Enzymes for improved hydrolysis of lignocellulosics

Doctoral dissertation

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Department of Agricultural Chemical Technology
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Hungary
2005
ABSTRACT

The considerably low β-glucosidase activity : FPA ratio of 0.5-0.6 represented by the enzyme complex of *Trichoderma reesei* RUT C30 results in poor hydrolysis potential. To overcome this problem culture conditions were developed to enhance β-glucosidase production. Mandels’ medium supplemented with tris-maleate buffer proved to be efficient for cellulase production with elevated β-glucosidase activity. β-Glucosidase activity : FPA ratio of the obtained enzymes were around the optimal on Solka Floc, lactose and glucose carbon sources. Furthermore, there were no considerable changes in maleic acid concentration of cultivations throughout the fermentations, therefore it is tempting to speculate that maleic acid is a kind of β-glucosidase inductor. In addition, in cultures with tris-maleate buffer pH values varied in a narrow range, thus tris-maleate buffered cultures can be used in shake flask experiments for modeling fermentations running at constant pH.

Tris-maleate buffer system was applied in shake flask experiments to test steam pretreated corn stover, spruce and willow for cellulase production. On steam pretreated corn stover higher cellulase activities could be obtained than on Solka Floc that was used as reference. However, the achieved β-glucosidase activity was higher on Solka Floc compared to steam pretreated corn stover. On the other two steam pretreated materials lower enzyme activities were reached than on Solka Floc. The produced enzymes and two commercial cellulases (Celluclast 1.5L and Econase CE) were tested in hydrolysis experiments using the three pretreated materials and Solka Floc as substrates. Generally, the highest yields were obtained using the enzyme produced on steam pretreated corn stover. Moreover, the highest sugar yield was reached on steam pretreated corn stover using the enzyme produced on steam pretreated corn stover. Similar results were obtained in the case of chemically pretreated corn fiber. Significantly higher sugar yields were obtained in the hydrolysis of corn fiber using the enzyme produced on the same substrate i.e. on corn fiber than applying commercial cellulases.

It was shown that mannanase and endoglucanase expressed coordinately on the examined carbon sources, since a good correlation was observed between the two enzyme activities. This observation might be valuable information to understand the regulation mechanism of hemicellulase genes in *Trichoderma reesei*.

Results of mixed cultivation of *Aspergillus niger* and *Trichoderma reesei* indicate that *Trichoderma* can be used to produce glucose for *Aspergillus* from lignocellulosic wastes, which might be advantageous if cheap raw material is needed for large-scale production of β-glucosidase by *Aspergillus* species.
PREFACE

This work was carried out at Budapest University of Technology and Economics, Department of Agricultural Chemical Technology between 2001 and 2005. I thank to Prof. Béla Sevella, head of department, for giving me the opportunity to work at the department.

My most sincere thanks are due to my supervisor associate professor Dr. Kati Réczey for providing the excellent working facilities at BUTE. I am very grateful for her support, encouragement, constructive criticism and help during this work. I also express my thanks to Dr. Zsolt Szengyel who helped me a lot. A great deal of appreciation goes to Professor Liisa Viikari and Dr. Matti Siika-aho for giving me the opportunity to do part of my research at VTT Biotechnology (Technical Research Centre of Finland).

I want to express my thanks for the pleasant collaboration to Dr. Béla Simándi, Dr. Edit Székely, Dr. Erika Vági and Dr. Ildikó Kmetz at BUTE Department of Chemical Engineering; to Prof. Dr. Guido Zacchi, Dr. Beatriz Palmarola-Adrados, Dr. Mats Galbe, Dr. Malek Alkasrawi and Karin Öhgren at University of Lund; to Michael J. Bailey, Juha Tähtiharju, Satu Homan and Jan Sclenar at VTT Biotechnology.

I also wish to thank all the personnel at BUTE Department of Agricultural Chemical Technology for the positive and creative working atmosphere: Dr. Enikő Varga, Nóra Szijártó, Zsófia Kádár, Dóra Dienes, Melinda Gáspár, Zsuzsanna Babics, Bálint Kupcsulik, Áron Németh, Laura Leitgib, Krisztina Kozma, Gergely Kálmán, Ágnes Sárdi, Róbert Fülöp, Judit Ádám, Eszter Balogh, Norbert Soós, Balázs Kiss, Krisztina Loksa, Veronika Lajtos and Bernadett Kondor.

Financial supports from the EU "TIME" project (ENK6-CT-2002-00604); Hungarian Ministry of Education (NKFP-OM-00231/2001); The National Research Fund of Hungary (OTKA T025234); Varga József Foundation; Rubik Foundation, Hungarian Academy of Engineering; Pro Renovanda Cultura Hungariae, Student Scientific Foundation and Bizáki Puki Péter Foundation are gratefully acknowledged.
LIST OF PUBLICATIONS

This work is based on the following publications (Appendices I-V.), which are referred to in the text by their Roman numerals. Additional unpublished data are also presented.


Other related papers by the same author:


<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AXE</td>
<td>Acetyl xylan esterase</td>
</tr>
<tr>
<td>β-Glu</td>
<td>β-glucosidase</td>
</tr>
<tr>
<td>CBHI</td>
<td>Cellobiohydrolase I</td>
</tr>
<tr>
<td>CE</td>
<td>Celluclast 1.5 L enzyme (Novozymes, Bagsværd, Denmark)</td>
</tr>
<tr>
<td>CO</td>
<td>Enzyme complex produced on steam pretreated corn stover (SPCS)</td>
</tr>
<tr>
<td>CPCF</td>
<td>Chemically pretreated corn fiber</td>
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<tr>
<td>EC</td>
<td>Econase CE enzyme (ROAL Oy, Finland)</td>
</tr>
<tr>
<td>EG</td>
<td>Endoglucanase</td>
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<tr>
<td>FPA</td>
<td>Filter paper activity</td>
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1. Introduction

Due to the impact of greenhouse effect on the Earth’s climate, biomass originated alternative fuels have gained remarkable attention during the past decades. Ethanol seems to be a potential substitute of gasoline in internal combustion engines due to its advantageous physical properties. Lignocellulosics are ideal raw materials that could support large-scale fuel ethanol production, since vast quantities of lignocelluloses are produced every year, constituting the most abundant renewable natural resource available on this planet (1). As it is presented in Figure 1, ethanol produced from biomass i.e. lignocelluloses do not increase the carbon-dioxide concentration in the air, since the released CO$_2$ is consumed by photosynthesizing plants. Furthermore, ethanol production from biomass has several other advantages. For instance, new fuel ethanol plants can reduce unemployment and dependency on fuel import. In addition, agricultural overproduction can also be reduced by growing energy plants instead of food crops.

![Figure 1 Closed carbon-dioxide circle](image-url)

One of the process alternatives suggested for the production of fuel ethanol from lignocellulosic materials is based on the enzymatic saccharification of cellulose (see Figure 2). For efficient conversion of the cellulose fraction, large enzyme dosage per unit of raw material has to be applied, which due to the high market price of cellulases significantly increases the overall production cost of ethanol (1, 2).
Research on cellulolytic enzymes began in the 1950s and in the past decades cellulase based technologies have aroused worldwide research activity (3). To improve cellulase production many efforts have been made in genetic modification of strains, optimization of culture conditions, characterization of enzymes in genetic and biochemical levels to produce effective enzyme system for enzymatic hydrolysis of cellulose (4). In spite of the extensive research work, the real breakthrough has not yet succeeded (3, 4). Therefore, the production of enzymes for improved hydrolysis still remains an issue in the next decades. However, Novozymes claims according to their research program that their cellulase complex will be available around 2012, which will be cheap and suitable enough for economical cellulose hydrolysis (5).

In spite of that, it is important to investigate further cellulase production, since its production on site ("in situ") can reduce ethanol production costs compared to processes, where commercial cellulases are applied (1-3). The main aim of present study was to investigate conditions that enable the efficient hydrolysis of lignocellulosics. On the one hand, parameters of enzyme production were studied to produce enzymes with high hydrolysis capacity. On the other hand, pretreated lignocellulosics were examined to select a raw material that is suitable both for enzyme production and hydrolysis.
2. Literature background

2.1. Lignocelluloses

Main components of lignocelluloses, which are the most common biopolymers in nature, are cellulose, hemicelluloses and lignin. Lignocelluloses also contain a few percent of mainly terpenoid compounds that can be extracted by various solvents. Therefore, they are usually referred as extractives (6).

Cellulose (see Figure 3) is a glucohomopolymer and its linear, unbranched chains contain up to 10,000 1,4-β-linked β-D-glucopyranose units, which are in chair conformation (6). The repeating units are cellobiose, since every other unit is rotated 180° around the main axis of cellulose chain. The parallel chains are held together by hydrogen bonds to form fibrils. Despite of cellulose is a hydrophilic molecule, it is insoluble in water (6, 7). Cellulose has crystalline and less ordered i.e. amorphous parts, which exhibit less resistance (7).

Hemicelluloses are more heterogeneous, highly branched polysaccharides and composed of several different monosaccharides and some other biomolecules (6). There are four types of hemicelluloses: xylans, mannans, arabinans and galactans regarding to the main sugar components in their backbones (8, 9).

Xylans have 1,4-linked β-D-xylopyranose backbones. In hard wood 10% of xylose units contain 4-O-methyl-α-D-glucuronic acid and 60-70% of the xylose residues are acetylated (10). In contrast, in soft wood xylans backbones are further substituted by α-L-arabinofuranoside and no acetylated xylan are observed (10). In addition, both in soft and hard wood some arabinofuranoside units are esterified by ferulic acid and p-coumaric acid (11).
Backbones of mannans consist of β-1,4-linked glucose and mannose units, which are distributed randomly and substituted with α-D-galactose units by 1,6 linkage. O-acetyl-galactoglucomannans are the main hemicelluloses in soft wood, while hard wood contains only a few percent of mannans with no galactose and acetyl groups (10). The main chain sugars of arabinans and galactans are L-arabinose and D-galactose, respectively (9). These hemicelluloses are also referred as pectic polysaccharides and they are usually not significant components of wood lignocelluloses (9, 12).

Lignin is presented in Figure 4. It is a highly branched macromolecule and composed of several types of aromatic acids such as guajacylpropan and p-hydroxyphenylpropan. Lignin has great role in preventing lignocellulosic materials against biological attacks (13).

![Figure 4 Structure of lignin](image)

**2.2. Pretreatments of lignocelluloses**

Usually various pretreatments are applied to break down the intact structure of lignocellulosics. Lignin is closely associated with hemicellulose, thus preventing enzymes accessing cellulose. Therefore, the aim of pretreatments is to degrade the lignocellulosic material to enhance enzymatic degradation (14). In many pretreatment methods most of the hemicelluloses are removed and the remaining fraction, i.e., mixture of degraded lignin and cellulose is used as substrate either for hydrolysis or for cellulolytic enzyme production (14). It is important to perform pretreatments in such way to avoid the degradation of hydrolysis products and the formation of inhibitors. These inhibitors, for instance furfurol, hydroxymethyl-furfural, acetic acid etc., can hinder the consequent enzyme or ethanol production by causing toxic environment to fungi or yeasts (14, 15).
Pretreatment methods can be classified into physical, chemical, biological and combined pretreatments regarding to the applied method for the disruption of lignocellulosic matrix. Physical pretreatments, such as grinding, radiation and pyrolysis are found to be ineffective (14, 15). Biological pretreatment by lignin-solubilizing filamentous fungi is relatively slow and the substrate is partly consumed by the microbes. Chemical pretreatments applying acids, alkalis, peroxides and organosolvents are reported to be effective methods. However, the consumed chemicals can be expensive and might cause environmental problems (15). Combined pretreatments are found to be the most suitable methods. Currently, steam explosion and wet oxidation, which is a reaction involving oxygen and water at high temperature and pressure, are the most studied methods (15, 16). Detailed results on pretreatments can be found elsewhere (14-19).

2.3. Lignocellulolytic enzymes

2.3.1. Cellulase enzyme complex

For the complete hydrolysis of cellulose, cellulolytic microorganisms produce a whole set of enzymes i.e. cellulases, which act synergistically in the degradation process (20). Cellulase enzyme complex can be classified into of three types of enzymes. Regarding to an older theory, exoglucanases or cellobiohydrolases (CBH) cleave cellobiose units from the end of cellulose chain. Endoglucanases (EG) randomly attack cellulose in the middle of the chain, which results in the release of cello-oligosaccharides and new chain ends that enables exoglucanases to express their hydrolytic action (21). The mechanism that is most accepted today is presented in Figure 5 according to Enari et al. (22).

![Figure 5 Mechanism of cellulose hydrolysis (22)](image-url)
The difference is that cellulose is first attacked by cellbiohydrolases and the released cellodextrines are further degraded by endoglucanases. The resultant cellobiose is converted to glucose by β-glucosidases (21, 22). Strictly speaking, β-glucosidase is not a cellulase since this enzyme does not act on water insoluble cellulose. However, it shares a common feature with endo- and exoglucanases, namely the specificity towards β-1,4-glucosidic bonds (22, 23).

In culture filtrates of *Trichoderma reesei*, of which cellulase complex is the best characterized, there are at least four different endoglucanases (EGI, EGII, EGIII, EGV), two different cellbiohydrolases (CBHI, and CBHII) (24) and at least two, an intra- and an extracellular β-glucosidase (24, 25). CBHI and CBHII are the major cellulases secreted by *T. reesei*, constituting approximately 60-70% and 20% of the total cellulolytic protein, respectively (24, 26, 27). CBHI is typically active on crystalline cellulose and releases cellobiose units from the reducing end of cellulose, while CBHII has also activity against amorphous cellulose and acts from the non-reducing end of the cellulose chain (28, 29).

EGI is an enzyme that exhibits high activity on soluble cellulose derivatives and has relatively lower activity towards crystalline cellulose (30). Furthermore, EGI also catalyzes transglycosylation reactions (31, 32) and it can hydrolyze xylan (33). EGII is a specific endoglucanase that attacks insoluble cellulose and it is the only enzyme that can hydrolyze cellotriose (34). The other endoglucanases (III-V) constitute around 1% of the total cellulolytic protein and are usually referred as low molecular weight EGs (35).

2.3.2. Hemicellulases

As hemicelluloses are more heterogeneous polymers than cellulose, therefore several enzymes play role in their degradation. The main enzymes needed for hemicellulose hydrolysis are endo-enzymes *i.e.* endo- xylanase and mannanase. Xylanase enzymes degrade 1,4-β-D-xylosidic linkages in xylans, while mannanases hydrolyze backbones of mannan, resulting in the solubilization of short oligosaccharides (36). Accessory enzymes like β-xylosidases and side group cleaving enzymes like α-arabinosidase and α-galactosidase, which release arabinose and galactose molecules, respectively, further degrade these oligosaccharide fragments (36, 37). The acetyl xylan esterase (AXE) cleaves acetyl groups from sugar units of xylan (37,38).
2.3.3. Application of cellulolytic enzymes

Cellulases and hemicellulases can be used in the extraction and clarification process of vegetable juices (39), for production of fruit nectars (40) and extraction of olive oil (41). Moreover, the enzymes can improve the quality of bakery products (42). Cellulolytic enzymes are used in beer brewing (43) and wine production technologies (44). In addition, the enzymes serve as animal feed (45) and washing powder (46) components. Furthermore, cellulases and hemicellulases are used in textile (47), pulp and paper industry (48). Last but not least cellulolytic enzymes can be used in the process of bioethanol production for the degradation of lignocellulosic residues, which application is hopefully going to be part of our every day life in the near future (3).

2.4. Measurement of cellulase and hemicellulase activities

To determine the activity of various components of cellulase complex, different substrates are used. However, in most cases not only one but several enzyme components play role in the degradation of the applied model substrate. In spite of that, enzyme activities depend on model substrates can be well evaluated and used to characterize enzyme complexes.

Overall cellulase activity is measured against Whatman No I filter paper substrate and the enzyme activity is expressed in FPU/mL (filter paper unit). The enzyme complex hydrolyzes cellulose to oligo-, di- and monosaccharides that are determined colorimetrically by dinitro-salycilic acid (DNS) (49). Mannanase is measured by locust bean gum substrate that mostly comprises of mannan (50). The released saccharides are measured by DNS method. CMC (carboxy-methyl-cellulose) and HEC (hydroxy-ethyl-cellulose) are suitable substrates for endoglucanase measurements (51). Endoglucanases cleave free chain ends that can be determined by the DNS method. Both endo- and exoglucanases (see Materials and Methods) can be measured against 4-methylumbelliferyl-β-D-lactoside (52). Enzymes cleave bonds between lactose and 4-methylumbelliferyl and the released aromatic compound i.e. 4-methylumbelliferyl can be measured by photometer. α-Galactosidase, β-xilosidase, α-arabinosidase and β-glucosidase assays have the same principle as endo- and exoglucanase measurement (53-56). These enzymes release p-nitro-phenyl molecules from model substrates, which can be measured colorimetrically (see Materials and Methods). Acetyl xylan esterase is measured against an acetylated xylo-oligo-saccharide and the released acetic acid is assayed either by HPLC or by test kits (57).
2.5. Production of fungal cellulases and hemicellulases

Cellulolytic and hemicellulolytic microbes play a key role in the carbon cycle of Earth by decomposing lignocellulosic residues. Both bacteria and fungi produce cellulase enzymes (58). However, for industrial purposes filamentous fungal species are the most suitable cellulase producers, since most of their enzymes are extracellular (59). Furthermore, fungi produce a whole set of cellulolytic enzymes, while bacteria usually produce only few components of cellulases and hemicellulases (58, 59).

2.5.1. Why Trichoderma reesei RUT C30?

The most extensively studied filamentous fungus for cellulase fermentation is Trichoderma reesei, which was discovered during the II. World War, due to the degradation of tents and other textiles. In 1951, after the isolation of the wild type strain (T. reesei QM 6a), the research continued in strain-development (see Figure 6) that led to the isolation of the hypercellulolytic Trichoderma reesei RUT C30 mutant strain at Rutgers University in 1977 (59, 60).

![Figure 6 Strain-development of Trichoderma reesei (60)]

In the research T. reesei RUT C30 is the most applied microbe for cellulase fermentation (59). It is a cre1 mutant strain, which means that monosaccharides do not cause carbon catabolite repression.
Therefore, cellulases can also be produced in the presence of glucose (61). *T. reesei* RUT C30 produces cellulases with approximately three times higher activity than the wild type strain *T. reesei* QM 6a (62). It should be noted that the strain-development did not stopped at *T. reesei* RUT C30. However, significantly less is known about the new mutant strains compared to *T. r. RUT C30* (63).

### 2.5.2. Induction of enzyme production

In the early years it was of particular interest to explain how the synthesis of cellulases can be turned on in the presence of polymeric cellulose. Mandels et al. (64) suggested that *T. reesei* produces constitutive level of cellulase that are bound onto the surface of conidia (65). These bound enzymes enable the primer attack on cellulose. Later the cleaved cellobio-oligosaccharides can act as cellulase inducers (65). This theory was later proved by immunological methods (66). Moreover, it has been reported that the true inducer of cellulases is sophorose that is a glucose disaccharide with $\beta$-1,2 linkage (64, 67). Strongly supports this view that the constitutive cell-bound $\beta$-glucosidase can form sophorose from cellobiose by transglycolisation activity (68). It should be added that lactose can also induce cellulase production in smaller extent. However, the induction mechanism is still not clear (69, 70).

Transcription of cellulase genes starts approximately 20 minutes after induction. Regulation of genes are under transcription control, which is proved by mRNA probes (66, 68, 71). The levels of individual enzymes correlate with the steady state levels of respective transcripts (71). It has been shown that expression of different cellulase genes is coordinated. Thus, expression levels of cellulases are approximately the same in various conditions (68, 72). There is usually also a feed back control, which means that the produced glucose repress cellulase synthesis (73). This inhibition is usually referred as catabolite repression and it cannot be observed in the case of *T. reesei* RUT C30 (61, 73).

$\beta$-Glucosidase I is induced much lower level than the other cellulases. However, it seems that the highest $\beta$-glucosidase and other cellulase activities can be reached in the same culture conditions (36). It has been demonstrated that there are at least two types of $\beta$-glucosidases in *Trichoderma reesei* (74). Recently, Takashima et al. (75) have provided evidence for $\beta$-glucosidase II of *Trichoderma reesei*, which is a constitutive and mostly intracellular enzyme that also has transglycolisation activity (19, 75).
Both β-glucoosidases are expressed on lactose, cellobiose, cellulose and on inert carbon sources i.e. on sorbitol and glycerin in the presence of sophorose (19). Furthermore, β-glucoosidases are constitutively produced on glucose by *Trichoderma reesei* RUT C30 (19, 75).

*T. reesei* produces two types of specific xylanases and an unspecific one i.e. endoglucanase I (76). All three enzymes are induced by the presence of xylan. Sophorose induces xylanase and endoglucanase, while induction by xylobiose lead to the formation of only the two specific xylanases (76-78). On glucose a basal production of xylanase can be observed, which suggest that xylanase production is partially under carbon catabolit repression in *Trichoderma reesei* RUT C30 (36).

There seems to be evidence for low constitutive level of xylanase expression, which is the responsible for the formation of inducer from xylan i.e. xylobiose. In contrast to cellulase system, it has been reported that the inactivation of surface bound xylanase activity did not affect the ability of *T. reesei* to form xylanase in the presence of xylan (79). Therefore, it can be argued that the secreted constitutive level of xylanase has great role in formation of xylanase inducers (79).

Very few data are available on the induction of other hemicellulases. It has been shown (36) by mRNA analysis that on various carbon sources various enzymes were produced and no clear correlations were observed. However, it has been demonstrated that arabinose is the most suitable monosaccharide inducer of α-arabinosidase (80), while α-galactosidase was induced by galactose and arabinose (81). β-Xylosidase I is produced if xylanase formation can be observed (36). Biely et al. have found that acetyl xylan esterase is co-expressed with endoglucanase (82). In addition, it is well known that in *T. reesei* RUT C30 not only xylanase but also acetyl xylan esterase and α-galactosidase are under carbon catabolite repression caused by cre1 gene (36). Induction of *Trichoderma reesei* mannanase has been least studied. However, Margolles-Clark et al. (36) have showed that its was rather poorly produced except on cellulose.
2.5.3. Culture conditions

It is a well-established fact that parameters of cultivation affect significantly the production of cellulases and hemicellulases (83). The most important factors are the quality and quantity of carbon source. The applied media should be also supplemented with suitable nitrogen source, salts and trace elements. Applied pH strategy and temperature also have considerable effects on enzyme production (83).

2.5.3.1. Carbon sources for cellulase production

Carbon sources can be classified into soluble and insoluble materials. The most studied soluble carbon sources are glucose and lactose. On glucose low cellulase activities can be obtained, since only constitutive enzyme synthesis can be observed. Glucose as carbon source is usually used in scientific experiments, for instance to obtain information on regulation of cellulase genes or testing inhibitory effect of compounds etc. (84). Lactose can be applied as carbon source for larger-scale enzyme production. Chaudhuri et al. (85) have produced 1.72 FPU/mL and 2.84 FPU/mL filter paper activity on 20 g/L and 40 g/L lactose containing media, respectively. These results illustrate also the concentration effect of carbon source. The higher is the applied concentration, the higher enzyme activity can be obtained. However, enzyme yields on the basis of 1 g applied carbon source usually decrease with the increasing concentration (83, 85, 86).

Carbon source concentration cannot be increased over a certain level, especially in the case of insoluble carbon sources. Stirring, rheological and mass transfer problems can be occurred if high concentration insoluble carbon source is used (86, 87). Application of insoluble carbon sources for enzyme production, for instance Solka Floc (88, see Tables 1-3), waste paper (89, 90), wheat bran (91), wheat straw (92), corn stover (83), wood (93) and corn fiber (94) have been extensively studied. The results have shown that pure cellulose (Solka Floc, Avicel, Sigmacel etc.) is the best carbon source among insoluble materials. However, pure cellulose is quite expensive. Agricultural and forestry byproducts would be more economical. However, to get good enzyme yields, lignocellulosics should be pretreated prior to enzyme fermentation. Therefore, for larger-scale enzyme production usually pretreated materials are used. Tables 1-3 summarize the most relevant results obtained by *Trichoderma reesei* RUT C30 in shake flask experiments.
<table>
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<td>FPU/g carb. s</td>
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<td>2.80</td>
<td>-</td>
<td>30.0</td>
<td>62.2</td>
<td>95</td>
</tr>
<tr>
<td>15 g/L lactose</td>
<td>1.50</td>
<td>-</td>
<td>15.0</td>
<td>100.0</td>
<td>95</td>
</tr>
<tr>
<td>10 g/L lactose</td>
<td>1.50</td>
<td>-</td>
<td>6.9</td>
<td>150.0</td>
<td>96</td>
</tr>
<tr>
<td>10 g/L lactose + 5 g/L Alon*</td>
<td>3.00</td>
<td>-</td>
<td>13.9</td>
<td>300.0</td>
<td>96</td>
</tr>
</tbody>
</table>

Table 1 Soluble carbon sources for cellulase fermentation by *Trichoderma reesei* RUT C30 in shake flasks (P=productivity, Y= yield, on the basis of 1 g carbon source) * colloid additive

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>FPA</th>
<th>β-Glu. a.</th>
<th>P</th>
<th>Y</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IU/mL</td>
<td>FPU/L*h</td>
<td>FPU/g carb. s</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 g/L cellulose (Sigmacell)</td>
<td>1.20</td>
<td>0.60</td>
<td>10.0</td>
<td>120.0</td>
<td>63</td>
</tr>
<tr>
<td>50 g/L Solka Floc</td>
<td>14.40</td>
<td>-</td>
<td>74.0</td>
<td>240.0</td>
<td>83</td>
</tr>
<tr>
<td>10 g/L cellulose (Whatman CF-11)</td>
<td>2.50</td>
<td>-</td>
<td>11.6</td>
<td>250.0</td>
<td>96</td>
</tr>
<tr>
<td>10 g/L cellulose + 5 g/L Alon*</td>
<td>4.50</td>
<td>-</td>
<td>20.8</td>
<td>450.0</td>
<td>96</td>
</tr>
<tr>
<td>20 g/L cellulose (Whatman CF-11)</td>
<td>5.90</td>
<td>-</td>
<td>27.3</td>
<td>295.0</td>
<td>96</td>
</tr>
<tr>
<td>20 g/L cellulose + 5 g/L Alon*</td>
<td>7.60</td>
<td>-</td>
<td>35.2</td>
<td>380.0</td>
<td>96</td>
</tr>
<tr>
<td>30 g/L cellulose (Whatman CF-11)</td>
<td>7.10</td>
<td>-</td>
<td>32.8</td>
<td>236.7</td>
<td>96</td>
</tr>
<tr>
<td>30 g/L cellulose + 5 g/L Alon*</td>
<td>9.50</td>
<td>-</td>
<td>44.0</td>
<td>316.6</td>
<td>96</td>
</tr>
<tr>
<td>10 g/L cellulose + 10 g/L starch</td>
<td>3.50</td>
<td>0.70</td>
<td>14.6</td>
<td>175.0</td>
<td>97</td>
</tr>
<tr>
<td>60 g/L cellulose + 36 g/L corn steep l.</td>
<td>3.20</td>
<td>-</td>
<td>13.3</td>
<td>53.3</td>
<td>98</td>
</tr>
<tr>
<td>20 g/L Solka Floc</td>
<td>5.60</td>
<td>0.30</td>
<td>23.0</td>
<td>280.0</td>
<td>100</td>
</tr>
<tr>
<td>20 g/L Solka Floc</td>
<td>4.20</td>
<td>-</td>
<td>29.0</td>
<td>210.0</td>
<td>101</td>
</tr>
<tr>
<td>50 g/L Solka Floc</td>
<td>8.00</td>
<td>-</td>
<td>55.0</td>
<td>160.0</td>
<td>101</td>
</tr>
<tr>
<td>100 g/L Solka Floc</td>
<td>8.00</td>
<td>-</td>
<td>26.0</td>
<td>80.0</td>
<td>101</td>
</tr>
<tr>
<td>40 g/L cellulose (Whatman CF-11)</td>
<td>4.10</td>
<td>0.50</td>
<td>17.0</td>
<td>102.5</td>
<td>102</td>
</tr>
</tbody>
</table>

Table 2 Cellulose carbon sources for cellulase fermentation by *Trichoderma reesei* RUT C30 in shake flasks (P=productivity, Y= yield, on the basis of 1 g carbon source) * colloid additive
<table>
<thead>
<tr>
<th>Carbon source</th>
<th>FPA FPU/mL</th>
<th>β-Glu. a. IU/mL</th>
<th>P FPU/L*h</th>
<th>Y FPU/g carb. s</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 g/L grain husk</td>
<td>0.37</td>
<td>0.20</td>
<td>3.1</td>
<td>18.5</td>
<td>63</td>
</tr>
<tr>
<td>10 g/L hydrolyzed hard wood saw dust</td>
<td>0.93</td>
<td>0.87</td>
<td>7.8</td>
<td>165.0/cel</td>
<td>99</td>
</tr>
<tr>
<td>10 g/L hydrolyzed soft wood saw dust</td>
<td>0.29</td>
<td>0.53</td>
<td>2.4</td>
<td>53.0/cel</td>
<td>99</td>
</tr>
<tr>
<td>10 g/L hydrolyzed and SO₂ impregnated soft wood saw dust</td>
<td>0.68</td>
<td>0.80</td>
<td>5.7</td>
<td>126.0/cel.</td>
<td>99</td>
</tr>
<tr>
<td>40 g/L steamed aspen</td>
<td>1.60</td>
<td>0.20</td>
<td>6.8</td>
<td>40.0</td>
<td>102</td>
</tr>
<tr>
<td>38 g/L steamed aspen + 2 g/L cellulose</td>
<td>3.10</td>
<td>0.30</td>
<td>12.9</td>
<td>77.5</td>
<td>102</td>
</tr>
<tr>
<td>36 g/L steamed aspen + 4 g/L cellulose</td>
<td>3.60</td>
<td>0.40</td>
<td>14.9</td>
<td>90.0</td>
<td>102</td>
</tr>
<tr>
<td>34 g/L steamed aspen + 6 g/L cellulose</td>
<td>3.90</td>
<td>0.40</td>
<td>16.3</td>
<td>97.5</td>
<td>102</td>
</tr>
<tr>
<td>10 g/L steam pretreated willow</td>
<td>1.04</td>
<td>0.31</td>
<td>5.4</td>
<td>104.0</td>
<td>103</td>
</tr>
<tr>
<td>5 g/L steam pretreated willow + 5 g/L hemicellulose fraction</td>
<td>1.33</td>
<td>0.47</td>
<td>6.9</td>
<td>133.0</td>
<td>103</td>
</tr>
<tr>
<td>20 g/L steam pretreated willow</td>
<td>1.58</td>
<td>0.44</td>
<td>8.2</td>
<td>79.0</td>
<td>103</td>
</tr>
<tr>
<td>10 g/L steam pretreated willow + 10 g/L hemicellulose fraction</td>
<td>1.79</td>
<td>0.43</td>
<td>9.3</td>
<td>86.0</td>
<td>103</td>
</tr>
<tr>
<td>Steam pretreated willow (10 g/L cellulose)</td>
<td>1.30</td>
<td>0.20</td>
<td>7.4</td>
<td>130.0/cel.</td>
<td>104</td>
</tr>
<tr>
<td>Steam pretreated willow (10 g/L cellulose)</td>
<td>0.66</td>
<td>-</td>
<td>3.9</td>
<td>66.0/cel.</td>
<td>105</td>
</tr>
<tr>
<td>20 g/L steam pretreated oak wood</td>
<td>4.25</td>
<td>-</td>
<td>17.7</td>
<td>212.5</td>
<td>106</td>
</tr>
<tr>
<td>20 g/L acid percolated oak wood</td>
<td>3.25</td>
<td>-</td>
<td>13.5</td>
<td>162.5</td>
<td>106</td>
</tr>
</tbody>
</table>

**Table 3** Pretreated carbon sources for cellulase fermentation by *Trichoderma reesei* RUT C30 in shake flasks (P=productivity, Y= yield, on the basis of 1 g carbon source)

When comparing the results in Tables 1-3 it should be taken into account that cellulase researchers all over the world use a large variety of methods for cellulase production and enzyme activity measurements. Therefore, in most cases enzyme activity values alone do not definitely cover results of experiments. Thus, comparing results of cellulase fermentations found in literature needs due foresight.
It should be also added that nowadays solid-state fermentation is the most studied method (2.5.4.) of cellulase production. Recent results published on submerged cultures are rather focused on genetic and molecular biological aspects of cellulolytic enzyme production (70).

2.5.3.2. Carbon sources for hemicellulase production

Cellulase production is usually coupled with hemicellulase, especially with xylanase production. *T. reesei* RUT C30 can produce high levels of xylanase on xylan containing media (107). Hence, the aforementioned pretreated materials are good carbon sources for xylanase production (107, 108). Detailed information on xylanase production can be found in articles presented by Gamerith et al. (107), Bailey et al. (108) and Haapala et al. (109).

In contrast to xylanase production, very little is known about mannanase production by *Trichoderma*. Interestingly, it seems that mannanase can be produced better on cellulose than on locust bean gum, which represents the main substrate of the enzyme, since it mostly consists of galactoglucomannan (36).

*Trichoderma reesei* produces low levels of accessory hemicellulases compared to other species. Therefore, only few data are available on the production of α-arabinosidase and α-galactosidase. Roche et al. (110) produced α-arabinosidase on sugar beet pulp grown in solid-state cultures, while Zeilinger et al. (81) carried out submerge fermentation using locust bean gum for the production and characterization of α-galactosidase. Biely et al. (82) have studied the carbon source requirements for production of acetyl xylan esterase. The highest activity was reached when *T. reesei* RUT C30 was cultivated in a medium containing deacetylated larchwood that was a mixture of 0.9% xylan and 0.1% cellulose. Almost the same results were obtained using deacetylated birchwood and pure cellulose (1.0%), while very low acetyl xylan esterase activities were reached on glucose, xylose and cellobiose (82).

2.5.3.3. Nitrogen sources

Besides carbon sources, nitrogen sources have also considerable effect on the production of cellulolytic and hemicellulolytic enzymes. It has been demonstrated that higher enzyme activities could be obtained using elevated nitrogen source concentrations. Tangnu et al. (83) have reported that higher urea concentration led to higher filter paper activity and shorter fermentational time.
Lu-Kwang et al. (111) have continued Tangnu’s work and demonstrated that the highest enzyme activities could be achieved by applying carbon and nitrogen sources in the concentration ratio of 1:1 (g/L). However, carbon source was limiting even in the system of 1:0.25 (C:N) (111). Doppelbauer et al. (112) have studied the effect of peptone concentration. They obtained 1.0 FPU/mL and 1.7 FPU/mL filter paper activities using 0.5 g/L and 2.0 g/L peptone, respectively.

Finding a cheap substitute of peptone for larger-scale enzyme production is also of particular interest. Unfortunately, on most natural nitrogen sources, for instance on rape or soy extracts, lower enzyme activities could be reached than on peptone (113). However, peanut extract proved to be suitable for cellulase production. On Avicel carbon source doubled concentration of peanut extract increased FPA two fold (113). Corn steep liquor is also a suitable and widespread nitrogen source (98).

2.5.3.4. Effect of cultivation pH

It has been indicated that pH and pH controlling strategies have great effect on the amount of cellulase produced (62, 83, 101, 112, 114-122). It has been shown that depending on the nature of the carbon source used to induce cellulase production of *Trichoderma* strains, different initial pH of the cultivation may be optimal for maximum cellulase yield. Ryu and Mandels (62) have reported that pH range 3.0-4.0 was optimal for pure cellulose carbon source, however higher initial pH (5.0-6.0) was recommended for lignocelluloses. For sugar cane bagasse containing media (free of water soluble sugars), pH between 5.0 and 6.0 was observed to be optimal using *T. reesei* QM 9123 (114). In other studies maximum yield of cellulases was obtained in the range of pH 3.0 to 5.0 (115-117).

In shake flask cultures the pH control is usually limited to either addition of buffering salts such as phosphates, or periodic manual pH adjustment, which is obviously tedious and less effective. The use of ammonium sulfate as the major nitrogen source in the Mandels’ medium (119) requires a more compelling buffering system.

Without buffering, the pH drops quickly during the first stage of the fermentation due to the depletion of ammonia and liberation of protons (86, 120, 121). Application of KH₂PO₄ – K₂HPO₄ buffer system (4 g/L) for controlling the pH in shake flasks proved to be inefficient in compensation of the acidification. However, higher cellulase activity was obtained in the buffered system than in the basal Mandels’ medium (91).
Kadam and Keutzer (117) have investigated several organic acid buffer systems, \textit{i.e.} acetate, succinate, phthalate and citrate, unfortunately the efficiency of the various buffer systems was not reported.

Tangnu et al. (83) have studied the influence of pH on cellulase production of \textit{T. reesei} RUT C30 in pH-controlled fermentor on cellulose carbon source. In the pH range of 4.0-6.0, no significant effect on the production rate and final cellulase yield was observed, however the $\beta$-glucosidase production was affected to a large extent. At pH 4.0 and 5.0 the $\beta$-glucosidase activity was gradually increasing until it reached its maximum value by the 8\textsuperscript{th} day of cultivation. Controlling the pH in the fermentor to 6.0 increased the production rate of $\beta$-glucosidase considerably and approximately 30\% higher enzyme yield was achieved by the 4\textsuperscript{th} day than at lower pH values. However, during the final stage of the fermentation the level of $\beta$-glucosidase activity decreased to the same value as measured at lower pH levels. Hendy et al. (101) have reported that performing the fermentation above pH 5.0 resulted in a significant loss of cellulase activity on Solka Floc carbon source.

Instead of keeping the pH at a constant value during the whole fermentation process pH profiling was recommended by Doppelbauer et al. (112). On lignocellulosic waste carbon sources, for the growth phase of \textit{T. reesei}, the pH of cultivation was suggested to be maintained at 4.0, while in the later stage of production and secretion of cellulases, an elevated pH level of 5.0 was recommended. In another study, pH cycling coupled with temperature profiling increased the amount of cellulases by 13\% compared to the control case during which the pH was maintained at a constant value (118).

### 2.5.3.5. Effect of temperature

Temperature has also significant effect on enzyme production. Tangnu et al. (83) have performed experiments to find the optimal temperature for enzyme production on cellulose carbon source. They have reported that keeping the cultivations in the beginning at 31\degree C and after a few days at 28\degree C resulted in the highest enzyme activities. On lactose carbon source Merivuori et al. (122) have partly confirmed Tangnu’s findings by proving that the optimal temperature for cellulase production is 28\degree C.
2.5.4. Fermentation methods

The most studied and simplest fermentation method is the batch technique. However, applying fed-batch cultivation higher activity, productivity and yield could be obtained (see Table 4). As presented in Table 4 cellulase production in continuous cultivations usually lead to lower enzyme activities, thus it is very rarely applied (59, 101).

<table>
<thead>
<tr>
<th>Fermentation method</th>
<th>Carbon source</th>
<th>FPA (FPU/mL)</th>
<th>P (FPU/L*h)</th>
<th>Y (FPU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 168 h</td>
<td>50 g/L Solka Floc</td>
<td>8.00</td>
<td>55.0</td>
<td>160.0</td>
</tr>
<tr>
<td>Batch 336 h</td>
<td>100 g/L Solka Floc</td>
<td>8.00</td>
<td>26.0</td>
<td>80.0</td>
</tr>
<tr>
<td>Fed-batch 300 h</td>
<td>100 g/L Solka Floc</td>
<td>26.10</td>
<td>109.0</td>
<td>261.0</td>
</tr>
<tr>
<td></td>
<td>Final conc.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Continuous (One stage) D=0.046 h⁻¹</td>
<td>Feed: 20 g/L Solka Floc</td>
<td>2.10</td>
<td>97.0</td>
<td>105.0</td>
</tr>
<tr>
<td>Continuous (Two stages) D=0.017 h⁻¹</td>
<td>Feed: 20 g/L Solka Floc</td>
<td>3.30</td>
<td>56.0</td>
<td>165.0</td>
</tr>
</tbody>
</table>

Table 4 Effect of fermentation method on cellulase production (59, 101)

Solid-state fermentations (SSF) are also applied for cellulase production. However, in SSF processes to control fermentation parameters and produce enzyme in larger-scale can be extremely difficult. In spite of that SSF is extensively studied and it is a widespread method, especially in the Far East. More information on SSF for cellulase production can be found elsewhere (123-125).

2.6. β-Glucosidase production by Aspergillus species and cocultures

Aspergillus strains are known for their ability to produce β-glucosidase with significantly higher yields than Trichoderma species. In most cases, where the augmentation of β-glucosidase activity is needed, Aspergillus β-glucosidase is applied, for instance Novozyme 188 (Novozymes, Bagsværd). Several Aspergillus species produce β-glucosidase, such as A. niger, A. wentii, A. fumigatus, A. phoenicis, A. foetidus (126, 127). However, A. niger is the most studied filamentous fungus for β-glucosidase production (128-130).
Cofermentations of *Aspergillus* and *Trichoderma* species were examined by Duff *et al.* on starch (97) and starch-cellulose mixture (121). The highest β-glucosidase activity obtained was 2.9 IU/mL while the enzyme mixture had a cellulolytic activity of 3.0 FPU/mL. Maheshwari *et al.* (131) investigated the cofermentation of the two species on cellulosic waste. They have found that yields in cellulose hydrolysis were two or three times higher if enzymes derived from cofermentations were used compared to hydrolysis experiment, where enzymes form monocultures were applied. Madamwar *et al.* (132) performed semi-solid state cofermentations of the two fungal species and obtained 20–40 % higher cellulytic enzyme activities with mixed cultures than with monocultures.

In the abovementioned cases the aim of mixed cultures was to produce high β-glucosidase activity and FPA at the same time. However, *Aspergillus* strains usually have shorter adaptation and generation time. Thus, in most submerged fermentations, especially if not pure cellulose is the carbon source, *Aspergillus* strains suppress *Trichoderma*, which lead to lower levels of FPA values. Therefore, it can be advantageous if cofermentations are designed such way that *Trichoderma* produces only low levels of cellulases, just enough for the breakdown of carbon source to supply *Aspergillus* with mono-, di- and oligosaccharides. This method might lead to higher β-glucosidase activity than it can be achieved with the previous method. The produced β-glucosidase can be added to *Trichoderma* culture filtrates, which elevates the hydrolysis potential of the enzyme (see below). It should be worked out by several experiments if the previous or the latter method gives better enzyme yield.

### 2.7. Hydrolysis of lignocellulosics and the hydrolysis potential of cellulases

Either acid or enzymes can hydrolyze lignocelluloses (133). Acid hydrolysis can be classified into one-step concentrated and two-step dilute acid processes. Acid hydrolysis played significant role during World War II by substituting gasoline (134, 135). However, fuel ethanol was not competitive with gasoline when the fuel supply chain was rebuilt after the war. Main drawbacks of acid hydrolysis are sugar degradation that cause low process yields, corrosion and environmental problems and high cost of acid consumption and recovery (136).

In the enzymatic process much milder conditions are used, due to the specificity of applied enzymes. However, cellulosic residues should be pretreated prior to hydrolysis, for instance using conditions similar to acid hydrolysis. Cellulases are very susceptible to end-product inhibition caused by cellobiose and partly glucose (137).
Furthermore, part of endo- and exoglucanases adsorb irreversible to their substrate and to lignin that causes the loss of cellulase activity (138). Castanon et al. (139) have indicated that these cellulases are bound to inactive sites of cellulose and they cannot be recycled at all. On the other hand, cellulases adsorb reversible with the help of their cellulose-binding domain (CBD) onto the surface of the solid cellulose where they catalyze the hydrolysis. After several sequential hydrolytic reactions the enzyme desorbs and returns to the bulk fluid (140). Velkovska et al. (141) have proven that cellulase adsorption can be best characterized by a Langmuir adsorption isotherm that is presented in Figure 7.

![Figure 7 Example of adsorption isotherm of cellulases (141)](image)

Considering the aforementioned phenomena, in order to get high sugar yield high enzyme dosage should be used and all factors should be optimized that affects the hydrolysis (142). Generally, the applied cellulase activity varies between 7 FPU/g and 33 FPU/g substrate depending on the used substrate (133, 142). Usually, it is necessary to augment β-glucosidase activity to avoid inhibitory effect of formed cellobiose. β-Glucosidase augmentation is performed by external β-glucosidase addition, which further increases the cost of hydrolysis (142). Thus, it is highly important to use an enzyme mixture that exhibits good hydrolysis ability. In contrast, the cellulytic enzyme produced by most fungi, including *Trichoderma reesei* RUT C30, contains inadequate amount of β-glucosidase, which leads to poor hydrolysis potential (59). Considering cellulases, the optimal hydrolysis potential of the enzyme complex corresponds to 1.0-1.5 β-glucosidase activity : FPA ratio (143). In contrast, in *Trichoderma* culture filtrates β-glucosidase and filter paper activity ratio is only around 0.7 (59). Hence, to develop a method that enables the production of cellulases with adequate amount of β-glucosidase is of particular interest. On the other hand, end-product inhibition can be avoided by sugar removal.
This can be accomplished by applying ultrafiltration or simultaneous saccharification and alcohol fermentation (SSF) (142). SSF is a comprehensively studied one-step method for alcohol production using starch or lignocelluloses as raw materials. Detailed results on SSF can be found elsewhere (144-146).

Hydrolysis is usually carried out at elevated temperature (45-50°C) above the growth temperature of most microorganism to avoid the loss of carbon substrate. Generally, the optimal pH of applied enzymes is used that is around pH 4.8 in most cases (142). Substrate concentration exceeds 10% very rarely, due to rheological problems (87). Hydrolysis is usually conducted for a few days resulting in 70-80% conversion (142). To improve the yield and fasten hydrolysis speed, it has been suggested to use surfactants by lowering surface tension of solid substrate and enhancing enzyme adsorption. However, surfactant-mediated enhancement in hydrolysis is substrate dependent and its mechanism is still not clear, thus the optimal application of surfactants for hydrolysis still remains an issue to develop (147).

It is well known that there are synergisms among various cellulase components. However, several results showed that the synergism is not as simple as it was indicated in earlier studies (20, 23, 142). On the other hand, it is possible that some interactions exist between cellulase and hemicellulase enzymes. For instance, as it was mentioned above, EGI has also xylanase activity (76). Interestingly, it seems that acetyl xylan esterase has particular role in the degradation of lignocellulosics by cleaving ester-linked non-carbohydrate residues, thereby increasing biodegradability. Grohman et al. (148) have shown that due to deacetylation xylan became 5-7 times more digestible, which in turn made cellulose three times more digestible. Poutanen and Puls (149) have demonstrated that partial enzymatic deacetylation of birchwood xylooligomers enhanced xylan degradation three-fold.
2.8. Background and outline of the thesis

At the Department of Agricultural Chemical Technology, Budapest University of Technology and Economics, extensive research is being done since the beginning of the 1990s on the production of fuel ethanol from lignocellulosic residues. The research work is to be classified into pretreatment of lignocelluloses, cellulase production and alcohol fermentation. This thesis represents a small part of the scientific work carried out on cellulase production.

The general aim of the present work was to find alternative methods to improve the hydrolysis of lignocellulosics. The first part summarizes the experiments carried out to produce cellulase enzyme complex with high β-glucosidase activity. A buffer system was developed that enables *Trichoderma reesei* shake flask experiments at constant pH and at the same time enhances β-glucosidase production to obtain cellulase enzyme complex with adequate amount of β-glucosidase. The second part deals with experiments performed to test steam pretreated corn stover, spruce and willow for enzyme production and hydrolysis, while the third part studies a new approach to β-glucosidase production on waste paper carbon source by mixed cultures of *Trichoderma reesei* and *Aspergillus niger*. 
3. Materials and Methods

3.1. Substrates and Carbon sources

Solka Floc (delignified cellulose), glucose, lactose, steam pretreated and willow (SPW), spruce (SPS), corn stover (SPCS) and chemically pretreated corn fiber (CPCF) were used as carbon sources for cellulase enzyme production and as substrates for hydrolysis. After pretreatments most of the hemicellulose fraction was removed by thorough washing and the remained solid fraction mostly consisted of cellulose was used as substrate or carbon source.

Pretreated wood and corn stover samples were obtained from Sweden while corn fiber was obtained from Hungary. Prior to steam pretreatments materials were impregnated with SO₂. Steam pretreatments were performed by loading the samples into a 1 L steam gun. After the treatment time at a certain temperature (see Table 5), the pretreated materials were released by rapid depressurization to let the material to explode into a cyclone. Chemical pretreatment was performed by keeping corn fiber at 120°C for 120 minutes in 2.5% NaOH and 0.006 H₂O₂ solution. Pretreated materials were collected, filtrated, washed with distilled water and analyzed using a modified Hägglund’s method (150).

<table>
<thead>
<tr>
<th>Substartes / carbon sources</th>
<th>Solka Floc</th>
<th>SPCS</th>
<th>SPS</th>
<th>SPW</th>
<th>CPCF</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pretreatment conditions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time (min)</td>
<td>-</td>
<td>5</td>
<td>5</td>
<td>12</td>
<td>120</td>
<td>-</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>-</td>
<td>190</td>
<td>215</td>
<td>190</td>
<td>120</td>
<td>-</td>
</tr>
<tr>
<td>SO₂ (%)</td>
<td>-</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>-</td>
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<td>NaOH (%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.5</td>
<td>-</td>
</tr>
<tr>
<td>H₂O₂ (%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.006</td>
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<tr>
<td><strong>Composition (%)</strong></td>
<td></td>
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<tr>
<td>Cellulose</td>
<td>78.8</td>
<td>56.9</td>
<td>50.4</td>
<td>52.9</td>
<td>49.4</td>
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<tr>
<td>Xylan</td>
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<td>6.8</td>
<td>0.0</td>
<td>3.8</td>
<td>13.8</td>
<td>16.6</td>
</tr>
<tr>
<td>Dry matter</td>
<td>97.0</td>
<td>20.2</td>
<td>22.0</td>
<td>25.2</td>
<td>100.0*</td>
<td>100.0*</td>
</tr>
</tbody>
</table>

* carbon sources were kept in a drier until they were used for fermentation or hydrolysis

Table 5 Composition of carbon sources (based on dry matter) and pretreatment conditions
Waste wrapping paper was used as carbon source for β-glucosidase production by mixed cultures of *Aspergillus niger* and *Trichoderma reesei* (see Table 5). The paper was cut into about 2X2 cm pieces and put into water. A kitchen blender was used to disintegrate paper and produce paper slurry that was filtrated on a 150 µm mesh nylon filter via vacuum and dried at 105°C. The paper was kept in drier until it was used for fermentation.

3.2. Inoculum and enzyme production in shake flasks

3.2.1. *Trichoderma reesei*

*Trichoderma reesei* RUT C30 (ATCC#: 56765) stock cultures were maintained on agar slants containing: 20 g/L malt extract, 5 g/L glucose, 1 g/L proteose peptone and 20 g/L bacto agar. After few weeks at 28°C conidia were suspended in 5 mL of sterile water and 1 mL of this spore suspension was transferred aseptically to 750 mL Erlenmeyer flask containing 200 mL of the sterile medium prepared referred as modified Mandels’ medium (119) in which the concentrations of nutrients were: 0.40 g/L urea, 1.87 g/L \((\text{NH}_4)_2\text{SO}_4\), 2.67 g/L \(\text{KH}_2\text{PO}_4\), 0.53 g/L \(0.3 \text{ g/L} \) \(\text{CaCl}_2 \times 2\text{H}_2\text{O}\), \(\text{MgSO}_4\), 0.33 g/L yeast extract, 1.00 g/L proteose peptone together with 10 g/L Solka Floc cellulose powder. The medium was also supplemented with the following trace elements: 7 mg/L \(\text{FeSO}_4 \times 7\text{H}_2\text{O}\), 20 mg/L \(\text{CoCl}_2\), 2 mg/L \(\text{MnSO}_4\), 2 mg/L \(\text{ZnSO}_4\). After 4 days at 28°C and 350 rpm the inoculum was ready.

A total volume of 20 mL mycelium suspension, obtained from the inoculum cultures, was used to initiate growth in a 750 mL E-flask containing 200 mL of a modified Mandels’ medium (119, Paper I), which was supplemented with the components of buffers (Paper I, II). The carbohydrate concentrations of the aforementioned carbon sources were equivalent with 11 g/L glucose. After inoculation flasks were incubated in an orbital shaker at 28°C and 350 rpm.

3.2.2. *Aspergillus niger*

*Aspergillus niger* BKMF-1305 stock cultures were maintained on agar slants containing: 20 g/L malt extract, 5 g/L glucose, 1 g/L proteose peptone and 20 g/L bacto agar. After few weeks at 30°C conidia were suspended in 5 mL of sterile water and 1 mL of this spore suspension was transferred aseptically to 750 mL Erlenmeyer flask containing 150 mL of sterile and pH adjusted (pH=5.5) medium prepared according to Mandels (119).
Mass concentrations of nutrients were: urea 0.3 g/L, (NH₄)₂SO₄ 1.4 g/L, KH₂PO₄ 2.0 g/L, CaCl₂ 0.3 g/L, MgSO₄ 0.3 g/L, yeast extract 0.25 g/L, proteose peptone 0.75 g/L together with 10 g/L glucose. The medium was also supplemented with the following trace elements: FeSO₄ x 7H₂O 5 mg/L, CoCl₂ 20 mg/L, MnSO₄ 1.6 mg/L, ZnSO₄ 1.4 mg/L. After 3 days at 30 °C and 350 rpm the inoculum was ready.

3.2.3. Aspergillus niger and Trichoderma reesei

10 mL of Trichoderma and 5 mL of Aspergillus mycelium suspensions, obtained from inoculum cultures, were used to initiate growth in 750 mL Erlenmeyer flasks containing 150 mL of modified Mandels’ medium, as described above, at pH=5.5. The applied carbon source was 20 g/L waste paper that was equivalent with 11.4 g/L glucose and 3.8 g/L xylose and arabinose (analyzed by Hägglund’s method (150)). After the inoculation culture flasks were incubated in an orbitary shaker at 30°C and 350 rpm for 7 days. Samples were withdrawn every day and at the same time pH was adjusted to 6.0 by addition of either sterile NaOH or H₂SO₄ solutions. The samples were centrifuged at 8000 rpm for 5 min and the supernatants were collected for the analysis of β-glucosidase activities.

3.2.4. Comparing the applied media

For production of Aspergillus niger inoculum original salt and nitrogen source concentrations were used (119), while carbon source (glucose) concentration was elevated from 7.5 g/L to 10 g/L because Aspergilli grew faster than Trichoderma and it needed more carbon source to obtain good inocula after 3 days. In contrast, in Trichoderma experiments, where 10 g/L cellulosic carbon source was used (equivalent with 11 g/L glucose), salt concentrations were also elevated by 1.33 fold (=10/7.5) to avoid the limitation of nitrogen source and other salts. In cofermentations the amounts of salts and nitrogen sources were doubled to cover nutrition demands of both microbes. However, later it was proven (unpublished data) that modification of salt and nitrogen source concentrations did not have significant effect on cellulase and β-glucosidase activites in all sorts of fermentation.
3.3. *Trichoderma reesei* and *Aspergillus niger* cofermentation in Biostat CDSU-3 laboratory fermentor

Upscale enzyme production experiments were performed in a 31-L double-walled stainless steel laboratory fermentor (Biostat CDCU-3; B Braun Biotech, Germany). Mixed fermentation of *A. niger* BKMF-1305 and *T. reesei* RUT C30 was performed in modified Mandels’ medium (119) (20 g/L waste wrapping paper as carbon source) as described in the previous section. Prior to sterilization at 121°C for 20 min, the nutrients, carbon source, and trace elements required for 20 L of production medium were dissolved in 19.5 L of tap water. During sterilization, vapor equivalent to about 500 mL of water was bled through the gas exhaust system in order to achieve sterile conditions. After sterilization, the temperature of the fermentor was decreased to 30°C and 1 L of starter culture (670 mL of *Trichoderma* and 330 mL of *Aspergillus* inoculum) was aseptically added to initiate growth and enzyme production. The fermentation was carried out with an agitation rate of 250 rpm for 168 h. Samples were withdrawn regularly and centrifuged at 8000 rpm for 5 min. Supernatants were collected and enzyme activities were measured. The initial pH was 5.5 and it was let to drop down to 3.5. Afterwards it was continuously set to pH of 6.0 by automatic addition of sterile, 10 wt% solutions of either H$_2$SO$_4$ or NaOH. The aeration was 0.5 VVM throughout the fermentation. To avoid the formation of foam, silicon oil–based Sigma-Aldrich Antifoam A (Munsch, Germany) in 30% ionic emulsion was added manually four times a day at about 6-hour intervals.

3.4. Protein and enzyme assays

All samples were analyzed in triplicates and the mean values were calculated. The relative standard deviation of enzyme activity measurements were always below 5%. Protein content of samples was determined using BSA as standard by either the Coomassie (151) or the Lowry assay (152). Overall cellulase activity of samples was measured as filter paper activity (FPA) expressed in filter paper units (FPU) using Mandels’ procedure (49). Part of FPA measurements were repeated by setting β-glucosidase activity to 1.4 IU/mL by adding commercial β-glucosidase (Novozyme 188, Bagsvær, Denmark) to avoid the effect of different β-glucosidase levels on FPA values (153). β-Glucosidase activity was measured using essays based on Berghem’s method (56). 4-nitrofenil-β-D-glucopyranoside was applied as substrate in concentrations of 5 mM (pH 4.8) and 1 mM (pH 5.0) in chapters 3.1. and 3.2., respectively.
Endoglucanase activity was assayed against HEC (hydroxy-ethyl-cellulose) as substrate using a method based on IUPAC’s procedure (51). 1 mL substrate (2% HEC, dissolved in citrate buffer pH 4.8, 0.1 M) and 0.5 mL sample was incubated at 50°C for 10 minutes. The reaction was terminated by adding 3 mL of 3,5-dinitrosalicylic acid (DNS) reagent and boiling for 5 minutes. After cooling, 16 mL distilled water was added and the absorbance was measured at 540 nm. One international unit (IU) was defined as the release of 1 µmol glucose / minute. EG I and CBH I activities were measured against 4-methylumbelliferyl-β-D-lactoside (MUbL) either with or without 1 mM cellobiose (52).

The obtained activity without cellobiose was referred as EG I & CBH I activity, while the activity in the presence of cellobiose was considered as EG I activity, since cellobiose is a competitive inhibitor for CBH I. CBH I activity was calculated by subtracting EG I activity from EG I & CBH I activity.

Xylanase activity was assayed against xylan (Roche Co.) by Bailey’s method (154). β-Xylosidase, α-galactosidase and α-arabinosidase activities were determined using 4-nitrophenyl-β-D-xylanopyranoside, 4-nitrophenyl-α-D-galactopyranoside and 4-nitrophenyl-α-L-arabinofuranoside substrates, respectively (53-55). Acetyl xylan esterase (AXE) activity was measured against an acetylated xylo-oligomer (kindly supplied by Jürgen Puls) and the liberated acetic acid was assayed using acetic-acid test kit (Boehringer Co.) (57). Locust bean gum was used as substrate for mannanase activity determination (50). Enzyme activities were expressed in both IU and nkat (1 nkat = 0.06 IU).

3.5. Hydrolysis

In the case of Solka Floc, steam pretreated corn stover, spruce and willow the hydrolysis was performed in 50 mL test tubes with a working volume of 30 mL at 45 °C. The substrate containing 0.6 g cellulose was suspended in 0.1 M acetate buffer (pH 5.0) and the slurry was supplemented with either the produced or commercial cellulase enzymes (15 FPU/g cellulose). The applied commercial enzymes were Celluclast 1.5 L (Novozymes, Bagsværd Denmark) and Econase CE (ROAL, Finland). Samples were withdrawn after 24 h of hydrolysis, centrifuged and the supernatants were analyzed by HPLC.
In the case of corn fiber substrate the hydrolysis was performed in 100 mL E-flasks with a working volume of 50 mL at 50 °C. 2.5 g of dried chemically pretreated corn fiber was suspended in 0.05 M acetate buffer (pH 4.8) and the slurry was supplemented with the enzyme produced on corn fiber or with industrial cellulase enzymes (30.4 FPU/g cellulose and 15.2 IU/g cellulose, filter paper and β-glucosidase activities, respectively).

The industrial enzymes were Celluclast 1.5 L and Novozyme 188 (Novozymes, Bagsværd Denmark). Samples were withdrawn five times, centrifuged and the supernatants were analyzed by HPLC.

3.6. HPLC analysis

For sugar and organic acid determinations an Aminex HPX-87H (Bio-Rad, Hercules, CA, USA) organic acid column was used at 65°C. The mobile phase was 5 mM sulfuric acid at a flow rate of 0.5 mL/min. For the detection of various components separated on the analytical column a refractive index detector (Shimadzu) was used.
4. Results and Discussion

4.1. Production of cellulase complex with high \(\beta\)-glucosidase activity

The considerably low \(\beta\)-glucosidase activity : FPA ratio of 0.5-0.6 represented by the enzyme complex of \textit{Trichoderma reesei} RUT C30 results in poor hydrolysis potential. To overcome this problem culture conditions are needed to be developed in order to enhance \(\beta\)-glucosidase production (59, 142, 143). Experiments are presented here that were carried out to test buffer systems for production of cellulases with elevated \(\beta\)-glucosidase activity, particularly considering the effect of pH, which is known as a significant factor that affects \(\beta\)-glucosidase production.

4.1.1. pH effect - selection of buffers

The effect of pH on \(\beta\)-glucosidase production by \textit{Trichoderma reesei} RUT C30 has been comprehensively studied on Solka Floc, which is delignified cellulose powder and the most suitable and widespread carbon source on which cellulase production has been studied.

When planning shake flask experiments for investigating the effect of pH, it should be taken into consideration that constant pH cannot be kept in cultivation flasks. pH drops quickly during the first stage of fermentation owing to the depletion of ammonia and liberation of protons. Therefore, periodic manual pH adjustment is needed, which is tedious, less effective, time consuming and results in a saw tooth-like pH profile instead of constant pH (Paper I, 119).

To overcome this problem media can be supplemented by buffer components. The applied buffers should have a sufficiently high buffering capacity to compensate the rapid pH drop and it should be capable of controlling the pH in the range of our interest. Inorganic phosphate salts were excluded during the selection of potential buffer systems, because they have already been proved to be insufficient (91). Although, citric acid and acetic acid have already been examined in a previous study, the pH range in which they were applied was much lower than our primary interest (107). Thus, these organic acids were also tested in experimental series along with tris-maleate, maleate, and succinate as shown in Table 6.
All buffer systems were applied in 0.1 M concentration at pH 5.0 or 6.0, at which the most of cellulase fermentations have been performed (83, 101, 114-117). The concentration of various organic acids in fermentation broth was determined using HPLC.

<table>
<thead>
<tr>
<th>Buffer System</th>
<th>Cellulase</th>
<th>β-Glucosidase</th>
<th>pH Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric Acid – Citrate, pH 5.0</td>
<td>Poor</td>
<td>Poor</td>
<td>Poor</td>
</tr>
<tr>
<td>Acetic Acid – Acetate, pH 5.0</td>
<td>—</td>
<td>—</td>
<td>Very good</td>
</tr>
<tr>
<td>Succinic Acid – NaOH, pH 5.0</td>
<td>Very good</td>
<td>Very good</td>
<td>Poor</td>
</tr>
<tr>
<td>Tris-maleate, pH 6.0</td>
<td>Good</td>
<td>Very good</td>
<td>Very good</td>
</tr>
<tr>
<td>Maleate, pH 6.0</td>
<td>Good</td>
<td>Very good</td>
<td>Very good</td>
</tr>
</tbody>
</table>

Table 6 Application of organic acid buffer systems in shake flask cultures on Solka Floc

Acetic acid : acetate buffer system applied at pH 5.0 kept the pH constant throughout the fermentation. No changes in acetic acid concentration could be observed in the medium. However, no enzyme activities were detected after 7 days of cultivation. Inhibition of microbial growth by acetic acid could be a reasonable explanation for the poor performance of *T. reesei*. In contrast, employing succinic acid in the fermentation medium at pH 5.0 resulted in rather high enzyme activities compared to other buffer systems used. However, the pH fluctuated over a wide range. At the beginning of the cultivation, buffering capacity was not high enough to prevent acidification caused by ammonia depletion, while in the final stage, succinic acid was taken up by the fungus and a dramatic pH shift to alkaline region was observed (Paper I.)

In contrary to previous results reported by Kadam and Kautzer (107), citric acid buffer could not keep pH at 5.0. Similarly to succinic acid buffer system, citric acid was also consumed by *T. reesei* RUT C30, and an extensive basification of the medium was observed. In this case cellulases and β-glucosidase enzymes were produced in moderate amounts.

Cultivation of *T. reesei* RUT C30 in tris-maleic acid, maleic acid-NaOH buffer systems were promising. In the case of these buffers pH 6.0 was applied, since these buffers can be used at higher pH values than 5.2. Both FPA and β-glucosidase activities were considerably high in the fermentation broth using these systems. Furthermore, these buffer systems managed to maintain the pH around the desired value with great stability. In addition, *Trichoderma* did not consume maleic acid (Paper I, II). Therefore, these buffer systems were chosen to quantitatively compare them with four other pH-controlling strategies to investigate which pH profile gives the highest β-glucosidase activity. The experimental setup is outlined in Table 7.
<table>
<thead>
<tr>
<th>Cultures</th>
<th>pH-controlling strategy</th>
<th>Average pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Daily pH adjustment to 5.0</td>
<td>4.7</td>
</tr>
<tr>
<td>II</td>
<td>pH adjustment in case pH drops below 4.0, starting pH: 5.0</td>
<td>4.1</td>
</tr>
<tr>
<td>III</td>
<td>Daily pH adjustment to 6.0</td>
<td>5.7</td>
</tr>
<tr>
<td>IV</td>
<td>pH adjustment in case pH drops below 4.0, starting pH: 6.0</td>
<td>4.6</td>
</tr>
<tr>
<td>V</td>
<td>0.1 M, tris–maleic acid buffer, pH 6.0, no manual adjustment</td>
<td>6.0</td>
</tr>
<tr>
<td>VI</td>
<td>0.1 M, Maleic acid–NaOH buffer, pH 6.0, no manual adjustment</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Table 7 pH-Controlling Strategies Followed in Shake-Flask Cultivation of *T. reesei* RUT C30

Filter paper activities about 1.2 – 1.4 FPU/ml were measured on day 7 of cultivation in each condition (Paper I). In contrast, β-glucosidase activities varied in the wide range of 0.4 and 1.5 IU/ml. The results are presented on Figure 8. It seems the higher is the average pH, the higher is the obtained β-glucosidase activity. It is obvious that pH 6.0 is better initial pH than pH 5.0. In addition, the highest β-glucosidase activities were obtained in buffered cultures. Moreover, 25% higher activities were reached in cultivations supplied by tris-maleate buffer than in cultures with maleate. Thus, tris-maleate buffer system was selected to evaluate it on three carbon sources against two other pH strategies (Table 8, Paper II).

![Figure 8](image-url)

**Figure 8** β-Glucosidase activity vs time for shake-flask cultivations using different pH-controlling strategies. (---■---), Condition I; (---○---), condition II; (---▲---), condition III; (---△---), condition IV; (---●---), condition V; (---○---), condition VI.
4.1.2. Testing of tris-maleate buffer

All together nine experimental setups were tested against each other as shown in Table 8.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Carbon source</th>
<th>pH control</th>
<th>Average pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Solka Floc</td>
<td>daily</td>
<td>5.7</td>
</tr>
<tr>
<td>B</td>
<td>Solka Floc</td>
<td>-</td>
<td>3.9</td>
</tr>
<tr>
<td>C</td>
<td>Solka Floc</td>
<td>buffer (0.10 M)</td>
<td>5.9</td>
</tr>
<tr>
<td>D</td>
<td>glucose</td>
<td>daily</td>
<td>5.7</td>
</tr>
<tr>
<td>E</td>
<td>glucose</td>
<td>-</td>
<td>3.7</td>
</tr>
<tr>
<td>F</td>
<td>glucose</td>
<td>buffer (0.10 M)</td>
<td>6.0</td>
</tr>
<tr>
<td>G</td>
<td>lactose</td>
<td>daily</td>
<td>5.7</td>
</tr>
<tr>
<td>H</td>
<td>lactose</td>
<td>-</td>
<td>4.9</td>
</tr>
<tr>
<td>I</td>
<td>lactose</td>
<td>buffer (0.10 M)</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Table 8 Experimental setup and average pH values in fermentations on various carbon sources using different pH strategies

To study pH effect in detail, experiments were performed not only on Solka Floc, but on glucose and lactose, as well (Paper II). Effect of pH might be dependent on the applied carbon source, since *Trichoderma* produces cellulase not in the same extent on various carbon sources. Glucose, for instance does not induce cellulase production, while lactose induces enzyme formation. However, lactose is not as strong inducer as cellulose i.e. Solka Floc.

The three applied pH-controlling strategies were as follows. In experiments A, D, G daily manual pH adjustment was carried out, while in experiments B, E and H pH control was not performed. Cultivations C, E and I were supplemented by the components of tris-maleate buffer system (0.1 M, pH 5.8) to keep pH in the range of 5.6 and 6.0.

The results are presented in Figure 9 A and 9 B. The obtained enzyme activities are in good accordance with results published elsewhere (59). The highest FPA values were around 1.15 FPU/mL that were reached on Solka Floc, which is constituted by cellulose and xylan. Therefore, it is rather good carbon source for cellulase production. Lactose can also induce the production of cellulases in smaller extent (69, 95). The obtained filter paper activities on lactose were 40% less than reached on Solka Floc. Glucose does not repress cellulase production in *Trichoderma reesei* RUT C30, since it is cre1 mutant strain (4). However, only low levels of cellulases can be produced on glucose, because of the lack of inducers (4). Thus, only around 0.40 FPU/mL cellulase activities were obtained on glucose.
Figure 9 A Filter paper activities (4th day, 7th day, 7th day with added β-glucosidase)

Figure 9 B Endoglucanase activities (7th day)
Filter paper activities measured with additional \( \beta \)-glucosidase were higher than determined without \( \beta \)-glucosidase and there were minor differences within the experiments where the same carbon source was used. In addition, there were also minor differences regarding to endoglucanase activities using different pH strategies on the same carbon source (see Figure 9 B). The highest endoglucanase activities were obtained on Solka Floc, while on lactose and glucose 30% and 75% lower activities were reached, respectively.

Figure 10 shows that the highest \( \beta \)-glucosidase activities after 7 days of fermentation were obtained on each carbon source in experiments with tris-maleate buffer. The highest activities (1.4 IU/mL) were reached on Solka Floc and lactose with tris-maleate buffer, while 60% lower \( \beta \)-glucosidase activity was obtained on buffered glucose media. In buffered cultures the reached activities were 140% higher on Solka Floc and glucose, 550% higher on lactose compared to non-buffered, pH-adjusted cultures, respectively. The obtained \( \beta \)-glucosidase activities in pH-adjusted cultures were two and three times higher than in cultures without pH adjustment, which is in good accordance with the observation that higher pH enhances \( \beta \)-glucosidase production. It can be seen in Table 7 that pH adjusted cultures had higher average pH (5.6-5.7) than non-pH-adjusted cultivations (3.7-4.9). In buffered cultures the average pH values varied between 5.9 and 6.0.
The reached $\beta$-glucosidase activities were the highest in buffered cultures. Therefore, it is tempting to speculate that buffer components provoke $\beta$-glucosidase production. Strongly support this view that in buffered cultures not only $\beta$-glucosidase activities, but also extracellular protein concentrations were considerably higher compared to non-buffered cultivations. According to Table 8 in experiments with tris-maleate buffer the reached protein concentrations were almost two times higher than obtained in non-buffered cultures. Moreover, in buffered cultures major changes were in protein concentrations between the 4th and 7th days, which were believed to be mainly due to $\beta$-glucosidase production.

<table>
<thead>
<tr>
<th></th>
<th>Protein concentration (mg/mL)</th>
<th>4th day</th>
<th>7th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.83</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.53</td>
<td>0.87</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1.01</td>
<td>1.46</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.24</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>0.13</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>0.41</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>0.31</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>0.26</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.72</td>
<td>0.91</td>
<td></td>
</tr>
</tbody>
</table>

**Table 9** Protein concentrations (4th and 7th day)

However, the average pH values of buffered cultures were slightly higher than non-buffered cultivations. Therefore, to prove that not the higher pH, but the buffer components themselves provoke enhanced $\beta$-glucosidase production, tris-maleate buffer was tested in the concentration range of 0.05 M and 0.15 M using Solka Floc carbon source. Time profiles of obtained filter paper and $\beta$-glucosidase activities are presented in Figures 11 A-B. It seems that because of buffer salts in buffered cultivations *Trichoderma reesei* needs longer adaptation time compared to non-buffered cultures. Therefore, the higher is the concentration of buffer, the lower is the initial enzyme production rate. However, until the seventh day of experiments the differences in enzyme activities were equalized. Therefore, it can be concluded that the concentration of buffer components in the range of 0.05-0.15 M have no effect on final $\beta$-glucosidase and filter paper activities.
Figure 11 A  Time courses of filter paper activities in experiments with various buffer concentrations (Buffer concentration: ♦ 0.05 M, ■ 0.10 M, ▲ 0.15 M, x without buffer)

Figure 11 B  Time courses of β-glucosidase activities in experiments with various buffer concentrations (Buffer concentration: ♦ 0.05 M, ■ 0.10 M, ▲ 0.15 M, x without buffer)
Average pH values were in the range of 5.6-6.0, since lower buffer concentrations had insufficient buffering capacity to compensate pH changes. In cultures with 0.05 M tris-maleate buffer the average pH was lower than in non-buffered, pH-adjusted cultivations, which proves that the considerably high β-glucosidase activity is not caused by the high pH. In addition, previous experiments indicate that maleic acid is the buffer component that enhances cellulase production, since similar results could be obtained without tris-hydroxymethyl-amino-methane by applying maleate buffer (Paper I).

4.2. Substrate selection for enzyme production and hydrolysis

It has been proven that Solka Floc is a suitable carbon source (Paper I, II) for cellulase production. However, it is uneconomical for large-scale enzyme production. Instead of expensive Solka Floc, pretreated lignocellulosics, derived from agricultural and forestry wastes or from energy crops should be used.

It seems that considerably higher β-glucosidase activity can be obtained on all three tested carbon sources if cultivations are supplemented by tris-maleate buffer. The buffer system was also tested on a few pretreated lignocellulosic carbon sources in experiments as follows. The produced enzymes were tested against each other by their composition and hydrolysis potential. It has been reported that hemicellulolytic activities also affect cellulose hydrolysis (76, 148). Thus, hemicellulolytic activities were also studied.

4.2.1. Fermentation on various substrates

Cellulase enzymes were produced by *Trichoderma reesei* RUT C30 on steam pretreated spruce, willow, corn stover and on Solka Floc as a reference. The same cellulose concentration was applied at each carbon source. The used medium was supplemented with buffer components according to experiments presented in the previous chapter. The obtained enzymes were SP (enzyme produced on steam pretreated spruce), WI (enzyme produced on steam pretreated willow), CO (enzyme produced on steam pretreated corn stover) and SF (enzyme produced on Solka Floc), respectively. The produced enzymes and two commercial cellulases, CE (Celluclast 1.5 L) and EC (Econase CE) were characterized with respect to the various activities and protein content (Paper III).

Figure 12 shows the main enzyme activities and protein concentrations obtained on the seventh day of fermentations. As revealed by the graph both filter paper and endoglucanase activities of CO were the highest.
In addition, the highest protein concentration was also reached on steam pretreated corn stover. However, the $\beta$-glucosidase activity of CO was lower compared to the reference enzyme \textit{i.e.} SF. The activities and protein concentrations of both WI and SP were lower than that of SF.

\textbf{Figure 12} Main enzyme activities and protein concentrations (7\textsuperscript{th} day)

It should be noted that in this set of experiments the obtained $\beta$-glucosidase activity on Solka Floc was lower compared to the abovementioned experiments. This is probably due to the biological variability. In addition, it is certain that the slight differences in $\beta$-glucosidase assays (see Materials and Methods) had also effect on the calculated $\beta$-glucosidase activity. Furthermore, little variations in fermentation conditions, for instance in temperature, agitation, sampling time etc. also have effect on final enzyme activity. Nevertheless, the reached $\beta$-glucosidase activity : FPA ratio was still considerably higher than the basal 0.5-0.6. However, on steam pretreated corn stover and willow the ratios of enzyme activities were only around 0.6. To evaluate these results it should be taken into consideration that usually on pretreated lignocellulosic substrates lower $\beta$-glucosidase activity can be reached than on cellulosic carbon source (see Table 2 and 3). The ratio of 0.5-0.6 is typical only in experiments with pure cellulose. For instance on 10 g/L cellulose (Sigmacell) (63) 1.20 FPU/mL filter paper and 0.60 IU/mL $\beta$-glucosidase activities were reached, respectively.
While on steam pretreated willow, with cellulose concentration of 10 g/L, 1.30 FPU/mL and 0.20 IU/mL β-glucosidase activity were achieved, respectively (104). Considering the aforementioned results, it is believed that buffer components still enhanced β-glucosidase production, however in lower extent. On the other hand, on steam pretreated spruce the obtained enzyme ratio was higher than 1.0.

4.2.2. Comparing the spectra of obtained enzymes

Table 10 shows the obtained specific enzyme activities. There were minor differences among the overall cellulase activities (FPA) and the activities of main cellulase components i.e. exoglucanases and endoglucanases. Generally, the activities of cellulase produced on spruce were slightly lower than those produced on the other carbon sources. Within the hemicellulases, there were more differences among the produced enzymes. As shown in Figure 13, the higher was the xylan concentration of substrate, the higher was the obtained xylanase activity, corresponding to both absolute and specific activities. Thus, it seemed obvious that xylan induced xylanase production. However, even in the absence of xylan, i.e. in the case of steam pretreated spruce as carbon source, a low constitutive xylanase production could be observed. β-Xylosidase activity was also related to xylan content of carbon source. SP had the lowest, while SF had the highest β-xylosidase activity. These findings are in good accordance with the results reported by Kubicek and Penttilä (4). They showed that xylanase and β-xylosidase production is clearly induced by xylans (4).

![Figure 13](image)

**Figure 13** Xylose content of substrates versus measured xylanase activities (7th day)
<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Protein</th>
<th>Specific enzyme activities (FPU/mg, nkat/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/mL</td>
<td>FPA</td>
</tr>
<tr>
<td>Produced enzymes (supernatants of fermentation broths)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SF</td>
<td>1.89</td>
<td>0.58</td>
</tr>
<tr>
<td>CO</td>
<td>2.23</td>
<td>0.52</td>
</tr>
<tr>
<td>SP</td>
<td>0.83</td>
<td>0.45</td>
</tr>
<tr>
<td>WI</td>
<td>1.05</td>
<td>0.56</td>
</tr>
<tr>
<td>Industrial enzymes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CE</td>
<td>125</td>
<td>0.55</td>
</tr>
<tr>
<td>EC</td>
<td>110</td>
<td>0.63</td>
</tr>
</tbody>
</table>

*Table 10* Specific enzyme activities and protein concentrations (7th day)
(Relative standard deviations of specific activities were below 10%)
As it can be seen in Figure 14, acetyl xylan esterase was probably co-produced with endoglucanase. Biely et al. (82) have reported that acetyl xylan esterase was co-produced with both endoglucanase and xylanase. In contrast, I did not detect any correlation between xylanase and acetyl xylan esterase activities.

![Specific enzyme activity (nkat/mg)](image)

**Figure 14** Specific acetyl xylan esterase, mannanase, endoglucanase activities (7th day)

As it is presented in Figure 14, a good correlation could be observed between specific mannanase and endoglucanase activities. Thus, there is a good possibility that mannanase expression is regulated coordinately with endoglucanases. However, Margolles-Clark et al. (36) indicated that mannanases were not co-produced with cellulases on most of the carbon sources (36). Moreover, it was reported that mannanase is regulated coordinately with α-galactosidase (53). In contrast, I conclude that no correlation could be observed between mannanase and α-galactosidase activities. There were minor differences among the produced enzymes regarding specific α-galactosidase and α-arabinosidase activities. However, SF had very low, almost zero specific α-galactosidase activity. There were major differences among the produced enzymes in terms of specific β-glucosidase activities. SF had the highest and CO had the lowest specific β-glucosidase activity.

It is almost certain that differences among β-glucosidase activities were caused by the nature of carbon sources, since all culture conditions were the same and the effect of pH was excluded by buffering the cultures with tris-maleate buffer system. As a result of buffering, the pH was kept in the range of 5.8 – 6.1.
These results show that steam pretreated spruce and willow were considerably worse carbon sources compared to Solka Floc, which was used as a reference. In contrast, on steam pretreated corn stover higher cellulolytic enzyme activities were reached than on Solka Floc.

Finally, to compare the commercial and produced enzymes, it can be seen that the specific xylanase, mannanase and $\beta$-glucosidase activities of commercial enzymes were generally lower than those of the produced enzymes. On the other hand, acetyl xylan esterase activities of the commercial enzymes were the highest. Celluclast and Econase have almost similar enzyme spectra, especially in terms of cellulases. However, some differences could be observed concerning hemicellulases. The specific $\alpha$-galactosidase activity of Celluclast was almost zero, while Econase had rather high specific $\alpha$-galactosidase activity. There were smaller differences between the two enzymes with respect to specific $\alpha$-arabinosidase, xylanase and acetyl xylan esterase activities.

### 4.2.3. Hydrolysis test of produced and commercial enzymes

Solka Floc, steam pretreated spruce, willow and corn stover were hydrolyzed by the four produced and the two commercial enzymes (Paper III). The enzymes were dosed in such way that the filter paper activities per gram of cellulose in the substrate were the same in all cases to ensure constant enzyme/substrate ratio. The obtained sugar yields are presented in Table 11.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Glucose yield (%)</th>
<th>Xylose yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Solka F</td>
<td>SPCS</td>
</tr>
<tr>
<td>SF</td>
<td>36</td>
<td>55</td>
</tr>
<tr>
<td>CO</td>
<td>46</td>
<td>59</td>
</tr>
<tr>
<td>SP</td>
<td>31</td>
<td>30</td>
</tr>
<tr>
<td>WI</td>
<td>40</td>
<td>34</td>
</tr>
<tr>
<td>CE</td>
<td>52</td>
<td>41</td>
</tr>
<tr>
<td>EC</td>
<td>47</td>
<td>34</td>
</tr>
</tbody>
</table>

**Table 11** Glucose and xylose yields after 24 h of hydrolysis (expressed in percent of theoretical) (Relative standard deviations were around 10%)
It can be seen that the performance of CO was the best in almost all cases. Table 12 summarizes the enzyme activities used in hydrolysis, it seems that the good hydrolysis ability of CO cannot be explained by its higher enzyme activities.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>FPA</th>
<th>β-Glu.</th>
<th>AXE</th>
<th>CBH I</th>
<th>Xylanase</th>
<th>EG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FPU/g c.S.</td>
<td>nkat/g cellulose in substrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SF</td>
<td>15.0</td>
<td>228</td>
<td>158</td>
<td>523</td>
<td>56843</td>
<td>4269</td>
</tr>
<tr>
<td>CO</td>
<td>15.0</td>
<td>144</td>
<td>45</td>
<td>530</td>
<td>32754</td>
<td>3555</td>
</tr>
<tr>
<td>SP</td>
<td>15.0</td>
<td>232</td>
<td>11</td>
<td>596</td>
<td>5384</td>
<td>2766</td>
</tr>
<tr>
<td>WI</td>
<td>15.0</td>
<td>117</td>
<td>45</td>
<td>466</td>
<td>17583</td>
<td>2745</td>
</tr>
<tr>
<td>CE</td>
<td>15.0</td>
<td>113</td>
<td>380</td>
<td>463</td>
<td>2710</td>
<td>3519</td>
</tr>
<tr>
<td>EC</td>
<td>15.0</td>
<td>107</td>
<td>184</td>
<td>389</td>
<td>6066</td>
<td>3636</td>
</tr>
</tbody>
</table>

**Table 12** Enzyme activities used in the hydrolysis experiments

The yields of xylose varied between 6% and 33% (see Table 11). Generally, the more xylanase was in the hydrolysate, the higher xylose yield was obtained.

Substrate accessibility was not the same regarding to the used enzyme. Steam pretreated corn stover was the best hydrolysable substrate in experiments with CO, SF and SP and the performance of CO was considerably better on steam pretreated corn stover compared to the other substrates. In contrast, WI degraded steam pretreated willow significantly better than the other substrates.

These results bear some similarity to experiments where chemically pretreated corn fiber was used for fermentation and hydrolysis (Paper IV). Cellulase enzyme produced on pretreated corn fiber was used for hydrolysis of the same substrate. The results of hydrolysis were compared to the performance of commercial enzymes. Celluclast 1.5 L and Novozyme 188 were applied to set the same filter paper and β-glucosidase activities. Hydrolysis conversion obtained after 24 h hydrolysis are presented in Figure 15. It seems that the enzyme produced on pretreated corn fiber could degrade corn fiber substrate approximately 15% more efficiently than commercial enzymes.
According to these observations, it can be argued that it might be advantageous, at least in the case of some substrates, to use the produced enzyme for the hydrolysis of the same substrate as it was used as carbon source for fermentation. Obviously, the efficiency of the enzyme production depends also on other factors than the bare chemical composition of the raw material, such as accessibility of various components and their chemical or physical associations.

**Figure 15** Glucose yields after 24 h of hydrolysis expressed in percent of theoretical, —■— enzyme produced on pretreated corn fiber (CPCF), —●— commercial enzymes, relative standard deviation is approximately 4%
4.3. Production of β-glucosidase by cofermentation of *Trichoderma reesei* and *Aspergillus niger*

The production of β-glucosidase enzyme has also great importance, since it can be used to elevate the hydrolysis potential of cellulase complex if it contains insufficient amount of β-glucosidase. β-Glucosidase is usually produced by *Aspergillus* species on glucose containing media. However, lignocellulosic materials can replace sugar if a microbial consortium is used for enzyme production (Paper V).

Enzyme production of a well-known β-glucosidase producer, *Aspergillus niger* (BKMF 1305), was examined in a culture, where it was cocultivated with *Trichoderma reesei* RUT C30. The experiments were designed in such way that cellulase production of *Trichoderma* was kept at low level, just enough for the breakdown of cellulosic carbon source, thereby supporting the β-glucosidase production of *Aspergillus*. This type of *Aspergillus* can produce only low amount of cellulase. Therefore, without *Trichoderma* it is hardly able to utilize cellulose.

The fermentation was optimized in shake flask experiments (Paper V.) and the optimized conditions were used to perform enzyme production in a laboratory scale fermentor. Mandels’ medium was used with 20 g/L waste paper as carbon source, which was equivalent with 11.4 g/L glucose and 3.8 g/L xylose and arabinose.

The obtained enzyme activities are summarized and compared with results published by Brumbauer et al. (128) in Table 13. Brumbauer et al. performed β-glucosidase fermentations by monocultures of *Aspergillus niger* using the same strain and almost the same conditions. The only significant difference was the applied medium. Brumbauer used 10 g/L glucose carbon source with basal level of Mandels’ salts and nitrogen source, while in mixed fermentations 20 g/L waste paper was applied as carbon source and the concentration of other nutrients was doubled compared to the basal level. It was suspected that the elevated level of salts and nitrogen source might have positive influence on β-glucosidase production in mixed cultivation. However, later it was proven, that the elevated nutrient concentration did not cause considerably higher β-glucosidase activity (unpublished data).
Table 13. Comparison of β-glucosidase production by mixed culture of *A. niger* & *T. reesei* and monoculture of *A. niger* (* Brumbauer et al. (128))

<table>
<thead>
<tr>
<th></th>
<th>Shake flask experiments</th>
<th>Fermentor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>A. n. + T. r.</em></td>
<td><em>A. niger</em></td>
</tr>
<tr>
<td>Fermentation time (h)</td>
<td>168</td>
<td>168</td>
</tr>
<tr>
<td>Activity (IU/mL)</td>
<td>3.07</td>
<td>2.80</td>
</tr>
<tr>
<td>Productivity (IU/l*h)</td>
<td>18.3</td>
<td>19.1</td>
</tr>
<tr>
<td>Yield (IU/g glucose)</td>
<td>269.3</td>
<td>280.0</td>
</tr>
<tr>
<td>Yield (IU/g carbohydrate)</td>
<td>202.0</td>
<td>280.0</td>
</tr>
</tbody>
</table>

Considering that the price of sugar is about 1 €/kg (155), while waste paper costs approximately 0.04 €/kg (156), it can be argued that this 25 fold difference between the prices of the two carbon sources might be advantageous in the case of large-scale enzyme production.

Furthermore, results of mixed fermentation experiments indicate a new approach to enzyme production. *Trichoderma* can be used to produce glucose for another microbe from lignocellulosic wastes, which might be advantageous if cheap raw material is needed for large-scale production of a valuable product fermented by *Aspergillus* species.
5. Conclusions – New scientific thesis

1. 0.1 M tris-maleate buffer could keep pH in the range of 5.8 and 6.1 (initial pH: 6.0) throughout shake flask fermentations by *Trichoderma reesei* RUT C30 on Solka Floc, glucose, lactose, steam pretreated corn stover, spruce and willow.

2. In cultivations with tris-maleate buffer β-glucosidase activities increased more than 100% compared to non-buffered cultures on Solka Floc, glucose and lactose. Moreover, the obtained β-glucosidase activity : FPA ratios were around the optimal in buffered cultures on all three carbon sources.

3. Steam pretreated corn stover was an excellent carbon source for cellulase fermentation and ideal substrate for hydrolysis. In tris-maleate buffered cultures of *Trichoderma reesei* RUT C30 10-40% higher cellulase activities (FPA, endo- and exoglucanase) could be obtained on steam pretreated corn stover than on Solka Floc that was used as reference. However, the reached β-glucosidase activity was higher on Solka Floc compared to steam pretreated corn stover, which was probably due to enzyme adsorption onto lignin.

4. Two industrial and the four produced enzymes were tested on the four substrates that were used as carbon source for enzyme production (steam pretreated corn stover, spruce and willow and Solka Floc). The highest yields were obtained using the enzyme produced on steam pretreated corn stover. Moreover, the highest sugar yield among all experiments was reached on steam pretreated corn stover using the “own enzyme” i.e. the enzyme produced on steam pretreated corn stover. It might be advantageous, at least in the case of some substrates (for instance corn stover and corn fiber), to use the “own enzyme” for hydrolysis. This observation is also supported by results obtained in the case of chemically pretreated corn fiber. Significantly higher sugar yields were reached in the hydrolysis of corn fiber using the “own enzyme” than applying commercial cellulases. This observation shows one benefit of on site enzyme production.

5. It was found that mannanase and endoglucanase expressed coordinately on the examined carbon sources, since a good correlation was observed between the two enzyme activities. This observation might be valuable information to understand the regulation mechanism of hemicellulase genes in *Trichoderma reesei*. However, to confirm this observation more experiments are needed.
6. Approximately the same amount of β-glucosidase enzyme could be produced by mixed fermentations of *Aspergillus niger* and *Trichoderma reesei* on cheap waste paper carbon source compared to fermentation of *Aspergillus niger* on glucose. The produced β-glucosidase can be added to cellulase complex to elevate its hydrolysis potential.
6. References


