the hydrolysis (Figure 10. a and b.). In every case RS concentration started to increase rapidly which after the velocity decreased depending on substrate availability. Although hydrolysis of bagasse pretreated at 121°C gave the highest final RS concentrations (in case of 2% NaOH and 2% KOH, 22.9 and 21.3 g/L, respectively, equal to a carbohydrate conversion of 94.6% and 89.2% based on the glucan and xylan content of pretreated materials), pretreatments at room temperature with 2% of alkalis (NaOH 17.0 g/L RS, 86.8% carbohydrate conversion and KOH 15.4 g/L RS, 83.2%
carbohydrate conversion) can be considered also efficient, however, yielding lower conversion rates. In case of low concentration (1%) only hydrolysis of NaOH pretreated bagasse showed comparable results especially when pretreated at 121°C (17.0 g/L, 90.5% and at room temperature 12.9 g/L, 68.4%) whereas the effect of the 1% KOH pretreatment on hydrolysis lagged behind (at elevated temperature 10.5 g/L, 57.6% and at room temperature 6.2 g/L, 33.1%) .

Figure 10a. – RS liberation during hydrolysis of at 25ºC pretreated materials

Figure 10b. – RS liberation during hydrolysis of at 121ºC pretreated materials.
These hydrolyzates were fermented to ethanol using the same conditions applied to the juice. Based on real time gas formation curves, constant gas formation rate was reached in less than an hour. It took only 3-5 hours for the yeast cells to consume glucose liberated during enzymatic hydrolysis. In this case the fermentable sugar content (RS<23 g/L) was significantly lower than the total consumable sugar content of the juice (163.8 g/L) what explains well the short fermentation time.

Once gas formation stopped final ethanol concentrations were measured. These concentrations were used in order to calculate combined hydrolysis and fermentation conversion values based on the glucan content of pretreated materials (Table 12.). Surprisingly, the highest conversion was not reached when applying 2% of alkalis at 121°C as it could have been expected from its efficiency in terms of composition of pretreated materials and results of hydrolysis. Even though, the highest ethanol concentration was reached on bagasse pretreated with 2% KOH at 121°C, the highest ethanol conversion factor was obtained after fermentation of bagasse pretreated with 2% NaOH at 25°C. Reason for this may be the presence of some acetic acid in the fermentation samples as detected by HPLC that may have arisen of hemicellulose deacetylation during hydrolysis.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Ethanol g/L</th>
<th>Conversion %</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% NaOH</td>
<td>2.777 ± 0.370</td>
<td>48.2</td>
</tr>
<tr>
<td>2% NaOH</td>
<td>4.158 ± 0.337</td>
<td>69.6</td>
</tr>
<tr>
<td>1% KOH</td>
<td>1.160</td>
<td>21.8</td>
</tr>
<tr>
<td>2% KOH</td>
<td>2.413 ± 0.047</td>
<td>41.6</td>
</tr>
<tr>
<td>121°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% NaOH</td>
<td>3.107 ± 0.556</td>
<td>52.5</td>
</tr>
<tr>
<td>2% NaOH</td>
<td>3.829 ± 0.199</td>
<td>48.0</td>
</tr>
<tr>
<td>1% KOH</td>
<td>1.910 ± 0.004</td>
<td>34.9</td>
</tr>
<tr>
<td>2% KOH</td>
<td>4.461 ± 0.355</td>
<td>57.6</td>
</tr>
</tbody>
</table>

Combined ethanol yield of juice and bagasse as expressed in L/ha was calculated for sweet sorghum variety ‘Monori Édes’. For the juice according to the ethanol concentration reached (68.7 g/L) an ethanol yield (L/ha) was
calculated equal to 3 729 L/ha assuming 45 t/ha juice yield comparable to the ones found in the literature (Almodares and Hadi, 2009; Byrt et al., 2011). Based on the results obtained with the bagasse an ethanol yield of 4 560 L/ha was calculated which corresponds to the highest ethanol conversion rate (69.9% with pretreatment 2% NaOH at room temperature) in case of 45 t/ha bagasse yield with 50% dry weight. Summing up of ethanol yields, an overall ethanol potential of 8 289 L/ha was obtained, which is an outstanding performance for a plant cultivable under moderate climate. Processing of sugar cane juice can yield 3 000-9 000 L/ha under tropical climate with multiple harvests annually (Byrt et al., 2011).

4.3 Onsite cellulase enzyme production on liquid fraction

The aim of this part was to introduce a substrate for cellulase production by *T. reesei* that is available onsite and thus can be merged into the biomass-to-ethanol process. Liquid fraction of steam pretreatment was chosen since it is an unavoidable byproduct in the process. Besides its valuable sugar content, mainly in the form of pentose monomers and oligomers, due to severe conditions applied in pretreatments liquid fractions also contains compounds inhibiting microbial growth.

4.3.1 Adaptation of *T. reesei* to liquid fraction of pretreated sweet sorghum bagasse (Paper III)

4.3.1.1 Effect of liquid fraction on *T. reesei* enzyme production

The liquid fraction of steam pretreatment was utilized for cellulase production according to option 1 on Figure 8. This liquid fraction (actually diluted with washing water) contained monomeric and oligomeric sugars, mainly xylose and xylooligosaccharides, and because of further degradation also furfural, HMF, acetic and formic acid and also other inhibitors not measured.

Preliminary shake flask experiments indicated that this liquid fraction was too toxic for *T. reesei* (neither enzyme production, nor RS consumption occurred). This was unexpected since, according the results of Szengyel and Zacchi (2000), sum concentration of furfural and acetic acid present in this liquid fraction should not have led to inhibition. However, results of Szengyel and Zacchi were obtained in a model medium not in liquid fraction coming from pretreatment. It was assumed that the difference in inhibition could be caused by other degradation products, originating mostly from lignin not measured here, and by possible interactions between inhibitors. In order to overcome this unexpected inhibition, *T. reesei* was adapted to the liquid fraction that contains
a wide variety of inhibitors of which only the four major ones (furfural, HMF, acetic and formic acid) were determined.

4.3.1.2 Pre-adaptation on agar plates

Another preliminary fermentation was carried out with applying inoculum already containing some liquid fraction (20%). It was observed that during the fermentation consumption of RS was quicker if inoculated with the *T. reesei* culture that had already contained some liquid fraction. Therefore, it was assumed that adaptation could be a good strategy to partially overcome the inhibitory effect of the liquid fraction. Pre-adaptation of *T. reesei* was carried out on agar plates containing liquid fraction in different dilutions and further subcultured decreasing the dilution of liquid fraction. Reference plates did not contain any liquid fractions.

![Image of T. reesei growing on plates containing different dilution of liquid fraction. From left side: 1:3, 1:1, undiluted 7th day](image)

This approach proved to be very representative since the size of colonies reflected well the composition of media (i.e. dilution of liquid fraction), as well as the inhibitory nature of liquid fraction and the ability of *T. reesei* to adapt (Figure 11.). Colony diameters were measured to quantify adaptation ability: diameters decreased with increasing ratio of liquid fraction and the differences between the plates were also diminishing over time (Table 13.). Moreover, the changes in growth were not proportional to the dilution of liquid fraction. For instance on day 5 the diameter of the culture growing on 1:3 and 1:1 dilution was 18.6% and 62.7% lower, respectively, than the diameter of the reference (not by 25% and 50% assuming proportional inhibition). In case of agar plates with undiluted liquid fraction measurable growth could be observed only in the second week when other cultures had already overgrown the plates making the comparison impossible. This finding implies the very effect of inhibitors on the lag phase.
Table 13. – Colony diameters (mm) of *T. reesei* cultures growing on agar plates containing liquid fraction in different dilutions.

<table>
<thead>
<tr>
<th>Day</th>
<th>Reference</th>
<th>1:3</th>
<th>1:1</th>
<th>Undiluted</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>40±4.1 (100%)</td>
<td>29±3.4 (72.5%)</td>
<td>12±2.1 (30.0%)</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>59±5.3 (100%)</td>
<td>48±4.4 (81.4%)</td>
<td>22±2.9 (37.3%)</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>78±5.1 (100%)</td>
<td>71±4.4 (91.0%)</td>
<td>40±2.9 (51.3%)</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td>31±7.1</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td>40±6.4</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td>60±7.1</td>
</tr>
</tbody>
</table>

The adaptation ability expressed as rate of growth (mm²/day) decreased also with the increasing ratio of the liquid fraction (Figure 12.). The differences of the growing rates were compared with t-test for independent samples (p>0.05) showing that differences between reference and 1:3 plates are not significant (day 4-5 p = 0.12, day 5-7 p = 0.5). This indicates that a comparable growth could be obtained with adaptation.

![Graph showing rate of growth of *T. reesei* on plates containing liquid fraction in different dilutions](image)

**Figure 12.** – Rate of growth of *T. reesei* on plates containing liquid fraction in different dilutions

In order to increase the adaptation-efficiency and approach the features of cultures growing on reference plate, a second step was applied. Colonies growing on plates containing liquid fraction in dilutions of 1:3 and 1:1 were
subcultured to plates containing undiluted liquid fraction (1:3 was also subcultured to 1:1). With this additional step, growth on undiluted plates got measurable already in the first week unlike in first round. These observations proved that adaptation ability can be maintained and further enhanced by subculturing onto media containing decreasing dilution of liquid fraction.

4.3.1.3 Pre-adapted cultures in shake flasks fermentations

Cultures pre-adapted on agar plates were used to prepare inocula for shake flasks fermentations. However, according to Bigelow and Wyman (2002) conidia do not possess the gained adaptation ability, therefore, a piece of agar containing hyphae was cut off from plates and added into the inoculum media. These media contained liquid fraction in a ratio agreeing with the dilution in the given plate (Table 14.). For reference a non-adapted culture of *T. reesei* was used. After 4 days of cultivation (e.g. prior to inoculating the fermentation medium) growth of different inocula was assessed by pH and FPA measurements. There were no significant differences observed among the broths. In every case pH dropped to near 3.5 and FPA reached almost 0.5 FPU/mL. This indicates that adaptation ability was successfully carried from solid media into liquid ones.

Table 14. – Ratio of liquid fraction in liquid medium and its agar plate counterpart

<table>
<thead>
<tr>
<th>ID</th>
<th>Dilution of liquid fraction in the inoculum</th>
<th>Dilution of liquid fraction in the respective plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nr. 1 Reference</td>
<td>no liquid fraction</td>
<td>no liquid fraction</td>
</tr>
<tr>
<td>Nr. 2</td>
<td>1:3</td>
<td>1:3</td>
</tr>
<tr>
<td>Nr. 3</td>
<td>1:1</td>
<td>1:1</td>
</tr>
<tr>
<td>Nr. 4</td>
<td>1:1→1:1</td>
<td>1:3→1:1</td>
</tr>
<tr>
<td>Nr. 5</td>
<td>Undiluted</td>
<td>1:3→undiluted</td>
</tr>
<tr>
<td>Nr. 6</td>
<td>Undiluted</td>
<td>1:1→undiluted</td>
</tr>
<tr>
<td>Nr. 7</td>
<td>Undiluted</td>
<td>Undiluted</td>
</tr>
</tbody>
</table>

The fermentation medium in all cases contained undiluted liquid fraction besides the other components as given in Materials and methods section. In order to evaluate the fermentation process RS and FPA measurements were carried out regularly. Regarding the RS profile, describing well fermentation in terms of sugar consumption and so indicating growth, in all cases a slight
increase was observed from the initial 9 g/L concentration on day 1 (Figure 13.). On one hand this is due to the action of cellulase enzymes present in precultures and carried over at inoculation. On other hand, accumulation of RS showed prolongation of lag phase that was effectively shortened by adaptation: while adapted cultures had already started growing and consuming sugar on day 2, in reference RS concentration had increased further. On day 3 reference culture also started growing and consuming sugar but in lower rate than cultures inoculated with adapted T. reesei did one day before. It could be concluded that prolongation of lag phase and delayed growth caused by inhibitors could be overcome with adaptation as it could be perceived from RS profiles. In case of adapted broths, RS content dropped sharply resulting in an average difference of 77% between them and the reference. In all cases except the reference, RS was equivalent to zero from day 4.

![RS profiles](image)

**Figure 13.** – RS profiles on medium containing undiluted liquid fraction (inoculated with differently adapted T. reesei cultures).

Moreover, differences could be observed not only between the adapted broths and the reference but also among the adapted broths. In line with the liquid fraction content of inocula and agar plates, RS consumption profiles could be arranged into the following groups: Nr. 2 (equal to 1:3 plate) – Nr. 3 & 4 (equal to 1:1 plates) – Nr. 5, 6 & 7. After averaging the RS values in these groups the differences from reference were growing with decreasing dilution of liquid fraction in inocula: on day 2 in broths of Nr. 2, group of Nr. 3 & 4 and group of Nr. 5, 6, & 7 RS content were 4.7±0.2%, 24.3±4.9% and 43.8±3.4% lower,
respectively, than in the reference. As indicated on Figure 13. and based on acceptable standard deviations of differences the grouping of different broths in line with liquid fraction contents was a valid decision. The observed different patterns in RS consumption and its connection to liquid fraction dilutions in preculture phase indicate that the dilution of liquid fraction in the last step of adaptation process is more crucial than in how many steps it had been reached.

FPA values also reflect this grouping; therefore broths Nr. 3 & 4 and Nr. 5, 6 & 7 are shown as averages (Figure 14.). Standard deviations of averages were found not to be different than in case of unique broths that also underpins that final concentration of inhibitors determinates the success of adaptation. The most significant difference was observed on day 3: broths inoculated with either Nr. 5, 6 or 7 (i.e., pre-adapted on undiluted agar plates) reached in average 71.3±1.4% higher FPA than the reference. Delayed initiation of growth could be observed here as well. From day 3 the reference culture also started to grow and produce enzyme as reflected on the decreasing differences between FPA values. However, after 11 days the group consisting of Nr. 5, 6 & 7 had an average FPA of 1.7 FPU/mL that was 22.6% higher FPA than those of the reference.

![Figure 14. – Secreted FPA on undiluted liquid fraction (inoculated with differently adapted T. reesei cultures).](image)

These results are in accordance with observed growing rates on plates indicating that with adaptation longer lag phase caused by inhibitors can be significantly reduced both in terms of growth and enzyme production. Furthermore, and contrarily to the literature stating that no increased enzyme yields can be
reached with adaptation (Hayward et al., 1999), in this case, the adapted strains also achieved a higher final FPA value when growing on the liquid fraction. This also means higher FPA yields, since the carbohydrate content of broths was the same.

4.3.2 Cellulase production and use of produced enzymes in a wheat straw based process (Paper IV)

Wheat grain is a wide-spread raw material for first generation (starch based) ethanol production. Therefore, when talking about wheat straw utilization for bioethanol integration possibilities into wheat grain processing should be investigated to bring the two technologies closer in order to benefit the existing infrastructure. For this reason in Paper IV the liquid fraction of steam pretreated wheat straw was investigated for onsite enzyme production with wheat grain or pretreated washed wheat straw (WIS) supplementation.

4.3.2.1 Effect of wheat grain on cellulase secretion

Effect of wheat grain on cellulase production by T. reesei RUT C30 was studied and assessed by measuring different enzyme activities and RS. Final values after 7 days of fermentation are presented in Table 15. The highest overall cellulase activity was measured in broths growing on Solka Floc plus ground wheat grain (SF+WG). This was 20% higher than in case of reference broths growing solely on Solka Floc (SF). β-glucosidase secretion was also the most effective on mixed carbon source, and surprisingly, the next highest value was measured from broths growing on solely ground wheat grain (WG). These were 25% and 23% higher, respectively, than that of the solely Solka Floc broths.

Table 15. – Enzyme activities and RS

<table>
<thead>
<tr>
<th></th>
<th>FPA</th>
<th>BGA</th>
<th>RS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 2</td>
<td>Day 7</td>
<td>Day 7</td>
</tr>
<tr>
<td>FPU/mL</td>
<td>IU/mL</td>
<td>g/L</td>
<td></td>
</tr>
<tr>
<td>SF</td>
<td>0.66±0.02</td>
<td>0.86±0.01</td>
<td>0.40±0.02</td>
</tr>
<tr>
<td>WG</td>
<td>0.36±0.01</td>
<td>0.63±0.01</td>
<td>0.49±0.03</td>
</tr>
<tr>
<td>SF+WG</td>
<td>0.77±0.01</td>
<td>1.03±0.04</td>
<td>0.50±0.02</td>
</tr>
</tbody>
</table>

In broths containing ground wheat grain, accumulation of RS was observed, which after RS concentration decreased and got equal to zero by day 4. Because the increase of RS was not found in broths containing solely Solka Floc, and Solka Floc with wheat grain did not yield any significantly more glucose,
hydrolysis of starch and consumption of its products occurred. Production of amylolytic enzymes by the *Trichoderma* species (including *T. reesei*) has already been reported (Benitez et al., 1998).

It was also assumed that the effect on enzyme activities caused by wheat grain addition was most likely related to its starch content. Nevertheless, a small amount of lignocellulosic carbohydrate from wheat distillers’ grain and wheat bran was also present in each case. Wayman and Chen (1992) also observed that untreated whole wheat flour was good at inducing cellulase synthesis in *T. reesei* RUT C30. They assumed that the reason for this induction involved some synergy between the effects of bran and starch in the whole wheat flour.

Differences in β-glucosidase activity can be explained by the mechanisms of fungal amylases that, also assuming the ones of *T. reesei* RUT C30, liberate maltose and glucose from chain ends of starch. β-glucosidase triggered by lignocellulosic components in the medium can form cellulase inducers via transglycolysation from this starch derived glucose. This ability of β-glucosidases from different species has already been proposed (Fujimoto et al., 1988; Chen and Wayman, 1992). It hypothesized that some of these compounds can trigger even more β-glucosidase, creating a positive feedback loop.

On the other hand, *T. reesei* RUT C30 does not possess any functional genes encoding maltose permease which is responsible for maltose uptake into the cell. As a consequence, its growth on media where the sole carbon source is starch, or other α-glucans, is strongly impaired (Seidl et al., 2008). This phenomenon is supported by an earlier finding that starch itself was a poor inducer of cellulase enzyme production (Chen and Wayman, 1992). Furthermore, the catabolite derepressive property of *T. reesei* RUT C30 (Ilmén et al., 1996) also has an important role in helping to avoid repression by glucose from starch. Both of these unique features of RUT C30 can be crucial in cellulase secretion triggered by starch or its derivates because no cellulolytic enzyme production was observed in *T. reesei* QM9414 cultures growing on acid-hydrolyzed potato starch (Marquina and Flores, 1997).

Since wheat grain had a very positive effect on β-glucosidase production and as β-glucosidase activity is a crucial factor in hydrolysis with *T. reesei* enzymes (Breuil et al., 1992), ground wheat grain was used as supplementary material in the experiments with the liquid fraction.
4.3.2.2 Enzyme production on liquid fraction of steam pretreated wheat straw

In preliminary experiments the liquid fraction of steam pretreated wheat straw was found to be too toxic for \textit{T. reesei}: no growth was initiated on it even in fourfold (1:3) dilution as perceived by constant RS concentration of the broths. The liquid fraction contained HMF and furfural, derivatives of glucose and xylose, respectively. Additionally, a large amount of acetic acid was present, which demonstrates the high degree of hemicellulose solubilization. Further degradation of furfural and HMF occurred, resulting in formation of formic acid (Table 16.). Besides the inhibitors listed in Table 16., other compounds, namely 4-hydroxybenzaldehyde, vanillin, syringaldehyde, coumaric and ferulic acids, were identified in minor concentrations (<0.1 g/L).

In this fourfold diluted liquid fraction both acetic acid and furfural concentrations were in ranges (2.20 g/L and 0.52 g/L, respectively) reported not to be inhibitory for \textit{T. reesei} (Szengyel and Zacchi, 2000). Moreover, approximately this concentration of acetic acid (1.0 and 2.0 g/L) appeared to reduce the inhibitory effect of furfural (0.4, 0.8 and 1.2 g/L) when both were present in low concentrations (Szengyel and Zacchi, 2000). Despite this, no growth could be observed. This demonstrates again the effect of interactions and other possible inhibitors present in the liquid fraction.

In order to overcome the inhibition a two-step detoxification process consisting of vacuum evaporation and overliming by Ca(OH)$_2$ was carried out as described in Materials and methods section. As the result of detoxification furans were almost completely eliminated, but an increase in acetic acid concentration was observed leading to increased total inhibitor concentration. The glucose and xylose concentrations increased but with slightly decreasing total sugar concentrations showing that during detoxification, further degradation of oligomers occurred (Table 16.). Interestingly, the total amount of inhibitors in the detoxified liquid fraction was higher than in the original one, but mostly composed of aliphatic acids.
Table 16. – Effect of detoxification on inhibitors and sugars concentrations.

<table>
<thead>
<tr>
<th></th>
<th>Untreated liquid fraction</th>
<th>After detoxification&lt;sup&gt;b&lt;/sup&gt;</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Furfural</strong></td>
<td>2.1±0.5</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td><strong>HMF</strong></td>
<td>0.7±0.0</td>
<td>0.1±0.1</td>
<td></td>
</tr>
<tr>
<td><strong>Acetic acid</strong></td>
<td>8.8±0.8</td>
<td>12.4±1.7</td>
<td></td>
</tr>
<tr>
<td><strong>Formic acid</strong></td>
<td>7.2±0.9</td>
<td>6.8±2.2</td>
<td></td>
</tr>
<tr>
<td><strong>Total inhibitors</strong></td>
<td>18.8</td>
<td>19.3</td>
<td></td>
</tr>
<tr>
<td><strong>Glucose</strong></td>
<td>0.6±0.1</td>
<td>2.1±0.2</td>
<td></td>
</tr>
<tr>
<td><strong>Total glucose</strong></td>
<td>7.2±0.7</td>
<td>7.1±0.3</td>
<td></td>
</tr>
<tr>
<td>(mono- &amp; oligomers)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Xylose</strong></td>
<td>5.5±0.2</td>
<td>6.4±0.2</td>
<td></td>
</tr>
<tr>
<td><strong>Total xylose</strong></td>
<td>40.5±3.1</td>
<td>37.6±3.3</td>
<td></td>
</tr>
<tr>
<td>(mono- &amp; oligomers)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Other sugar monomers</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.6±0.3</td>
<td>2.4±0.1</td>
<td></td>
</tr>
<tr>
<td><strong>Total other sugars</strong></td>
<td>4.5±0.6</td>
<td>4.8±0.1</td>
<td></td>
</tr>
<tr>
<td>(mono- &amp; oligomers)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total sugars</strong></td>
<td>52.2</td>
<td>49.5</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> galactose, mannose and arabinose
<sup>b</sup> adjusted to pH 5.8 as used in fermentations

Fermentations were performed with this detoxified liquid fraction in different dilutions. Enzyme production was only observed in the case where the liquid fraction was diluted to 1:3.

Inhibitors were measured also at the end of the 11 days fermentations (Table 17.). In case of broths containing undiluted or twofold (1:1) diluted detoxified liquid fraction, concentrations of inhibitors did not change during fermentation. Neither aliphatic acids, nor other inhibitors could be detected in broth containing detoxified liquid fraction in 1:3 dilution. Szengyel and Zacchi (2000) and Palmqvist et al. (1997) also found that *T. reesei* was able to consume acetic acid in media containing low overall levels of inhibitors, in this experiment consumption of formic acid by *T. reesei* was also confirmed.
4.3.2.3 Integrated enzyme production

The fourfold diluted detoxified liquid fraction was used as sole carbon source (LIQ) or supplemented with other carbon sources (washed pretreated wheat straw – LIQWIS, ground wheat grain – LIQWG) related to wheat processing to enhance cellulase productivity and to optimize the composition of the enzyme mixture for hydrolysis. Media compositions are presented in Materials and methods section. With these media, every compound, excluding salts and buffer, would be available onsite through an integrated wheat processing factory.

Fermentation was monitored by regular sampling, RS and FPA measurements. Accumulation of RS on the first two days illustrated the prolonged lag phase by inhibition (Figure 15.). This accumulation was due to enzymes present in the inocula while the consumption of produced sugars was hampered by inhibitors. Unlike this, no RS accumulation was observed in reference broths where cells were immediately able to utilize the released sugars from pretreated washed wheat straw. When *T. reesei* started to grow, concentration of RS dropped sharply. This event occurred at the same time in liquid fraction containing media, irrespective of supplementing carbon source. Thus, the supplementary substrates did not decrease the length of the lag phase.

### Table 17. – Inhibitor content of final fermentation broths

<table>
<thead>
<tr>
<th></th>
<th>Formic acid</th>
<th>Acetic acid</th>
<th>Furfural</th>
<th>HMF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/L</td>
<td>g/L</td>
<td>g/L</td>
<td>g/L</td>
</tr>
<tr>
<td>Fourfold diluted (1:3, 25%)</td>
<td>below detection limit</td>
<td>below detection limit</td>
<td>below detection limit</td>
<td>below detection limit</td>
</tr>
<tr>
<td>Twofold diluted (1:1, 50%)</td>
<td>4.6</td>
<td>6.1</td>
<td>below detection limit</td>
<td>below detection limit</td>
</tr>
<tr>
<td>Undiluted</td>
<td>8.0</td>
<td>11.5</td>
<td>below detection limit</td>
<td>below detection limit</td>
</tr>
</tbody>
</table>
It was hypothesized that the easily accessible monomeric sugars in the liquid fraction were consumed first (between days 2-4) to promote growth even in the presence of inhibitors. Supporting this, in *T. reesei* RUT C30 cultures growing on a mixture of xylose and cellulose, xylose was found to be consumed first to support growth (Mohagheghi et al., 1988).

As FPA values show the enzyme production had already started in the presence of RS (day 3 and 4) (Figure 16.). But, in line with the observed RS accumulation a lag phase in FPA production was also measured when the liquid fraction was present. Reference broths without any inhibiting substances showed obvious constant increase of produced FPA already from day 1, whereas a similar increase could only be observed in liquid fraction containing broths from day 3 and 4. After seven days the reference broths had already reached maximal FPA, while the other cultures still showed increasing activities reaching 25-69% higher volumetric FPA activities on day 11 than the control. The catabolite derepressive feature of RUT C30 became a crucial factor again by largely contributing to the enzyme production on liquid fraction. The liquid fraction contained soluble xylose and glucose in addition to their oligomers. Induction of cellulase secretion has been reported by water soluble glucose oligomers (n<6) in *T. koningii* (Wang et al., 1988) and also partially by xylose in *T. reesei* RUT C30 (Mohagheghi et al., 1988). In the liquid fraction used,
these soluble inducing oligomers were available to facilitate enzyme secretion, however, in a matrix toxic for microbes.

In order to evaluate the effectiveness of fermentation and investigate the composition of enzyme mixture secreted β-glucosidase and xylanase activities were measured from final broths (after 11 days of fermentation) (Table 18.). Xylanase activities were found to be notably higher in broths containing the liquid fraction than in the reference. Reason for this low activity of the reference may be the lower xylan content of the washed pretreated wheat straw as a sole carbon source. On the other hand presence of D-xylose di- and trisaccharides in liquid fraction may cause more powerful stimulation of xylanase secretion than monomers alone (Xiong et al., 2005). Moreover, in the used fourfold diluted detoxified liquid fraction other pentoses were also present. For instance the concentration of arabinose, that was found to be a more effective inducer of xylanase secretion than xylose (Xiong et al., 2004), was the third highest (0.5 g/L) among monomers. Among the broths with liquid fraction, xylanase activities were practically equal in LIQ and LIQWIS, while 30% higher xylanase activity was measured in LIQWG.

As result of wheat grain supplementation β-glucosidase activity was near doubled, whereas FPA was 35% higher, both compared to reference. Thus, a more favorable BGA/FPA ratio could be reached. This positive effect on
β-glucosidase activity of wheat grain may be related to the starch content because enhanced β-glucosidase activity was observed when *T. reesei* RUT C30 was cultivated on Mandels’ medium with different carbon sources supplemented with 1% starch (Taj-Aldeen, 1993).

### Table 18. – Final protein concentrations, volumetric activities and yields after 11 days of cultivation.

<table>
<thead>
<tr>
<th></th>
<th>Protein (g/L)</th>
<th>Xyla (IU/mL)</th>
<th>BGA (IU/mL)</th>
<th>FPA (FPU/mL)</th>
<th>BGA/FPA ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIQ</td>
<td>0.58±0.03</td>
<td>127±4</td>
<td>0.88±0.02</td>
<td>1.38±0.01</td>
<td>64</td>
</tr>
<tr>
<td>LIQWG</td>
<td>1.18±0.05</td>
<td>165±4</td>
<td>1.74±0.03</td>
<td>1.87±0.05</td>
<td>93</td>
</tr>
<tr>
<td>LIQWIS</td>
<td>0.69±0.02</td>
<td>130±5</td>
<td>0.91±0.06</td>
<td>1.67±0.04</td>
<td>55</td>
</tr>
<tr>
<td>REF</td>
<td>0.44±0.06</td>
<td>49±6</td>
<td>0.88±0.01</td>
<td>1.11±0.04</td>
<td>79</td>
</tr>
</tbody>
</table>

### 4.3.2.4 Enzymatic hydrolysis

In order to investigate the hydrolytic potential of produced enzymes hydrolysis experiments on washed pretreated wheat straw were carried out. Volumes to obtain 20 FPU/g glucan were calculated by activities measured in supernatants, however whole fermentation broths (after 11 days of fermentation) were actually used. Whole fermentation broth of *T. reesei* RUT C30 was reported to be more effective in hydrolysis in the aspect of glucose liberation than the supernatant (Kovács et al., 2009a). While the BGA/FPA ratio for the LIQ and LIQWIS samples were 64% and 55%, respectively, that of LIQWG was similar to the ratio of the reference (93% versus 100%, respectively) (Table 19.).

### Table 19. – Enzyme activities and protein content of produced and commercial enzymes representing 20 FPU cellulase activity.

<table>
<thead>
<tr>
<th></th>
<th>Volume (mL)</th>
<th>FPA (FPU)</th>
<th>Protein (mg)</th>
<th>BGA (IU)</th>
<th>Xyla (IU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIQ</td>
<td>14.49</td>
<td>20</td>
<td>8.40</td>
<td>12.75</td>
<td>1840</td>
</tr>
<tr>
<td>LIQWG</td>
<td>10.70</td>
<td>20</td>
<td>12.63</td>
<td>18.62</td>
<td>1766</td>
</tr>
<tr>
<td>LIQWIS</td>
<td>11.98</td>
<td>20</td>
<td>8.27</td>
<td>10.90</td>
<td>1557</td>
</tr>
<tr>
<td>Celluclast + Novozym 188a</td>
<td>0.34</td>
<td>20</td>
<td>13.04</td>
<td>20</td>
<td>243</td>
</tr>
</tbody>
</table>

aBGA supplemented to 20 IU/g glucan

Hydrolysis was monitored by regular sampling and glucose, cellobiose and xylose measurements. Concentration of released glucose, cellobiose and xylose was plotted against hydrolysis time (Figure 17. a and b). Cellobiose is an
intermediary of the cellulose hydrolysis; however its presence refers to actual β-glucosidase action. Therefore, the more favorable BGA/FPA ratios resulted in less accumulation, whereas suboptimal ones led to increased cellobiose concentration. The highest cellobiose accumulation of 3.3 g/L, was measured from samples LIQ after 24 hours, while a decline was already occurring in the other samples at this time. Contrary to the measured lower activity of β-glucosidase, LIQWIS showed less cellobiose accumulation than the aforementioned sample. Thus, it can be assumed that the LIQWIS broth had more enzymes in the hyphae or substrate-bound form (the LIQWIS broth contained a solid residue of the steam pretreated substrate resulting in higher enzyme adsorption).

Despite the near optimal BGA/FPA ratio of LIQWG temporary cellobiose accumulation was much higher than in the reference. Nevertheless, it was the most effective among the broths in terms of glucose liberation; at the end of the 72-hours hydrolysis, glucose both in the reference and samples with LIQWG broth also reached 11 g/L. This concentration is equal to 85% glucan conversion, while LIQWIS and LIQ reached 77% and 76%, respectively, compared to the theoretical maximum based on the initial glucan content of the washed steam pretreated wheat straw.

Liberation of xylose was similar in each case and more effective than with the Celluclast 1.5L-Novozym 188 combination, which had the lowest dose of xylanase. In terms of the dose of xylanase, there was no large difference between the broths. However, the xylan content of the substrate was rather low. On the other hand, higher xylanase activities may have contributed to the efficient glucose liberation achieved by increasing the accessibility of cellulose (Berlin et al., 2005). Final xylan conversion values were the following for each broth: 70% for the reference, and 81%, 85% and 85% for LIQ, LIQWG and LIQWIS, respectively, although LIQWIS contained the lowest xylanase activity among the broths.
Figure 17. – Hydrolytic performance on washed pretreated wheat straw of whole broths of *T. reesei* produced on liquid fraction (LIQ) supplemented with washed pretreated wheat straw (LIQWIS) or ground wheat grain (LIQWG). As reference (REF) commercial enzyme preparation was used.
4.4 Utilization of strains created by novel approach in up-scaled cellulase production (Paper V)

Effects of light on metabolic processes have been shown in numerous fungi and for many target mechanisms (Schmoll et al., 2010, Tisch and Schmoll, 2010). Although the most obvious functions of *Trichoderma* photoreceptors and related trans-factors (e.g. BLR1, BLR2 and ENV1) have been observed and studied in light, it has been also shown clearly that these proteins and their homologues additionally have functions in darkness (Schuster et al., 2007, Chen et al., 2009, Castellanos et al., 2010).

4.4.1 Main results of fermenter cultivations

In order to see whether the approach of modifying signal transduction could be exploited in creating more efficient industrial strains *T. reesei* QM9414 deletion mutants of *blr1*, *blr2* and *env1* were compared in 30 L laboratory scale fermenter on 10 g/L Solka Floc and 5 g/L wheat distillers’ grain supplemented with salts. Samples were withdrawn regularly to measure activities, protein and biomass content. Moreover, fermentation parameters such as pH, added volume of acid (10% H$_3$PO$_4$) and base (10% NH$_4$OH), pO$_2$ level, stirring velocity and air flow were recorded.

According to the recorded values of fermenter cultivations, each strain had similar patterns of pH adjustment and oxygen supply (data not shown). After approximately 5 hours of lag phase, pO$_2$ and pH began to decrease indicating growth of the fungi. These parameters suggested that modified strains have no increased oxygen and inorganic nitrogen demand (based on the pH as reflecting the ammonium consumption).

The enzyme activity profiles of strains (Figure 18.) show that missing function(s) of the regulatory mechanisms in deletion mutants causing enhanced cellulase production becomes most significant after 30 hours of fermentation. While for QM9414 and Δ*blr1* enzyme activities stagnated shortly thereafter, enzyme secretion as reflected by still increasing activities continues in Δ*blr2* and Δ*env1* (in most cases reaching the maximum between 48-54 hours of fermentation). The reason for the decrease of activities is presumably the protease activity in the medium.
Figure 18. – EG, FPA & BGA profiles of *T. reesei* QM9414, Δenv1, Δblr1 & Δblr2
The effect of deletion of \textit{blr1} and \textit{blr2} was different among the activities measured (Table 20.). For instance, despite its high efficiency on cellulose, \textit{Δblr2} showed approximately the same xylanase activity as QM9414 which might be the result of the different regulation pattern of hemicellulolytic enzymes. A comparable effect has already been observed in case of \textit{T. atroviride} mutants created by random mutagenesis that possessed enhanced FPA production properties but were deficient in xylanase secretion (Kovács et al., 2009b).

Table 20. – Comparison of strains by means of different enzyme activities. 0 – no significant difference to QM9414, + - higher (<30%) activity than that of QM9414, ++ - more than 30% difference compared to QM9414, -- - less activity than 70% of QM9414 produced

<table>
<thead>
<tr>
<th></th>
<th>Δenv1</th>
<th>Δblr1</th>
<th>Δblr2</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGA</td>
<td>++</td>
<td>0</td>
<td>++</td>
</tr>
<tr>
<td>FPA</td>
<td>+</td>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>BGA</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>XYLA</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
</tbody>
</table>

Nevertheless, the positive effect of \textit{env1} gene deletion was consistent among the different activities. This observation suggests that the regulatory targets of ENVOY include the pathways involved in plant cell wall degradation (Table 20.). The observed very significant enhancement of EGA production suggests that the increase of FPA in cases of Δ\textit{env1} and Δ\textit{blr2} is only in part due to enhanced expression of the cellobiohydrolases, but seems to be mainly caused by an improved overall efficiency of the endoglucanase mixture secreted (and in case of Δ\textit{blr2} BGA could also largely contribute).

It is interesting that different effects have been observed for deletion of \textit{blr1}, \textit{blr2} and \textit{env1}. On one hand this finding indicates that BLR1 and BLR2 do not act as a complex under our experimental conditions (mainly darkness) targeting cellulase gene expression, but rather have individual functions. In \textit{N. crassa} it was reported that the respective homologues, WC-1 and WC-2 can act as dimers (Ballario et al., 1998).

On other hand it also suggests that although induction of \textit{env1} transcription is abolished upon deletion of \textit{blr1} or \textit{blr2} (Castellanos et al., 2010), the consequences of deletion of \textit{env1} are not similar to those of deletion of these photoreceptors. Hence BLR1 and BLR2 do not exert their function via
ENVOY, which confirms the hypothesis proposed earlier (Castellanos et al., 2010).

4.4.2 Specific activities

Different regulatory backgrounds for enhanced enzyme activities could be proposed as perceived protein or biomass specific activities (Table 21.). In case of Δblr2 the enhanced FPA and endoglucanase activity were in line with the highest protein concentration measured among the strains. Considering also the similar biomass formation as of QM9414, this result indicates that the high cellulolytic activity found in the culture medium of this strain is mainly due to an enhanced protein secretion capacity.

Table 21. – Biomass and protein specific activities

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hour</th>
<th>QM9414</th>
<th>Δenvl</th>
<th>Δblr1</th>
<th>Δblr2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass, (mg GlcA)/mL</td>
<td>24</td>
<td>0.16±0.03</td>
<td>0.17±0.01</td>
<td>0.22±0.02</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.29±0.03</td>
<td>0.26±0.03</td>
<td>0.41±0.04</td>
<td>0.29±0.03</td>
</tr>
<tr>
<td>Cell specific FPA, FPU/(mg GlcA)</td>
<td>24</td>
<td>2.70±0.43</td>
<td>2.74±0.03</td>
<td>1.41±0.23</td>
<td>3.33±0.18</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4.09±0.16</td>
<td>5.59±0.09</td>
<td>1.99±0.21</td>
<td>4.80±0.23</td>
</tr>
<tr>
<td>Cell specific EGA IU/(mg GlcA)</td>
<td>24</td>
<td>32.70±3.61</td>
<td>24.35±0.15</td>
<td>22.69±2.93</td>
<td>55.87±3.94</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>40.54±1.47</td>
<td>64.80±3.92</td>
<td>26.83±3.88</td>
<td>66.19±2.60</td>
</tr>
<tr>
<td>Protein mg/mL</td>
<td>24</td>
<td>0.20±0.04</td>
<td>0.18±0.01</td>
<td>0.19±0.04</td>
<td>0.38±0.04</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.40±0.08</td>
<td>0.49±0.02</td>
<td>0.50±0.04</td>
<td>0.81±0.10</td>
</tr>
<tr>
<td>Cellulase efficiency, FPU/(mg protein)</td>
<td>24</td>
<td>2.27±0.36</td>
<td>2.74±0.03</td>
<td>1.63±0.26</td>
<td>1.40±0.07</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>2.98±0.12</td>
<td>2.93±0.05</td>
<td>1.61±0.17</td>
<td>1.70±0.08</td>
</tr>
<tr>
<td>EGA efficiency IU/(mg protein)</td>
<td>24</td>
<td>27.50±3.04</td>
<td>22.19±0.13</td>
<td>26.23±3.39</td>
<td>23.50±1.66</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>29.47±1.07</td>
<td>33.97±2.05</td>
<td>21.65±3.13</td>
<td>23.43±0.92</td>
</tr>
<tr>
<td>Cell specific proteins mg/(mg GlcA)</td>
<td>24</td>
<td>1.19±0.23</td>
<td>1.10±0.01</td>
<td>0.87±0.19</td>
<td>2.38±0.28</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1.38±0.28</td>
<td>1.91±0.07</td>
<td>1.24±0.09</td>
<td>2.83±0.37</td>
</tr>
</tbody>
</table>

Despite high FPA and endoglucanase activity values of Δenvl, only slightly increased total protein concentration was observed. Therefore, in contrast to Δblr2, the enzyme mixture secreted by Δenvl seems to be more efficient than that of the other strains investigated in this study. Light dependent adjustment of the proportions of respective enzymes could be one way to achieve this
enhanced efficiency, for instance, by binding of trans-factors involved in light response.

Regarding \( \Delta \text{blr1} \) lower efficiency of biomass specific protein secretion could be due to a counteracting effect of BLR1 compared to BLR2 in secretion. In \( N. \text{crassa} \) it has been shown that in darkness the homologue of BLR2, WC-2, is present in excess over WC-1 (Loros, 2005). Consequently, the effect seen for \( \Delta \text{blr1} \) is more likely to be due to an increased availability of BLR2, whose binding partner has been removed resulting in additional BLR2 proteins, now free to exert their negative function on secretion.

The assumption explained above on how the deletion of genes can contribute differently to the enhancement of cellulase secretion was also underlined by Western blotting. In case of \( \Delta \text{blr2} \) clearly higher abundance of CBH1 was observed, indicating that the increased cellulosylolytic efficiency of this strain is at least in part due to enhanced secretion of this enzyme. For \( \Delta \text{env1} \) the effect was less clear, suggesting that the improvement of the cellulase mixture found for this strain is likely to be caused by altered regulation of expression of the pool of enzymes contributing to cellulose degradation. Considering the data above, results of Western blotting correspond well with the enhanced protein secretion capacity of \( \Delta \text{blr2} \) and the evidently improved enzyme proportions for \( \Delta \text{env1} \).

### 4.4.3 Comparison of the effects of deletions on the level of transcription and secretion

Besides proving the effectiveness of signal transduction modification in up-scaled fermentation by means of improved secretion of cellulases another interesting observation was made. Considering the transcription data for \( \text{cbh1} \) in these strains (Castellanos et al., 2010) this higher efficiency confirmed in both preliminary shake flasks and in fermenter cultivations was surprising. In contrast to transcription of \( \text{cbh1/cel7a} \), which decreases in deletion mutants of \( \text{blr1} \) and \( \text{blr2} \) (Castellanos et al., 2010), cellulase activities secreted by the respective strains during submerged fermentations was nevertheless increased or at least remained at wild-type levels in case of \( \Delta \text{blr1} \). Interestingly, comparable effects have been observed for \( N. \text{crassa} \) (Schmoll et al., 2012). Light has previously been reported to influence protein secretion in \( N. \text{crassa} \) (Kallies et al., 1992), however a role of the photoreceptors in this process has not been known so far.
The finding of this discrepancy between transcription of major cellulases and detected cellulase activities suggests that cellulolytic enzymes may not be exclusively regulated on the transcriptional level (Schmoll and Kubicek, 2003), but also posttranscriptionally – presumably in response to light. Although the signal transduction pathway triggering cellulase gene expression is far from being well-established, first insights are already available. It has been revealed that signals arising from different environmental stimuli (sulphur metabolism (Gremel et al., 2008) as well as heterotrimeric G-protein signaling (Schmoll et al., 2009; Seibel et al., 2009)) affect cellulase gene transcription in a light dependent manner. Hence the relevance of the signals transmitted by G-proteins as well as the sulphur signal must be dependent on the light status, which is perceived and transmitted by the photoreceptors BLR1 and BLR2 (Castellanos et al., 2010).

The results could be interpreted in a way that the extent of cellulase transcription is set in response to environmental signals, but that the distribution of resources for the energy consuming process of translation and secretion is at least in part governed by the photoreceptors BLR1 and BLR2 and possibly by ENVOY. The fact that these proteins and their orthologs regulate multiple targets (Rosales-Saavedra et al., 2006, Schuster et al., 2007, Chen et al., 2009) supports this hypothesis.
5. Summary

Novel fermentation monitoring device

A novel device and software was developed that is suitable to follow ethanol fermentation or any other process with gas formation in real time and on parallel channels. Advantage of this approach is the immediate results and the possibility to study the kinetics and the impact of different experimental designs. Statistical evaluation of test measurements showed that the operation of the device is reliable and repeated runs of same samples deliver the same results regardless the channel used. An equation was also formulated to calculate ethanol concentration from the gas volume produced, however, a growing discrepancy was found with the number of displacement event between HPLC measured and calculated concentrations. Nevertheless, based on the experiments it cannot be stated that this discrepancy is obviously because of an error in the device. Nevertheless, based on the statistical evaluation, the device is suitable for monitoring of ethanol fermentation.

Whole sweet sorghum plant as bioethanol feedstock

Sweet sorghum whole plant can be effectively converted into ethanol with an outstanding high overall potential of 8 300 L/ha of which 45% comes from the juice. Pretreatment of bagasse with alkalis was found to be a proper method for good conversion values, NaOH proved to be the most efficient (90.5% and 94.6% RS liberation after pretreatment with 1% and 2% NaOH, respectively) at 121°C in both concentrations. However, possibly because of the presence of acetic acid as the result of hemicellulose deacetylation during hydrolysis, higher ethanol yields were reached on a substrate pretreated at room temperature (2% NaOH, 69.6%).

The liquid fraction as carbon source for T. reesei

Dilution of liquid fractions of pretreated sweet sorghum bagasse and wheat straw were found to be proper substrates to produce enzymes on. However, either the fungus needed to be adapted to the liquid fraction or the liquid fraction had to be detoxified. The findings highlight that not only the discrete concentrations of inhibitors but the interactions between them and possible other compounds not measured are also relevant to enzyme production of T. reesei.

During the solid state pre-adaptation the colony diameters reflected the inhibitory nature of the liquid fraction of steam pretreated bagasse well. Gained
adaptation ability could be transferred into shake flasks cultures where not only the lag phase was shortened but higher final FPA was also obtained compared to non-adapted cultures. Inhibitors usually affect microbial growth in the initial phase therefore it is noteworthy that adaptation of *T. reesei* can not only lead to quicker initiation of growth but also to higher final FPA. Furthermore, results of shake flask cultivations showed that sequential steps of pre-adaptation towards the lowest dilution of liquid fraction do not matter, and thus only the concentration of inhibitors in the last step has influence. These results explain that *T. reesei* can overcome at least partially the inhibition caused by inhibitors present in liquid fraction; however, an increasing interval was needed for adaptation with decreasing liquid fraction dilution.

Detoxified liquid fraction of pretreated wheat straw was supplemented with wheat grain that resulted in near optimal BGA/FPA ratio produced by *T. reesei*. As a result the produced enzymes competed efficiently the commercial enzyme preparation in hydrolysis. Using starchy materials like wheat grain for onsite enzyme production by *T. reesei* RUT C30 can be a good integration opportunity for biomass-to-ethanol process in first generation factories. Due to using liquid fraction as carbon source the amount of produced xylanases increased significantly and this enhanced the xylan conversion in the subsequent hydrolysis (70% in case of reference, while 81-85% in case of broths with liquid fraction).

**Up-scaled cellulase enzyme production by signaling pathway modified *T. reesei* mutants**

It was shown that different *T. reesei* mutants modified in elements of the light signaling pathways can deliver enhanced enzyme production compared to wild type QM9414. However, different regulatory patterns were suggested for the strains: while the deletion of *env1* led to the secretion of a more effective enzyme mixture, as a result of the deletion of *blr2* the amount of secreted protein increased significantly.

A discrepancy was found between transcription of the major cellulases and detected cellulase activities in case of deletion mutants of *blr1* and *blr2*. This finding implies that cellulase secretion of *T. reesei* is not only regulated on the level of transcription but possibly at other posttranscriptional targets. Therefore, transcript level of a given gene may not reflect well the actual cellulase enzyme secretion.
These results provided new insights into the regulatory machinery of *T. reesei*, which can be exploited to enhance biotechnological fermentation at different stages of regulation in fungi. Hence investigation of the light response machinery, and also of its downstream targets, with respect to industrial fermentations (i.e. likely in darkness), opens up a new strategy for strain improvement aimed at more efficient biofuel production.

5.1 New scientific findings

1. Together with Stereo Vision Ltd. a device was developed that is able to track gas formation reliably as tested on several raw materials. Statistical evaluation of test runs showed reliable operation. Based on the final gas volume, ethanol concentration can be estimated, however, a growing discrepancy appeared with the increase of the total gas volume. (Paper I and II)

2. Dilute alkali pretreatment of sweet sorghum bagasse is an efficient method, considering both the hydrolysis by means of RS liberation and the whole SSF process as described by ethanol yield. However, the pretreatment parameters yielding the best results are different in the two processes. (Paper II)

3. Adaptation of *T. reesei* to the liquid fraction of steam pretreated sweet sorghum bagasse can be a successful approach to enhance the cellulase production efficiency compared to non-adapted cultures. Adaptation ability of *T. reesei* gained during solid state pre-adaptation (on agar plates) could be transferred into submerged cultures where the adapted strains started producing enzymes earlier and also secreted higher final enzyme activities than non-adapted ones. (Paper III)

4. The effectiveness of *T. reesei* cultivation on the liquid fraction cannot be preliminary defined based on measurement of the major inhibitors (acetic and formic acids, furfural and HMF). Possible other substances and/or interaction between inhibitors influence more the growth and enzyme production of fungus. (Paper III and IV)

5. Diluted and detoxified liquid fraction of steam pretreated wheat straw is an appropriate carbon source for *T. reesei* fermentation available onsite. Besides its availability, the main advantage of using the liquid fraction is the highly enhanced xylanase activity produced by *T. reesei*. (Paper IV)
6. Starch, as supplementary carbon source to liquid fraction (of steam pretreated wheat straw) can improve beta-glucosidase production significantly, thus optimizing the composition of enzyme mixture (close to a BGA/FPA ratio of 1), yet supplementation cannot facilitate overcoming the inhibition. (Paper IV)

7. It was demonstrated that modification of the light signaling pathways of *T. reesei* could be a fruitful approach in obtaining higher enzyme activities also in up-scaled fermentations (30 liters). However, different patterns of regulation could be suggested for the enhanced activities. While deletion of *blr2* resulted in a significantly increased amount of secreted total protein content, deletion of *env1* leads to the secretion of a more efficient enzyme mixture. (Paper V)
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STATEMENT

I the undersigned, hereby declare, that this PhD thesis was written by me, and I only used the sources listed in the reference list.

NYILATKOZAT

Alulírott Gyalai-Korpos Miklós kijelentem, hogy ezt a doktori értekezést magam készítettem és abban csak a megadott forrásokat használtam fel. Minden olyan részt, amelyet szó szerint, vagy azonos tartalomban, de átfogalmazva más forrásból átvettem, egyértelműen, a forrás megadásával jelöltetem.

Budapest, 2012

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Gyalai-Korpos Miklós
Evaluation of an online fermentation monitoring system
- manuscript -

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Abstract

In order to introduce promising bioethanol production technologies new kind of feedstocks and fermentation processes with high ethanol yield are necessary. This need calls for advanced laboratory techniques those help to study experiment designs and obtain their results in a quick and reliable way. Real time monitoring of fermentation avoids time consuming steps, long lasting analyses and delivers information about the process directly. Method development and monitoring devices based on general principles of ethanol fermentation, such as effluent CO₂ volume measurement could be used in many related research areas including food industry (for example brewery). In this paper a novel device developed by the authors is described that possesses the above features. Based on statistical evaluation it was found that the calibration method is reliable, but when verifying the method versus ethanol measured by HPLC a constant discrepancy was observed growing with ethanol concentration, however, it cannot be stated that the error comes from the monitoring device. The device proved to be advantageous in monitoring and with further clarification it can become also applicable in estimating ethanol concentration.

Keywords
Ethanol, fermentation, monitoring, device, CO₂

Introduction

Ethanolic fermentation is an enzymatic disassembly of organic matters. Bioethanol can be produced from a wide range of raw materials built up of either sugar or starch or lignocellulose. Whatever is the raw material, it must be decomposed first to simple sugars of six carbons of which ethanol can be produced by yeast strains, usually by common baker’s yeast (Saccharomyces cerevisiae) with exclusion of oxygen (anaerobic conditions). Based on the
Stoichiometry of fermentation one mol of six carbon sugar delivers 2 moles of ethanol and CO₂ (equation 1). This basic natural process has been utilized by the mankind for many thousand years mostly in food related industries. The traditional food fermentation industries such as winery, brewery, and bakery apply yeast as well (Johnson and Echavarri-Erasun, 2010).

\[ C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2 \]

New application area for ethanol is in the transport sector, where depending on the origin of the glucose basically there are two technologies. In case the six carbon sugar originates from starchy (mostly wheat or corn grain) or sugar (mostly sugar beet or sugar cane) materials the technology is called first generation bioethanol production which can be considered as a mature technology. (Sanchez and Cardona, 2008). Globally the most common sugar plant to produce ethanol is sugar cane growing on tropical climate. Its alternative for moderate climate could be sweet sorghum those stem juice contains sugar up to 20%, which compounds 60% sucrose, 33% fructose and 7% glucose (Prasad et al., 2007).

Second generation bioethanol production utilizes lignocellulose as feedstock in form of agricultural/forestry residues (like corn stover, wheat straw, sweet sorghum bagasse) or dedicated energy crops. The utilization of byproducts or energy crops makes second generation technology advantageous over first generation where the feedstock portfolio may compete with food uses. However, due to the complex structure of lignocelluloses, it has to be mechanically disassembled, pretreated to loosen the structure and enzymatically hydrolyzed by cellulase enzymes in order to liberate glucose and other sugars before fermentation (Yulin and Mosier, 2008). Due to this complex pattern, although a lot of research has been done so far the process requires further research.

This research and development needs dedicated, easy-to-use and reliable devices to monitor ethanol fermentation in a direct and comparable manner. During ethanol fermentation concentrations of both substrates and products can provide important information about the state of the process. To follow fermentations basically three methods can be used.

The first and most common one is sampling, when concentrations of both substrates and products can be measured from the fermentation broth after withdrawal and analysis of the sample. It is usually a time and work consuming chemical analysis that necessitates a proper and selective method. The results are usually obtained after timely analyses when the actual state of the system is.
has already changed, thus it is not possible to deliver immediately information about the system. Moreover, with the regular sampling the opening of the system is inevitable that can cause infection and changes in the environmental parameters (pH, temperature).

The second possibility is to measure the solved substances by an online monitoring method where the selective detector is part of the fermentation system. Biosensors with enzymatic detection are able to measure glucose and/or ethanol directly from the reactor, however, while these biosensors posses good sensitivity and stability, they usually have poor selectivity (Lei et al., 2006). Biosensors can be used in fermentors but may not be applicable in small flask reactors where several experiments are run in parallel.

Numerous ways are known – including volume displacement devices, lubricated syringes, manometer-assisted syringes, calibrated pressure manometers and automatic respirometers – to measure the produced CO\textsubscript{2} that leaves the reactor, as being the third method.

As the stoichiometry of fermentation is well known substrate consumption and ethanol production can be calculated from the volume of CO\textsubscript{2} produced. The most sophisticated method to measure the amount of the gas is operating on the principle of displacement. These gas volume measuring devices can be classified in two types (Gyalai-Korpos et al., 2007). The first type is when the fluid is in a cylindrical device, and the gas periodically displacing with the fluid (Walker et al., 2009; Macías et al., 1995). The second type is the conventional Mariotte bottle. Disadvantage of these two types is that only given volume of gas can be measured with these devices, then the bottle must be refilled (Young et al., 1991), (Veiga et al., 1990). Continually functioning devices are known, as well. In this case gas is allowed to leave the system to the atmosphere, and the device can return to the starting position (Smith and Stöckle, 2008).

Such methods and equipments are needed where concentrations of either substrate or products can be measured easily at a low price in laboratory scale. The aim of our study was to develop and statistically evaluate an online monitoring system for ethanol fermentation, which is able to track the fermentation in real time delivering an immediate feedback of the process. Moreover, as monitoring is based on the simple method of measuring effluent gas, other processes with gas formation (e.g. biogas production) can be tracked as well.
Materials and methods

Raw materials and strains

Synthetic medium containing glucose in different concentrations (10 g/L, 20 g/L, 30 g/L, 40 g/L, 60 g/L, 80 g/L) and the following salts 0.3 g/L MgSO₄, 2.0 g/L NH₄Cl, 1.0 g/L K₂HSO₄ were used for ethanol fermentation experiments by baker’s yeast. For fermentation experiments fresh blocks of baker’s yeast (S. cerevisiae), product of Lesaffre Magyarország Élesztőgyártó és Kereskedelmi Kft., Budafok, Hungary were purchased in a local store on the day of use.

Fermentation

Batch fermentations on the synthetic media were carried out at 30 ºC pH 4.8 with the addition of 2 g DM per liter yeast cell in 250 mL screw-capped bottles with magnetic stirring (250 rpm) connected to the monitoring device. Fermentation was tracked by measuring CO₂ production by a novel online fermentation module device developed by Nonfood group (at Budapest University of Technology and Economics) and Stereo Vision Ltd. In order to correctly carry out the statistical evaluation of the device, fermentations were performed at least in duplicates in parallel and also with repetition at other times.

At the end of fermentation when CO₂ production ceased, flasks were sampled. Samples were centrifuged in 50 mL centrifuge tubes at 9000 rpm for 5 min. Supernatant was analyzed for sugar and ethanol concentration by HPLC.

Analytical methods

Samples for HPLC analysis were prepared by filtering through a regenerated cellulose syringe filter with 0.45 μm pore size (ProFill, Langerwehe, Germany). The residual sugar and ethanol contents of the fermented samples were separated on an Aminex ion exclusion HPX-87H cation-exchange column (BioRad, Hercules, CA USA) running at 65°C with 5 mM sulfuric acid as mobile phase at a flow rate of 0.5 mL/min. After separation, compounds were detected by a Shimadzu RID-I0A refractive index detector (Shimadzu, Kyoto, Japan).

Evaluation of the results

Determination of the concentration of ethanol with online fermentation monitoring system is based on the produced amount of CO₂ according to equation 1 ethanol and CO₂ are stochiometrically equal in the reaction. Eq. 2 based on the ideal gas law was used to calculate the ethanol concentration.
\[ c_{\text{EIOH}} [\text{g/L}] = \frac{(p_{\text{air}} [\text{Pa}] + 0.09 \cdot 1000 \cdot 9.81) \cdot V_{\text{CO2}} [\text{mL}] \cdot 10^{-6}}{R \cdot T_{\text{air}} [\text{K}]} \cdot \frac{46}{V_{\text{sample}} [\text{L}]} \] (2)

Where \( p_{\text{air}} \) is the average atmospheric pressure during the measurement [Pa], 0.09 is the maximum deflection in the U-tube just before inversion [m], 1000 is the density of 10^{-2} M HCl which was applied to minimize CO₂ loss by solution [kg/m³], 9.81 is the acceleration of gravity [m/s²], \( V_{\text{CO2}} \) is the total volume of the produced gas [mL], 10^{-6} is a factor to convert mL to m³, \( R \) is the Regnault constant [8,314 J/mol K], \( T_{\text{air}} \) is the average air temperature during the measurement [K], 46 is the molar weight of ethanol [g/mol], \( V_{\text{sample}} \) is the volume of the sample [L].

Simplification can be made in Eq. 2 using standard conditions (25 °C, 100 000 Pa) that approximates well actual laboratory conditions during the study (Eq. 3).

\[ c_{\text{EIOH}} [\text{g/L}] = \frac{0.0019 \cdot V_{\text{CO2}} [\text{mL}]}{V_{\text{sample}} [\text{L}]} \] (3)

Statistical analyses were performed with STATISTICA 9.0 software (StatSoft, Inc., Tulsa, OK, USA). The significance level for t-test for independent samples was set to 0.05.

In order to estimate the error due to this simplification error propagation calculation was tested with possible maximal variances from the standard conditions under the conditions of the experiments (Eq. 4)

\[ \sigma_{\text{EIOH}}^2 = \left( \frac{\partial c_{\text{EIOH}}}{\partial P_a} \right)^2 \times \sigma^2 P_a + \left( \frac{\partial c_{\text{EIOH}}}{\partial T_a} \right)^2 \times \sigma^2 T_a + \left( \frac{\partial c_{\text{EIOH}}}{\partial m} \right)^2 \times \sigma^2 m \] (4)

Where \( \sigma \) is the variance, first term (\( P_a \)) describes the maximal variance of the standard air pressure during the measurement based on data of the Hungarian Meteorological Service [15 hPa], second (\( T_a \)) the variance of the standard air temperature during the measurement [3 K], while third (\( m \)) the variance derived from the error of the scales (mass of feedstock in each fermenter) [0.01 g], \( c_{\text{EIOH}} \) is the concentration of ethanol [g/L].

The result of the calculation is ± 0.0052, which is minor so the simplification of equation 2 is valid and does not result in error propagation.
Results and discussion

Description and operation principle

In order to evaluate and draw correct conclusions from experiments the existence of proper methods and dedicated devices is necessary. First, a device was developed that is capable to monitor ethanol fermentation in real time. With this feature and the eight parallel sample sites it is possible to see the differences between substrates, pretreatments and media as soon as fermented and not only after timely analyses.

The system is basically divided into two parts, the measuring device and the processing software. The main component part of the device is a U-tube filled with $10^{-2}$ M HCl solution to prevent CO$_2$ loss and equipped with two copper electrodes of different lengths. The end of the longer electrode is permanently immerses in the acid solution while the shorter one does only when the device is under pressure, i.e. effluent gas enters the tube.

The U-tube has a 15 mm internal diameter, and a height of about 25 cm. Top of the left leg is closed, and the gas inlet tube from the fermenter is connected also here. The right leg is open to atmosphere and the electrodes are placed here. Between the two legs a transfer tube is placed with 3 mm internal diameter and connection points to the main legs at different heights (Figure 1).
This structure of the U-tube and thus the operation principle are originally described by Veiga et al. (1990) in order to measure the volume of biogas in aerobic digestion. Compared to that design some minor differences were applied to the U-tube used in our setup. First, the size was decreased resulting in discharge events with volume around 12 mL instead of the 50 mL as described by Veiga et al. Second, the original design was supplied by a voltage of 220 V, while in our case it was only 4.4 V supplied trough the USB port of a PC. Nevertheless, the major difference is the method of signal processing: with being connected to a PC, it became possible to follow the process in real time and obtain a view into the reaction kinetics. This real time tracking is possible on 8 channels simultaneously – to measure more samples at the same time is not mentioned by Veiga et al. Furthermore, we carried out a statistical evaluation and attempted to define the accuracy of our device specific for ethanol fermentation.

The operation principle is the following: the produced CO$_2$ enters in either leg of the tube and leaves from the other while the same volume of liquid as its own is moving through the legs. The gas leaves the tube in fixed increments through an inverted siphon (transfer tube) placed between the two legs of the U-tube. The different lengths of the electrodes and thus their changing contact with the solution depending on the level make possible to detect changes in the liquid surface level and thereby count the number of discharge events. These events get registered on a personal computer by a software, also developed by Stereo Vision Ltd. As the measuring device was calibrated, the exact volume leaving the tube during a discharge event is known so by cumulating the number of these events the volume can be calculated. As the main feature the software displays a cumulated CO$_2$ versus time plot in real time. The measured data and the plot can be exported to an Excel file for further editing and studying.

Statistical verification of calibration

As the event volume is of crucial importance for reliable results, the reliance and repetitiveness of the calibration method was investigated. For this reason calibration on each channel was repeated many times even at different days and testing whether the same units got obtained (Table 1). Principle of calibration is also displacement using an empty bottle instead of fermentation sample connected to the device. The 8 channel was parallel calibrated with 500 mL, about 31°C (the temperature of fermentation) water running through the system and displacing the some volume of air leaving the bottle via the U-tube
imitating displacing events. The calibration unit was calculated from the volume of the transferred water measured with a measuring cylinder divided with the number of displacement events (mL/event).

Table 1 – Evaluation of calibration measures

<table>
<thead>
<tr>
<th>Channel</th>
<th>Average volume of displacement event mL</th>
<th>Standard deviation mL</th>
<th>Number of repetitions</th>
<th>t value (p = .05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.24</td>
<td>0.08</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>11.50</td>
<td>0.17</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>11.45</td>
<td>0.48</td>
<td>3+3</td>
<td></td>
</tr>
<tr>
<td>3 (day A)</td>
<td>11.75</td>
<td>0.36</td>
<td>3</td>
<td>0.077</td>
</tr>
<tr>
<td>3 (day B)</td>
<td>11.03</td>
<td>0.06</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>12.22</td>
<td>0.38</td>
<td>3+3</td>
<td></td>
</tr>
<tr>
<td>4 (day A)</td>
<td>12.14</td>
<td>0.26</td>
<td>3</td>
<td>0.826</td>
</tr>
<tr>
<td>4 (day B)</td>
<td>12.21</td>
<td>0.50</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>11.95</td>
<td>0.23</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>12.09</td>
<td>0.28</td>
<td>3+3</td>
<td></td>
</tr>
<tr>
<td>6 (day A)</td>
<td>12.36</td>
<td>0.25</td>
<td>3</td>
<td>0.137</td>
</tr>
<tr>
<td>6 (day B)</td>
<td>11.83</td>
<td>0.40</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>11.66</td>
<td>0.31</td>
<td>3+3</td>
<td></td>
</tr>
<tr>
<td>7 (day A)</td>
<td>11.67</td>
<td>0.33</td>
<td>3</td>
<td>0.909</td>
</tr>
<tr>
<td>7 (day B)</td>
<td>11.64</td>
<td>0.40</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>11.97</td>
<td>0.22</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

In case of repetitions were also made on different days (3+3) the averages of each day (day A versus B) were compared by t-tests hypothesizing no effect of date on calibration unit. As shown by t-test the difference between days is not significant at a significance level 0.05. This means that the date (and thus possible changes of conditions) of calibration is not relevant.
The ranges of calibration units are also illustrated on Figure 2. The calibration unit is inside a range of 2 mL in case of each channel what may be because of the volume of water used for the calibration is not a whole number multiple of the calibration unit and thus resulting in remaining level deflection not calculated into calibration unit.

The value of the medians varies from channel to channel. However, taken into account that the U-tubes are hand-made and the level of solution is different, this variation is normal and thus the calibration can be verified only for a channel not in between channels.

Figure 2 – Ranges of displacement events by channels

No outliers and extremes were obtained showing that the calibration regardless of its day and channel is restricted to a range. The lack of extreme values and outliers was also confirmed by Grubbs test with testing whether the values in the series of calibration on each channel are part of the series or outliers (Eq 5)
\[ Z = \frac{x_q - \bar{x}}{s} \] (5)

where \( Z \) is the Grubbs test result, \( x_q \) is the measurement’s minimum or maximum value, \( \bar{x} \) is the average of the calibration unit on each channel, \( s \) is the standard deviation of the calibration unit.

Based on the repeated calibration and Grubbs test the method of calibration was accepted, as well as the calibration unit was considered as reliable and accurate. The calibration units in Table 1 were used through the tests. In case of all channels the calibration units are about 12 mL that is – according to eq. 2 – equal to a resolution of 0.045 g of hexose substrate or 0.023 g of ethanol assuming a method with good sensitivity.

**Verification of the device with different samples**

Based on the verified calibration unit the device was used to track real fermentations running on synthetic substrate. Similar to calibration the fermentation runs were repeated many times on different days and also same samples were run on different channels to test the compatibility of channels.

The result of the tracking is the real time cumulated gas volume plots (Figure 3.). As these plots are available during the whole fermentation it is easy to follow the process and see immediately the actual state of the fermentation, as well as direct comparison between the 8 channels with different experimental designs also possible.

![Figure 3](image)

Figure 3 – Real time gas volume spots on different channels and glucose concentration
The device is also suitable for monitoring the kinetics of fermentation. For instance it can be observed that fermentation kinetics does not depend on the concentration of glucose in case of the synthetic media, since each curve has the same profile. In the initial phase, the volume of the produced gas is proportional with the glucose concentration. The curves flatten before reaching the plateau of which time point depend on the amount of sugar. As an advantage of the real time tracking, the end of fermentation can be recognized immediately once the plateau is reached meaning gas formation had stopped.

The compatibility of results (gas volumes) obtained on different days and channels was tested by analysis of covariance. The eligibility criteria for ANCOVA were also tested and the results proved that the variation of obtained gas volumes (represented by residuals) follow normal distribution (normal probability plot) and the homogeneity of variances among the samples as tested by Cochran test also is also accepted. However, because of some microbial phenomenon baker’s yeast consumed glucose in 80 g/L (16 g) with very poor efficiency resulting in nonlinear relationship between glucose and gas volume. When removing the results on 16 g glucose (only including results of 12 g and below) the linear connection was established, thus only these results were used for ANCOVA. As a result of ANCOVA standard deviations of 58.8 mL and 82.1 mL as the impact of channels and days, respectively, were obtained. Based on this a combined standard deviation including all effect of 116.2 mL was calculated which is equal to approximately 10 displacement event or 0.23 g of ethanol. In view of these results it can be stated that the choice of channel did not influence the measurement; thus all channels are compatible with the others and channels can reliably repeat measurements.

*Verification of equation to calculate the amount of ethanol formed*

At the end of the fermentation, the concentration of ethanol in each sample was determined by calculation based on Eq. 3 and by HPLC technique. It was found that the amount of ethanol measured by HPLC was slightly different from the amount of ethanol calculated on the basis of eq. 3.

The reason for this difference was examined by plotting the ethanol concentration measured by HPLC against recorded gas volumes (Figure 4). Moreover, the more events were detected (the higher was the glucose/ethanol concentration), the higher was the difference of the measured (by HPLC) and calculated (by Eq. 3) ethanol amount. This dependence of the bias on glucose
concentration (i.e. on produced volume) was confirmed by residual plotting too (data not shown).

According to eq. 3 a slope of 0.0019 is expected (1 mL of CO$_2$ is equal to 0.0019 g ethanol) but based on measurements a value of 0.0016 was obtained, however, the second member of the equation describing the slope was not zero leading to a constant bias. In order to approach the hypothetical equation, a correction was employed to the displacement volume (mL/event) that considers the discrete nature of displacement events. Even though some improvements were reached by this (Figure 4) there were still growing discrepancy.

Figure 4 – Measured ethanol versus gas volume: hypothetical and measured samples

The ratio of calculated ethanol based on the modified displacement event volume and the HPLC calculated ethanol was compared. When the 2 g glucose was present in this ratio was above 1 showing that in this cases a higher ethanol concentration was calculated based on gas volume. In larger glucose amount the ratio was in almost all cases below 1 but above 0.8. Taking into account of all samples an average ratio of 0.91 was obtained, meaning that the difference between HPLC and calculated ethanol values is in 10%.

Reason for this systematic discrepancy may be the discrete value of calibration unit, the non-verified HPLC technique or any other conditions not taken into account. Hence, it is too early to state that the device is not suitable for more
exact measurement and concentration determination, however, a correction factor can be necessarily to approach the actual ethanol concentration.

Conclusions

A device and software were developed that is suitable to follow ethanol fermentation or any other process with gas formation in real time and on parallel channels. Advantage of this approach is the immediate results and the possibility to study the kinetics and the impact of different experimental designs. An equation was also formulated to calculate ethanol concentration from the gas volume produced, however, a growing discrepancy was found with the number of displacement event between HPLC measured and calculated concentrations. Nevertheless, based on the experiments it cannot be stated that this discrepancy is obviously because of an error in the device. Consequently, without any correction factor the device is still suitable for monitoring of ethanol fermentation due to the advantages of real time parallel tracking of samples.

Acknowledgements

János Geier and Stereo Vision Ltd. are gratefully acknowledged for the assistance in developing the device and for his valuable comments on drafting this manuscript. Sándor Kemény is gratefully acknowledged for his assistance on the statistical evaluation.

References


SWEET SORGHUM JUICE AND BAGASSE AS A POSSIBLE FEEDSTOCK FOR BIOETHANOL PRODUCTION

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The aim of our study was to estimate the overall ethanol potential of a promising Hungarian sweet sorghum variety called ‘Monori Édes’ developed by Agroszemek Ltd. For ethanol production following parts of the plant can be utilized: the stem juice containing sucrose and the bagasse built up mainly from lignocellulose. As lignocellulosics have to be pretreated and hydrolyzed prior to fermentation, another purpose of our research was to apply weak alkaline pretreatment methods to enhance enzymatic digestibility of bagasse, thus, to improve the ethanol yield. In our study the effect of two bases (NaOH and KOH) in two concentrations (1\% and 2\%) and at two temperatures (room temperature and 121 °C) was investigated on the efficiency of enzymatic hydrolysis. Every pretreatment type affected positively the hydrolysis efficiency but in different degrees. Best results were achieved with 2\% NaOH at 121 °C. However highest ethanol conversion based on the glucan content of pretreated material was reached using 2\% NaOH at room temperature. Summarizing the ethanol potentials of juice and bagasse an overall potential of about 8 300 L/ha was estimated.

\textbf{Keywords:} sweet sorghum, enzymatic hydrolysis, alkaline pretreatment, ethanol fermentation

Introduction

Sorghum is the forth most important forage crop in the world with a cultivation area over 40 million hectares [1]. In the dry zones of tropical and subtropical areas it is used for food purposes too while under the moderate climate the utilization as feed has priority. Two different sorts of sorghum are cultivated, the grain sorghum and the sweet sorghum which is a sugar cane-like plant with sucrose-rich juice in the stem. In contrast to sugar cane, sweet sorghum can be cultivated in nearly all temperate climatic areas in Europe, also in regions possessing weak arable land conditions.

Besides using sweet sorghum as animal feed there is another possibility to cultivate it as energy crop getting more and more attention. From the extracted juice fuel ethanol can be produced, in Asia there are already industrial scale factories based on this technology. In 2007 4.92 billion liters of ethanol were produced from sweet sorghum juice in China and India. The first factory of the USA utilizing sweet sorghum juice alone will be built in Florida by Renergie which has recently received a 1.5 million dollars grant to start the project [2].

In our study the ethanol potential of a Hungarian sweet sorghum variety called ‘Monori Édes’ developed by Agroszemek Ltd was estimated. Since this variety has been improved in Hungary, it has adapted to the climatic conditions and it can produce high green biomass yield, i.e. 80–100 t/ha. It is harvested in September-October when the sugar content of the stem is the highest. The sugar concentration of the juice can reach 14–20\%. Nowadays, it is mostly used as cattle feed but there is a growing interest to utilize it as ethanol fermentation feedstock. In this case the harvested stems get pressed to extract the juice (40–50 t/ha); the by-product of the process is the bagasse, the leftover of the stem (40–50 t/ha, 50\% dry weight) built up mainly from lignocellulose. Although bagasse could be used as raw material for second generation bioethanol production, regularly it is burnt to supply the energy demand in the juice-to-ethanol process.

While the technology based on juice is already available on industrial scale, the lignocellulose conversion is still in experimental phase. There are only a few papers available on this alternative utilization. Gnansounou et al. [3] studied the theoretical possibility for a sweet sorghum biorefinery under circumstances in North China. The purpose of our work was to analyse and utilize the bagasse of ‘Monori Édes’ as a possible raw material for ethanol production.

Before fermentation the lignocellulosic bagasse has to be pretreated and hydrolyzed to liberate glucose molecules. The aim of pretreatment is to break down the complex and resistant structure of lignocellulose and hereby increase the efficiency of subsequent enzymatic hydrolysis. Usually after grinding a chemical or physico-chemical method is applied. In case of chemical treatment acid, base or organic solvent is used. Based on previous
results achieved with other agricultural by-products alkaline pretreatment was used in our experiments [4]. Their effect on the efficiency of enzymatic hydrolysis was evaluated.

The pretreated and hydrolyzed samples as well as the juice were fermented by baker’s yeast to determine the ethanol potential of the whole plant.

Materials and methods

Raw materials

Both the frozen, with nitric acid acidified juice (pH = 3.0 – 3.5) and the bagasse were obtained from the site of Agroszemek Ltd. near to Hódmezovásárhely. Harvest and pressing were performed during the autumn of 2007. Before use the bagasse was chopped, dried and ground.

HPLC analysis

Water soluble sugar (cellobiose, glucose, xylose and arabinose) and ethanol content were determined by high-performance liquid chromatography (HPLC). Samples for HPLC analysis were prepared by filtration through a 0.45 µm pore size regenerated cellulose syringe filter (La-Pha-Pack, ProFill™, Langerwehe, Germany). An Aminex HPX-87H (BioRad, Hercules, CA, USA) column was used at 65°C with 5 mM sulphuric acid mobile phase at 0.5 mL/min flow rate. Separated compounds were detected by a Shimadzu RID-10A refractive index detector (Shimadzu, Kyoto, Japan).

Raw material analysis

Initial sugar content of the juice was determined in triplicate by ‘Sucrose, D-fructose and D-glucose’ kit (Megazym). Cellulose- and hemicellulose content of bagasse were determined in triplicate before and after pretreatments by a two-step sulfuric acid hydrolysis. The principle of this method was originally described by Hägglund [5]. Firstly 0.5 g of dry ground bagasse was hydrolysed in 2.5 mL of 72% sulfuric acid for 2 hours at room temperature. After that 77 mL of distilled water was added into it and further hydrolysed for 1 hour at 121°C. After separation the reaction mixture on a G4 glass filter HPLC analysis was carried out from the liquid fraction. The solid fraction was washed with hot distilled water and dried. This residue was defined as the lignin content and was determined gravimetrically.

Pretreatments

Bagasse pretreatments were carried out on eight different ways. The effect of three parameters was investigated on two levels: type of base (NaOH versus KOH), concentration of base (1% versus 2%) and temperature – time combination (25°C, 3 days versus 121°C, 1 hour). Ground bagasse (0.3 – 1.4 mm) at 10% dry weight content (40 g DM in 400 g total mass) was suspended into NaOH or KOH solution in 1000 mL screw-capped bottles and left at room temperature for 3 days or autoclaved at 121°C for 1 hour. After pretreatments the mixtures were separated and the solid phase was washed with hot distilled water to remove solubles. Filter cake was dried at 50°C and used for raw material analysis and for enzymatic hydrolysis.

Hydrolysis

Pretreated bagasse samples were hydrolyzed at 50°C at 2% dry weight content in 0.05 M acetate buffer (pH 4.8) by commercially available enzymes Celluclast 1,5L and Novozym 188 (Novozymes) at 20 and 40 IU/ g DM, respectively. As control untreated bagasse was also hydrolyzed. Process was carried out in 250 mL screw-capped bottles containing 4 g DM pretreated bagasse in 200 g total mass with stirring (250 rpm). 1.8 mL of samples were taken at start of hydrolysis and after 1, 3, 4, 6, 24 and 48 hours. Samples were centrifuged in Eppendorf tubes at 15 000 rpm for 5 minutes, subsequent reducing sugar determination was carried out from the supernatant. After 48 hours the hydrolysates were cooled down to 30°C and moved to the fermentation step.

Reducing sugar determination

Hydrolysis was tracked by reducing sugar content determination according to Miller’s colorimetric method [6]. A suitable volume (containing reducing sugar inside the applied calibration range) was pipetted from the samples into test tubes and completed to 1.5 mL by adding distilled water. 3 mL of DNS (3,5-dinitrosalicylic-acid) reagent was added and the mixture was boiled for 5 minutes. After cooling down to room temperature 16 mL of distilled water was added, mixed and the absorbance was measured at 550 nm against a blank sample. Results were obtained due to the calibration of the DNS reagent.
Batch fermentations were carried out at 30°C in 250 mL stirred flasks (250 rpm) with measuring CO₂ production by an online fermentation module device, developed by Nonfood group (BME) and Stereo Vision Ltd. Baker's yeast suspension was added to 2 g dry weight per liter. At the end of fermentation when CO₂ production ceased, flasks were sampled. Samples were centrifuged in 50 mL centrifuge tubes at 9 000 rpm for 5 minutes. Supernatant was analyzed for sugar and ethanol concentration by HPLC.

Two different carbon sources were investigated:
- Enzymatic hydrolysates of sweet sorghum bagasse pretreated in different ways.
- Sweet sorghum juice (prior to fermentation the pH was adjusted to 5 by 10% NaOH solution).

**Results and discussion**

**Sweet sorghum juice**

Total sugar content of sweet sorghum juice was 163.8 g/L with a standard deviation of 7.3 g/L. Distribution of di- and monosaccharide molecules can be seen in Table 1. Sweet sorghum juice contains primary sucrose but due to acidification and storage sucrose hydrolyzed partially to fructose and glucose.

<table>
<thead>
<tr>
<th>Component</th>
<th>Initial</th>
<th>After fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose</td>
<td>14.2 ± 0.3</td>
<td>7.6 ± 0.1</td>
</tr>
<tr>
<td>Glucose</td>
<td>16.5 ± 1.6</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>133.1 ± 5.8</td>
<td>N/A</td>
</tr>
<tr>
<td>Ethanol</td>
<td>N/A</td>
<td>68.7 ± 2.1</td>
</tr>
</tbody>
</table>

160 mL of sweet sorghum juice was inoculated with yeast suspension in a volume to correspond to 2 g DM/L. After approximately 20 minutes to inoculation the gas production started. It reached a constant velocity in 70 minutes for a 8 hour interval which was slowly declining and gas formation stopped in the 24th hour of fermentation, Fig. 1.

Tracking was stopped and broths were sampled for HPLC analysis; Table 1. HPLC detected also some remaining fructose which corresponds to the results of Phowchinda and Strehaiano [7], they have found that sugar remains in low concentration at the end of the fermentation. Based on the initial sugar content and final ethanol concentration conversion value of 78.9% was calculated. This high value corroborates that no complementary salt addition and pH regulation are necessary during fermentation to reach good conversion.

**Sweet sorghum bagasse**

Prior to pretreatment ground bagasse contained 41.30±0.07% glucan which can be considered as cellulose content, 17.41±0.15% xylan and 17.62±0.11% lignin, on dry weight basis. Pretreatment resulted in an enhanced glucan and xylan content of bagasse. The sum of these was the basis for conversion calculations. Glucan content increased from 41% to 45-67% on dry weight basis depending on pretreatment. Xylan content reached a maximum of 29.37% using 2% KOH at elevated temperature. Detailed results are summarized in Table 2. Highest increase of glucan content was caused by bases in 2% concentration at elevated temperature. At room temperature 1% NaOH solution had the same effect on glucan content as the 2% KOH. Generally it can be said that NaOH is a more efficient pretreating agent than KOH in both concentrations and at both temperatures.
Table 2: Composition of pretreated bagasse samples

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Glucan average</th>
<th>Glucan std dev</th>
<th>Xylan average</th>
<th>Xylan std dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 ºC 1% NaOH</td>
<td>48.24</td>
<td>0.57</td>
<td>25.16</td>
<td>0.35</td>
</tr>
<tr>
<td>2% NaOH</td>
<td>50.02</td>
<td>0.52</td>
<td>23.34</td>
<td>0.13</td>
</tr>
<tr>
<td>1% KOH</td>
<td>44.59</td>
<td>1.19</td>
<td>24.25</td>
<td>0.29</td>
</tr>
<tr>
<td>2% KOH</td>
<td>48.63</td>
<td>0.68</td>
<td>24.44</td>
<td>0.30</td>
</tr>
<tr>
<td>2% NaOH</td>
<td>49.57</td>
<td>0.67</td>
<td>25.75</td>
<td>0.46</td>
</tr>
<tr>
<td>1% KOH</td>
<td>48.63</td>
<td>0.68</td>
<td>24.44</td>
<td>0.30</td>
</tr>
<tr>
<td>2% KOH</td>
<td>45.78</td>
<td>0.59</td>
<td>24.56</td>
<td>0.74</td>
</tr>
<tr>
<td>2% KOH</td>
<td>64.85</td>
<td>0.36</td>
<td>29.37</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Decrease of the lignin content was observed, which is in line with what had been expected. In case of alkaline pretreatment it is supposed that the lignin fraction get partly solved. Lignin content of sweet sorghum bagasse showed 2-7% decrease depending on pretreatment conditions.

During hydrolysis of pretreated material in every case the reducing sugar contentration started to increase rapidly and then the velocity decreased depending on substrate availability. Hydrolysis curves of the room temperature pretreated bagasse can be seen on Fig. 2 while the hydrolysis data of the pretreatment experiment performed at 121 ºC are summarized on Fig. 3.

In pretreatment at room temperature KOH was not effective in 1% concentration the reached reducing sugar content was nearly the same as the one for control sample (6.5 g/L). Although the pretreatment with 2% NaOH gave the highest reducing sugar concentration (17.0 g/L), the pretreatments with 1% NaOH (RC = 12.9 g/L) and 2% KOH (RC = 15.4 g/L) can be considered also efficient.

Hydrolysis of the bagasse pretreated at 121 ºC resulted in a more diverse pattern where hydrolysis on bagasse pretreated by 2% NaOH and 2% KOH gave the
highest reducing sugar concentrations, 22.9 and 21.3 g/L respectively.

For the calculation of conversion the actual reducing sugar content was divided by the monomer equivalent of the xylan and glucan content of pretreated material. Since there were differences in the composition of the pretreated bagasse depending on the applied pretreatment, the order of conversion values is not always the same as that of the reducing sugar contents. The final (at 48 hours) conversion values can be seen in Table 3.

Based on conversion results the bagasse pretreated with 2% bases (both KOH and NaOH) gave the highest values under room temperature pretreated materials. Among on elevated temperature pretreated materials the bagasse treated with NaOH showed better results than the ones with KOH in both concentrations. The highest conversion value (94.6%) was achieved in case of 2% NaOH pretreatment performed at elevated temperature.

In ethanol fermentation of various hydrolyzates a short lag phase could be observed, the constant gas formation rate was reached in less than an hour. It took only 3 – 5 hours for the yeast cells to consume the liberated glucose which after no more CO₂ gas formation could be detected, Fig. 4.

Table 3: Conversion values at the end of hydrolysis (48 h) - based on reducing sugar measurements

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Conversion %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% NaOH</td>
<td>68.4</td>
</tr>
<tr>
<td>2% NaOH</td>
<td>86.8</td>
</tr>
<tr>
<td>1% KOH</td>
<td>33.1</td>
</tr>
<tr>
<td>2% KOH</td>
<td>83.2</td>
</tr>
<tr>
<td>1% NaOH</td>
<td>90.5</td>
</tr>
<tr>
<td>2% NaOH</td>
<td>94.6</td>
</tr>
<tr>
<td>1% KOH</td>
<td>57.6</td>
</tr>
<tr>
<td>2% KOH</td>
<td>89.2</td>
</tr>
<tr>
<td>Control</td>
<td>20.4</td>
</tr>
</tbody>
</table>

![Fig 4: Ethanol fermentation tracked by CO₂ production in Online Fermentation Module device](image)

The curves recorded by the Online Fermentation Module device show a good parity between the duplicates, indicating that measuring the gas volume is an appropriate method to track ethanol fermentation. Largest difference between parallels was about 40 mL (data not shown) which corresponds to less than 0.1 g ethanol (calculated according to the ideal gas law and stochiometry of hexose fermentation).

According to the final ethanol concentrations measured by HPLC combined hydrolysis and fermentation conversion values were calculated based on the glucan content of the pretreated materials, Table 4. as Saccharomyces cerevisiae used in the experiments was unable to utilize xylose. These conversion values vary in a wide range, but no correspondence could be observed between them and the hydrolysis efficiency (Table 4. versus Table 3.). Some acetic acid formation was detected in nearly every sample suggesting by-product formation.

Table 4: Results of ethanol fermentation and combined hydrolysis and fermentation conversion values

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Ethanol conc. g/L</th>
<th>Conversion %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% NaOH</td>
<td>2.777 ± 0.370</td>
<td>48.2</td>
</tr>
<tr>
<td>2% NaOH</td>
<td>4.158 ± 0.337</td>
<td>69.6</td>
</tr>
<tr>
<td>1% KOH</td>
<td>1.160</td>
<td>21.8</td>
</tr>
<tr>
<td>2% KOH</td>
<td>2.413 ± 0.047</td>
<td>41.6</td>
</tr>
<tr>
<td>1% NaOH</td>
<td>3.107 ± 0.556</td>
<td>52.5</td>
</tr>
<tr>
<td>2% NaOH</td>
<td>3.829 ± 0.199</td>
<td>48.0</td>
</tr>
<tr>
<td>1% KOH</td>
<td>1.910 ± 0.004</td>
<td>34.9</td>
</tr>
<tr>
<td>2% KOH</td>
<td>4.461 ± 0.355</td>
<td>57.6</td>
</tr>
</tbody>
</table>
**Ethanol potential**

Sweet sorghum juice has high and directly fermentable carbohydrate content which can be fermented to ethanol without any salt addition effectively. According to the ethanol concentration reached in our fermentation (68.7 g/L) an ethanol yield (L/ha) was calculated equal to 3 729 L/ha assuming 45 t/ha juice yield.

Based on the results 4 560 L/ha ethanol yield was calculated for bagasse which corresponds to the highest ethanol conversion rate (69.9% – pretreatment: 2% NaOH at room temperature) in case of 45 t/ha bagasse yield with 50% dry weight.

Adding up the ethanol yields of juice and bagasse an overall ethanol potential of 8 289 L/ha can be calculated.

**Summary**

Sweet sorghum juice fermented during our research had an initial sugar concentration of 163.8 g/L and which a conversion value of 78.9% it yielded 68.7 g/L ethanol.

In order to improve the degradability of sweet sorghum bagasse eight different alkaline pretreatments were performed. Best hydrolysis conversion based on reducing sugar measurement was reached in case of pretreatment at 121°C in 2% NaOH solution, namely 94.6%.

Enzymatic hydrolyzates were fermented to ethanol using *Saccharomyces cerevisiae*. Highest ethanol concentration, 4.461 g/L was reached when bagasse pretreated with 2%KOH at 121°C was used. Concerning conversion, based on the glucan content of pretreated material, the highest combined hydrolysis and fermentation conversion was 69.9% by the pretreatment 2% NaOH at room temperature.

The overall ethanol yield (L/ha) of the whole plant is about 8 300 L/ha.

Our research demonstrated that the Hungarian sweet sorghum variety ‘Monori Édes’ is promising feedstock for bioethanol production with a high overall ethanol yield.

**Acknowledgement**

The authors gratefully acknowledge to OTKA TS 049849 and NKFP3-00006/2005 for their financial support. Novozymes (Denmark) is kindly thanked for providing enzymes.

**REFERENCES**

Processing sweet sorghum into bioethanol – an integrated approach

Miklós Gyalai-Korpos / Tünde Fülöp / Bálint Sipos / Katalin Réczey

Abstract
Numerous evidences have been provided that juice of sweet sorghum and the leftover after squeezing, the bagasse can be a proper feedstock for bioethanol production. The possibility to integrate a side stream of sweet sorghum processing into the biomass-to-ethanol process was investigated in this study. The liquid fraction, a side stream of the necessary pretreatment of the bagasse was utilized as carbon source for Trichoderma reesei RUT-C30 to produce cellulase enzymes for biomass conversion. However, to overcome the inhibitory nature of the liquid fraction, pre-adaptation of the fungus on solid media was carried out previous to submerged fermentations. The results show that with this approach the lag phase caused by the inhibitors could be markedly shortened and an enhancement of the final enzyme production could be achieved when comparing the pre-adapted strains to reference.

Keywords
sweet sorghum · steam pretreatment · Trichoderma reesei · cellulase · bioethanol

Acknowledgement
The Hungarian National Research Fund (OTKA – K 72710) and the New Hungary Development Plan (Project ID: TÁMOP-4.2.4.B-09/1/KMR-2010-0002) are kindly acknowledged for their financial support. Guido Zacchi is gratefully acknowledged for letting use the steam pretreatment unit.

Enhanced utilization of sweet sorghum in bioethanol production by adaptation of Trichoderma reesei RUT-C30

Background
Different cultivars of sweet sorghum (Sorghum saccharatum), especially the ones that have been bred in Hungary and adapted to the local environmental conditions, can be viable solutions for rural fuel supply. Sweet sorghum is a sugar cane-like plant, containing juice with high concentration of sucrose in the stem that can be effectively extracted by squeezing and thereafter readily fermented to ethanol by baker’s yeast. The leftover, like in case of sugar cane processing, is called bagasse. In contrast to sugar cane, sweet sorghum can be grown on continental climate, however with only one harvest per year – the frost terminates the growth of the plant. In this case the cultivation period is between April and mid September-October when the sugar content is the highest. Advantages of sweet sorghum cultivation in Hungary are the high sugar yield (5-6 t/ha), the drought tolerance (no irrigation is needed) and the modest demand for soil (that are not appropriate for corn or wheat). With these properties the cultivation of sweet sorghum will not face the food versus fuel debate often related to bioethanol production.

Feasibility of ethanol production from sweet sorghum juice has already been presented in many studies [1] furthermore several attempts have also been made to use the bagasse for second generation ethanol production [7, 19, 21]. The technological difficulty of sweet sorghum processing is the short harvest period making the juice available only for 1-2 months in the year: the juice cannot be stored because the microbes including its natural microbial flora are degrading the easily fermentable sugar content.

The utilization of the bagasse and any other lignocellulosic by-product could balance the annual short availability of the juice. A theoretical scheme for the integrated sweet sorghum to ethanol process is demonstrated on Fig. 1. This process is capable to utilize the whole plant as well as other lignocellulose based residues for ethanol production all year round. This would mean a great opportunity for the integration of first and second generation technologies as well as to balance the main disadvan-
Contrarily to first generation ethanol production that uses starch or sucrose containing feedstock, second generation ethanol production is based on lignocellulosic biomass [11]. However, because of the more resistant structure of lignocellulosic materials, the enzymatic hydrolysis must be preceded by pretreatment that aims to set cellulose chains free of the lignin matrix to be available for enzymes and eliminate the other component of lignocelluloses, namely the hemicelluloses (composed mostly of pentoses) in a soluble form [2]. The pretreatment is often a physical-chemical process, like steam explosion that applies severe pressure and temperature conditions for an exact time which after the pressure is rapidly expanded to atmospheric causing destruction in the lignocellulosic structure. Due to the severe conditions the breakage of the useful sugar molecules is also expected creating soluble compounds with inhibitory effect on microbial growth [14]. After pretreatment the slurry is separated to the cellulose containing fiber fraction subsequently exposed to enzymatic hydrolysis and the liquid fraction containing inhibitory compounds making the further utilization difficult. Despite of this difficulty the utilization of liquid fraction attains interest because of its high sugar content mostly in forms of pentoses available on-site. However, before any kind of utilization the liquid fraction most likely needs to be detoxified to decrease its inhibitory property. Chandel and coworkers [4] recently delivered a review on the possibilities to overcome inhibition covering several physical, chemical and biological methods. There are two possible utilisations of the liquid fraction that could be integrated into the biomass-to-ethanol process. One is the utilization of it by different microbes, mostly by ethanologenic bacteria and yeast strains in a separate fermentation step to increase the overall ethanol yield. Extensive work has been done on developing inhibitor tolerant and pentose utilizing yeast strains [5, 9]. The other option is to use this fraction for on-site cellulase production by Trichoderma reesei [8, 20, 23]. However, these efforts with Trichoderma have mostly failed when the total concentration of the inhibitors exceeded the level of tolerance of T. reesei.

The effect of acetic acid, one of the compounds present in the liquid fraction, on cellulase production by T. reesei RUT C30 was investigated up to 3 g/L on washed steam pretreated willow as carbon source at pH 6.0 and no inhibitory effect was found (FPA of 1.3 FPU/mL was reached on day 7). However, when both furfural and acetic acid were added into the medium a clear inhibitory effect was observed. Interestingly, it was also found that the acetic acid at low concentrations appeared to reduce the inhibitory effect of the furfural [24]. Similar effect was observed with the liquid fraction of steam pretreated wheat straw. T. reesei was not able to utilize the liquid fraction without detoxification, even though after detoxification the total concentration of inhibitors was the same with eliminating furfural and 5-hydroxymethyl furfural (HMF) but increasing the concentration of aliphatic acids [8]. This explains well that the synergies between compounds with possible inhibitory effect may be more relevant than their discrete concentration. Many sources report that T. reesei is able to consume several inhibitors when concentrations are below the inhibitory level [8, 12, 17, 24].

These findings indicate that T. reesei possesses a relative resistance to inhibitors that prompted us to find a biological way to enhance this property. This study concentrates on on-site cellulase enzyme production using the liquid fraction as carbon source that could be easily and at low cost integrated into the
process to overcome the barriers related to second generation ethanol production. As other parts of the process have been demonstrated earlier to be feasible [7,19,21], neither the ethanol fermentation of juice nor the enzymatic hydrolysis of pretreated bagasse were investigated this time.

Materials and methods

Strain and raw materials

For enzyme production experiments T. reesei RUT C30 (ATCC56765) strain purchased from the American Type Culture Collection was used. It was maintained on malt agar slants at 30°C composed of 20 g/L malt extract, 20 g/L agar, 5 g/L glucose and 1 g/L peptone. Slants were subcultured biweekly.

Sweet sorghum variety Berény was cultivated at Research Institute, Karcag (Centre for Agricultural Sciences and Engineering, University of Debrecen, Hungary) in 2006. Sweet sorghum juice was extracted from the fresh stem with leaves on by squeezing. Bagasse was collected, chopped and dried at 50°C to 85-90% dry matter content. Before composition analyses it was ground to fine powder.

Steam pretreatment

Sweet sorghum bagasse was steam pretreated at the Department of Chemical Engineering, Lund University, Sweden. The material was steamed at atmospheric pressure for 1 hour in order to reach an approximately 50% moisture content, and then impregnated with 2% SO2 (based on moisture content) in plastic bags for 30 min. Steam pretreatment was performed in a reactor with 10 L working volume [16]. Temperature was set and maintained by injection of saturated steam. After 10 min of residence time at 190°C the pressure was released and the material was collected in a flash-cyclone. Parameters of steam pretreatment were applied according to Sipos and coworkers [21] who found this combination of temperature and time to be the most effective for increasing cellulose conversion among the studied parameter-combinations.

The slurry after pretreatment was collected from the cyclone and washed with triple amount of warm (60-70°C) distilled water to remove the majority of the water soluble substances, then the liquid and fiber fractions were separated. Therefore, the fraction nominated as “liquid fraction” in present study is a dilution by washing water of the fraction obtained by filter-pressing of the original slurry. The liquid fraction was analyzed for sugar and inhibitor content and the washed fiber fraction was analyzed for structural carbohydrates and lignin content. Both were used as carbon sources in the enzyme production experiments.

Adaptation

T. reesei was cultured on agar plates containing liquid fraction in different dilutions. This solid medium composed of glucose in 5 g/L, peptone in 1 g/L and agar-agar in 20g/L suspended in either tap water or liquid fraction or mixture of these. pH was set to 6.0 by adding solid NaOH. The medium was sterilized on 121°C for 20 minutes and after that plates were prepared. In the water-liquid fraction mixture the dilution of the liquid fraction was 2 or 4 (1:1 or 1:3 liquid fraction:water ratio).

Plates were inoculated by placing a piece of agar from a previously cultured plate in the middle of the new plate. The plates were grown at room temperature simply in the laboratory. The diameter of the cultures was measured daily in the same time. The rate of growth was expressed as the difference of the areas of the circles covered by the culture on two days and divided by the numbers of days between the two readings (mm²/day). The concentric rings in the conidia formation – caused by the day night cycle of the fungus – made the reading even more exact.

Inoculum preparation

Conidia from 14-day-old slants were harvested with sterile distilled water. Either this suspension or a piece of the preadapted, T. reesei grown agar plates were used to inoculate Erlenmeyer flasks containing 200 mL of sterile modified Mandels’ medium to obtain a final concentration of 10³ conidia/mL. This medium contained 1.87 g/L (NH4)2SO4, 2.67 g/L KH2PO4, 0.53 g/L CaCl2·2H2O, 0.81 g/L MgSO4·7H2O, 0.40 g/L urea, 5.0 mg/L FeSO4·7H2O, 1.7 mg/L MnSO4·H2O, 1.4 mg/L ZnSO4·7H2O, 2.0 mg/L CoCl2·6H2O, 1.00 g/L peptone, 0.33 g/L yeast extract and 10 g/L Solka Floc as carbon source. These components were suspended in either tap water or liquid fraction or a mixture of these in dilutions as indicated later. Inoculated flasks were closed with cotton plugs and incubated at 30°C and 300 rpm on a rotary shaker for 4 days.

Shake flask cultivation

In each case the medium for cellulase production was composed of (NH4)2SO4 and KH2PO4 both in concentration of 0.83 g/L and 10 g/L washed pretreated bagasse as carbon source. Each medium contained an additional 5 g/L wheat distillers’ grain as a nitrogen source (32.2% protein) that is also composed of lignocellulosic carbohydrates (18.6% glucan and 14.6% xylan). Solid substances were suspended in 0.1 M TRIS-maleic acid buffer (pH 5.8) to avoid pH changes prepared in either tap water or liquid fraction in dilutions indicated later.

Cellulase producing media were inoculated with an aliquot of 4-day-old inoculum at 10% (V/V), and cultures were propagated at 30°C and 300 rpm on a rotary shaker. Samples were withdrawn regularly and centrifuged (3400 g, 5 min) to separate supernatants for further analysis. Fermentations were terminated after 9 or 11 days.

Enzyme assay

Filter Paper Activity (FPA) measurement was carried out according to Mandels et al. [6], with the modification, that an enzyme dilution releasing 1 mg glucose was used. FPA was expressed as FPU/mL, where FPU was defined as the amount of liberated glucose given in micromoles per minute.
Lignin and carbohydrate analysis

Lignin and carbohydrate content of raw and pretreated materials were analyzed using NREL protocol [22] with some modification. 0.5 g of oven dried (105°C) sample was hydrolyzed with 2.5 ml 72% sulfuric acid at room temperature for 2 hours. After that, 75 ml of distilled water was added and the hydrolysis was continued at 121°C for 60 min. The samples were filtered through G4 filter crucibles and washed with hot distilled water several times. The remaining lignin on the filter was dried at 105°C, weighted and placed in furnace at 550°C for 6 hours. The Klasson lignin content was taken as the ash free residue after hydrolysis.

Sugar analysis

Reducing sugar contents (RS) during the enzyme fermentation were analyzed using the 2,4-dinitrosalicylic acid reagent as described by Miller [13].

Liquid samples upon their sugar and inhibitor concentrations were analyzed with a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) using an Aminex HPX-87H column (Bio-Rad, Hercules, CA) at 65°C. The eluent, 5 mM H2SO4 was used at a flow rate of 0.5 ml/min. Total sugar content (including both monomers and oligomers) was determined after mild acid hydrolysis (4% (V/V) H2SO4, 120°C and 30 min). All samples were filtered through a 0.2 µm pore size filter before HPLC analysis to remove solid particles.

Results and discussion

Mass balance of the pretreatment

Fig. 2 shows the mass balance of the pretreatment in terms of hexoses, pentoses and lignin, respectively. The hexose recovery in the fiber fraction was 89%, whereas it was 36% in case of pentoses suggesting that the hemicelluloses were removed sufficiently. 9% and 46% of the hexoses and pentoses, respectively were solubilized, and were present in the liquid fraction after pretreatment. Consequently, the hemicellulose content decreased due to the solubilization, while the ratio of lignin and cellulose increased. This represents the aim and the well chosen parameters of the pretreatment, namely the pretreated material contains more and presumably easier accessible cellulose. The composition of the pretreated material is in accordance with the mass balances (Table 1).

As expected some solubilized sugars, both hexoses and pentoses, were further degraded during the pretreatment to non-sugar compounds. These compounds such as furfural, HMF and aliphatic acids (mostly formic and acetic acid) can inhibit microbial activity under certain circumstances. According to the composition of the separated liquid fraction the carbohydrates, as well as the inhibitors resulted mainly from the hemicellulose (Table 2). The acetic acid and the furfural are decomposition products of hemicellulose. Formic acid arises with the further decomposition of furfural and HMF [15]. The presence of glucose, glucan and HMF shows that due to the severe conditions the cellulose has also degraded to some extent in line with the mass balance.

Liquid fraction as carbon source for T. reesei

The inhibitory effect of the liquid fraction and the possibility of adaptation were investigated already in the inoculum phase: 20% of the water was replaced by liquid fraction, while the parallel reference run contained solely tap water (further referred as reference). It was assumed that in both cases the growth had been initiated based on the dropped pH values (after 4 days of cultivation). The fall of pH reflects well the growing of T. reesei and it is mainly caused by the consumption of ammonium salts acting as nitrogen source. From the same starting value of pH 5.70 the final was 3.17 in case of no liquid fraction, while in the broths containing 20% liquid fraction the pH was 3.29.

According to Szengyel and Zacchi [24] the sum concentration of furfural and acetic acid as present in the liquid fraction would not lead to growth inhibition. However, this result was obtained in a model medium not in liquid fraction resulting after pretreatment. It is assumed that this difference of inhibition is caused by the other degradation products, mostly from lignin not measured.
here and the synergies between inhibitors.

Both sorts of precultures were used to inoculate medium containing one part of liquid fraction and one part of water (substituted in 50%). The fermentation was monitored by RS and FPA measurements. Regarding RS the initial 100% content dropped already in the first 24 hours in both cases. However, there was significant difference between the broths prepared with the different precultures. In case of the broths inoculated with the reference a concentration of 4.6 g/L was measured, while in case of the broths prepared with inoculum containing 20% liquid fraction the RS was 17.3% lower, 3.8 g/L. For the 48th hour this difference nearly disappeared and in both cases the RS zeroed for the 4th day. This profile indicates that the utilization of sugar originating from the liquid fraction is quicker if T. reesei had already been adapted already in the inoculum to the liquid fraction.

In FPA profiles similar trends were observed. The difference between the two groups was 12% observed on day 3 but activities leveled off on day 4 (1.07 FPU/mL in both cases). However, while in the reference broths only minor amount of FPA was produced after day 4 (peaking with 1.27 FPU/mL at day 9), in the broths initiated with pre-adapted inoculum 1.69 FPU/mL was reached by day 9.

The inhibitors usually affect the microbial growth in the initial phase. Therefore it is noteworthy that adaptation of T. reesei can not only lead to quicker initiation of growth but also higher final FPA yield. Due to this significant enhancement of enzyme production in the fermentation experiment, it was assumed that adaptation could be a good strategy to partially overcome the inhibitory effect of the liquid fraction. Therefore, in view of this promising result, our aim was to develop a strain maintenance strategy that results in the utilization of the liquid fraction more effectively.

1 Pre-adaptation

Adaptation has been widely investigated in case of yeast strains growing on hydrolysates [18], but the possible adaptation of T. reesei has rarely been studied. Bigelow and Wyman [3] reported that adaptation is an encouraging option but because of the difficulty of maintaining the adapted strains no detailed study including enzyme activity measurements was carried out. Hayward and co-workers [10] found that despite adaptation leads to the increased growth, it was not succeed to obtain strains with increased enzyme production. It is noteworthy that the conclusions of both above cited studies came from submerged fermentations.

We aimed to investigate the effect of the liquid fraction on the growth of T. reesei in an easy to monitor manner. Therefore T. reesei was inoculated on agar plates containing liquid fraction in different dilutions and further subcultured decreasing the dilution of liquid fraction. The reference plates did not contain any liquid fraction. This pre-adaptation was characterized by the diameter of the colonies.

The daily reading of this parameter expresses well the inhibitory nature of the liquid fraction and the ability of the strain to adapt. The measured diameters decreased with increasing ratio of liquid fraction in the media (Table 3). In case of the agar plates prepared with 100% liquid fraction measurable growth could be observed only in the second week when the colonies on other plates had already overgrown the plates. The changes of cell mass as expressed in diameters is not proportional with the ratio of liquid fraction, for instance on the 5th day the diameter of the culture growing on 25% and 50% liquid fraction was 18.6% and 62.7% lower, respectively, than the diameter of the reference. Moreover, the difference between the plates was also diminishing over time, on 7th day it was 9.0% and 48.7% respectively.

The adaptation ability expressed in the rate of growth
With this approach the inhibitory effect of the liquid fraction on the growth of T. reesei, as well as the adaptation and the viability can be visualized well (Fig. 4). In the next step these pre-adapted cultures were investigated in shake-flask cellulase fermentation using the pretreated bagasse and other compounds as specified in the Material and methods section suspended in the liquid fraction as additional carbon source.

2 Enzyme fermentations

The cultures pre-adapted on agar plates were used to prepare the inocula. However, according to Bigelow and Wyman the conidia do not possess the gained adaptation ability [3], therefore, a piece of agar containing hyphae was cut off from the plates and added into the inoculum media. These media contained the liquid fraction in a ratio agreeing the ratio in the given plate (Table 5). For reference a non-adapted culture of T. reesei was used. After 4 days of cultivation (e.g. prior to inoculating the fermentation medium) the growth of the different inocula was assessed by pH and FPA. There were no significant differences observed between the broths. In every case the pH had dropped to near 3.5 and the FPA reached almost 0.5 FPU/mL. This indicates that the adaptation ability was successfully carried from the solid media into the liquid ones.

The fermentation medium in all cases contained undiluted liquid fraction (in 100%) besides the other components as given in Materials and methods section. Regarding the RS profile that describes well the fermentation in terms of sugar consumption and so indicating the growth in all cases a slight increase was observed from the initial 9 g/L concentration on day 1 (Fig. 5). This is due to the action of cellulase enzymes already present in precultures and carried over at inoculation. While the adapted cultures had already started growing and consuming sugar on day 2, in reference case it had increased further. The difference could be observed best on day 3 when reference culture started growing and consuming sugar but in smaller extent than the cultures inoculated with adapted T. reesei one day before. It could be concluded that the lack of pre-adaptation causes prolongation of lag phase and delays growth as it could be perceived from RS profile of the reference. In case of the pre-adapted broths the RS

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**Tab. 3.** Colony diameters (mm) of T. reesei cultures growing on agar plates containing liquid fraction in different dilutions. Mean values of three plates and standard deviations are presented.

<table>
<thead>
<tr>
<th>Day</th>
<th>Reference</th>
<th>25%</th>
<th>50%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>40±4.1 (100%)</td>
<td>29±3.4 (72.5%)</td>
<td>12±2.1 (30.0%)</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>59±5.3 (100%)</td>
<td>48±4.4 (81.4%)</td>
<td>22±2.9 (37.3%)</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>78±5.1 (100%)</td>
<td>71±4.4 (91.0%)</td>
<td>40±2.9 (51.3%)</td>
<td>–</td>
</tr>
<tr>
<td>11</td>
<td>–</td>
<td>31±7.1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>–</td>
<td>40±6.4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>–</td>
<td>60±7.1</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

**Tab. 5.** Ratio of liquid fraction in liquid Mandels’ medium and its agar plate counterpart.

<table>
<thead>
<tr>
<th>ID number</th>
<th>Ratio of liquid fraction in the liquid inoculum</th>
<th>Ratio of liquid fraction in the respective agar plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nr. 1</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nr. 2</td>
<td>25%</td>
<td>25%</td>
</tr>
<tr>
<td>Nr. 3</td>
<td>50%</td>
<td>50%</td>
</tr>
<tr>
<td>Nr. 4</td>
<td>50% (25%→50%)</td>
<td>50%</td>
</tr>
<tr>
<td>Nr. 5</td>
<td>100%</td>
<td>25%→100%</td>
</tr>
<tr>
<td>Nr. 6</td>
<td>100%</td>
<td>50%→100%</td>
</tr>
<tr>
<td>Nr. 7</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

The fermentation medium in all cases contained undiluted liquid fraction (in 100%) besides the other components as given in Materials and methods section. Regarding the RS profile that describes well the fermentation in terms of sugar consumption and so indicating the growth in all cases a slight increase was observed from the initial 9 g/L concentration on day 1 (Fig. 5). This is due to the action of cellulase enzymes already present in precultures and carried over at inoculation. While the adapted cultures had already started growing and consuming sugar on day 2, in reference case it had increased further. The difference could be observed best on day 3 when reference culture started growing and consuming sugar but in smaller extent than the cultures inoculated with adapted T. reesei one day before. It could be concluded that the lack of pre-adaptation causes prolongation of lag phase and delays growth as it could be perceived from RS profile of the reference. In case of the pre-adapted broths the RS
dropped sharply resulting in an average difference of 77% between them and the reference. In all cases except the reference RS was equivalent to zero from day 4.

Moreover, not only between adapted broths and reference but also among the adapted broths differences were found. In RS profile a grouping (Nr. 2 – Nr. 3 & 4 – Nr. 5, 6 & 7) could be observed according to the liquid fraction content of the inoculum and agar plates. The difference from reference was growing with decreasing dilution of the liquid fraction in the inocula: on day 2 in broths of Nr. 2, of the group of Nr. 3 & 4 and of the group of Nr. 5, 6, & 7 the RS content were 5%, 24% and 44% lower, respectively, than in the reference. The observed different patterns in RS consumption and its connection to the liquid fraction ratios in the preculture phase indicate that the final ratio of liquid fraction in the adaptation process is more crucial than in how many steps it had been reached.

The FPA profile also reflects this grouping and the most significant difference was observed on day 3 (Fig. 6). The broths inoculated with either Nr. 5, 6 or 7 (e.g. pre-grown on 100% liquid fraction agar plates) reached in average 72.3% higher FPA than the reference. The delayed initiation of growth could be observed here as well. From day 3 the reference culture also started to grow and produce enzyme as reflected on the decreasing differences between FPAs. However, after 11 days the group of Nr. 5, 6 & 7 had an average FPA of 1.7 FPU/mL that is 22.6% higher FPA than those of the reference.

These results are in accordance with the observed growing rates on the plates indicating that with adaptation the longer lag phase caused by the inhibitors can be significantly reduced both in terms of growth and enzyme production. Furthermore
and contrarily with the literature stating that no increased enzyme yields can be reached with adaptation [10], in our case the adapted strains achieved also higher final FPA value when growing on liquid fraction resulting also in higher FPA yields, since the carbohydrate content of the broths was the same.

Numbers refer to the different pre-adaptation conditions on agar plates (percent of liquid fraction in the solid media and in case of multi step adaptation the previous ratio too): Nr. 2 25%, Nr. 3 50%, Nr. 4 25%→50%, Nr. 5 25%→100%, Nr. 6 50%→100%, Nr. 7 100%. The same ratio of liquid fraction as that of the agar plate was applied in each inoculum while fermentations were run in 100% liquid fraction in all cases. The reference agar plate and inoculum contained no liquid fraction. For further information see text and Table 5.

3 Conclusions

Our results demonstrated that the by-product stream of the pretreatment, called liquid fraction can be used for on-site cellu- lase production by T. reesei despite its inhibitory feature. However, due to its inhibitor content either detoxification is necessary or the strains have to be pre-adapted. By choosing this latter approach we have proved that T. reesei can utilize better the liquid fraction in submerged fermentation after a pre-adaptation on solid media. It was found that adaptation could stimulate the initiation of growth and the enzyme production and thus reducing the lag phase. Furthermore, final enzyme activities were found to be higher in case of adapted strains were grown.

Fig. 5. RS profiles on medium containing undiluted liquid fraction (inoculated with differently pre-adapted T. reesei cultures). Numbers refer to the different pre-adaptation conditions on agar plates (percent of liquid fraction in the solid media and in case of multi step adaptation the previous ratio too): Nr. 2 25%, Nr. 3 50%, Nr. 4 25%→50%, Nr. 5 25%→100%, Nr. 6 50%→100%, Nr. 7 100%. The same ratio of liquid fraction as that of the agar plate was applied in each inoculum while fermentations were run in 100% liquid fraction in all cases. The reference agar plate and inoculum contained no liquid fraction. For further information see text and Table 5.

References

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Fig. 6. The initial FPA profiles on undiluted liquid fraction (inoculated with differently adapted T. reesei cultures). Numbers refer to the different pre-adaptation conditions on agar plates (percent of liquid fraction in the solid media and in case of multi step adaptation the previous ratio too): Nr. 2 25%, Nr. 3 50%, Nr. 4 25%→50%, Nr. 5 25%→100%, Nr. 6 50%→100%, Nr. 7 100%.

Cellulase production using different streams of wheat grain- and wheat straw-based ethanol processes

Miklós Gyalai-Korpos · Réka Mangel · Pablo Alvira · Dóra Dienes · Mercedes Ballesteros · Kati Réczey

Abstract Pretreatment is a necessary step in the biomass-to-ethanol conversion process. The side stream of the pretreatment step is the liquid fraction, also referred to as the hydrolyzate, which arises after the separation of the pretreated solid and is composed of valuable carbohydrates along with compounds that are potentially toxic to microbes (mainly furfural, acetic acid, and formic acid). The aim of our study was to utilize the liquid fraction from steam-exploded wheat straw as a carbon source for cellulase production by Trichoderma reesei RUT C30. Results showed that without detoxification, the fungus failed to utilize any dilution of the hydrolyzate; however, after a two-step detoxification process, it was able to grow on a fourfold dilution of the treated liquid fraction. Supplementation of the fourfold-diluted, treated liquid fraction with washed pretreated wheat straw or ground wheat grain led to enhanced cellulase (filter paper) activity. Produced enzymes were tested in hydrolysis of washed pretreated wheat straw. Supplementation with ground wheat grain provided a more efficient enzyme mixture for the hydrolysis by means of the near-doubled β-glucosidase activity obtained.

Keywords Trichoderma reesei RUT C30 · Wheat straw · Steam pretreatment · Cellulase fermentation · Hydrolyzate

Introduction Lignocellulotic materials, in the form of agricultural and forestry residues, are produced in abundant amounts worldwide. To utilize their carbohydrate content for ethanol fermentation, the feedstock needs to be pretreated and hydrolyzed by cellulase enzymes to liberate sugars. One of the drawbacks of the industrial-scale spread of the process is the high price of cellulase enzymes, which accounts for approximately 15% of the total ethanol production costs [21]. One possibility for reducing this cost is on-site enzyme production using the liquid stream of pretreatment as the carbon source.

Pretreatment is necessary to disrupt the resistant lignocellulosic matrix consisting of cellulose, hemicellulose, and lignin. The main aim of the pretreatment is to make the cellulose surface accessible to cellulase enzymes during the subsequent hydrolysis [2]. However, because of the harsh conditions, some components are removed from the matrix and even degraded. In the case of steam explosion, partial hydrolysis and solubilization of the hemicellulose fraction occur [16], and therefore these substances remain in the liquid fraction after separation of the pretreated slurry. This liquid fraction, also referred to as the hydrolyzate, contains valuable sugars, mostly oligomeric forms of pentoses, along with degradation products that have possible inhibitory effects on the growth of microbes. This inhibitory property makes further utilization of the hydrolyzate difficult. The predominant degradation products present in the liquid fraction of steam explosion are hydroxymethylfurfural (HMF) and furfural, derived from glucose and xylose.
respectively [1]. Further breakdown of these inhibitors also occurs, leading to formic acid formation. Another aliphatic acid, acetic acid, originates from the acetyl groups on the xylan and mannan backbone of the hemicellulose. Besides these inhibitors, phenolic compounds derived from lignin degradation are also detected. Importantly, levels of these inhibitors depend on substrate composition and pretreatment conditions [31].

Although there are several options for utilizing the hydrolyzate from different raw materials and pretreatments, a preliminary detoxification is often required, regardless of the raw material and the type of pretreatment. The most investigated alternative involves producing ethanol from the sugar content of the liquid fraction using naturally occurring yeast strains (e.g., *Pichia stipitis* or engineered bacteria or yeast strains (e.g., *Escherichia coli* or *Saccharomyces cerevisiae*) that are able to convert both hexoses and pentoses to ethanol [29, 40]. Xylool production by different yeast strains has also been examined by using xylene-rich acid hydrolysates as substrates [32]. Hydrolyzate of hydrothermally treated wheat straw was also used for production of biogas [19] and biohydrogen [18]. The liquid fraction of steam-pretreated willow was investigated as carbon source for β-glucosidase production by different *Aspergillus* and *Penicillium* strains [33]. Some attempts were also made to employ this fraction for cultivating *Trichoderma reesei*. One option for utilizing this fraction is detoxification; *T. reesei* is able to consume some of the inhibitors in low concentrations under certain conditions [22, 30]. The second possibility is using it as carbon source for cellulase production [7, 12, 23, 33, 36].

The filamentous fungus *T. reesei* (anamorph of *Hypocrea jecorina*) is one of the most studied cellulase-producing organisms. Cellulase secretion in *Trichoderma* is an inducible phenomenon, but the mechanism of cellulose sensing is still unclear, mostly due to its water insolvency. Several small compounds, like sophorose [35] and sorbose [28], possess the capability to induce cellulase secretion, but an industrial application of these compounds, as carbon sources, is unfeasible because they are expensive to purchase. Lactose, another inducer, could be a preferable choice for industry because whey, a by-product of cheese processing, contains lactose. However, whey is not readily available on-site as it is not biomass related [10, 42]. The liquid fraction obtained from steam pretreatment contains both glucose and xylose oligomers which may induce cellulase secretion in *Trichoderma*; owing to its availability, this liquid fraction can be a good substrate for on-site enzyme production.

Although starch is usually not recognized as an inducer for cellulase production in *Trichoderma* species, an inductive effect was reported for starch-derived compounds in *T. reesei* RUT C30 [10, 43]. One possible explanation for this finding is that during the acidic or enzymatic hydrolysis of starch, reversion products, such as sophorose, are also formed, and these products can act as inducers of cellulase transcription [10]. However, the same positive effect was observed in the case of utilizing native starch as partial carbon source supplemented with cellulosic materials [38, 43]. Using starchy materials like wheat grain for on-site enzyme production by *T. reesei* RUT C30 can be a good integration opportunity for the biomass-to-ethanol process in first-generation factories.

In our study, the potential utilization of the liquid fraction of steam-exploded wheat straw, a possible raw material for second-generation ethanol [39], was tested in shake flask cultures of *T. reesei* RUT C30. Additionally, the medium was supplemented with dried distiller’s grain as nitrogen source and either with washed pretreated wheat straw or wheat grain as extra carbon source to examine their effect on specific activities. Produced fermentation broths were tested in enzymatic hydrolysis of washed pretreated wheat straw.

**Materials and methods**

**Strain and raw materials**

For enzyme production experiments, *T. reesei* RUT C30 (ATCC56765) strain obtained from the American Type Culture Collection was used. It was maintained at 30°C on malt agar slants composed of 20 g/l malt extract, 20 g/l agar, 5 g/l glucose, and 1 g/l peptone. Slants were subcultured biweekly.

The following materials were utilized as carbon sources for enzyme production alone or in combination: pretreated wheat straw separated into liquid and water-insoluble fractions, ground wheat grain with or without α-amylase treatment, wheat distiller’s grain, and Solka Floc 200 FCC commercial cellulose product (International Fiber Corporation, North Tonawanda, NY, USA).

The wheat straw was harvested in 2008 in Soria, Spain. It was milled using a laboratory hammer mill to obtain a chip size of 10 mm, and it was stored at room temperature until use.

Mazurka-type wheat grain was harvested in the fall of 2009 in Somodorpuszta, Hungary. Dried grain was ground, and the following size distribution was obtained: 49.8% (related to total weight) was between 1.00 and 0.32 mm, while 82.2% was between 1.40 and 0.10 mm. For the α-amylase treatment, ground wheat grain was suspended in 0.1 M citrate–phosphate buffer (pH 5.6) to make a 30% solution. Liquefaction was performed for 1 h at 85°C using Termamyl SC DC (Novozymes A/S, Bagsvaerd, Denmark) at an enzyme dosage of 0.2 g/kg dry solid.
**Table 1** Composition of wheat straw before and after steam pretreatment (210°C, 2.5 min)

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Glucan, %</th>
<th>Xylan, %</th>
<th>Lignin, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat straw</td>
<td>40.7 ± 2.7</td>
<td>27.6 ± 2.4</td>
<td>17.0 ± 0.8</td>
</tr>
<tr>
<td>Pretreated whole slurry</td>
<td>42.4 ± 1.3†</td>
<td>18.6 ± 0.5†</td>
<td>25.5 ± 2.2</td>
</tr>
<tr>
<td>WIS (washed solid)</td>
<td>57.5 ± 0.8</td>
<td>6.0 ± 0.1</td>
<td>30.1 ± 0.1</td>
</tr>
</tbody>
</table>

Data are presented as mean values of triplicates with standard deviations.

† Monomers, oligomers, and polymers are presented together as polymers.

Before composition analyses occurred, all samples were ground into a fine powder.

**Pretreatment**

Steam explosion pretreatment was performed in a 10-l reactor at 210°C for 2.5 min using Masonite technology [24]. These conditions were established according to previous experiments based on optimal sugar recovery and hydrolysis yields [3]. Under these circumstances, most of the xylan content in the wheat straw was solubilized (Table 1). Following the pretreatment, a slurry with 31.0% total solid was recovered. One portion of the slurry was vacuum-filtered to separate water-insoluble solids and the liquid fraction. The solid fraction was thoroughly washed with tap water resulting in washed pretreated wheat straw (WIS), and both fractions were stored at −20°C until further use. The ratio of WIS and soluble solid content was 21.1 and 9.9%, respectively, related to the whole slurry. The obvious lack of xylan content in the slurry is due to some loss during the recovery process after pretreatment and to degradation into furfural, which was present in the liquid fraction.

**Detoxification**

The liquid fraction was vacuum-evaporated at 50°C for 10 min, during which the starting volume of 250 ml decreased by 60 ml. This loss was replaced by distilled water to obtain the initial volume again. The solution was then overlimed at 35°C by adding Ca(OH)₂ powder to pH 10 with continuous stirring. Once a pH of 10 was achieved, the solution was stirred for an additional 1 h. To remove any precipitate, the overlimed liquid was centrifuged (3,400 g, 5 min); the supernatant was stored at 4°C until use.

**Inoculum preparation**

Conidia from 14-day-old slants were harvested with sterile distilled water. This suspension was used to inoculate Erlenmeyer flasks containing 200 ml of sterile modified Mandels’ medium to obtain a final concentration of 10⁶ conidia/ml. This medium contained 1.87 g/l (NH₄)₂SO₄, 2.67 g/l KH₂PO₄, 0.53 g/l CaCl₂·2H₂O, 0.81 g/l MgSO₄·7H₂O, 0.40 g/l urea, 5.0 mg/l FeSO₄·7H₂O, 1.7 mg/l MnSO₄·H₂O, 1.4 mg/l ZnSO₄·7H₂O, and 2.0 mg/l CoCl₂·6H₂O; it was supplemented with peptone to 1.00 g/l, yeast extract to 0.33 g/l, and Solka Floc as carbon source to 10 g/l. Inoculated flasks were closed with cotton plugs and incubated at 30°C and 300 rpm on a rotary shaker for 4 days.

Shake flask cultivation

The medium for cellulase production was composed of (NH₄)₂SO₄, KH₂PO₄, and carbon sources at different concentrations. Concentrations of the two salts were equal and were based on the amount of the carbon source. When the carbohydrate content was 15 g/l or lower, 0.83 g/l of both salts were used; this amount was doubled (1.66 g/l) when the carbohydrate content was above 15 g/l. Each medium contained an additional 5 g/l wheat distiller’s grain, which also included lignocellulosic carbohydrates (18.6% glucan and 14.6% xylan); although it was added to the medium as a nitrogen source, it contained 32.2% protein. To avoid pH changes 0.1 M TRIS and maleic acid were also added; it had been previously discovered that TRIS–maleic acid buffer can stabilize the pH during cultivation, and, under these conditions, some positive effects on cellulase production were also observed [17]. All of the substances were suspended either in tap water or in the liquid fraction of steam-pretreated wheat straw that had been undiluted or diluted with tap water (1:1 and 1:3). The pH was set to 5.8 with NaOH, resulting in a 0.1 M TRIS–maleic acid buffer system (pH 5.8).

Cellulase-producing media were inoculated with an aliquot of 4-day-old preculture at 10% (v/v), and cultures were propagated at 30°C and 300 rpm on a rotary shaker. Samples were withdrawn regularly and centrifuged (3,400 g, 5 min) to separate supernatants for further analysis. Fermentations were terminated after 7 or 11 days, and whole fermentation broths were stored at −20°C to test some of them in hydrolysis assays.

Reducing sugar, enzyme activity, and protein assays

For measurement of reducing sugars (RS), 3 ml of the dinitrosalicylic acid (DNS) reagent was added to 1.5 ml of an appropriately diluted sample, and the mixture was placed in boiling water for 5 min. After cooling and adding 16 ml of distilled water, the absorbance was measured at 550 nm to determine the concentration of the RS. Glucose was used to prepare the standard curve.
Filter paper activity (FPA) measurements were performed to describe the overall potential for saccharification. Appropriately diluted supernatant was used to liberate 1 mg of glucose under assay conditions; the volume was increased to 1.5 ml by adding 0.05 M acetate buffer (pH 4.8). A strip of Whatman No. 1 filter paper (1 × 6 cm), equal to 50 mg of substrate, was added and incubated for 1 h at 50°C. Three milliliters of DNS reagent was added to each tube to stop the reaction. The amount of released RS was measured as discussed above. FPA was expressed as FPU/ml, where FPU was defined as the amount of liberated glucose given in micromoles per minute.

To measure xylanase activity, 0.1 ml of the appropriately diluted supernatant, to liberate approximately 0.25 μg xylose, was added to the mixture of 0.4 ml 0.05 M citrate buffer (pH 5.3) and 0.5 ml of 1% (w/v) birchwood xylan (Sigma–Aldrich, St. Louis, MO, USA) solution prepared in citrate buffer pH 5.3. After incubating at 50°C for 10 min, the reaction was terminated by adding 1.5 ml of the DNS reagent, and the mixture was placed in boiling water for 5 min. After cooling, the absorbance was read at 550 nm. A xylose calibration curve was used to calculate the activity unit, which was defined as the amount of xylose released, given in micromoles per minute.

β-Glucosidase activity was assayed according to Berghem and Pettersson with a 5 mM 4-nitrophenyl-β-D-glucopyranoside solution as the substrate [5].

The concentration of the extracellular protein in the fermentation supernatants was determined by the Bradford method using Coomassie Blue G250 reagent [8]. A calibration curve was obtained by using bovine serum albumin as the standard.

Hydrolysis

Hydrolysis experiments were carried out in a 5-ml reaction volume in test tubes with magnetic stirring at 50°C. Dry WIS content was 2% suspended in 0.05 M acetate buffer (pH 4.8). The enzyme to substrate ratio used achieved a glucan dosage of 20 FPU/g. As a control, the substrate was hydrolyzed by commercial enzyme preparation, Celluclast 1.5L (Novozymes A/S, Bagsvaerd, Denmark) supplemented with Novozym 188 (Novozymes A/S, Bagsvaerd, Denmark) in 20 FPU/g glucan cellulase and 20 IU/g glucan total β-glucosidase dosages. Hydrolysis was run for 72 h with withdrawals at 0, 3, 6, 9, 24, 48, and 72 h. At each time point, three tubes were taken (representing triplicates) and boiled for 5 min to inactivate the enzymes. They were then centrifuged, and the supernatant was prepared for high-performance liquid chromatography (HPLC) analysis to measure cellobiose, glucose, and xylose concentrations. The conversion values were calculated based on the initial glucan and xylan content of the WIS.

Analytical methods

The glucan and xylan contents of wheat straw before and after steam pretreatment were determined in triplicates using a two-step sulfuric acid hydrolysis, which was based on a method described by Högglund [13]. In our analysis, a modified method was performed in which 0.5 g raw material was dispersed in 2.5 ml 72% sulfuric acid and maintained at room temperature for 2 h with occasional stirring. Next, 75 ml of distilled water was added and autoclaved for 1 h at 121°C. The reaction mixture was separated on preweighed, dried G4 glass filtering crucibles. The filtrate was analyzed for carbohydrates by HPLC. The filter cake was washed with hot distilled water and dried at 105°C. This residue was defined as the lignin content, and it was corrected for its ash content, which was determined after incineration at 550°C for 6 h.

To determine the starch content of wheat grain, a 0.5-g sample was suspended in a mixture of 25 ml distilled water and 10 ml 2 M NaOH. It was then incubated at 90°C for 20 min, followed by cooling to a temperature below 50°C. The sample was neutralized through the addition of 10 ml 2 M HCl. Then 10 ml of 0.1 M sodium acetate buffer (pH 4.2) and AMG 300L amyloglucosidase at 600 U/g solid were added for a 12-h hydrolysis at 40°C. This mixture was centrifuged at 3,400 × g for 5 min, and the supernatant was analyzed for glucose via HPLC. Measured glucose concentrations were reduced by the glucose content of the AMG 300L. The remaining solid was washed to remove residual glucose, dried, and analyzed for lignocellulose content as described above.

Samples for HPLC analysis were prepared by filtering through a regenerated cellulose syringe filter with 0.45-μm pore size (ProFill, Langerwehe, Germany). The glucose, cellobiose, and xylose contents of hydrolysis and the composition analysis samples were separated on an Aminex ion exclusion HPX-87H cation-exchange column (Bioread, Hercules, CA USA), which was run at 65°C with 5 mM sulfuric acid as mobile phase at a flow rate of 0.5 ml/min. After separation, compounds were detected by a Shimadzu RID-I0A refractive index detector (Shimadzu, Kyoto, Japan).

Sugars and degradation compounds in the liquid fraction were also measured. Total sugars (including both monomers and oligomers) were determined after mild acid hydrolysis (4% (v/v) H2SO4, 120°C and 30 min). Monomeric and total sugar concentrations in the liquid fraction were measured by HPLC (Waters, Milford, MA, USA) with a 2414 refractive index detector (Waters, Milford, MA, USA). A Transgenomic CarboSep CHO-682 carbohydrate analysis column was employed for the separation, and it operated at 80°C with deionized water as mobile phase (0.5 ml/min).
Furfural, HMF, vanillin, 4-hydroxybenzaldehyde, coumaric acid, and ferulic acid were analyzed on an Aminex HPX-87H column at 65°C. For the mobile phase, 89% 5 mM H2SO4 and 11% acetonitrile, at a flow rate of 0.7 ml/min were used. For detection, a 1050 photodiode-array detector (Agilent, Waldbronn, Germany) was employed. Acetic and formic acid were also quantified by HPLC (Waters, Milford, MA, USA) with a 2414 refractive index detector and an Aminex HPX-87H column maintained at 65°C with a 5 mM sulfuric acid mobile phase at a flow rate of 0.6 ml/min.

Results and discussion

Effect of wheat grain addition on cellulase production

To investigate the effect of wheat grain on cellulase production by T. reesei RUT C30, the following experiment was performed. As a carbon source, 5 g/l Solka Floc was supplemented with 6.25 g/l ground wheat grain (80% carbohydrate content). As controls, 12.5 g/l ground wheat grain and 10 g/l Solka Floc were used as sole carbon sources.

After 7 days of cultivation, the highest overall cellulase activity, 1.03 FPU/ml, was measured from supernatant samples produced on Solka Floc plus ground wheat grain (SFWG). The cellulase activity of this sample was 20% higher than that in the Solka Floc control (Fig. 1). Broths growing on medium containing wheat grain afforded the lowest activity (0.63 FPU/ml). β-Glucosidase secretion (data not shown) was the most effective on the mixed carbon source (0.50 IU/ml), and, surprisingly, the next highest value was measured from broths on solely ground wheat grain (0.49 IU/ml). These samples had 25 and 23% higher β-glucosidase activity, respectively, than that of the Solka Floc control (0.40 IU/ml). Because the main component of wheat grain is starch (65.9%), it was assumed that the effect caused by the wheat grain addition was most likely related to its starch content. Nevertheless, a small amount of lignocellulosic carbohydrate from wheat distiller’s grain and wheat bran was also present in each case. Wayman and Chen [43] also observed that untreated whole wheat flour was good at inducing cellulase synthesis in T. reesei RUT C30. They assumed that the reason for this induction involved some synergy between the effects of bran and starch in the whole wheat flour.

In the broths containing ground wheat grain, accumulation of RS content was observed with peaking on day 2 (1.11 and 1.05 g/l for wheat grain with and without Solka Floc, respectively) and was consumed by day 4. Because RS liberation was not found in broths containing Solka Floc alone, and Solka Floc with wheat grain did not yield significantly more glucose, hydrolysis of starch and consumption of its products occurred. Production of amylolytic enzymes by the Trichoderma species (including T. reesei) has already been reported [4].

Fermentations were also performed on the α-amylase-treated ground wheat grain in combination with Solka Floc. This carbon source mixture resulted in a higher FPA but a lower β-glucosidase activity, compared to the results with untreated ground grain (data not shown). Because β-glucosidase activity is a crucial factor in hydrolysis with T. reesei enzymes [9], untreated ground grain was used in the following experiments. Higher cellulase activity obtained on amylase-treated grain agreed with the theory of Chen and Wayman [10], who assumed that during enzymatic or acidic hydrolysis of starch, reversion products, such as sophorose, are formed under certain circumstances. These products can act to induce cellulase expression. Differences in the β-glucosidase activity can be explained by the mechanisms of bacterial amylases (Thermamyl SC from Bacillus licheniformis) or those of fungal (produced by Trichoderma itself) origin. While the amylases from bacteria randomly attack the α-1–4 bonds of starch to create dextrins, the fungal amylases, presumably also those of T. reesei RUT C30, liberate maltose and glucose from chain ends. β-Glucosidase triggered by lignocellulosic components in the medium can form cellulase inducers via transglycolysation from this starch-derived glucose. This ability of β-glucosidases from different species has already been proposed [10, 11]. It was hypothesized that some of these compounds can trigger even more β-glucosidase, creating a positive feedback loop.

On the other hand, T. reesei RUT C30 does not possess any functional genes encoding maltose permease, which is responsible for maltose uptake into the cell. As a consequence, its growth on media where the sole carbon source is starch, or other α-glucans, is strongly impaired [34]. This
phenomenon is supported by an earlier finding that starch itself was a poor inducer of cellulase enzyme production [10]. Furthermore, the catabolite derepressive property of \textit{T. reesei} RUT C30 [15] also has an important role in helping to avoid repression by glucose from starch. These unique features of RUT C30 are crucial in cellulase secretion triggered by starch or its derivatives because no cellulolytic enzyme production was observed in \textit{T. reesei} QM9414 cultures growing on acid-hydrolyzed potato starch [25].

Liquid fraction as carbon source for cellulase production

The liquid fraction separated after steam pretreatment of wheat straw was tested in cellulase fermentations with no dilution, twofold or fourfold dilutions supplemented with other medium components at a pH of 5.8. The concentration of the RS remained constant during the 11-day cultivation (12.5 g/l for 1:3 dilution, 22.4 g/l for 1:1 dilution, and 36.5 g/l for undiluted liquid fraction), indicating that there was no utilization of the carbon source even at the highest dilution; this result also indicates that no growth occurred.

Based on these results, a two-step detoxification process of the liquid fraction was executed. Importantly, there was no optimization of the detoxification process; the primary aim of the current process was to make the liquid fraction useable. Originally, the liquid fraction contained HMF and furfural, derived from glucose and xylose, respectively. Additionally, a large amount of acetic acid was present, which demonstrates the high degree of hemicellulose solubilization. Further degradation of furfural and HMF occurred, resulting in formation of formic acid (Table 2). Besides the inhibitors listed in Table 2, other compounds, namely 4-hydroxybenzaldehyde, vanillin, syringaldehyde, coumaric and ferulic acids, were identified at minor concentrations (<0.1 g/l).

During evaporation, volatile compounds were partially eliminated from the mixture. Approximately 61, 10, and 4% of the initial amount of furfural, acetic acid, and formic acid, respectively, was found in the condensate (Table 2). With a prolonged boiling time, it is possible that more acids could be stripped out. For acetic acid and HMF, the mass balances are complete within error intervals; 89 and 100% were left in liquid fraction. In the case of furfural (24% left in liquid fraction) and formic acid (88%), the mass balances are only partial, and therefore it was assumed that even more evaporated from the liquid fraction, leaving the cooler without condensation. As a result of the two-step detoxification process, furan aldehydes were almost completely eliminated, but an increase in acetic acid concentration was observed. The results of repeated evaporation showed a good correspondence with the first test in terms of the concentrations of each compound. However, the results of repeated overliming were in line with the first experiment only for the furfural, HMF, and acetic acid. In contrast to the other compounds, the concentration of formic acid decreased during the first overliming, but it increased during the second; this difference can also be detected in the high standard deviation of the mean value. Effectiveness of overliming with Ca(OH)\textsubscript{2} depends on its severity, which can be described with three parameters: final pH, temperature, and treatment time. Aliphatic acids are usually unaffected, but under harsher conditions, their concentration can increase. Horváth et al. investigated the effect of these factors on the composition, and only one setup was found to decrease the concentration of formic acid, but even in this case the concentration of acetic acid increased; in all other investigated setups, increases in both concentrations were observed [14]. The parameters of that one exception (pH 10, 35°C, and 1 h) are quite similar to the overliming process applied in our experiments (pH 10, 35°C, and 1 h). However, some oscillations in temperature may result in an increased formic acid concentration as well.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Untreated</th>
<th>After evaporation(^{a})</th>
<th>Condensate</th>
<th>After overliming(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furfural</td>
<td>2.1 ± 0.5</td>
<td>0.5 ± 0.0</td>
<td>5.3 ± 0.8</td>
<td>0.0</td>
</tr>
<tr>
<td>HMF</td>
<td>0.7 ± 0.0</td>
<td>0.7 ± 0.0</td>
<td>0.0</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>8.8 ± 0.8</td>
<td>7.8 ± 0.1</td>
<td>3.8 ± 0.1</td>
<td>12.4 ± 1.7</td>
</tr>
<tr>
<td>Formic acid</td>
<td>7.2 ± 0.9</td>
<td>6.3 ± 0.0</td>
<td>1.1 ± 0.1</td>
<td>6.8 ± 2.2(^{c})</td>
</tr>
</tbody>
</table>

Mean values of two detoxifications with standard deviations are presented. During evaporation from an initial volume of 250 ml, approximately 60 ml of volume was lost, and it was replaced by distilled water.

\(^{a}\) Refilled to original volume with distilled water

\(^{b}\) Adjusted to pH 5.8 as used in fermentations

\(^{c}\) The values of two detoxification trials showed different trends, for details see text

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Interestsingly, an elevated concentration of ferulic acid was detected, from the initial 0.04 to 0.49 g/l after over-liming. One possible reason for this increase is that at the low pH of the original liquid (around 3.4), ferulic acid can form polymers, which were not determined. However, at higher pH values, such as those following over-liming and the pH adjustment to 5.8, these polymers are degraded and can be measured as monomers.

As an effect of detoxification, the content of glucose and xylose increased but with slightly decreasing total sugar concentrations, which shows that during detoxification, further degradation of oligomers occurred (Table 3). The concentrations of other carbohydrates remained constant, within error intervals.

Fermentations were performed with the detoxified liquid, and enzyme production was only observed in the case of the fourfold-diluted liquid fraction, which reached an activity of 1.18 FPU/ml after 11 days. At the other dilutions, the concentration of the RS was constant during fermentation. Therefore, in the subsequent parts of the study, the detoxified liquid fraction was applied at a 1:3 dilution with tap water.

In the case of this dilution, the initial acetic acid concentration of the treated liquid fraction was 3.1 g/l in the culture broth. The effect of acetic acid on cellulase production by T. reesei RUT C30 was investigated up to 3 g/l at a pH of 6.0 using Mandels’ medium with washed steam-pretreated willow as carbon source and no influence was observed [37]. However, there is no information about the effect of acetic acid at higher concentrations. When furfural (0.4, 0.8, and 1.2 g/l) was also added into the same medium containing acetic acid at low concentrations (1.0 and 2.0 g/l), the acetic acid appeared to reduce the inhibitory effect of the furfural [37]. Both the acetic acid and the furfural concentrations were in these ranges in the fourfold-diluted, untreated liquid fraction (2.20 and 0.52 g/l, respectively), but no sugar consumption was observed during cultivation, demonstrating an effect of other inhibitors present in the liquid fraction. This synergy between inhibitors was also confirmed by the fact that the total amount of inhibitors in the treated liquid was higher than in the original one, but mostly contained only aliphatic acids and enzyme production occurred when it was diluted fourfold.

Inhibitors were also measured in fermentation broths at the end of cultivation. In the case of broths containing undiluted or twofold dilutions of the liquid fraction, the concentrations of the inhibitors did not change during cultivation. Neither aliphatic acids, nor the other inhibitors were detected in the broth with fourfold-diluted treated liquid fraction. Palmqvist et al. [30] and Szengyel et al. [37] also found that T. reesei was able to consume acetic acid in media containing low overall levels of inhibitors. In the current study, consumption of formic acid by T. reesei was also detected.

### Integrated enzyme production

The fourfold-diluted, treated liquid fraction was supplemented with other carbon sources related to wheat processing to enhance cellulase productivity and to optimize the composition of the enzyme mixture for the hydrolysis. Media compositions are presented in Table 4. With these media, every compound, excluding salts and buffer, would be available in-house through an integrated wheat processing factory. As reference fermentation, WIS of steam-pretreated wheat straw was used as carbon source and suspended in 0.1 M TRIS–maleic acid buffer prepared with tap water.

The presence of inhibitory compounds in the liquid fraction prolonged the lag phase of enzyme production compared to the reference. Therefore, when the reference had already reached maximal FPA after 7 days, the other cultures still showed increasing activities (Fig. 2).

The prolonged lag phase could be perceived on the RS curves as well. RS concentration increased during the first 2 days of fermentation, which was due to the enzymes present in the inocula and the presence of inhibitors. No RS accumulation was observed in REF where cells were immediately able to utilize the released sugars from the WIS. When T. reesei started to grow, the concentration of RS dropped sharply. This event occurred at the same time in the media containing the liquid fraction, irrespective of the supplementing carbon source. Thus, the supplementary substrates did not decrease the length of the lag phase. During RS consumption, enzyme production had also started, possibly even in the presence of monomers. In this case, the catabolite derepressive feature of the RUT C30 strain became a crucial factor again because it can contribute largely to this observed enzyme production. It has been determined that in T. reesei RUT C30 cultures growing on a mixture of xylose and cellulose, xylose was consumed first to support growth [27]. However, when

### Table 3 Sugar concentrations in liquid fraction before and after detoxification (given in g/l)

<table>
<thead>
<tr>
<th></th>
<th>Untreated Monomer</th>
<th>Untreated Total</th>
<th>Detoxified Monomer</th>
<th>Detoxified Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0.6 ± 0.1</td>
<td>7.2 ± 0.7</td>
<td>2.1 ± 0.2</td>
<td>7.1 ± 0.3</td>
</tr>
<tr>
<td>Xylose</td>
<td>5.5 ± 0.2</td>
<td>40.5 ± 3.1</td>
<td>6.4 ± 0.2</td>
<td>37.6 ± 3.3</td>
</tr>
<tr>
<td>Others*</td>
<td>2.6 ± 0.3</td>
<td>4.5 ± 0.6</td>
<td>2.4 ± 0.1</td>
<td>4.8 ± 0.1</td>
</tr>
</tbody>
</table>

Data are presented as mean values of triplicates with standard deviations

* Galactose, mannose, and arabinose

---

[Table 3](#)
glucose (2.5 g/l) was present in the medium with xylose (7.5 g/l), in the form of a conditioned hydrolyzate and supplemented with 1% Solka Floc, the glucose was utilized first, resulting in its depletion by the second day, followed by depletion of xylose on the third day, and no increase was detected in either concentration [12]. Therefore, we hypothesized that these easily accessible sugars can promote growth of the fungus even in the presence of inhibitors.

An experiment was also carried out with the twofold dilution of the liquid fraction supplemented with 12.5 g/l ground wheat grain to determine whether additional substrates could decrease the inhibitory effect. However, no RS consumption occurred.

Beside the presence of inhibitors, a different matrix of carbohydrates in the media could also cause differences in FPA profiles compared to the reference. The liquid fraction only contained soluble xylose and glucose in addition to their oligomers derived from lignocellulosic material. Induction of cellulase secretion by water-soluble glucose oligomers (n < 6) in T. koningii [41] has been reported and also partially by xylose in T. reesei RUT C30 [27]. In the liquid fraction used in this study, these soluble inducing oligomers are readily available. However, in the case of WIS, only water-insoluble polymers were present, and they needed to be degraded before utilization.

After 11 days, the highest volumetric activities of the cultures were obtained in the medium supplemented with wheat grain, which was also reflected in the protein content (Table 5). Supplementation with wheat grain caused a 35% increase in FPA, which was 21% in the case of WIS compared to the liquid fraction alone. The highest FPA yield was obtained by the reference containing no inhibitors, followed by the unsupplemented liquid fraction (Table 5). In the case of supplementations, the enhanced carbohydrate content did not result in increased FPA yields. Xylanase activities were practically equal in the LIQ and LIQWIS samples, while 30% higher xylanase activity was measured by using wheat grain supplementation. Final xylanase activity of REF was 62% lower than that obtained in the LIQ sample. On one hand, the reason for this low activity may be the lower xylan content in the washed pretreated wheat straw (Table 4). On the other hand, presence of D-xylose di- and trisaccharides in the liquid fraction may cause more powerful stimulation of xylanase secretion than the monomers alone [45]. Moreover, L-arabinose was found to be a more effective inducer of xylanase secretion than xylose [44]. In the liquid fraction used for this study, the presence of arabinose was significant because it was the third highest concentration (0.5 g/l) in the fourfold-diluted, treated liquid fraction.

Fig. 2 FPA and RS profile for LIQ, LIQWG, LIQWIS, and REF (for abbreviations see Table 4). Mean values and standard deviations are shown for two flask cultures that were grown in parallel. FPA values of the liquid fraction containing media for the first and second days are not shown because they are highly affected by the amount of RS background.
Fermentation was repeated to confirm the results, and the repetitions yielded good correspondence with the initial data.

Hydrolysis

For hydrolysis experiments, whole fermentation broths from 11-day cultures in LIQ, LIQWG, and LIQWIS media were used. Hereafter, these abbreviations refer to enzymes produced in the corresponding medium. Whole fermentation broth of \textit{T. reesei} RUT C30 has been reported to be more effective than the supernatant in hydrolysis in terms of glucose liberation\cite{20}. The reason for this finding is likely that \( \beta \)-glucosidase mostly appears in hyphae-bound form\cite{26}, and the presence of this enzyme component at a sufficient concentration is crucial for an elevated glucose yield.

Hydrolysis of the pretreated and washed wheat straw (WIS) was performed with equal enzyme doses, 20 FPU/g glucan of WIS for each broth. For a reference, a commercial enzyme preparation from \textit{T. reesei}, Celluclast 1.5L, was used at the same dosage. However, it was supplemented with \( \beta \)-glucosidase from \textit{Aspergillus niger} (Novozym 188) to obtain 20 BG IU/g glucan. This addition was necessary because the \( \beta \)-glucosidase level of Celluclast 1.5L is suboptimal\cite{9}. Other activities of the culture broths (supernatants) in the volume containing 20 FPU are presented in Table 6. While the BGL/FPA ratio for the LIQ and LIQWIS samples was 64 and 55%, respectively, that of LIQWG was similar to the ratio of the reference (93 vs. 100%, respectively). The doses of xylanase were similar in the cases of LIQ and LIQWG, whereas LIQWIS presented the lowest xylanase and \( \beta \)-glucosidase activities. However, activities by the bound and intracellular enzymes were not included in the dosages because the activity values used for calculating the needed volume were measured from the supernatants.

Hydrolysis time curves for released glucose (cellobiose included) and xylose are presented in Fig. 3. Some cellobiose accumulation was observed with each fermentation broth but to a different extent. The highest cellobiose concentration, 3.3 g/l, was measured from samples hydrolyzed with LIQ broth after 24 h, while a decline was already occurring in the other samples at this time. Although less \( \beta \)-glucosidase activity was added with broth LIQWIS (Table 6), it showed less cellobiose accumulation than the aforementioned sample. This finding is somehow contrary to the measured amount of \( \beta \)-glucosidase, but these measurements were taken from the supernatant. Thus, it can be assumed that the LIQWIS broth had more enzymes in the hyphae or substrate-bound form (the LIQWIS broth contained a solid residue of the steam-pretreated substrate resulting in higher enzyme adsorption). Although LIQWG had the optimal BGL/FPA ratio (Table 5), cellobiose accumulation was much higher than in the reference. However, LIQWG was the most effective among the broths tested (Fig. 3), in terms of glucose liberation. By the end of the 72-h hydrolysis, differences between broths and the reference decreased. Even the LIQWG broth reached the level of the commercial enzymes.

In connection with the concentration of cellobiose, the glucose in the reference sample increased fastest, but at the

Table 5 Final protein concentrations, volumetric activities, and yields after 11 days of cultures for the LIQ, LIQWG, LIQWIS, and REF samples

<table>
<thead>
<tr>
<th></th>
<th>Protein (g/l)</th>
<th>BGL (IU/ml)</th>
<th>XYL (IU/ml)</th>
<th>FPA (FPU/ml)</th>
<th>Yield (FPU/g CH)</th>
<th>BGL/FPA ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIQ</td>
<td>0.58 ± 0.03</td>
<td>0.88 ± 0.02</td>
<td>127 ± 4</td>
<td>1.38 ± 0.01</td>
<td>98.6</td>
<td>64</td>
</tr>
<tr>
<td>LIQWG</td>
<td>1.18 ± 0.05</td>
<td>1.74 ± 0.03</td>
<td>165 ± 4</td>
<td>1.87 ± 0.05</td>
<td>77.9</td>
<td>93</td>
</tr>
<tr>
<td>LIQWIS</td>
<td>0.69 ± 0.02</td>
<td>0.91 ± 0.06</td>
<td>130 ± 5</td>
<td>1.67 ± 0.04</td>
<td>72.3</td>
<td>55</td>
</tr>
<tr>
<td>REF</td>
<td>0.44 ± 0.06</td>
<td>0.88 ± 0.01</td>
<td>49 ± 6</td>
<td>1.11 ± 0.04</td>
<td>122.1</td>
<td>79</td>
</tr>
</tbody>
</table>

For abbreviations see Table 4. Data are presented as mean values and standard deviations from two parallel flask cultures.

Table 6 Enzyme activities and protein content of produced and commercial enzymes representing 20 FPU cellulase activity

<table>
<thead>
<tr>
<th></th>
<th>Volume (ml)</th>
<th>FPA (FPU)</th>
<th>Protein (mg)</th>
<th>BGL (IU)</th>
<th>XYL (IU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIQ</td>
<td>14.49</td>
<td>20</td>
<td>8.40</td>
<td>12.75</td>
<td>1,840</td>
</tr>
<tr>
<td>LIQWG</td>
<td>10.70</td>
<td>20</td>
<td>12.63</td>
<td>18.62</td>
<td>1,766</td>
</tr>
<tr>
<td>LIQWIS</td>
<td>11.98</td>
<td>20</td>
<td>8.27</td>
<td>10.90</td>
<td>1,557</td>
</tr>
<tr>
<td>Celluclast + Novozym 188*</td>
<td>0.34</td>
<td>20</td>
<td>13.04</td>
<td>20</td>
<td>243</td>
</tr>
</tbody>
</table>

Although whole culture broths were used in hydrolysis, enzyme activities were measured from supernatants

* BGL supplemented to 20 IU/g glucan
end of the 72-h hydrolysis, glucose in samples with LIQWG broth also reached 11 g/l. Surprisingly, the liberated glucose in samples with broths LIQ and LIQWIS did not reflect the difference between their respective accumulation of cellobiose (approximately 1 g/l after 72 h). During the 72-h hydrolysis, both the reference sample and that from broth LIQWG attained 85% glucan conversion and LIQWIS and LIQ, 77 and 76%, respectively, compared to the theoretical maximum.

Liberation of xylose was similar in each case and more effective than with the Celluclast 1.5L-Novozym 188 combination, which had the lowest dose of xylanase. In terms of the dose of xylanase, there was no large difference between the broths. However, the xylan content of the substrate was rather low. On the other hand, higher xylanase activities may have contributed to the efficient glucose liberation achieved by increasing the accessibility of cellulose [6]. Final xylan conversion values were the following for each broth: 70% for the reference, and 81, 85, and 85% for LIQ, LIQWG, and LIQWIS, respectively, although LIQWIS contained the lowest xylanase activity among the broths.

Conclusions

In this study, we investigated the possibility of using the liquid fraction of steam pretreatment in on-site enzyme production by T. reesei RUT C30. As a result of the presence of inhibitors, no enzyme production was observed with an untreated liquid fraction, even when it was diluted fourfold. Successful fermentation could be performed only in a fourfold-diluted, detoxified liquid fraction. In this treated liquid furfural and HMF were eliminated, but concentrations of aliphatic acids increased. Therefore, the total amount of inhibitors remained unchanged. Our findings demonstrate that up to a specific level, the synergy of the inhibitors is more important than their discrete concentrations.

The main advantage of using the liquid fraction as carbon source was the highly enhanced xylanase activity compared to the reference (WIS in tap water). Wheat grain supplementation resulted in nearly doubled β-glucosidase activity compared to the original liquid fraction alone. However, FPA yields from supplemented media (LIQWIS and LIQWG) were considerably lower than those of the references.

Steam-pretreated and washed wheat straw was found to be a good substrate for enzymatic hydrolysis, for both commercial and in-house-produced enzymes. Contrary to expectations, the enzyme produced in medium supplemented with washed pretreated wheat straw (broth LIQWIS) did not yield any more glucose. However, adding wheat grain into the medium resulted in enhanced efficiency of hydrolysis, which is most likely due to the elevated β-glucosidase activity. This sample eventually reached the same final conversion as the commercial enzyme mixture supplemented with β-glucosidase.

With this study, we demonstrated that after detoxification, the liquid fraction of steam-pretreated wheat straw is a reasonable substrate for T. reesei RUT C30, which makes on-site enzyme production available using a less expensive carbon source. Moreover, supplementing this medium with ground wheat grain also carries apparent advantages and provides the possibility for the integration of second-generation ethanol technologies with on-site cellulase production in wheat grain processing ethanol factories.

Acknowledgments Hungarian National Research Fund (OTKA K72710) is gratefully acknowledged for the financial support.

References


Fig. 3 Concentrations of released sugars during hydrolysis of pretreated and washed wheat straw by 20 FPU/g glucan dosages of LIQ, LIQWG, and LIQWIS broths. A reference hydrolysis was performed by using commercial enzyme preparations (Celluclast 1.5L supplemented with Novozym 188). Data are presented as mean values of three parallel tubes with standard deviations. a Glucose (Glu) and cellobiose (Glu2) concentrations. b Xylose concentration.


34. Szengyel Z, Zachari G, Réczey K (1997) Cellulase production based on hemimcellulosic hydrolysate from steam-pretreated...
Relevance of the light signaling machinery for cellulase expression in *trichoderma reesei* (*hypocrea jecorina*)

Miklós Gyalai-Korpos1, Gáspár Nagy1, Zoltán Mareczky1, André Schuster2, Kati Réczey1, Monika Schmoll2*

**Abstract**

**Background:** In nature, light is one of the most important environmental cues that fungi perceive and interpret. It is known not only to influence growth and conidiation, but also cellulase gene expression. We therefore studied the relevance of the main components of the light perception machinery of *Trichoderma reesei* (*Hypocrea jecorina*), ENV1, BLR1 and BLR2, for production of plant cell wall degrading enzymes in fermentations aimed at efficient biosynthesis of enzyme mixtures for biofuel production.

**Findings:** Our results indicate that despite cultivation in mostly dark conditions, all three components show an influence on cellulase expression. While we found the performance of the enzyme mixture secreted by a deletion mutant in env1 to be enhanced, the higher cellulolytic activity observed for ∆blr2 is mainly due to an increased secretion capacity of this strain. ∆blr1 showed enhanced biomass accumulation, but due to its obviously lower secretion capacity still was the least efficient strain in this study.

**Conclusions:** We conclude that with respect to regulation of plant cell wall degrading enzymes, the blue light regulator proteins are unlikely to act as a complex. Their regulatory influence on cellulase biosynthesis involves an alteration of protein secretion, which may be due to adjustment of transcription or posttranscriptional regulation of upstream factors. In contrast, the regulatory function of ENV1 seems to involve adjustment of enzyme proportions to environmental conditions.

**Findings**

The ascomycete *Trichoderma reesei* (anamorph of *Hypocrea jecorina*) is one of the most prolific cellulase producing microorganisms, its efficient enzyme mixture being used in several processes of textile, food and pulp and paper industries [1-3]. Moreover a new market potential is arising with the commercialization of cellulosic ethanol plants: however, a main bottleneck for the economic success of the production of the second generation biofuels is the price of cellulolytic enzymes [4]. Strain improvement in *T. reesei* for plant cell wall degrading enzyme production can become more efficient with the use of the genome sequence [5,6]. Interestingly, analysis of the genome of *T. reesei* revealed an unexpectedly low number of genes encoding cellulolytic enzymes - despite the high efficiency of the cellulase mixture produced by this fungus. Besides improving the produced enzymes themselves or the efficiency of the promotors by which their expression is controlled, one strategy to elucidate the underlying mechanisms responsible for this high efficiency of *T. reesei* can be the investigation and exploitation of signal transduction processes [7,8] during growth on cellulosic substrates. Signaling mechanisms greatly contribute to successful adaptation and survival by receiving and interpreting numerous biotic and abiotic factors one of which is light. In contrast to plants, which utilize light as energy, for fungi light is merely a source of information. Blue light affects or initiates a number of physiological processes in fungi in general and also in *Trichoderma*, e.g. growth, conidiation and numerous metabolic pathways [9,10]. Many effects of light are common within the fungal kingdom and also the pathways of light sensing and its elements often share significant homology [11].

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The photobiology of *Trichoderma* spp. has been investigated in considerable depth for decades [12]. Orthologues of the well studied *Neurospora crassa* photoreceptor genes *wc-1* and *wc-2* [13] genes were described in *Trichoderma atroviride* [14] and subsequently also in *T. reesei* [15]. The *T. reesei* blue light regulators (BLR1 and BLR2) have similar structural domains (PAS/LOV) and light independent regulatory roles were also reported for these proteins [15]. In *T. atroviride* also BLR independent light sensing routes have been proposed [16]. ENVOY, a PAS/LOV domain protein in *T. reesei*, which shares similarity with the *N. crassa* photoreceptor VIVID [17–19] is crucial in light tolerance and modulates cellulase transcription in a light dependent manner [20]. Recently, also an influence of the two photoreceptors BLR1 and BLR2 on cellulase gene transcription has been shown [15] suggesting that these regulators act positively on this process. In *Trichoderma*, also cAMP levels are responsive to light [21] and cAMP is involved in regulation of cellulase levels [22], which indicates an action via phosphorylation of transcription factors by cAMP dependent protein kinase A. Moreover, two G-protein alpha subunits (GNA1 and GNA3, which impacts cAMP levels) have been shown to exert a considerable light dependent influence on transcription of the major cellulase gene *cbh1/cel7a* [23,24]. However, since these high levels of transcription did not result in an equally high production capacity of the respective mutant strains (M. Schmoll, unpublished results), further (presumably light-dependent) regulatory checkpoints at the level of translation and/or secretion can be expected.

Based on these findings we assumed that the major components of the light response pathway (BLR1, BLR2 and ENV1) could be crucial regulators or checkpoints in (light dependent) production of extracellular enzymes. Therefore we aimed to investigate the relevance of the light signaling machinery, which obviously plays an important role in cellulase regulation, for industrial fermentations. Analysis of strains defective in light sensing showed that the blue light regulatory proteins BLR1, BLR2 and ENV1 are indeed involved in regulation of expression and secretion of plant cell wall degrading enzymes, even in the predominantly dark conditions of a biotechnological steel fermentor. Consequently, our study revealed new targets for improvements of cellulase production by modification of the light signaling machinery.

Presence of promoter motifs associated with light response in genes encoding plant cell wall degrading enzymes

Because of the reported influence of light as well as of ENVOY, BLR1 and BLR2 on regulation of cellulase expression [15,20] a direct regulation of cellulolytic genes by the transcription factors BLR1 and BLR2 or and indirect impact of ENVOY can be assumed. In order to get a first guideline, whether the genes encoding plant cell wall degrading enzymes could be subject to direct regulation by the two photoreceptors or other factors associated to light response, we screened the promoters of genes encoding proteins presumably involved in plant cell wall degradation for the presence of light responsive promoter motifs.

The region within 1000 bp upstream of the ATG codon was searched for specific motifs (Table 1). Gene sequences were obtained from the *Trichoderma reesei*, v2.0 genome database http://genome.jgi-psf.org/Trire2/Trire2.home.html. A complex consisting of *N. crassa* WC-1 and WC-2, the homologues of *T. reesei* BLR1 and BLR2, was reported to bind light-response elements (LREs) with the consensus sequence of GATNC–CGATN, where N can be any nucleotide but the same in both repeats [25]. An LRE motif was only accepted if its length did not exceed 50 bp. As both BLR1 and BLR2 have the characteristic function as zinc-finger GATA factors, we also screened for the HGATAR (H = C, T, A) consensus sequence [26]. However, binding of these transcription factors to such GATA-sequences was not reported so far. The EUM1 motif which had been identified in the *env1* as well as the *N. crassa* vvd promoters and thereafter detected in the cellobiohydrolase promoters *cbh1 (cel7a)* and *cbh2 (cel6a)* [20] was searched because of the light-dependent complex formation detected on EUM1 in both the *env1* and *gna3* promoter [23,27]. The proteins constituting the EUM1 binding complex are currently unknown. The analyzed genes were associated with possible activities (Table 1), however, in some cases the lack of knowledge on cellular localization of beta-glucosidases implicates uncertainty as to their involvement in extracellular substrate degradation.

As presented in Table 1 the EUM1 sequence was found in the promoters of genes for all types of cellulolytic activities. These findings may be interpreted to correlate with the effect of light on cellulase transcription as detected earlier. The highest number of EUM1 motifs is present in the promoter of *cel74a*, which encodes a xylanase.

LRE motifs were only found in the promoters of three genes, two of which encode beta-glucosidases (*cel1b*, *cel3c*), albeit the localization of the encoded proteins is unknown. The third LRE was found in the promoter of a xylanase gene (*xyn3*).

GATA binding sites were found in most promoter regions. Since GATA factors are wide spread regulation elements and due to the high number of motifs found it is hard to predict any relation to light modulated cellulase gene expression, although binding of the photoreceptor complex cannot be excluded. Interestingly, the
promoter of cel5b encoding an endo-glucanase was the only one where none of these consensus sequences associated with light response was found.

Consequently, while in some cases LRE motifs would render a direct regulation by the photoreceptors BLR1 and BLR2 supposable and several EUM1 motifs suggest binding of (as yet uncharacterized) light regulatory factors, other mechanisms regulating the production of cellulolytic enzymes are likely to contribute to the modulated output in light and darkness.

Cultivation of mutants in env1, blr1 and blr2 for investigation of cellulase gene expression

In order to obtain preliminary information on whether the transcriptional data on the influence of env1, blr1 and blr2 on cellulase regulation [15,20] would correspond to the cellulolytic efficiency of the enzyme mixture secreted by the respective deletion strains, we first performed shake flask cultures. We found that all three strains showed a significantly higher specific cellulase activity on Mandels-Andreetti medium with 1% (w/v) microcrystalline cellulose than the wild-type strain up to 120 hours of cultivation (data not shown). Considering the transcription data for cbh1 in these strains [15] this higher efficiency was surprising. Therefore we chose to use all three strains for analysis of their efficiency in a laboratory scale fermenter and again tested them in shake flask cultures for cellulase expression in the medium to be used for fermentation, which confirmed the results above. In fermenter cultivations, similar patterns of pH adjustment and oxygen supply were observed for each strain. After approximately 5 hours of lag phase, pH and pH began to decrease indicating growth of the fungus. The fermentation parameters recorded suggested that the modified strains have no increased oxygen and inorganic nitrogen demand. Fermentations were aborted after 73 hours of run when the oxygen level had reached 50 - 60% and acid addition was detected.

Enhanced enzyme production by strains with defects in the light response pathway

The degradative potential of the enzyme mixtures secreted by wild-type and mutant strains was first characterized using dyed Azo-CM-cellulose (Megazyme),

### Table 1 Results of promoter analysis (1000 bp upstream of ATG) of genes coding cellulolytic enzymes

<table>
<thead>
<tr>
<th>Gene</th>
<th>EUM1</th>
<th>GATA</th>
<th>LRE</th>
<th>Enzyme</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>cbh1/cel7a</td>
<td>-37r</td>
<td>-186r, -182r, -258r, -890r</td>
<td>CBH</td>
<td>FPA</td>
<td></td>
</tr>
<tr>
<td>cbh2/cel6a</td>
<td>-159r, -1032r</td>
<td>-273r, -838r</td>
<td>CBH</td>
<td>FPA</td>
<td></td>
</tr>
<tr>
<td>egl1/cel7b</td>
<td>-28r, -232r, -421r</td>
<td>-25f, -807r, -1161r, -1229r</td>
<td>EG</td>
<td>FPA, EGA</td>
<td></td>
</tr>
<tr>
<td>egl2/cel5a</td>
<td>-168r</td>
<td>-1186r, -1281r</td>
<td>EG</td>
<td>FPA, EGA</td>
<td></td>
</tr>
<tr>
<td>cel5b</td>
<td>-87r</td>
<td>-827r</td>
<td>EG</td>
<td>FPA, EGA</td>
<td></td>
</tr>
<tr>
<td>egl3/cel12a</td>
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<td>-727r, -1023r</td>
<td>EG</td>
<td>FPA, EGA</td>
<td></td>
</tr>
<tr>
<td>egl4/cel61a</td>
<td>-61f, -109r, -815f</td>
<td>-508f, -378r</td>
<td>EG</td>
<td>FPA, EGA</td>
<td></td>
</tr>
<tr>
<td>egl5/cel45a</td>
<td>-508r, -378r</td>
<td>-606f</td>
<td>BG</td>
<td>FPA*, BGA*</td>
<td></td>
</tr>
<tr>
<td>cel74a</td>
<td>-80r, -736r, -948r</td>
<td>-548r, -1041f</td>
<td>EX-BG</td>
<td>FPA, BGA</td>
<td></td>
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<tr>
<td>bg1/cel5a</td>
<td>-889r, -1122r</td>
<td>-71r, -262r, -683r</td>
<td>BG</td>
<td>FPA*, BGA*</td>
<td></td>
</tr>
<tr>
<td>cel5c</td>
<td>-275f, -318r</td>
<td>-82r</td>
<td>BG</td>
<td>FPA*, BGA*</td>
<td></td>
</tr>
<tr>
<td>cel3d</td>
<td>-767r, -1222r</td>
<td>-823f</td>
<td>BG</td>
<td>FPA*, BGA*</td>
<td></td>
</tr>
<tr>
<td>cel5e</td>
<td>-772r</td>
<td>-137r, -202r, -402r, -710r</td>
<td>IN-BG</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>bg2/cel1a</td>
<td>-194f, -485r</td>
<td>-12f, -528r</td>
<td>IN-BG</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>xyn1</td>
<td>-986r, -868f</td>
<td>-986f, -868f</td>
<td>XYL</td>
<td>XYL</td>
<td></td>
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<tr>
<td>xyn2</td>
<td>-1092r</td>
<td>-1092r, -473f</td>
<td>XYL</td>
<td>XYL</td>
<td></td>
</tr>
<tr>
<td>xyn3</td>
<td>-77r</td>
<td>-741f, -473f</td>
<td>B-XYL</td>
<td>XYL</td>
<td></td>
</tr>
</tbody>
</table>
which revealed the effectiveness of the whole cellulase enzyme mixture, especially of endo-1,4-β-D-glucanases. Peaks of endo-glucanase activity in the culture medium were reached between 48 and 54 hours and after that slightly declined. Highest activity was measured in case of strain ∆blr2 followed by ∆env1, which are 59.0% (p-value 3·10^{-6}) and 44.2% (p-value 8.5·10^{-5}) higher than the wild-type QM9414, respectively. Between ∆blr1 and wild type no significant difference was found (p-value 0.33).

Analysis of filter paper activity (FPA), which determines the amount of glucose liberated from cellulose, was performed in order to obtain indications as to the extent of exo-cellulase/cellobiohydrolase activity. Maximal activity values were reached after 48 hours in case of each strain, thereafter activity values slightly decreased which is presumably due to protease activity in the medium (Figure 1B). Highest activities were reached by ∆env1 and ∆blr2, (1.45 and 1.37 FPU mL^{-1}), respectively, which are 21.9% (p-value 4.4·10^{-5}) and 15.3% (p-value 2.5·10^{-4}) higher than those of the parental strain QM9414 (1.218 FPU mL^{-1}). ∆blr1 produced 31.9% (p-value 3·10^{-6}) less FPU mL^{-1} than that observed with QM9414. Hence the increased degradative potential of ∆env1 and ∆blr2 is only in part due to enhanced expression of the cellobiohydrolases, but seems to be mainly caused by an improved overall efficiency of the endoglucanase mixture secreted. Nevertheless, it must be considered that FPA also involves the action of endoglucanases and β-glucosidase and can thus not be seen as exclusive determination of cellobiohydrolase activity.

Since the preferred substrate for yeast needed for the final step of conversion of plant material into ethanol is glucose, we also determined β-glucosidase activity (on pNPG).

Initially, curves had the same slope for each strain, but ∆blr2 reached an 88.4% (p-value 1.2·10^{-5}) higher peak value than the wild-type. Activities of ∆blr1 and ∆env1 were practically equal (p-value 0.29), approximately 10% higher than QM9414 (p-values 3.3·10^{-4} and 8.5·10^{-4}, respectively; Figure 1C). This result is in accordance with the findings described above, although it should be kept in mind that also cell wall bound β-glucosidase [28,29] may contribute to endoglucanase activity and FPA in vivo.

Finally, we also assessed the potential of the light response mutant strains to degrade the hemicellulosic part of a given plant material. Highest xylanase activities were observed between 48 and 52 hours of fermentation. Surprisingly, despite its high efficiency on cellulose, ∆blr2 (101.2 ± 7.5 IU/ml) showed approximately the same xylanase activity as QM9414 (103.8 ± 1.8 IU/ml; p-value 0.52) which may be because of the different regulation pattern of hemicellulolytic enzymes. A comparable effect has already been observed in case of T. atroviride mutants created by random mutagenesis that possessed enhanced FPA production properties but were deficient in xylanase secretion [30]. Peaks of ∆env1 (123.2 ± 2.0 IU/ml) and ∆blr1 (116.7 ± 5.9 IU/ml) were 18.7% (p-value 2.6·10^{-4}) and 12.4% (p-value 6·10^{-3}) higher than that of QM9414.

The positive effect of env1 gene deletion was consistent with the different activities suggesting that one downstream pathway of the regulatory output of ENVOY are the pathways involved in plant cell wall degradation. The fermentation profiles of the strains...
show that the function of the regulatory mechanisms causing enhanced cellulase production in the mutant strains becomes most significant after 30 hours of fermentation. While for QM9414 and ∆blr1 enzyme activities stagnate shortly thereafter (possibly because a critical level of activity for sustaining certain nutrient levels is reached), enzyme production/secretion as reflected by still increasing activities continues in ∆blr2 and ∆env1.

**Specific performance of enzyme mixtures**

In order to enable a correlation of the performance of the secreted enzyme mixture (i.e., U/mg of secreted protein) with growth and total protein secretion capacity of the individual strains, we determined biomass and protein content of the culture medium during fermentation (Table 2). Biomass production of strain ∆blr1 was considerably (37.5% (p-value 0.05) after 24 h and 41.4% (p-value 0.015) after 48 h) higher than that of the wild-type QM9414 throughout the fermentation. In contrast, neither ∆blr2 nor ∆env1 showed a significant difference to the wild type (p-values > 0.1). Biomass specific filter paper activity was the highest for ∆env1, followed by ∆blr2.

Both BLR1 and BLR2 have been reported to regulate growth of *Trichoderma atroviride* and *T. reesei* on solid media [15,31], however similar effects of deletion of either of these genes have been observed. Nevertheless biomass formation has not been studied on cellulose containing liquid media in *T. reesei*. Our results show that biomass formation of ∆env1 is only marginally reduced (p-value 0.11) compared to that of the wild type, which is consistent with the finding that growth is not affected by the deletion in darkness [32].

To illustrate the performance of the cellulase mixture (U/mg of total secreted protein), protein specific endoglucanase activity and FPA was also calculated. The enhanced FPA of ∆blr2 was in line with the highest protein concentration measured among the strains (Table 2). Considering the biomass formation of this strain, this result indicates that the high cellulolytic activity found in the culture medium of this strain is mainly due to an enhanced protein secretion capacity. An alternative interpretation would be that the higher levels of extracellular proteins observed for this strain are due to autolysis. However, the differences in secretion in terms of proteins secreted per biomass are already detectable for all strains after 24 hours of cultivation, when the fungi are clearly in their production phase and are actively growing (Table 2), which renders this hypothesis unlikely.

Despite the high endoglucanase activity values of ∆env1, only slightly increased protein concentration was observed. Therefore, in contrast to ∆blr2, the enzyme mixture secreted by ∆env1 seems to be more efficient than that of the other strains investigated in this study. Since in several promoters of plant cell wall degrading enzymes of *T. reesei* promoter motifs known to be involved in light response in other fungi have been detected (Table 1), light dependent adjustment of the proportions of the respective enzymes could be one way to achieve this enhanced efficiency.

For the photoreceptor mutants even decreased endoglucanase and FPA performance (EG activity per mg of secreted protein) was obtained. Interestingly, while the biomass-specific FPA for ∆blr1 is clearly lower than for all other strains, including wild-type, the protein specific

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hour</th>
<th>QM9414</th>
<th>∆env1</th>
<th>∆blr1</th>
<th>∆blr2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass (mg gluA) mL</td>
<td>24</td>
<td>0.164 ± 0.026</td>
<td>0.165 ± 0.010</td>
<td>0.215 ± 0.015</td>
<td>0.159 ± 0.002</td>
</tr>
<tr>
<td>Cell specific FPA</td>
<td>24</td>
<td>2.701 ± 0.429</td>
<td>2.739 ± 0.030</td>
<td>1.409 ± 0.228</td>
<td>3.327 ± 0.176</td>
</tr>
<tr>
<td>Cell specific EG</td>
<td>24</td>
<td>32.70 ± 3.61</td>
<td>24.35 ± 0.15</td>
<td>22.69 ± 2.93</td>
<td>55.87 ± 3.94</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hour</th>
<th>QM9414</th>
<th>∆env1</th>
<th>∆blr1</th>
<th>∆blr2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins (mg gluA) mL</td>
<td>24</td>
<td>0.195 ± 0.038</td>
<td>0.181 ± 0.001</td>
<td>0.186 ± 0.040</td>
<td>0.378 ± 0.044</td>
</tr>
<tr>
<td>Cellulase efficiency</td>
<td>24</td>
<td>2.271 ± 0.118</td>
<td>2.931 ± 0.045</td>
<td>1.629 ± 0.263</td>
<td>1.399 ± 0.074</td>
</tr>
<tr>
<td>FPU (mg proteins) mg mL</td>
<td>24</td>
<td>2.975 ± 0.118</td>
<td>2.931 ± 0.045</td>
<td>1.606 ± 0.173</td>
<td>1.701 ± 0.082</td>
</tr>
<tr>
<td>EG efficiency</td>
<td>24</td>
<td>27.50 ± 3.04</td>
<td>22.19 ± 0.13</td>
<td>26.23 ± 3.39</td>
<td>25.50 ± 1.66</td>
</tr>
<tr>
<td>IU (mg proteins) mg mL</td>
<td>24</td>
<td>29.47 ± 1.07</td>
<td>33.97 ± 2.05</td>
<td>21.65 ± 3.13</td>
<td>25.43 ± 0.92</td>
</tr>
</tbody>
</table>

Mean values of triplicate measurements are presented including standard deviation.
FPA is comparable to Δblr2. Consequently, the rather low efficiency of this strain despite its enhanced growth may be due to a decreased protein secretion efficiency caused by deletion of blr1.

Analysis of proteins secreted by mutant strains
In order to analyze whether the increased efficiency is due to an altered expression of the major cellobiohydro-lase of *T. reesei* CBH1/Cel7a, Western blotting was performed (Figure 2). In case of Δblr2 clearly higher abundance of CBH1 was observed, indicating that the increased cellulolytic efficiency of this strain is at least in part due to this enzyme. For Δenv1 the effect was less clear, suggesting that the improvement of the cellulase mixture found for this strain is likely to be caused by altered regulation of expression of the pool of enzymes contributing to cellulose degradation. Considering the data above, these results correspond well with the enhanced protein secretion capacity of Δblr2 and the obviously improved enzyme proportions for Δenv1.

Discussion
Transcriptional responses given to light reflect the changes in the ecological niche that surrounds fungi. *T. reesei* as a saprophyte mainly resides in the inside of decaying plant material, that is, in darkness. Presence of light means the open surface of the habitat where the successful dissemination of conidia is expected or existence of possible mating partner is assumed, but also where (energy consuming) measures for protection from harmful UV-light or desiccation have to be taken. The different physiological requirements of growth on the surface or within its substrate are reflected by the metabolic differences between these conditions [10,33], which can be exploited for strain improvement.

One of our most surprising results in this study was the finding that in contrast to transcription of cbh1/cel7a, which decreases in deletion mutants of blr1 and blr2 [15], cellulase activity in the culture medium of the respective strains was nevertheless increased or at least remained at wild-type levels in case of Δblr1. Interestingly, comparable effects have been observed for *N. crassa* (M. Schmoll, manuscript in preparation). Hence, this study will be the basis for further research to reveal the molecular basis for this phenomenon. In fact, in accordance with the results presented here, light has previously been reported to influence protein secretion in *N. crassa* [34], however a role of the photoreceptors in this process was not known so far. These results also decreased the applicability of our initial hypothesis that several cellulase genes (other than cbh1/cel7a, which is positively regulated by BLR1 and BLR2 [15]) might be directly regulated by binding of BLR1 and/or BLR2 to their promotors as transcription factors. Nevertheless, at present such a direct regulation cannot be fully ruled out.

The finding of a discrepancy between transcription of the major cellulases and detected cellulase activities in the culture medium is particularly interesting because it suggests that cellulolytic enzymes may not be exclusively regulated on the transcriptional level [35] and references therein, but also posttranscriptionally - presumably in response to light.

Although the signal transduction pathway triggering cellulase gene expression is far from being well-established, first insights are already available. Besides the light response pathway, signals related to sulphur metabolism as well as heterotrimeric G-protein signaling and the cAMP-pathway are involved in regulation of cellulase gene transcription. Addition of the organic sulphur source methionine decreases transcription of cbh1/cel7a below detection limits only in light [36]. Deletion of the G-protein alpha subunit GNA1 leads to strongly increased transcription of cbh1/cel7a in darkness [24].

**Figure 2** Western blot showing CBH1/Cel7a abundance in the culture filtrate. Samples were taken during fermentation at the time points indicated and equal amounts were loaded onto SDS-gels.
and constitutive activation of GNA3 causes considerably increased transcription of this gene in light [23]. Hence the relevance of the signals transmitted by GNA1 and GNA3 as well as the sulphur signal must be dependent on the light status, which is perceived and transmitted by the photoreceptors BLR1 and BLR2 [15]. The results presented here could be interpreted in a way that the extent of cellulase transcription is set in response to environmental signals (as transmitted for example by G-proteins via cAMP and phosphorylation to transcription factors), but that the distribution of resources for the energy consuming process of translation and secretion is at least in part governed by the photoreceptors BLR1 and BLR2 and possibly by ENVOY. The fact that these proteins and their orthologues regulate multiple targets [16,32,33] supports this hypothesis. Therefore it will be interesting to learn, whether or not the efficiency of strains engineered for high transcriptional activity of cellulase promotors - such as for example by deletion of gna1 - but also strains resulting from random mutagenesis, can still be improved by deletion of blr2, which might act negatively on secretion. Additionally, elucidation of the mechanism responsible for the impact of components of the light signaling pathways on the secretion machinery warrants further investigations.

It is interesting that different effects have been observed for deletion of blr1, blr2 and env1. On the one hand this finding indicates that BLR1 and BLR2 do not act as a complex under our experimental conditions (mainly darkness) in their regulatory function targeting cellulase gene expression, but have individual functions. On the other hand it also suggests that although induction of env1 transcription is abolished upon deletion of blr1 or blr2 [15], the consequences of deletion of env1 are not similar to those of deletion of these photoreceptors. Hence BLR1 and BLR2 do not exert their function via ENVOY, which confirms the hypothesis proposed earlier [15].

The increased efficiency of the enzyme mixture secreted by Δenv1 further renders a closer investigation of the postulated coregulation of cellulases [35] and references therein interesting, especially with respect to different light conditions and the regulators involved in transmission of this signal.

The lower efficiency of Δblr1 in terms of protein secretion could be due to a counteracting effect of BLR1 compared to BLR2 in secretion. However, in N. crassa it has been shown that in darkness the homologue of BLR2, WC-2, is present in excess over WC-1 [37]. Consequently, the effect seen for Δblr1 is more likely to be due to an increased availability of BLR2, whose binding partner has been removed resulting in additional BLR2 proteins, now free to exert their negative function on secretion.

Effects of light on metabolic processes have been shown in numerous fungi and for many target mechanisms [10,12]. Therefore the function of the main components of the light perception machinery in regulation of plant cell wall degrading enzymes is not without precedent. A connection between carbon sensing and the function of BLR-1 and BLR-2 has been suggested for T. atroviride [31] and different roles in these metabolic functions have been detected: While both proteins are required to adjust the intensity of the response to a certain carbon source, BLR-1 is responsible for carbon source selectivity [38]. For ENV1 a regulatory function in cellulase transcription had been shown in T. reesei [20]. Although the most obvious functions of BLR1, BLR2 and ENV1 have been observed and studied in light, it also has been shown clearly that these proteins and their homologues in other fungi additionally have functions in darkness [15,32,33,38]. Hence the investigation of the light response machinery as done in this study, but also of its downstream targets, with respect to industrial fermentations opens up a new strategy for strain improvement aimed at more efficient biofuel production.

**Conclusions**

With this study we demonstrated that components of light signaling pathways have an important role also in darkness related to carbon sensing, and highlight that factors which have been yet not associated with industrial cellulase expression still can have impact on it. From a practical point of view these modified strains can be used to enhance productivity, thus lower price of enzyme production which is essential for second generation biofuel breakthrough. Further studies to elucidate the complex signaling and regulatory network through which cellulase transcription and consequently expression can be triggered are already in progress. Our study thus provides new insights into this machinery, which can be exploited to enhance biotechnological fermentation at different stages of regulation in fungi.

**Materials and methods**

**Strains**

*Trichoderma reesei* QM9414 (ATCC26921) and the following descendents lacking parts of light sensing pathways were used through this study: Δenv, Δblr1 and Δblr2, missing the open reading frames of the respective genes [15]. Strains were maintained on malt agar plates (30 g L⁻¹ malt extract, 1 g L⁻¹ peptone, 20 g L⁻¹ agar) in darkness at 30°C.

**Preculture preparation and shake flask cultures**

Conidia from two weeks old plates were harvested with sterile distilled water. An adequate volume of this
conidia L
Solka Floc and 5 g L
KH and 0.83 g L
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final concentration
Solka Floc 200 (International
Fiber Corporation, North Tonawanda, NY, USA) was added. After 3 days of cultivation in constant darkness at 30°C on a rotary shaker (250 rpm) flasks were used to inoculate fermentation medium at 10% (v/v).

**Fermentation**
Every strain was analyzed in a 30 L double-walled stainless steel laboratory fermenter (Biostat C-DCU 3, B Braun Biotech, Germany) equipped with pO2, pH, temperature, pressure and foam sensors in 20 L working volume. Agitation was provided by a top-drive agitator with 3 Rushton impellers each with 6 blades.

Medium components (10 g L
Solka Floc and 5 g L
wheat distiller's grain, 0.83 g L
K
H
PO
and 0.83 g L
(NH
)\textsubscript{2}SO
were suspended in tap water sterilized in the mounted device and subsequently ten flasks of the precultures were combined aseptically into the sterile inoculating equipment resulting in 2 L of inoculum, which was transferred into the fermentor.

pH was continuously controlled and adjusted to 5.8 by addition of 10% (v/v) NH\textsubscript{4}OH or 10% (v/v) H\textsubscript{2}PO\textsubscript{4}. PO\textsubscript{4} level was kept on 30% by cascade control of air flow rate as first priority, varying between 5 and 12 L min\textsuperscript{-1} and velocity of agitation as secondary, shifting between 250 and 600 rpm. Beyond 30% of dissolved oxygen content (DO), air flow rate and agitation worked on the minimal values for both variables. Temperature was set to 28°C. Operation parameters (pH, volume of added base and acid, pO\textsubscript{2}, stirring velocity and air flow rate) were recorded by MFCS/win 2.0 (B Braun Biotech) software. To control foaming, ionic antifoam emulsion (Sigma-Aldrich Antifoam A) was added automatically. Fermentations were carried out in duplicate.

Samples were withdrawn regularly, centrifuged (3400 g, 5 minutes) and supernatants were analyzed for enzyme activities and extracellular protein content. Biomass measurements were carried out daily from fermentation broth (see below).

**Western blot analysis**
Proteins from 0.5 ml fermentation supernatant were precipitated by adding 1 ml of 96% ethanol. Western blotting was performed according to standard protocols [40]. Briefly, after centrifugation the pellet was dissolved in 0.2 ml SDS buffer; 10 μl representing equal amounts of culture filtrate were loaded into a 7.5% SDS-PAGE gel and run at 15 mA. Transfer to nitro-cellulose membranes (Hybond-C Extra, Amersham Biosciences, UK) was done by semidy electroblotting and antibodies against the major cellulase CBH1 as well as horseradish peroxidase-conjugated anti-mouse IgG (Promega, Madison, US) were used for analysis of expression of cellulases.

**Analytical assays**
For the Filter Paper Activity (FPA) assay 0.5 ml suitably diluted supernatant to liberate approximately 1 mg glucose equivalent was mixed with 1.0 ml of 0.05 M Na-acetate buffer (pH 4.8) and a 6 × 1 cm strip of Whatman grade 1 filter paper was added. After incubation for 1 hour at 50°C, 3 ml of dinitrosalicylic acid reagent [41] was added, kept at 100°C for 5 minutes, diluted with 16 ml of distilled water and reducing sugar content was measured at 550 nm. The filter paper unit (FPU) was defined as the amount of glucose released given in μmol min\textsuperscript{-1}.

To measure xylanase activity, 0.1 ml properly diluted supernatant to liberate approximately 0.2 μg xylene was added to the mixture of 0.4 ml 0.05 M citrate buffer (pH 5.3) and 0.5 ml of 1% (w/v) birchwood xylan (Sigma Aldrich) solution prepared the same buffer. After incubation at 50°C for 10 minutes the reaction was terminated by adding 1.5 ml of dinitrosalicylic acid reagent and then the mixture was kept at 100°C for 5 minutes. Absorbance was measured at 550 nm. A xylene calibration curve was used to calculate the activity which was defined as the amount of xylene released given in μmol min\textsuperscript{-1}.

β-glucosidase activity was assayed according to the procedure of [42] using 4-nitrophenyl-β-D-glucopyranoside (Sigma-Aldrich) as substrate.

For endo-glucanase activity measurement tenfold diluted supernatant was added to Azo-CM-cellulose solution (S-ACMC; Megazyme International Ltd., Ireland). Procedure was carried out according to the manufacturer’s instructions. Extracellular protein content was determined by Coomassie Blue G250 reagent [43] using Bovine Serum Albumin as standard.

All measurements were carried out at least in duplicate.

For biomass determination 100 ml whole fermentation broth was filtered onto a dry and preweighed filter cloth and washed with distilled water. Filtration was carried out in triplicate. After drying at 105°C for 6 hours the filter cloth containing filter cake was weighed again. For further analysis 0.5 g homogenous sample from each filter cake was subjected to a two step sulfuric acid hydrolysis to release N-glucosamine from fungal cell wall: the sample was incubated with 60% (w/v) sulfuric acid at room temperature for 24 hours with subsequent dilution to 1 N and hydrolysis at elevated temperature (121°C for 1 hour) [44]. The cooled mixture was then neutralized.
by addition of 1 N solution of NaOH and total volume was measured. A portion was centrifuged at 3400 g for 5 minutes and supernatants were analyzed for N-glucosamine by the method of Blix [45] as described by Bussari et al. [46]. N-glucosamine content was calculated according to a calibration curve prepared with reagent grade N-glucosamine.HCl (Sigma-Aldrich). Cell mass is expressed as mg N-glucosamine/ml fermentation broth.

Statistical analyses were performed with STATISTICA 8.0 software (StatSoft, Inc., Tulsa, OK, USA). The significance level for t-test for independent samples was set to a p-value of 0.05.

Acknowledgements
We want to thank Alfredo Herrera-Estrella for the gift of strains ΔΔβ1 and ΔΔβ2 and we gratefully acknowledge Christian P. Kubicek for critically reading the manuscript. This work was supported by the Austrian Research Fund (FWF), grant P21072 to MS. MGK was supported by the COST action BI0602.

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Authors’ contributions
MGK performed fermentations (together with ZM and GN) and sequence analysis and participated in drafting the manuscript. AS performed Western analysis and participated in drafting the manuscript. All authors read and approved the manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 15 November 2010 Accepted: 7 December 2010

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