PRODUCTION AND CHARACTERIZATION OF CELLULOSE-DEGRADING ENZYMES FOR VARIOUS APPLICATIONS

Ph.D. Thesis

Nóra Szijártó

Department of Applied Biotechnology and Food Science
Budapest University of Technology and Economics

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PREFACE

The work outlined in this Thesis was carried out at the Department of Agricultural Chemical Technology (currently Department of Applied Biotechnology and Food Science) at Budapest University of Technology and Economics (*Budapesti Műszaki és Gazdaságtudományi Egyetem*, BME) as the home organization in Hungary, under the encouraging supervision of Dr. Kati Réczey, whom I greatly appreciate and acknowledge for all her efforts *she* made to get *my* doctoral studies completed. A large portion of the experimental work was however done at several institutions abroad, which were *(i)* the Agrotechnological Research Institute (*Instituut voor Agrotechnologisch Onderzoek*, ATO), currently the Agrotechnology and Food Sciences Group at Wageningen University in the Netherlands, under the strict guidance of Dr. Pieternel Claassen, *(ii)* the Department of Chemical Engineering at Lund University (*Lunds Universitet*, LU) in Sweden, with the enthusiastic supervision of Prof. Gunnar Lidén, *(iii)* the Department of Applied Chemistry and Microbiology at University of Helsinki (*Helsingin Yliopistoon*, HY), and *(iv)* the Technical Research Center of Finland (*Valtion Teknillinen Tutkimuskeskus*, VTT) in Finland, under the kind supervision of Prof. Maija Tenkanen, Dr. Matti Siika-aho and Prof. Liisa Viikari; all deeply acknowledged for their innovative ideas and the fruitful discussions we shared.

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...

És végül - bár az efféle felsorolásban méltatlanul rendre az utolsó helyre kerülnek, pedig elsőként illeti őket elismerés végtelen türelmükért, kitartásukért, évtizedeken átívelő segítő gondoskodásukért - soha nem múló tisztelettel és köszönettel tartozom:

SZÜLEIMNEK...

LIST OF PUBLICATIONS

This study is based on the following papers, referred to by their Roman numerals in the text as given below:

I. **Szijártó, N.**, Lidén, G., Réczey, K. Effect of medium nitrogen content on cellulase production by *Trichoderma reesei*. Manuscript before submission.

II. **Szijártó, N.**, Szengyel, Zs., Lidén, G., Réczey, K. (2004) Dynamics of cellulase production by glucose grown cultures of *Trichoderma reesei* Rut C-30 as a response to addition of cellulose. *Appl. Biochem. Biotechnol.* 113-116, 115-124.

III. Juhász, T., Szengyel, Zs., **Szijártó, N.**, Réczey, K. (2004) The Effect of pH on the cellulase production of *Trichoderma reesei* Rut C-30. *Appl. Biochem. Biotechnol.* 113-116, 201-211.

IV. **Szijártó, N.**, Varga, E., Réczey, K., Thomsen, A.B. Cellulase enzyme production by *Trichoderma reesei* Rut C-30 on the separated liquid stream from wet oxidation of corn stover. Submitted for publication to *Enzyme Microbiol. Technol.*

V. **Szijártó, N.**, Faigl, Zs., Mézes, M., Bersényi, A., Réczey, K. (2004) Cellulase fermentation on a novel substrate (waste cardboard) and subsequent utilization of home-produced cellulase and commercial amylase in a rabbit feeding trial. *Ind. Crops Prod.* 20, 49-57.

VI. **Szijártó, N.**, Siika-aho, M., Tenkanen, M., Alapuranen, M., Vehmaanperä, J., Réczey, K., Viikari, L. Hydrolysis of amorphous and crystalline cellulose by heterologously produced cellulases of *Melanocarpus albomyces*. Submitted for publication to *J. Biochem*.

Other related papers by the same author:

Contreras, A.M.L., Martens, A.A., **Szijártó, N.**, Mooibroek, H., Claassen, P.A.M., van der Oost, J., de Vos, W.M. (2003) Production by *Clostridium acetobutylicum* ATCC 824 of CelG, a cellulosomal glycoside hydrolase belonging to family 9. *Appl. Envir. Microbiol.* 69, 869-877.

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Eiben, Cs., Mézes, M., **Szijártó, N.**, Kustos, K., Gódor, S. (2004) Dose-response of dietary cellulase enzyme inclusion on the performance of fattening rabbits. *World Rabbit Science* 12, 55.

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Szijártó, N., Varga, E., Réczey, K., Thomsen, A.B. Utilization of hemicellulose fraction of pretreated corn stover for cellulase enzyme production. *Days of Chemical Engineering '06.* Veszprém, Hungary, April 25-27, 2006, pp. 113-116.

Szijártó, N., Varga, E., Réczey, K., Thomsen, A.B. Integrated process scheme for on-site production of cellulose degrading enzymes in the lignocellulose to ethanol process using wet oxidised corn stover as the substrate. *14th European Conference and Technology Exhibition on Biomass for Energy, Industry and Climate Protection*. Paris, France, October 17-21, 2005, pp. 1851-1854.

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CONTENTS

LIST OF ABBREVIATIONS

CBH	cellobiohydrolase
CBM	cellulose binding module
Cel45A	20-kDa EG from *M. albomyces*
Cel45A(CBM)	20-kDa EG from *M. albomyces* carrying the CBM of *T. reesei* CBHI
Cel7A	50-kDa EG from *M. albomyces*
Cel7A(CBM)	50-kDa EG from *M. albomyces* carrying the CBM of *T. reesei* CBHI
Cel7B	50-kDa CBH from *M. albomyces*
Cel7B(CBM)	50-kDa CBH from *M. albomyces* carrying the CBM of *T. reesei* CBHI
CER	CO_2 evolution rate
CMC	carboxymethyl cellulose
CO_2	carbon dioxide
DNS	dinitrosalicylic acid
DO	dissolved oxygen
DP	degree of polymerization
DW	dry weight
E-flask	Erlenmeyer flask
EG	endoglucanase
FPA	filter paper activity
FPU	filter paper unit
G1	glucose
G2	cellobiose
G3	cellotriose
G4	cellotetraose
G5	cellopentaose
HEC	hydroxyethyl cellulose
HPAEC	high performance anion exchange chromatography
HPLC	high performance liquid chromatography
IU	international unit
LMW	low-molecular-weight (marker)
M. albomyces	*Melanocarpus albomyces*
N	nitrogen, as medium nitrogen
N_{est}	estimated stoichiometric nitrogen need
OCC	old corrugated cardboard
OD_{660}	optical density read at 660 nm
PASC	phosphoric acid swollen cellulose
RC	reducing carbohydrate(s)
rpm	revolution per minute (agitation rate)
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SF	Solka Floc
T	temperature
t	time
T. reesei	*Trichoderma reesei*
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
vvm	volume of gas per volume of liquid per minute (aeration rate)
WO	wet oxidation
WOCS	wet-oxidized corn stover

1 INTRODUCTION

Long-term scenarios that result in sustainable economic growth with decreased dependence on fossil resources at reduced environmental impact compulsorily rely on the increasing utilization of renewable materials to satisfy the commodity needs of our society [1,2,3,4,5,6].

Due to their great potential to replace various bulk products of the petrochemical industry, lignocellulose and its derivatives are seen to have a capital share as feedstocks in the sustainable production of environmentally friendly fuels, chemicals, and other commodities soon [7,8]. Processes under discussion will, in principle, focus on the careful degradation of the lignocellulose complex to its building compounds and subsequent chemical and/or biological processing of the different subunits to various value-added products [9,10]. The term "careful degradation" means that the major substructures: cellulose, hemicellulose and lignin are first separated from each other by a physical, chemical, or physico-chemical treatment, and thereafter processed individually by the most suitable - often enzymatic - method. For the degradation of the polymers under discussion enzymatic methods are superior to chemical techniques to many aspects and consequently are in the focus of current lignocellulose research [11,12]. In particular, the enzymatic conversion of cellulose to glucose attracts a superb attention, thanks to the tremendous alternatives that glucose as the "precursor" of various value-added products has [13,14].

1.1 The cellulose

Green biomass has been a major contributor to the ecosystem of our planet as long as plants have flourished. Even though it has been observed and used by humans for thousands of years, it was only in 1838 when the major constituent of plant material was identified. This was the year when the distinguished French scientist, Anselme Payen (1795-1871), a pioneer in wood chemistry, first isolated a fibrous substance commonly found in wood, cotton, and other plants: the cellulose, as he coined and introduced it into the scientific literature in 1839 [15].

Cellulose is a high-molecular-weight homopolysaccharide of β-1,4-linked *D*-glucopyranose residues. The adjacent monomer units are arranged so that glucosidic oxygens point in opposite directions to form cellobiose, the actual repeating unit of cellulose. The cellobiose units are linked to form an extended, straight chain, as shown schematically in Figure 1.

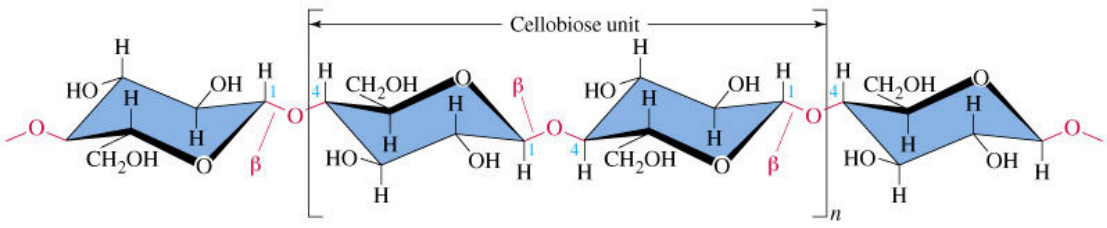

Figure 1 Structure of a cellulose chain. The glucosidic oxygens are marked by a "β".

The length of the cellulose chain, denoted as the degree of polymerization (DP), in nature, approximates 10,000-15,000 glucopyranose residues. The parallel chains are held together by hydrogen bonds making bundles of cellulose molecules to aggregate together in so-called microfibrils, in which highly ordered (crystalline) regions are combined with less ordered (amorphous) regions. The microfibrils form up fibrils which in turn form the cellulose fibers. This compact structure makes cellulose highly inert and resistant [16]. The tendency to form inter-chain bonds may also explain why oligomers with DP of 6 or higher are practically insoluble, despite that the molecule itself has a hydrophilic character.

In plant biomass cellulose fibrils are usually embedded in the matrix of hemicellulose and lignin to make up the solid framework of the plant cell wall: the lignocellulose. Hemicellulose is composed of branched polysaccharides, with xylans and mannans as the main components. Lignin is a highly crosslinked, random polymer of condensed aromatic alcohols coupled to hemicellulose through aromatic acids, which are linked by ester bonds to the side chains of xylans. Hemicellulose appears to act as a binding agent between cellulose fibrils and lignin.

1.2 Cellulose degrading enzymes

Cellulose, the major structural polysaccharide of plant cell wall is by far the most abundant organic material on earth [17] and therefore the enzymes able to degrade cellulose (cellulases), or rather the microorganisms able to excrete such enzymes, play a substantial role in the global carbon cycle. Due to its physical properties, however, cellulose is a highly recalcitrant substrate for enzymatic degradability and the capacity to completely hydrolyze the cellulose macromolecule is restricted to a relatively select but diverse group of microorganisms. In a typical cellulose degrading ecosystem, a variety of cellulolytic bacteria (*e.g., Clostridium*, *Bacillus*) and fungi (*e.g., Penicillium*, *Aspergillus*, *Trichoderma*) work in concert with related microorganisms to convert insoluble cellulosic substrates to soluble sugars, primarily glucose, which is then assimilated by the cell [18].

Cellulases are inducible enzymes synthesized only in the presence of cellulosic materials or other appropriate inducers [19,20,21,22,23,24,25]. The efficient decomposition of cellulose requires the complementary action of cellulolytic enzymes with different characteristics, such an endoglucanase (EG) capable to hydrolyze the amorphous regions of the cellulose fibrils by random cleavage of β-glucosidic bonds, a cellobiohydrolase (CBH) capable to release cellobiose from the chain ends, and a β-glucosidase capable to complete the degradation by splitting cellobiose to glucose units [26,27]. Only a high level of synergism between such enzymes can guarantee the complete degradation of cellulosic materials [28,29].

Due to its excellent ability to produce and secrete a complete set of cellulose degrading enzymes in such amounts, that makes their production viable at industrial scale, the soft rot fungus *Trichoderma reesei* has been in the focus of cellulase research for decades [30]. Even though dozens of novel strains with improved characteristics have been engineered and successfully applied in industrial production since the 1980s [31,32,33,34], the most studied cellulolytic organism to date *T. reesei* Rut C-30 developed by Montenencourt and Eveleigh in 1979 [35] still preserves its leading role as the major test organism in fundamental cellulase research.

1.3 Cellulases in the industry

Commercial production of cellulase enzymes by submerged fermentation, known to be superior to the solid state method to many aspects, began in the early 1970s, with cellulase made by *Trichoderma* sold for use in research and pilot studies. The mid 1980s saw the first large industrial uses of cellulases for stonewashing denim and as an additive for animal feeds. This was accompanied by the introduction of commercial cellulases made by fungi of the genera *Aspergillus*, *Penicillium*, and most importantly *Humicola*. Growth in cellulase use has continued ever since with many novel applications. For a summary see the next chapter based on the excellent review of Bhat [36].

1.3.1 Current industrial application of cellulases

In paper production cellulases are used *(i)* to decrease the viscosity of the processed material during the pulping process (bio-mechanical pulping) thus saving energy during refining, *(ii)* to improve drainage of recycled fibers thus increasing the runnability of paper mills, *(ii)* to release ink from fiber surface found in used paper material by partial hydrolysis (bio-deinking), *(iv)* to improve sheet-strength properties of the end-product, and (*v*) to characterize fibers by selective solubilization of pulp carbohydrates.

In textile industry they are extensively used in the bio-stoning of denim, as a sustainable alternative for stone-washing to gain faded, worn, aged appearance, by the careful removal of excess dye from the fabric. Cellulases, in fact, are also efficient in softening the textile without fiber damage thereby minimizing the production of low-quality garments. Short treatment times associated with cellulase use result in increased productivity of washing machines, which undergo less wear and tear in comparison to stonewashing.

Cellulases are extensively used in the production of environmentally friendly washing powders. They improve the detergent performance as they restore the softness and brightness of cotton fabric by selectively removing small and fuzzy fibrils from the surface. The same phenomenon occurs during cellulase action in bio-polishing: by the removal of excess microfibrils from fabric surface the treatment by cellulases results in improved color brightness and uniformity, and a smooth, glossy appearance. In addition, pilling of fabrics can also be prevented by the smart use of special cellulases.

In beer brewing cellulase use is restricted to improve the brewing process starting form a poor quality barely. Their role is to avoid gel formation that may cause poor filtration, slow run-off times, and low extract yields. In wine production cellulases are applied to obtain better skin degradation, improved color extraction, easier must clarification, better extraction, and improved quality and stability of the end product. Similar considerations apply when cellulases are used to produce fruit and vegetable juices, nectars and purees.

Cellulases are widely used to supplement monogastric and ruminant feed. Their role is *(i)* to eliminate anti nutritional factors present in grains and vegetables, *(ii)* to degrade certain cereal compounds to improve the nutritional value of feed, *(iii)* to supplement the own digestive enzymes of animals when those are inadequate (*e.g.,* in the post-weaning period), *(iv)* to improve feed conversion rate, and *(v)* to enable the utilization of less expensive feed components.

1.3.2 Cellulases in the lignocellulose to ethanol process

Long-term strategies towards a sustainable future often rely on the increasing role of clean, secure and affordable biofuels in the transportation sector, known to account for a major share in the total energy consumption worldwide [37,38]. This share is approx 30% in the European Union (EU) where the road transport is almost completely dependent on oil with approx 70% of the demand ensured from imports [39], making us dangerously vulnerable to the fluctuations in the world oil market. In addition, the rapidly growing transport sector is seen as one of the main reasons for the EU failing to meet the Kyoto targets on the reduction of greenhouse gases [40]. Therefore the need to develop innovative biofuel technologies in order to improve the energy security and the overall carbon dioxide (CO_2) balance is immediate.

The principle renewable fuel predicted to displace petrol in road transport soon is bioethanol, which can be produced by fermentation with yeast from practically any feedstock that contains plentiful natural sugars [41]. In some parts of the world it is already produced at large-scale and commercialized either pure or as a blend with gasoline. Popular raw materials include sugar cane (Brazil), sugar beet (Europe), and corn (United States), which are relatively easy to convert to ethanol. However, on the longer run and the larger scale the use of food crops for industrial purpose may cause confrontations. Indeed, in the present era with increasing world population and shortage in food, the question whether one should fill-up his car or afford his groceries instead, is easy to answer… A sustainable alternative with no ethic considerations involved would be the use of lignocellulose biomass for fuel ethanol production [42,43], which is already in effect at pilot scale at several sites, *e.g.,* in Sweden, Canada, and Spain [44]. Possible lignocellulosic raw materials include residues and wastes from agricultural, horticultural, industrial and municipal sources, as well as short rotation trees and grasses (energy crops) primarily grown to exploit their energy content [45].

Corn stover, with its worldwide annual production of approx 520 million dry tons is by far the most promising of such residues readily available today at temperate latitudes at minimal cost [46,47,48]. Currently, it is mainly left on field after corn grain harvest with only five percent collected and used as animal feed and bedding [49]. In a sustainable world we wish to live in we can not afford to leave this huge amount of carbohydrate rich material unutilized (N.B., a large proportion of it has to be left on field to prevent soil erosion [50] leaving approx 80-100 million dry tons per year available for industrial use [51]) and predictions are serious that the most likely alternative for its efficient utilization on the large scale will be in the lignocellulose to ethanol process [52,53]. According to the general protocol, the technology under discussion is comprised of an initial hydrolysis step to convert the cellulose content of the lignocellulose biomass to glucose, which is subsequently metabolized by yeasts with ethanol being the main product. From industrial point of view, the enzymatic hydrolysis of cellulose is superior to the acidic process to many aspects (*i.e.,* milder conditions, higher selectivity, higher conversion, less byproduct, lower environmental impact) and therefore the role of cellulose degrading enzymes in the bioconversion of lignocellulosics to ethanol is substantial [54].

Because of the various physical and chemical barriers that largely inhibit their susceptibility to hydrolysis and fermentation, lignocellulose feedstocks have to be pretreated before bioconversion [55,56,57,58]. Among the numerous methods available to get the rigid lignocellulose structure loosened thus making the cellulose content accessible to cellulose degrading enzymes, steam-explosion enjoys the best reputation, which was successfully applied to corn stover by our co-workers [59,60,61,62,63]. Wet oxidation (WO), a relatively novel development employing water, oxygen, mild alkali or acid, elevated temperature and

pressure, represents a promising alternative for the pretreatment of lignocellulosics [64,65,66]. When WO was applied to corn stover [67], our colleagues observed that the enzymatic hydrolysis of pretreated cellulose fibers was easier when they were separated from the slurry obtained after WO, compared to the case when the whole slurry was treated with enzymes [Enikő Varga, Risø National Laboratory, personal communication]. Therefore, it would be reasonable to collect the pretreated solids for further processing and in parallel to develop an effective way of utilization for the liquid side fraction rich in hydrolyzed hemicellulose (hereafter referred to as the hydrolyzate) thus generated. One possibility to utilize the hydrolyzate would be for ethanol production by pentose-fermenting yeasts or bacteria [65, 68,69,70] as far as they can tolerate the inhibitory compounds usually generated during the physico-chemical pretreatment of lignocellulosics such as carboxylic acids and furan derivatives from sugar degradation and phenol monomers from lignin degradation [71,72]. Unfortunately, the bioconversion processes under discussion are rather sensitive to and hampered by these compounds, which is especially worrying because by the recirculation of process streams necessarily employed to minimize the need for freshwater, the so-called inhibitors tend to accumulate in the technology [73,74,75,76].

Due to its ability to metabolize pentose sugars [77,78,79] while being able to degrade certain inhibitory compounds, *T. reesei* might be an attractive tool for the rational utilization and parallel detoxification of the hydrolyzate [80,81,82,83]. In this manner, the enzyme required to hydrolyze the pretreated fibers could be produced within the same process, which via the elimination of the need for high-cost commercial cellulases would mean a substantial improvement in the overall process economics (Figure 2).

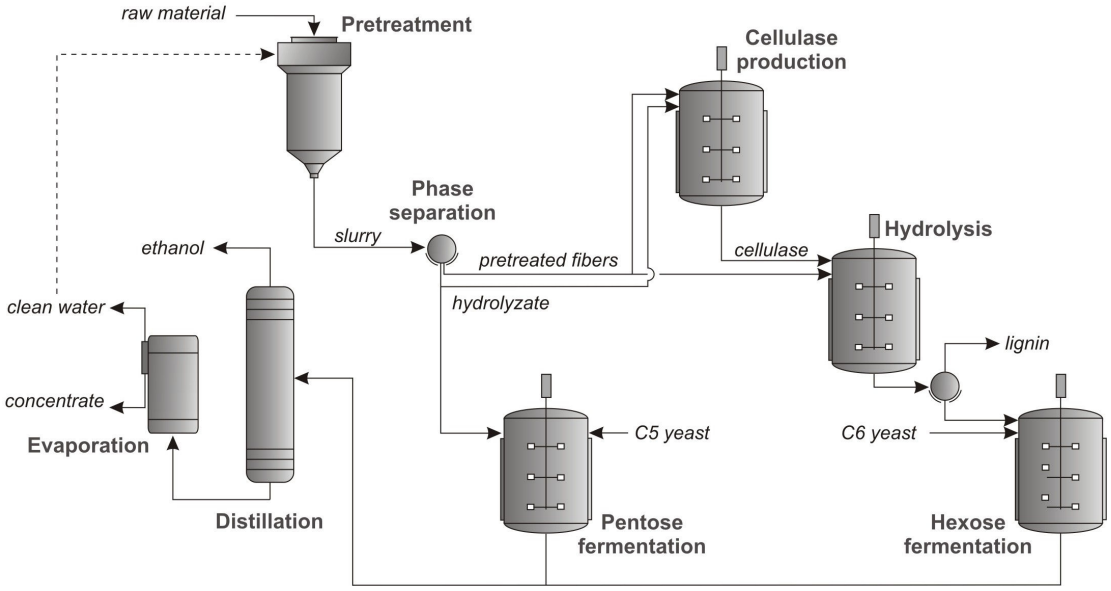

Figure 2 A schematic flowsheet for ethanol production from lignocellulosics.

1.3.3 The novel cellulases of *Melanocarpus albomyces*

Screening for and development of novel enzymes with special characteristics are key issues in current cellulase research [84]. Increased thermostability and tolerance of alkaline pH are some of the most common objectives of the enzyme improvement to provide suitable tools for various cellulase-assisted processes performed at elevated temperature (*e.g.,* high-temperature biomechanical pulping in paper production [85,86]), or pH (*e.g.,* alkaline biostoning during denim treatment [87]), or both (*e.g.,* using enzyme-supplemented detergents in textile and laundry [88,89]). In many of the target processes the essential role of thermostable and/or alkaline cellulases is clearly confirmed, while in other applications, like in the hydrolysis of lignocellulosics, the availability of such cellulases has just recently been identified as a prerequisite for a feasible process design [90,91]. Compared with enzymes from mesophilic sources, higher reaction temperature associated with thermostable cellulases may radically reduce the substrate viscosity in bulk processes, leading to higher reaction velocities and better substrate conversion at lower energy consumption. Contrary to using acid cellulases alone, the integrated use of various cellulases with overlapping pH optima may allow a higher flexibility in pH control during the enzyme treatment leading to substantial savings of chemicals. In addition, because of the non-physiological conditions applicable, the risk of microbial contamination is reduced during the enzymatic process.

At the time being, commercial supply of cellulases to meet these criteria is very limited and the efforts made to identify and characterize novel enzymes with improved features are substantial. In this view, the fungal ascomycete *M. albomyces*, recently reported to produce cellulases with relatively high temperature and pH optima by Miettinen-Oinonen *et al*. [87] is a promising candidate to provide suitable cellulases. However, a common problem that is often encountered with industrial exploitation of novel enzymes produced by natural isolates: the low production level by the native organism, was confirmed for the case. Therefore, heterologous production of the target proteins, a 20-kDa EG (Cel45A), a 50-kDa EG (Cel7A), and a 50-kDa CBH (Cel7B) in a host organism capable of producing high amounts of extracellular enzymes was essential. Single-copy transformants of *T. reesei* lacking its major native cellulases were already able to produce adequate levels of the *M. albomyces* proteins for industrial feasibility evaluation [92]. By the use of multicopy transformants a further improvement in the production level is expected.

Sequence analysis of the *Melanocarpus cel*45A, *cel*7A and *cel*7B genes (accession numbers: AJ515704, AJ515705, AJ515703, respectively) indicated that the encoded family 7 (Cel7A and Cel7B) and family 45 (Cel45A) glycosyl hydrolases - as identified according to the classification of Henrissat *et al.* [93] - lack a consensus cellulose binding module (CBM) and its associated linker [92]. In general, the CBM is known to enhance cellulose hydrolysis by increasing the effective enzyme concentration on the surface of the insoluble substrate [94] and/or promoting the release of a single cellulose chain from the crystalline cellulose surface thus making it available for the catalytic domain of the enzyme [95,96]. Because of this key role of the CBM, it would be interesting to see how these enzymes perform when they carry an exogenous CBM taken from a multidomain-structure cellulase.

2 AIM OF THE PRESENT STUDY

This Ph.D. Thesis, which is a summary of six research papers as introduced before, was meant to provide a broadened view on cellulose degrading enzymes. Therefore, it not only focuses on the well-known cellulase complex of the mesophilic soft rot fungus *Trichoderma reesei*, but also on the recently discovered cellulases of the thermotolerant ascomycete *Melanocarpus albomyces*. Covered fields involve *(i)* the characterization of growth dynamics and enzyme secretion by a real cellulase producer in conventional and modified media using traditional or alternative fermentation substrates, *(ii)* heterologous production and purification of novel cellulases, and *(iii)* characterization of the produced enzymes by their activity, substrate specificity and hydrolysis potential, as the major topics.

3 MATERIALS AND METHODS

3.1 Chemicals

Generic chemicals used for the preparation of solutions and media were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA) with the exception of bacto agar, yeast extract and proteose peptone obtained from Merck (Darmstadt, Germany), malt extract received as a kind gift from Dreher Breweries (Budapest, Hungary) and *D*-glucose obtained from VWR (West Chester, PA, USA). Specialty chemicals of other sources are introduced in the text at their occurrence.

Solutions and media were prepared using distilled water, unless otherwise stated. Those used in molecular biology or fermentation processes were sterilized at 121°C for 20 min before use. For the preparation of samples and solutions used in high performance liquid chromatography (HPLC) and high performance anion exchange chromatography (HPAEC) demineralized water was used.

3.2 Substrates

A selection of cellulose preparations and lignocellulose derivatives was used as the substrate of fermentation and hydrolysis experiments including model cellulose substrates (Solka Floc, Avicel, phosphoric acid swollen cellulose), and technically interesting lignocellulose derivatives (old corrugated cardboard, wet-oxidized corn stover) as detailed below. In fundamental research the use of model substrates simplify the evaluation of results, however from technical point of view the use of various derivatives with less pronounced uniformity in their composition and behavior is essential.

3.2.1 Solka Floc

Solka Floc (SF), a commercial brand of delignified pine pulp with approx 200 μm particle size, was obtained from Fiber Sales & Development (Urbana, OH, USA). The major fragment of SF is highly amorphous and readily digestible by cellulases.

3.2.2 Avicel

Avicel, a commercial brand of microcrystalline cellulose of 50 μm particle size prepared from fibrous cellulose by acid treatment that largely eliminates the amorphous fraction leaving the crystalline particles behind, was obtained from SERVA (Heidelberg, Germany). In water, Avicel forms an extremely stable gel comprised of millions of insoluble microcrystals, digestible - in principle - by CBHs.

3.2.3 Phosphoric acid swollen cellulose

Phosphoric acid swollen cellulose (PASC), a highly amorphous water-soluble cellulose derivative easily accessible by EGs, was prepared from Avicel according to the method of Walseth [97].

3.2.4 Old corrugated cardboard

Old corrugated cardboard (OCC) was collected in the Budapest area of Hungary in 1999. The uncoated, ink-free paperboards were chopped to flocks with a particle size of approx 1 × 10 mm by an electric garden-shredder and stored at room temperature until use. The cellulose content of the air-dried material was 75.9% (wt/wt) as determined by the method of Hägglund [98]. Before utilization, a slurry containing 5% (wt/vol) of OCC was prepared in tap water and pulped for 1 min by a household mixer (750 W) in 500 mL aliquots. The resulting pulp suspension was dosed to culture vessels and thereafter diluted by tap water to final concentration and volume.

3.2.5 Wet-oxidized corn stover

Wet-oxidized corn stover (WOCS) was prepared from *Zea mais* grown in the Somogy area of Southern Hungary in 2000. Collected stovers (comprised of stalks (60%), leaves (25%), and husks (13%) of the maize plant left behind after harvest) were let to dry in a storage room to a dry weight (DW) of approx 95% (wt/wt). The composition of the air-dried material as described by the gravimetric method of Goering and van Soest [99] was (% of DW): cellulose, 41.0; hemicellulose, 33.7; lignin, 20.3; and ash, 4.9. Before processing, the stovers were chopped to get the rigid structure of the stalks broken and then ground to a particle size of approx 2-3 mm. Pretreatment of the ground material by WO was performed in a specially designed 2-L loop autoclave constructed at Risø National Laboratory (Roskilde, Denmark) [64] as described by Varga *et al.* [67]. Briefly, a series of pretreatments using six different combinations of three process variables such as reaction temperature (T), time (t), and pH (Table 1) was applied to corn stover, while two of the process parameters such as the concentration of the raw material (60 g (DW) added to 1 L of demineralized water) and the pressure of oxygen (12 bar at room temperature) were identical in each experimental setup.

Table 1 Applied WO conditions and corresponding nomenclature used to label individual treatments and the products derived from those.

pH	T (°C)	t (min)	Identification
Neutral (pH 7.3)[1]	185	15	Ntr-185-15
	195	5	Ntr-195-5
Alkaline (pH 9.2)[1]	185	5	Alk-185-5
	195	15	Alk-195-15
Acidic (pH 3.5)[1]	185	5	Ac-185-5
	195	15	Ac-195-15

[1] Effective pH in the reactor *before* processing.

Slurries obtained after the oxidative treatments were filtered through a 100-↘m pore-size nylon sieve. Separated filtrates and solids were stored frozen (- 20°C) until processing.

3.3 Production of *T. reesei* cellulases

3.3.1 The strain

The hypercellulolytic soft rot fungus *T. reesei* Rut C-30 (ATCC 56765) used in cellulase production experiments was obtained from the American Type Culture Collection as freeze dried conidia. The strain was maintained at the Department of Applied Biotechnology and Food Science (formerly Department of Agricultural Chemical Technology) at BME on malt agar slants containing 20 g/L of bacto agar, 20 g/L of malt extract, 1 g/L of proteose peptone, and 5 g/L of glucose, at 30°C, with regular subculturing.

3.3.2 Inoculum preparation

To prepare the inoculum a spore suspension was prepared first by adding 5 mL of sterile distilled water to a densely sporulated (15-30-d-old) agar slant and then suspending the conidia in the added water by a sterile loop. On average, the resulting conidial suspension contained approx 2×10^8 spores as determined by a Bürker Counting Chamber.

To initiate inoculum growth in 750-mL Erlenmeyer flasks (E-flasks), 1 mL of the spore suspension was added to 150 mL of growth medium similar to the basic mineral medium of Mandels and Weber [100] (hereafter referred to as Mandels medium) containing (g/L): urea, 0.3; $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2.0; $CaCl_2 \cdot 2H_2O$, 0.4; $MgSO_4 \cdot 7H_2O$, 0.3; and (mg/L): $FeSO_4 \cdot 7H_2O$, 5.0; $MnSO_4 \cdot 7H_2O$, 1.6; $ZnSO_4 \cdot 7H_2O$, 1.4; $CoCl_2 \cdot 6H_2O$, 2.0; together with complex nutrient sources such as proteose peptone (0.75 g/L) and yeast extract (0.25 g/L). As the carbon source 10 g/L of SF was used.

In those studies (hereafter referred to briefly as the optimization experiments) where *(i)* the mode of inoculum preparation, *(ii)* the effect of increasing the carbon supply, and *(iii)* the effect of medium nitrogen (N) were examined as process variables to influence the fermentation performance (Paper I), or when the dynamics of cellulase production by cultures pre-grown on glucose was monitored as a response to cellulose addition (Paper II), the inoculum was prepared in a smaller scale (1 mL of spore suspension added to 75 mL of growth medium in 300-mL E-fasks) with some minor modifications as follows. Urea was omitted from the medium while the concentration of $(NH_4)_2SO_4$ was increased to 2.36 g/L (shake flask experiments) or 1.68 g/L (bioreactor experiments). The carbon source was either 10 g/L of glucose (sterilized separately from the other medium components in calculated amount of water and thereafter fed to the main stream so that to avoid the undesired side reaction of Maillard [101]) or 10 g/L of SF. The nature of the used inoculum (*i.e.,* grown on glucose or SF) varied depending on the purpose of the experiment. For the cultivation conditions applied during the preparation of these inoculum cultures see the data in brackets in the next paragraph.

The pH of the sterilized medium was adjusted to 5.5 (5.0) by the addition of sterile 10% (vol/vol) H_2SO_4 (2 M H_2SO_4) before inoculation and not controlled any further during the cultivation. After 4 d (3 d) of incubation at 30°C (28°C) on a rotary shaker with an agitation rate of 300-350 rpm (200 rpm) the cultivation was completed and the produced inoculum was used to initiate growth in the experimental medium at a volumetric percentage of 10% (2.5%).

3.3.3 Cellulase production in shake flasks

The medium used in the experimental cultivations was - in principle - identical to that used to prepare the corresponding inoculum culture with the following exceptions.

i) In the optimization experiments (Paper I) the concentration of $(NH_4)_2SO_4$ varied between 0.47 to 4.72 g/L when SF was used at a fixed concentration of 10 g/L, or was fixed at the ratio of 2.36 g/L of $(NH_4)_2SO_4$ for every 10 g/L of carbon source when a varying concentration of SF (5 to 50 g/L) was applied. Medium components other than the ammonium-sulfate were added proportionally to the carbon source in all cases.

ii) When the effect of pH on cellulase production was in the focus of exploration (Paper III) Mandels medium was supplemented by various organic acid buffer systems at 0.1 M such as Na-citrate, Na-succinate, Na-maleate, Tris-maleate.

iii) In those experiments where the primary aim was to examine special raw materials as potential fermentation substrates, the medium was prepared using *(i)* the filtrate fraction of WOCS mainly consisting of hydrolyzed hemicellulose (hereafter referred to as WOCS hydrolyzate) in 0.1 M Tris-maleate buffer at 25%, 50%, and 75% (vol/vol) in the medium (Paper IV), or *(ii)* diluted vinasses at 5 g/L and 10 g/L (DW), which was used without supplementation by Mandels nutrients (unpublished results in relation to Paper V). Uniquely in these shake flask studies the media was prepared using tap water.

The carbon source was 10 g/L of SF except *(i)* when the effect of carbon supply on cellulase production was studied and a series of SF concentrations covering a range of 5 to 50 g/L was used (Paper I), and *(ii)* when 13.18 g/L of OCC (equals to 10 g/L of cellulose) to be tested as an alternative carbon source was applied (Paper V).

The cultivations were, in general, performed in 750-mL E-flasks at a total volume of 150 mL (Paper III, Paper IV and Paper V). Exceptions are the optimization experiments carried out in 300-mL baffled E-flasks with a final broth volume of 120 mL (Paper I). The pH of the growth medium was adjusted to 5.0 (Paper I) or 4.5-5.5 (Paper V) before inoculation and a daily control to the same pH was continued thereafter by adding sterile 10% (vol/vol) H_2SO_4 and 10% (vol/vol) NaOH solutions, respectively. Alternatively, various buffering systems were incorporated in the production media to keep the pH at the initially adjusted level of 5.0 or 6.0 (Paper III) and 5.5 or 6.0 (Paper IV) with no further adjustment of pH employed thereafter unless severe fluctuations in the daily measured values were observed. Flasks (duplicates or triplicates for each setup) were incubated on rotary shaker at 28-30➡C with an agitation rate of 200-350 rpm for 7-12 d. Aliquots of culture broth were withdrawn daily and assayed by various methods after the removal of cells and solids by centrifugation (4,000 rpm, 10 min). Reported results are means of independent measurements made to samples taken from duplicate or triplicate cultivations as far as the relative standard deviation was below 10%.

3.3.4 Cellulase production in a 3-L bioreactor

To study the dynamics of cellulase production (Paper I and Paper II) cultivations were preformed in a 3-L Biostat A-DCU300 stirred-tank laboratory bioreactor (B. Braun, Melsungen, Germany) with an operation volume of 2 L. The temperature control of the glass vessel was ensured through an outer belt for electric heating and an internal heat exchanger for water-cooling. The vessel was equipped with a pH electrode (Mettler Toledo, Greifensee, Switzerland), a polarographic oxygen electrode (Mettler Toledo), and a tandem dual gas sensor (Adaptive Biosystems, Luton, UK). Signals from the electrodes and the gas analyzer were logged to a computer every 30 sec.

Cultivations were initiated by inoculum cultures prepared on the same carbon source as found in the vessel, which was either 10 g/L of SF or 10 g/L of glucose. Cultivations were performed at 28°C, with 600 rpm as the agitation speed and 500 mL/min (0.25 vvm) as the aeration rate, at atmospheric pressure. The applied airflow was low enough to avoid excessive foaming but still sufficient to keep the tension of dissolved oxygen (DO) above 15% of the saturation value. The pH of the medium was initially adjusted to and further controlled at 5.0 through the automatic addition of sterile 2 M H_2SO_4 and 2 M NaOH solutions, respectively. Foaming was prevented by the manual addition of filter-sterilized antifoam agent (Sigma Antifoam 289) through a septum by a syringe. Cultivations were continued until the evolution of CO_2 gradually decreased to reach a steady level in the outlet gas of approx 0.2-0.3% (vol/vol). One-stage batch cultivations performed on 10 g/L SF were terminated at this point. Two-stage cultivations initialized on 10 g/L glucose were fed at the same point by 20 g of SF suspended in a calculated amount of water sufficient to bring the broth volume in the bioreactor up to the original volume of 2 L. Assuming that by the end of the first cultivation stage (*i.e.,* at the feeding point) the medium was already depleted for glucose, the added SF was considered to set the carbon source concentration in the vessel at the initial value of 10 g/L again. Nutrients required to support culture growth in the second cultivation stage were *(i)* supplied along SF, or *(ii)* added in a separate feed prior to SF addition in a calculated amount of water, or *(iii)* included in the original medium, which thus contained a doubled set of nutrients at the beginning of the bioprocess.

Frequently withdrawn samples were collected aseptically via a silicon tube driven to the very bottom of the bioreactor using a syringe. Prior to sample withdrawal an aliquot of culture broth was taken and discharged to flush the sampling tube and then a known amount (approx 10 mL) of sample was collected. With the exception of a small portion (approx 1 mL) occasionally taken to read the optical density of the mycelial suspension against distilled water at 660 nm (OD_{660}) [102] samples were subjected to phase separation (4,000 rpm, 10 min) and the collected supernatants were assayed by various methods.

3.3.5 Cellulase production in a 30-L bioreactor

Scale-up enzyme production experiments using OCC as a technically interesting substrate (Paper V) were performed in a 30-L Biostat C-DCU3 stirred-tank laboratory bioreactor (B. Braun) with an operation volume of 20 L. The double-walled stainless steel culture vessel constructed with a closed-loop water circulation system for temperature control was equipped with a combined pH electrode (Mettler Toledo) and a polarographic pO_2 electrode (Mettler Toledo). Signals from the electrodes were logged to a computer every 30 sec.

Cultivations were carried out on 13.18 g/L OCC (equals to 10 g/L cellulose) in Mandels medium using tap water. The pH was initially adjusted to 5.5 and after a transient period when it was not controlled and as a consequence of ammonia consumption and subsequent liberation of protons a drop to pH 3.0-3.5 occurred, the pH was adjusted to and further controlled at 5.5 by the automatic addition of 10% (vol/vol) H_2SO_4 and 10% (vol/vol) NaOH solutions, respectively. Foaming was prevented by the manual addition of sterile Struktol J633 polyoleate antifoam agent (Schill & Seilacher, Hamburg, Germany). Enzyme production was continued for 4 d at 28°C, with 250 rpm as the agitation speed and 5 L/min (0.25 vvm) as the aeration rate, under an overpressure of 1 bar. Samples were withdrawn regularly through a sampling valve that was flushed by an aliquot of fresh culture broth before sample withdrawal to remove the residues of the previously collected sample. Supernatants of centrifuged samples (10,000 rpm, 10 min) were assayed by various methods.

3.4 Heterologous production and purification of *M. albomyces* cellulases

Genes derived from *M. albomyces* (ALKO 4237) isolated by and maintained at Roal Oy (Rajamäki, Finland) coding for the proteins Cel45A, Cel7A, and Cel7B were expressed in a genetically modified strain of *T. reesei* (A33) deficient in its major native cellulases (Δ*cbh1/cel7A*, Δ*cbh2/cel6A,* Δ*egl1/cel7B*, Δ*egl2/cel5A*) as described by Haakana *et al*. [103]. Genes were cloned in two variants: *(i)* using their native coding sequence, and *(ii)* with an exogenous CBM sequence taken from *T. reesei* CBHI/Cel7A attached to the C-terminus of the native coding sequence. Enzyme preparations, hereafter referred to as Cel45A, Cel45A(CBM), Cel7A, Cel7A(CBM), Cel7B, Cel7B(CBM), produced by the corresponding *T. reesei* strains in a lactose based complex nitrogen source medium similar to that described by Joutsjoki *et al*. [104] were studied as culture filtrates (Paper VI) after purification carried out as follows.

Crude enzyme preparations (0.5 g as net protein) were subjected to a heat-treatment (60°C, pH 6.5, 2 h) in order to partially remove background activity that may be present because of the intact minor cellulases native to the expression host (*i.e.,* EGIII/Cel12A, EGIV/Cel61A and EGV/Cel45A of *T. reesei*). Proteins in Cel45A and Cel45A(CBM) preparations were precipitated by ammonium-sulfate added to 70% saturation, and thereafter resolved in 0.018 M Tris-HCl buffer (pH 7.2), which step was not necessary for the other studied enzymes. Samples were then equilibrated in 0.02 M sodium phosphate buffer, pH 6.0, (Cel7A and Cel7B proteins), or in 0.025 M Tris-HCl buffer, pH 7.5, containing 0.25 mM EDTA, (Cel45A proteins), by gel filtration (Sephadex G-25, GE Healthcare, Buckinghamshire, UK) and applied at 3 mL/min to a 100 × 16 mm, i.d., anion exchange chromatography column (HiPrep DEAE Sepharose FF, GE Healthcare) developed with the same equilibration buffer. Elution of Cel7A and Cel7B proteins was performed by an increasing concentration of NaCl using a stepwise gradient from 0 to 0.45 M with a plateau at 0.16 M in the conductivity range of 20-42 mS/cm. The Cel45A proteins were eluted using linear gradient of NaCl from 0 to 0.3 M in the conductivity range of 15-25 mS/cm. Proteins were recovered as a single peak each. Active fractions were pooled to samples and checked for their purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gelelectrophoresis (PhastSystem, GE Healthcare) according to the manufacturer's instructions. Proteins were stained with silver-nitrate (Silver Stain Kit, Bio-Rad Laboratories, Hercules, CA, USA). As the marker a low-molecular-weight calibration kit (LMW) obtained from GE Healthcare was used.

For comparison studies, *T. reesei* CBHI/Cel7A with its native CBM and its catalytic core without the CBM were produced and purified according to Suurnäkki *et al*. [105] and considered as analogues to *M. albomyces* Cel7B(CBM) and Cel7B, respectively.

3.5 Hydrolysis studies

Hydrolysis experiments by home-produced and commercial cellulases with various objectives were carried out in triplicates. Reported results are means of individual measurements made to samples collected from parallel hydrolysis reactions unless the relative standard deviation was over 10%.

3.5.1 Hydrolysis of SF by *T. reesei* cellulases

The dynamics of cellulose degradation by a cellulolytic organism is difficult to follow because of the subsequent consumption of the liberated sugars by the cultured cells. One possibility to do so is to mimic the circumstances (medium composition, concentration of carbon source, enzyme to substrate ratio, pH, T, agitation rate, etc.) found in the culture vessel at the points of interest in *in-vitro* hydrolysis studies using cell-free enzyme preparations.

In this view, two specific points of the two-stage bioreactor cultivations: the point of SF addition, and the point at which the metabolic activity of cultured cells peaked while grown on the added SF, were examined in such experiments (Paper II). Because the presence of the mycelia made the determination of the solid substrate in the fermentation broth impossible by dry weight measurement (other known methods are difficult to carry out), both points of interest were modeled by using 10 g/L of SF, even though it was obvious that by the peak point the substrate concentration was already lower. Comparative hydrolysis studies employing a home-produced enzyme that was prepared by collecting the supernatants (10,000 rpm, 10 min) of 4-d-old *T. reesei* cultures grown on 10 g/L SF in shake flasks under similar conditions to those of the two-stage cultivations (section 4.1.3), and a commercially available cellulase preparation from *T. reesei* (Celluclast 1.5 L, obtained from Novozymes, Bagsværd, Denmark) were carried out in capped flasks with shaking. Enzyme loadings were adjusted accordingly based on the overall cellulose degrading efficiency (section 3.7.1) of the preparations. Dynamics of SF degradation was evaluated through the initial rates of glucose and cellobiose formation as determined from the HPLC analysis (section 3.6.1) of frequently withdrawn samples.

3.5.2 Hydrolysis of WOCS by *T. reesei* cellulases

To study the applicability of WOCS hydrolyzate as a medium additive in cellulase production, the hydrolytic potential of the enzyme mixtures secreted by *T. reesei* while grown in such media prepared by a 75% (vol/vol) blend of Alk-195-15 and Ac-195-15, respectively, was evaluated and compared to that of Celluclast 1.5 L (Paper IV). The substrate to be hydrolyzed was WOCS prepared under previously optimized conditions (195°C, 15 min, alkaline pH) known to produce the easiest digestible solid fraction after WO of corn stover [67]. The dosage of enzymes was identical for the home-produced cellulase and the commercial preparation in terms of their overall cellulose degrading efficiency (section 3.7.1). Due to the lack of sufficient ability to split cellobiose to glucose, which is a common feature of *T. reesei* cellulases [106], both preparations were supplemented by a commercially available β-glucosidase (Novozyme 188 obtained from Novozymes) at a ratio of 1:1 in enzyme units (section 3.7.3).

To make up the hydrolysis experiment, separated WOCS fibers were suspended to 2% (wt/wt) (DW) using a mixture of 0.2 M acetate buffer (pH 4.8) and the pH-adjusted (pH 4.8) supernatant of the fermentation broth. Controls were prepared in a similar manner

but using Celluclast 1.5 L in the properly diluted and pH adjusted (pH 4.8) hydrolyzate (pH 4.8) instead of the home-produced enzyme fermented on the same hydrolyzate. Hydrolysis was performed in magnetically stirred 10-mL test tubes placed in a heating bath. After 24 h of incubation at 50°C the reaction was stopped by boiling the samples for 5 min and thereafter separating the liquid fraction from residual solids by centrifugation (4,000 rpm, 10 min). Concentration of released cellobiose, glucose, xylose and arabinose was determined by HPLC (section 3.6.1).

3.5.3 Hydrolysis of Avicel and PASC by heterologously produced cellulases of *M. albomyces*

Hydrolytic properties of the *M. albomyces* cellulases applied individually and in controlled mixtures were studied using crystalline cellulose (Avicel) and amorphous cellulose (PASC) as the substrates (Paper VI). Hydrolysis of Avicel and PASC was carried out in 50 mM sodium phosphate buffer (pH 6.0) at 50°C with intensive magnetic stirring in Eppendorf tubes at a final volume of 1.8 mL. The substrates (10 g/L) were dosed in the vials as thick suspensions using a pipette. Enzymes were applied as solutions prepared in 50 mM sodium-phosphate buffer (pH 6.0) and loaded as proteins in a dosage of 5, 10, 20 mg/g and 0.5, 1, 2, 5 mg/g protein per substrate when Avicel and PASC was used, respectively. For enzymes carrying a CBM, deduced molecular mass of catalytic cores (*i.e.,* enzymes without CBM) were taken to set enzyme loadings, which ensured an equal molar-based dosage for each enzyme pair (*i.e.,* lacking/carrying a CBM). Hydrolysis reactions carried out individually for each time point were terminated after 1 h, 5 h and 24 h by boiling the whole reaction mixtures for 5 min. Liquid fractions containing the released soluble sugars were separated from the solid residues by centrifugation (5,000 rpm, 5 min) and analyzed for their reducing carbohydrate content (section 3.6.3). Selected samples were checked for their product profile by HPAEC analysis (section 3.6.2).

3.6 Analytical assays

3.6.1 Carbohydrate and organic acid analysis by HPLC

Soluble cellulose derivatives such as cellobiose and glucose generated during cellulase enzyme production on SF (Paper II) and hydrolysis of SF (Paper II) and WOCS (Paper IV), and organic acids present in buffered fermentation media such as citric acid, succinic acid, and maleic acid (Paper III) were determined by HPLC after filter-purification of samples through a 0.2 μm pore-size mixed cellulose ester filter (Schleicher & Schuell, Dassel, Germany). Components were separated on an Aminex HPX-87H column (Bio-Rad Laboratories) at 65°C and detected by a refractive index detector (Shimadzu, Kyoto, Japan). The mobile phase was 5 mM H_2SO_4 used at a flow rate of 0.5 mL/min. For instrument control and data handling UniPoint software (Gilson Inc., Middletown, WI, USA) was used. Reported results are means of triplicate measurements with a relative standard deviation below 5%.

3.6.2 Carbohydrate analysis by HPAEC

Product profile of selected samples collected during the hydrolysis of PASC by *M. albomyces* Cel7A, Cel7B, and *T. reesei* CBHI (Paper VI) were examined by HPAEC after filter-purification of samples through a 0.45 μm Acrodisc syringe filter with nylon membrane (Pall Corporation, Ann Arbor, MI, USA). For the ion-exchange a CarboPac PA-1 column (250 × 4 mm, i.d.) was used (Dionex, Sunnyvale, CA, USA). The eluents for gradient analysis of glucose (G1), cellobiose (G2), cellotriose (G3), cellotetraose (G4), and cellopentaose (G5), were *A*: 150 mM NaOH, and *B*: 500 mM sodium-acetate in 150 mM NaOH pumped with a flow rate of 1 mL/min. The linear gradient was from 90% *A* in 10% *B* to 100% *B* in 15 min and thereafter back to the initial ratio (90% *A* and 10% *B*) in 2 min. The mobile phases were filtered with 0.45 μm GH Polypro membrane filters (Pall Corporation) and degassed using helium before use. The column was stabilized for 10 min between separate injections of 10 μL each. To detect the hydrolysis products, a digital electrochemical amperometric detector equipped with a gold electrode (Antec Leyden, Zoeterwoude, The Netherlands) was used. For the identification and quantification of G1-G5 carbohydrates authentic standards were used. For instrument control and data handling Millenium software (Waters Corporation, Milford, MA, USA) was used. Reported results are means of triplicate measurements with a relative standard deviation below 5%.

3.6.3 Analysis of WOCS hydrolyzate

Carbohydrates in the WOCS hydrolyzates were quantified as monosaccharides gained after dilute sulfuric acid hydrolysis by 4% (wt/wt) H_2SO_4 at 121°C for 10 min (Paper IV). Glucose, xylose and arabinose were quantified both in native and acid-treated samples by HPLC using the method described in section 3.6.1.

Formic acid, acetic acid, 2-furfural, and 5-hydroxy-methyl-2-furfural, were determined and quantified by HPLC using the same method as that employed for the carbohydrate analysis.

Phenols were selectively extracted on a polystyrene divinylbenzene polymer column (International Sorbent Technology, Mid Glamorgan, UK) and quantified by gas chromatography (Hewlett Packard GC 6890) using a HP-5 column (crosslinked 5% phenyl-methyl-siloxane, 30 m × 0.32 mm, i.d., 0.25 μm film thickness) obtained from Agilent Technologies (Palo Alto, CA, USA) with flame ionization detection as described by Klinke *et al*. [107].

3.6.4 Determination of reducing carbohydrates

Reducing carbohydrates (RC) were determined by the dinitrosalicylic acid (DNS) method as described by Miller [108]. The assay employed 3 mL of DNS reagent added to 1.5 mL of the appropriately diluted sample in aqueous solution. The color obtained after boiling the mixture for 5 min and then diluting with 16 mL of distilled water was evaluated by reading the absorbance at 550 nm. An alternative but very similar DNS procedure employed to measure reducing carbohydrates (Paper VI) was according to the method Sumner and Somers [109] assaying 2 mL of sample and employing a colorimetric evaluation of undiluted reaction mixtures at 540 nm. Total reducing carbohydrates were estimated as glucose equivalents by a comparison to adequate standards (containing 0.1-1.5 mg of *D*-glucose in a total volume of 1.5 mL) processed similarly to the samples.

3.6.5 Determination of glucose

Concentration of glucose in selected samples taken during the 3-L bioreactor cultivations initiated on glucose (Paper I and Paper II) was determined by an enzymatic glucose test kit (Boehringer, Ingelheim, Germany).

3.6.6 Determination of ammonia

Ammonia, and eventually nitrogen concentration in the experimental medium during the optimization experiments (Paper I) was determined by an enzymatic ammonia test kit (Boehringer).

3.6.7 Determination of proteins

Proteins in the enzyme preparations of *M. albomyces* origin were determined by the method of Lowry *et al*. [110] using bovine serum albumin as the standard after precipitation with 3 volumes of chilled acetone (4°C) and subsequent resolvation from the precipitate (Paper VI).

3.7 Enzyme assays

3.7.1 Determination of filter paper activity

To describe the overall (absolute, non-specific) potential of the produced cellulase complex to hydrolyze cellulose, filter paper degrading activity (FPA) of samples taken from shake flask and bioreactor cultures was determined by the method of Mandels *et al*. [111]. A 1 × 6 cm strip of Whatman No.1 filter paper (approx 50 mg cellulose) obtained from Whatman Laboratories (Kent, UK) was employed as the cellulosic substrate to be hydrolyzed by 0.5 mL of appropriately diluted sample (culture supernatant) in 1.0 mL of 0.05 M citrate buffer (pH 4.8). After 60 min of incubation at 50°C the hydrolysis was terminated by the addition of 3 mL of DNS reagent. Reducing carbohydrates liberated from the substrate during the assay were estimated as glucose equivalents by the method of Miller [108] against a blank prepared similarly to the samples but in the absence of the substrate. One filter paper unit (FPU) was defined as the amount of enzyme to release 1 μmol of glucose per minute under the assay conditions. Activities were reported as FPU/mL. The same assay was employed to determine the overall cellulase activity of the commercially available Celluclast 1.5 L used in comparative hydrolysis studies (Paper II and Paper IV).

3.7.2 Determination of cellulase activities

EG activity of the *M. albomyces* cellulases (Paper VI) was measured as the release of RC according to Sumner and Somers [109] from hydroxyethyl cellulose (HEC) and carboxymethyl cellulose (CMC) as described by Bailey and Nevalainen [112], except that pH 6.0 was used.

3.7.3 Determination of ß-glucosidase activity

The β-glucosidase activity of the home-produced enzymes (Paper III, Paper IV, and Paper V) was determined according to the method of Berghem and Pettersson [113] with some minor modifications using 1 mM *p*-nitrophenyl-β-*D*-glucopyranoside in 0.05 M citrate buffer (pH 4.8) as the substrate and *p*-nitrophenol as the standard. The assay mixture contained 100 μL of enzyme (or 100 μL of distilled water for the blank and the standards) added to 1.0 mL of the substrate preheated at 50°C for 1 min. After 10 min of incubation at 50°C the enzymatic reaction was terminated by the addition of 2 mL of 1 M Na_2CO_3 and the reaction mixture was diluted by 10 mL of distilled water. The amount of *p*-nitrophenol liberated during the assay was determined by reading the absorbance at 400 nm against a blank and evaluated by a comparison to adequate standards prepared with 1 mL of 0.2 to 1.0 mM of *p*-nitrophenol instead of the substrate solution, all processed similarly to the samples. One international unit of β-glucosidase activity was defined as the amount of enzyme to release 1 μmol of glucose per minute under the assay conditions. Activities were reported as IU/mL. The same assay was employed to determine the β-glucosidase activity of the commercially available Novozyme 188 preparation used in comparative hydrolysis studies (Paper IV).

4 RESULTS AND DISCUSSION

4.1 Cellulase production by *T. reesei*

The concepts and ideas leading to process advancements can be suitable tested in shake flasks and later confirmed in stirred bioreactors. For this reason, most of the presented experiments (many of them are preliminary investigations) were performed in shake flasks.

Regardless of the diversities in cellulase production profiles reported in this chapter as the consequence of various effects, the general pattern of enzyme secretion - except for the two-stage cultivations - was basically uniform and followed an ordinary sigmoid curve.

4.1.1 Effect of carbon source

Dilemma concerning the carbon source (also referred as the substrate) of cellulase production by *Trichoderma* is manifold. *(i)* One important issue to consider is that the applied carbon source should not cause carbon catabolite repression (like *e.g.*, glucose usually does via repressing the operon responsible for cellulose metabolism in favor of glucose utilization when glucose is the predominant carbon source in the environment of the cell), but should preferably act as an inducer instead (like *e.g.,* cellulose via cellobiose and sophorose, or lactose do) to trigger the synthesis of these inducible enzymes. *(ii)* Interestingly, the nature of the substrate (*i.e.,* inducing or non-inducing) may be crucial in the inoculum preparation too, as it has been reported to have a remarkable effect on the fermentation performance on the long run [114]. *(iii)* Another factor to keep in mind when thinking of cellulase production at larger scales is that substrate-related expenses should not ruin the economics of the process, which, in fact, can easily happen by using purified celluloses such as the frequently used SF, Avicel, or cotton [24,115,116], all being far too expensive to serve as the carbon source of a bulk technology to be commercialized. Soluble substrates such as glucose and lactose may be used if their cost is justified by sufficiently high yields and productivities, as far as their use is not hampered by other means. In this view, one should strongly consider the warning of Kadam pointing out that lactose may not be available in sustainable quantities for an industrial-scale production [117], or the fact that improved strains reported to be resistant to carbon catabolite repression are still much less efficient on glucose than on cellulose [30]. As most investigators agree, a viable and cost-effective alternative for cellulase production at large-scale would be the exploitation of cellulosic wastes and by-products that are available in large volume at low or zero price. *(iv)* Once, the carbon source to be used as the fermentation substrate is qualified, it needs to be quantified too. This task is especially tricky when a solid substrate is used, what is foreseen to be the general case in the processes under discussion, because the more pronounced mass transfer limitations attributable to higher solid contents do not allow the explicit improvement of process performance by the simple means of increasing the substrate concentration.

In correspondence to the considerations above, this chapter focuses on the effect of inoculum cultivation and substrate concentration on the performance of cellulase production, while novel investigations on the applicability of alternative raw materials to replace and/or supplement conventional substrates are summarized in section 4.1.2 and section 4.1.6.

The mode of inoculum cultivation had a clear effect on the dynamics of enzyme production and the obtained cellulase activities and yields (Figure 3). By all measures, the performance of cellulase production by cells pre-cultivated on an inducing substrate (SF) was better than by those pre-cultivated on a non-inducing substrate (glucose). Similarities in the dynamics of enzyme secretion were higher between cultivations initiated by similar inocula regardless that the applied substrate concentration was unequal (5-50 g/L), than between cultures cultivated at equal carbon source loadings but initiated by different inocula, which phenomenon was especially pronounced in initial enzyme production rates (Figure 3 A). Obtained cellulase activities improved significantly when the inoculum used to initiate growth in the production medium was grown on SF instead of glucose, with an increase in the obtained FPA by 18% being the lowest (at a SF concentration of 50 g/L) and by 43% being the highest (at a SF concentration of 20 g/L) improvement achieved after 6 d of cultivation (Figure 3 B). Interestingly, the inoculum effect was as strong as to modify the general array in obtained FPA, which normally (*i.e.,* when not affected by other means) followed the order of the applied substrate concentration. As an example, obtained FPA on 30 g/L SF by a culture initiated with SF grown inoculum was roughly equal to that obtained on 50 g/L SF by a culture initiated with grown inoculum (Figure 3 B). Further observations on the long-term effect of the inoculum cultivation are reported in section 4.1.4.

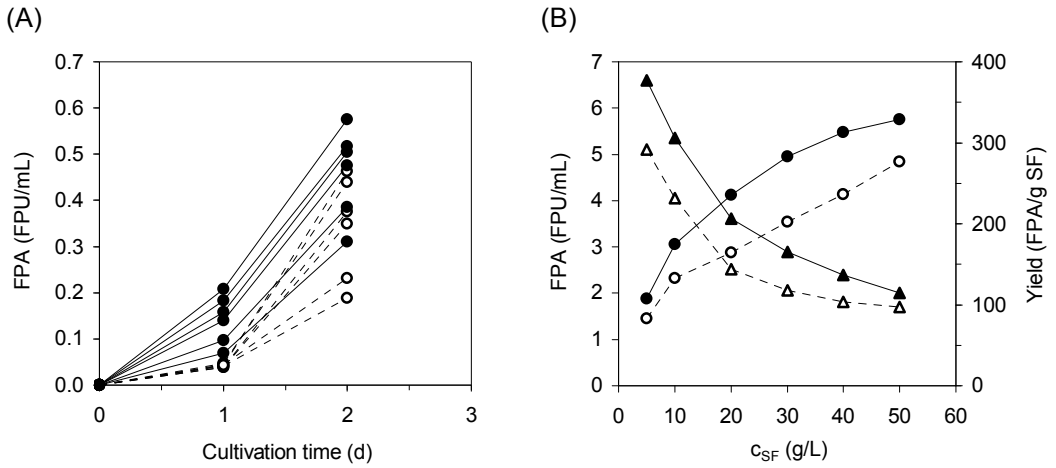

Figure 3 The effect of inoculum cultivation and substrate dosage on the dynamics (A) and performance (B) of cellulase production by *T. reesei* cultivated in shake flasks on 5 to 50 g/L SF in a modified Mandels medium (pH 5.0, 28°C) using glucose grown (open symbols, dashed line) and SF grown (closed symbols, solid line) inoculum cultures, respectively. (A): Initial enzyme production rate as demonstrated via produced FPA (---○---, —●—) plotted *vs* cultivation time. The order of curves within the same experimental series, *i.e.,* similar inculum cultures used, followed the order of the applied substrate concentration and therefore individual cultivations with varying substrate loadings are not distinguished by different labeling. (B): Cellulase activity (---○---, —●—) and corresponding yield (---Δ---, —▲—) obtained after 6 d of cultivation plotted *vs* SF concentration.

The improvement in obtained cellulase activities observed after 6 d of cultivation (in FPU/mL from 1.46 to 4.85, and from 1.89 to 5.75, for cultures initiated with glucose grown and SF grown inoculum, respectively) was not proportional to the increase in the applied SF concentration (from 5 to 50 g/L) causing a severe drop in obtained yields (in FPU/g SF from 292 to 97, and from 377 to 115, respectively) (Figure 3 B). To remain accurate, however, it is important to emphasize that cultivations performed at higher substrate loadings were not

completed in 6 d, and therefore the obtained results are adequate only for comparative analysis but not for quantitative evaluation. The reason for not running these cultures any longer was the significant loss of volume due to sampling and evaporation, which on the longer run would ruin the reliability of results at an even larger extent.

Not only because the product yield is one of the most important measures to label the efficiency of a fermentation process, but also because at higher solids mass transfer limitations due to insufficient mixing was obvious, which was in fact seen as the primary reason to cause the decline in yield, it was decided to set the substrate concentration at 10 g/L in further experiments. Nevertheless, the option of using an increased carbon source concentration is not to be completely rejected, especially because from a technological point of view it is a compulsory step towards maximizing the product output in a given volume within a given time-scale (productivity). What is more, at bioreactor-scale mass transfer limitations are less determinative and occur at higher solids. For example, Hayward *et al*. experienced that a suspension of 50 g/L of SF was already too thick to achieve effective mixing and aeration in shake flasks [118], while Hendy *et al*. reported that a similar suspension could successfully be applied in a bioreactor cultivation without limitation [119].

4.1.2 Substitution of solid carbon source with liquid carbon source

To improve mass transfer properties and thereupon increase the productivity, it would be desirable to obtain cellulase production on soluble oligomeric carbohydrates and/or monosaccharides. Relation between enzyme production and growth profile by the cultured organism is a key for designing such processes (section 4.1.3). Another issue to consider is the nature of the soluble carbohydrate to be used as the sole or supplementary carbon source of the fermentation process, which should be selected with a critical view on cost factors. Utilization of the solubilized hemicellulose gained after the pretreatment of lignocellulosics is recognized as an attractive alternative to produce cellulases, which would answer several other issues too, as detailed previously (section 1.3.2).

In accordance with these considerations, hemicellulose derived hydrolyzates gained after the pretreatment of corn stover by WO under six different combinations of reaction temperature, time, and pH (section 3.2.5), were examined and compared for their applicability as the supplementary carbon source in cellulase production by *T. reesei* grown on SF as the primary carbon source. To establish a suitable process scheme applicable to all WOCS hydrolyzates during the comparative enzyme production experiments, preliminary test fermentations were carried out with one sample appointed as the model, which was chosen upon the presumption that the cultivation parameters optimized for this sample would suit the other samples too. Due to the harsh pretreatment they went through (*i.e.,* higher reaction temperature, longer processing time, non-neutral conditions), Alk-195-15 and Ac-195-15 contained the highest concentration of inhibitory compounds such as carboxylic acids and furan derivatives from sugar degradation, and phenol monomers from lignin degradation (Table 2), and were therefore recognized as reasonable choices to serve as the model sample. The final decision of using Alk-195-15 in the preliminary fermentation tests was made with a practical consideration involved. Namely, it was shown previously by Varga *et al.* [67] that separated cellulose fibers from the corresponding pretreatment (Alk-195-15) performed the best in their enzymatic degradability to soluble carbohydrates by cellulases and subsequent fermentation of the liberated sugars to ethanol, and therefore this WO process is the most likely to gain commercial interest. Consequently, utilization of the corresponding WOSC hydrolyzate from the same pretreatment is the most likely to become a real issue.

Table 2 Concentration (mg/L) of inhibitory compounds in WOCS hydrolyzates gained after pretreatment under harshest conditions: Alk-195-15 and Ac-195-15, as determined by HPLC and GC, respectively (section 3.6.3).

	Alk-195-15	**Ac-195-15**
Formic acid	1,471	1,804
Acetic acid	1,863	2,289
2-furfural	173	203
5-hydroxy-methyl-2-furfural	6	33
Phenol	6	4
Guaiacol	12	13
Syringol	5	5
Total phenols	*23*	*22*
4-hydroxybenzaldehide	54	102
Vanillin	57	74
Syringaldehide	20	38
Total phenol aldehydes	*131*	*215*
4-hydroxy benzylalcohol	0	0
Vanillyl alcohol	0	0
Syringyl alcohol	0	0
Total phenol alcohols	*0*	*0*
4-hydroxyacetophenone	8	8
Acetovanillone	13	58
Acetosyringone	18	0
Total phenol ketones	*40*	*65*
2-furoic acid	7	4
4-hydroxy benzoic acid	51	54
Vanillic acid	55	57
Homovanillic acid	12	28
Syringic acid	28	42
Coumaric acid	32	35
Ferulic acid	7	9
Total phenol acids	*192*	*228*

Preliminary studies with 20% (vol/vol) Alk-195-15 in the medium suggested the necessity to use an inert buffer system for pH control, in order to avoid limitations in growth and enzyme production attributable to fluctuations in pH (Figure 4 A). Even though the employed 0.1 M Tris-maleate was not able to control the pH strictly in the desired range when the hydrolyzate content was increased to 75%, which, in part, caused a considerable drop in obtained FPA (in FPU/mL to 0.4 from 0.9 and 0.7, that were obtained with 25% and 50% Alk-195-15 in the medium, respectively), it was concluded that a hydrolyzate content of 75% was still tolerable by *T. reesei* under the applied experimental conditions (Figure 4 B) and can therefore be used in comparative fermentation studies. Although of interest from technological point of view, the complete replacement of water by WOCS hydrolyzate was rejected, as it caused a long adaptation period (3 d) and resulted in a low FPA obtained after 6 d of cultivation (0.25 FPU/mL), both considered as clear signs of poor process performance (graphs not presented). These observations are in perfect agreement with the findings of Thygesen *et al.* [120], who experienced a moderate enzyme production when cultivating *T. reesei* in undiluted WO hydrolyzate of wheat straw. Nevertheless, the complete replacement of process water by hydrolyzate may still remain a goal at production scale, where the pH issue due to the availability of automatic control techniques does not represent such a serious obstacle.

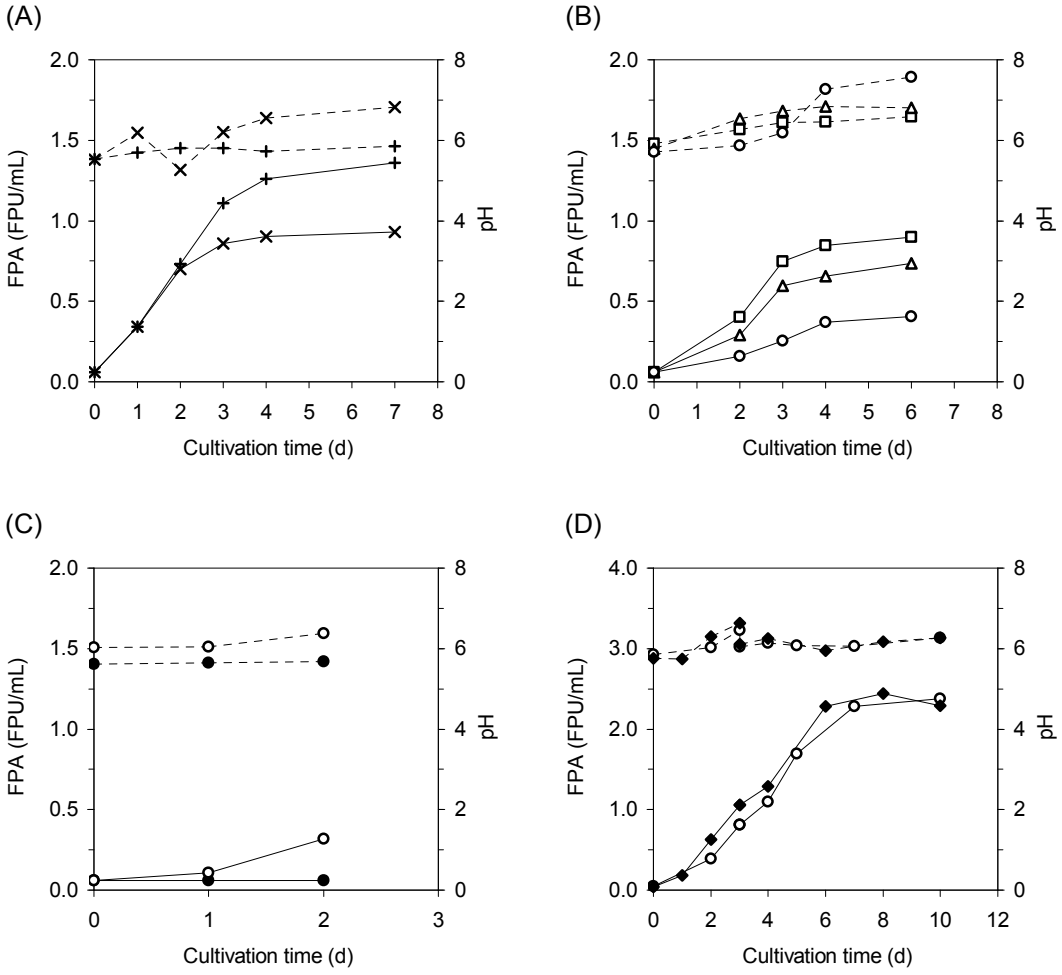

Figure 4 Cellulase production by *T. reesei* in shake flasks (30°C) on 10 g/L SF in Mandels medium supplemented by Alk-195-15, as monitored via obtained FPA (solid line) and pH (dashed line) plotted *vs* cultivation time. (A): The effect of using 0.1 M Tris-maleate buffer (+) in the fermentation medium (pH 6.0) compared to the non-buffered case (×), at a hydrolyzate content of 20% (vol/vol). (B): Fermentation performance using 25% (□), 50% (Δ), and 75% (○) hydrolyzate (vol/vol) in a buffered medium (0.1 M Tris-maleate, pH 6.0). (C): The effect of pH on the initial fermentation performance in a medium containing 0.1 M Tris-maleate and 75% (vol/vol) hydrolyzate at pH 5.5 (●) and pH 6.0 (○). (D): The effect of increasing the concentration of nutrient compounds in a buffered (0.1 M Tris-maleate, pH 6.0) medium proportionally to the increase in carbohydrate content due to the presence of 75% (vol/vol) hydrolyzate (♦), compared to the basic case where no increase was applied in the nutrient content (○).

To determine the most appropriate pH for the cultivation known to be dependent largely on the nature of the carbon source used [121], one should strongly consider the observations of Szengyel *et al.* [122], pointing out that the application of a higher pH is of favor by *T. reesei* when cultivated on derivatives of pretreated lignocellulosics. Nevertheless, in the given case it was out of option to control the pH at an even higher level, because the force to push the pH towards alkaline - as a possible consequence of the consumption of carboxylic acids from the hydrolyzate by the cultured cells - was as strong already at pH 6.0 as to unable the compelling buffer to save the culture from experiencing a hardly tolerable alkaline pH (Figure 4 B). The tendency towards turning alkaline is especially interesting if one considers that an opposite pH pattern (*i.e.,* growth-associated *drop* in pH due to the depletion of ammonia from the medium

and liberation of protons [100,123]) is the norm when *T. reesei* is cultivated in Mandels medium. Controlling the pH at a lower value was not a suitable alternative either, as no sign of microbial activity could be observed in 2 d of cultivation when pH 5.0 was employed (Figure 4 C). In contrast, the use of pH 6.0 resulted in an active secretion of cellulases already by the second day of cultivation (Figure 4 C) and because this alternative has previously proven its potential on the longer run too (Figure 4 B), pH 6.0 was chosen as the target condition in the comparative fermentation studies. The pH control was ensured by 0.1 M Tris-maleate buffer (pH 6.0) in the production medium and supplementary manual adjustment when necessary, *i.e.,* when severe fluctuations in pH were observed. This, if occurred at all, coincided with the mind-term of exponential phase of fermentation as it will be shown later (Figure 5). Even though the hydrolyzate represented an additional source of consumable carbohydrates in the production medium, the nutrients loaded according to the original recipe of Mandels and Weber [100] valid for 10 g/L carbon source were able to cover the need of the cultured organism and therefore no proportional increase in their concentration was necessary (Figure 4 D).

Comparative fermentation studies on 10 g/L SF using 75% (vol/vol) hydrolyzate in buffered (0.1 M Tris-maleate, pH 6.0) Mandels medium resulted in ordinary sigmoid curves for the produced cellulases, that were basically similar for all WOSC hydrolyzates (Figure 5). Major differences were however observed in the appearance of the adaptation period, which was not clearly detected (was less than 1 d) when using Alk-185-5 (Figure 5 C) and Ac-185-5 (Figure 5 E), but was extremely long in the case of using Ac-195-15 (4 d), as a possible consequence of the inhibitory compounds present in Ac-195-15 in the highest concentration (Table 2). The maximum FPA obtained was in the range of 2.1-2.7 FPU/mL for the various samples, which was significantly higher than that produced by the control (1.85 FPU/mL). Unfortunately, the FPA yield (Table 3) obtained in the experimental cultivations did not exceed the yield by the control, with one single exception (Alk-185-5).

Although the obtained FPA provides sufficient information on the absolute cellulose degrading efficiency of a cellulase preparation and was therefore seen as a reliable measure to evaluate and compare the applicability of WOCS hydrolyzates for the given task, it was also reasonable to check the produced enzyme preparations for their β-glucosidase activity due to the important role this enzyme has in the complete degradation of cellulose [124]. In general, the produced β-glucosidase activity was close to the achieved maximum (obtained in 14 d) already after 8 d of cultivation (graphs not presented), which was in the range of 1.3-1.7 IU/mL for the various samples (Table 3). As expected from the FPA results, the maximum yield for β-glucosidase was obtained with Alk-185-5 (121 IU/g carbon source), which was comparable to that obtained with the control (126 IU/g carbon source). This result is especially noteworthy when considered with a view on the findings of Thygesen *et al.*, who experienced a β-glucosidase activity close to zero when *T. reesei* was grown in the undiluted hydrolyzate of WO treated wheat straw using the suspended solids gained from the same pretreatment as the carbon source, while three other cellulolytic fungi produced significant amounts of β-glucosidase in similar experiments [120].

The fact that no clear correlation was seen between the maximum activities obtained and the amount of extra carbohydrate available from the hydrolyzate may be a consequence of the unwelcome coupling between the higher concentration of solubilized sugars in and the higher level of formed inhibitors during the WO treatments.

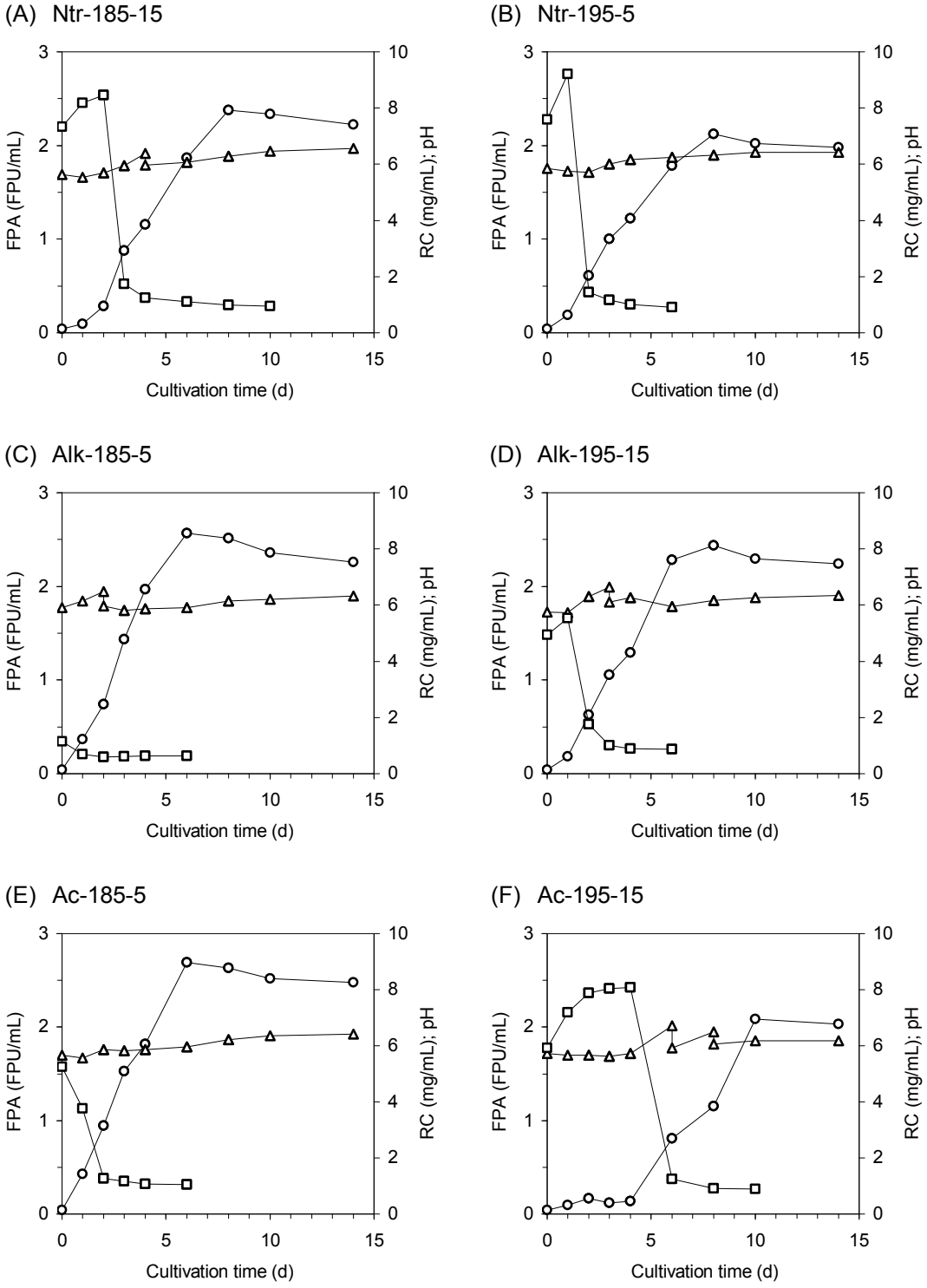

Figure 5 Produced FPA (—○—), concentration of reducing carbohydrates (—□—), and pH (—Δ—) plotted *vs* cultivation time during cellulase production by *T. reesei* in shake flasks (30°C) on 10 g/L SF in a buffered (0.1 M Tris-maleate, pH 6.0) Mandels medium supplemented by various WOCS hydrolyzates such as (A): Ntr-185-15, (B): Ntr-195-5, (C): Alk-185-5, (D): Alk-195-15, (E): Ac-185-5, and (F): Ac-195-15, at a volumetric ratio of 75%.

Table 3 The carbohydrate content of WOCS hydrolyzates as determined by HPLC and their performance during cellulase enzyme production by *T. reesei* on 10 g/L SF in shake flasks (30°C) in a buffered (0.1 M Tris-maleate, pH 6.0) Mandels medium prepared with 75% (vol/vol) hydrolyzate compared to a control containing no hydrolyzate.

	Ntr-185-15	**Ntr-195-5**	**Alk-185-5**	**Alk-195-15**	**Ac-185-5**	**Ac-195-15**	**Control**
Carbohydrate content of WOCS hydrolyzates							
Total soluble sugars (g/L)[1]	13.7	10.7	3.9	13.8	10.5	14.1	-
Glucose (g/L)	*1.8*	*0.9*	*1.1*	*1.9*	*0.7*	*1.4*	-
Xylose (g/L)	*10.0*	*7.8*	*2.0*	*10.1*	*9.0*	*10.7*	-
Arabinose (g/L)	*1.9*	*2.0*	*0.8*	*1.8*	*0.8*	*2.0*	-
Monomeric sugars in native hydrolyzate (g/L)[2]	2.5	1.8	0.4	2.0	1.9	3.3	-
Oligomeric sugars in native hydrolyzate (g/L)[3]	11.2	8.9	3.5	11.8	8.6	10.8	-
Substrates in the medium							
SF (g/L)	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Net carbohydrate concentration (g/L)[4]	21.2	19.1	13.9	21.3	18.9	21.6	11.0
FPA by secreted cellulases							
Maximum value obtained (FPU/mL)	2.38	2.12	2.57	2.44	2.69	2.09	1.85
Time point of maximum value reached (d)	8	8	6	8	6	10	8
Yield (FPU_{max}/g carbon source)	112.1	111.3	184.8	114.3	142.6	96.9	168.2
β-glucosidase by secreted cellulases							
Maximum value obtained (IU/mL)	1.38	1.31	1.69	1.49	1.52	1.25	1.39
Time point of maximum value reached (d)	14	14	14	14	14	14	14
Yield (IU_{max}/g carbon source)	65.0	68.7	121.5	69.8	80.6	58.0	126.4

[1] Total soluble sugar quantified as monosaccharides (glucose, xylose, and arabinose) after acid hydrolysis (4% (wt/wt) H_2SO_4, 121°C, 10 min) of WOCS hydrolyzates.
[2] Monomeric sugars in native (non-hydrolyzed) WOCS hydrolyzates as a sum of free glucose, xylose, and arabinose.
[3] Oligomeric sugars in the native WOCS hydrolyzates calculated as total soluble sugars in the acid treated hydrolyzate minus monomeric sugars in the native hydrolyzate.
[4] The cellulose (SF) content is considered in the calculations as glucose equivalents.

The profile obtained for the concentration of reducing carbohydrates correlated well to the cellulase secretion pattern for all WOCS hydrolyzates examined (Figure 5). In cultures with a clearly distinguished adaptation period an initial increase in the concentration of soluble sugars as monitored via RC measurement was observed, which is not surprising if one considers that the enzymes loaded with the inoculum (corresponding to an initial activity of approx 0.05 FPU/mL in the whole broth) presumably started their action immediately on the easily degradable fraction of SF and the soluble carbohydrates from the hydrolyzate (Table 3), but during this adaptation period the culture failed to consume the liberated sugar monomers at a comparable rate. A sharp drop in the concentration of reducing sugars after 1 to 4 d of cultivation indicated the completion of the adaptation period, which was followed by a switch to logarithmic growth and increased rate of cellulase secretion. In those experiments, where the adaptation period characterized by a moderate metabolic activity could not be detected by the employed daily control (*i.e.,* the adaptation period was shorter than 1 d), the uptake of consumable sugars by the cultured cells was more rapid for the entire length of fermentation than their release by the enzymatic action and therefore no accumulation of soluble sugars in the cultivation broth occurred (Figure 5 C and E). On the contrary, culture growth and cellulase production was rapid already from the very beginning of the cultivation resulting in the highest FPA and β-glucosidase activities and yields obtained (Table 3). Presumably, the lower pretreatment temperature and shorter processing time applied during WO made these hydrolyzates the most easily tolerable by the cultured *T. reesei*.

As mentioned previously, Varga *et al.* [67] found that WOCS fibers gained from Alk-195-15 performed the best in a lab-scale corn stover to ethanol process using commercial enzymes in the hydrolysis of pretreated solids and hence it was reasonable to check how the home-produced enzymes act on the same substrate in a similar experiment (section 3.5.2). A comparison test using the cellulase preparation obtained on the WOCS hydrolyzate derived from the same pretreatment (Figure 5 D) gave promising results; using an enzyme dosage of 25 FPU/g of substrate (DW) the conversion of cellulose to glucose was 71%, which was only a slightly lower than that obtained by the commercial preparation (79%).

4.1.3 Dynamics of culture growth and cellulase production on glucose and added cellulose

The relation between enzyme production and growth profile by the cultured organism must be well understood for both fundamental and applied research curiosities. The enzyme production is easy to follow thanks to the numerous assays available to determine the individual and sum activities of various cellulase components [111,125,126]. The growth of the culture growth is also simple to monitor via the measurement of optical density and/or determination of cell mass [127] as far as the fermentation broth remains free from solids other than the cultivated cells. According to the most likely process design this will not be the general case in future large-scale cellulase production technologies to be primarily performed on solid substrates of lignocellulose origin [128,129], and consequently, neither is it the case in corresponding research continued to explore industrial issues. However, predictions are serious that on the long monosaccharides may gain commercial interest to supplement or replace solid cellulosic substrates (see section 4.1.2 for considerations in concern) and therefore the use of a soluble carbon source in experimental cultivations is not only reasonable from a practical point of view (as it makes culture propagation easier to observe) but from a long-term technological perspective too. N.B., the primary carbon source used most widely at the moment for cellulase production, lactose, is also soluble.

To characterize the dynamics of culture growth and cellulase production by *T. reesei*, the strain was grown in batch cultures in two stages using glucose as the carbon source in the initial stage to produce cell mass and SF in the second stage to observe cellulase synthesis in response to the pulse-addition of an inducing substrate. The metabolic activity of cultured cells was followed via the on-line measurement of CO_2 evolution, which corresponded well to the DO level found in the vessel (data not presented) also known to be suitable to monitor the propagation of a microbial fermentation. Cellulase production was monitored via the FPA represented by the culture. A basic two-phase aerobic batch culture is shown in Figure 6 A.

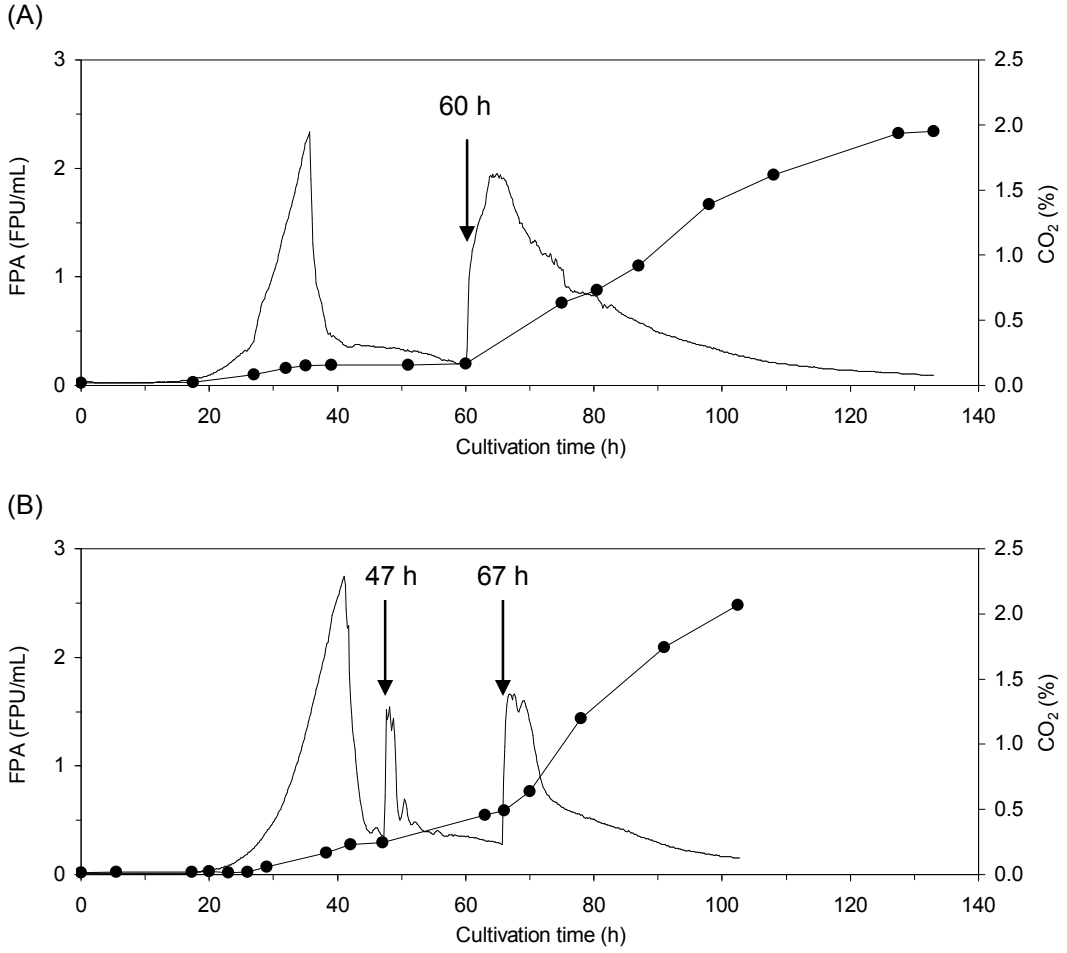

Figure 6 CO_2 concentration in the outlet gas (—) and produced FPA (—●—) plotted *vs* cultivation time during cellulase fermentation by *T. reesei* in a 3-L laboratory bioreactor in Mandels medium (28°C, pH 5.0). The initial growth medium contained 10 g/L of glucose as the carbon source. At (A): t = 60 h or (B): t = 67 h SF was added to a final concentration of 10 g/L. Nutrients required to support growth in the second fermentation stage were added (A): along SF at t = 60 h, or (B): prior to SF addition in a separate feed at t = 47 h.

Batch fermentations on glucose (first cultivation stage) followed a classic growth profile as evaluated via the off-gas CO_2 signal. After a relatively long *lag* phase of approx 20 h a sudden increase in metabolic activity occurred and the culture entered the exponential growth phase, identified by the rapid development of fungal biomass. After a sharp peak in the concentration of CO_2 recorded in the outlet gas within the next 20 h, a rapid decrease in CO_2 evolution occurred leaving some residual CO_2 formation. The decline of growth was considered as a consequence of the depletion of a key ingredient - in ideal case the carbon source - from the

fermentation broth. Pulse-addition of SF at t = 60 h together with the medium components required to support growth on the added carbon source triggered an immediate and extreme rapid increase in metabolic activity of cultured cells, which was accompanied by a switch to a higher enzyme production rate to that observed on glucose. One should remember that the clear evidence for cellulase production already during the first cultivation stage on glucose is not surprising, since the used *T reesei* isolate is known to be resistant to carbon catabolite repression [35,130]. However, the presence of some low levels of constitutively expressed cellulases in the culture broth already at the feeding point (FPA = 0.2 FPU/mL at t = 60 h) would not suggest such an immediate switch from glucose consumption to cellulose utilization, especially if one considers the strict regulatory mechanisms that rule cellulase production and carbon source utilization by *T. reesei* in general [114].

A possible explanation for the rapid growth observed immediately after the feed addition would be that in fact it was not the carbon source that had been depleted from the medium by the end of the first cultivation stage but a shortage in some other key element occurred, which element became available again from the fresh feed. This assumption suggests that carbon was continuously available and the switch from glucose consumption to cellulose utilization occurred some time after the feed addition. However, no transient behavior of the culture could be visualized during the second cultivation stage as an obvious sign for this switch in metabolism. It is also important to note that no significant amount of glucose could be measured in the broth after the first cultivation stage (data not presented).

When the experiment was repeated in a similar manner with the exception that the medium components necessary for the second batch of carbon source were fed apart from the SF, an extraordinary growth profile with two peaks in the second stage was observed (Figure 6 B). Addition of the medium components to the exhausted broth of the glucose grown and already declining culture (t = 47h) called forth an immediate increase in CO_2 evolution, just like the addition of SF did afterwards (t = 67 h). However, only this second peak in metabolic activity was accompanied by a remarkable increase in the enzyme production rate. Appearance of the first peak seemed to validate the previous theory suggesting that the fermentation broth of the first cultivation stage had been primarily depleted for a key ingredient other than the carbon source. The intensive metabolic activity triggered by the nutrients at t = 47 h peaked soon (in less than 1 h) and then a rapid decrease in CO_2 evolution occurred, suggesting that the broth finally got indeed depleted for glucose. Interestingly, when a new batch of carbon source was added (t = 67 h) a similar peak in CO_2 formation to that observed in the second fermentation stage of the previous experiment (Figure 6 A) was recorded. This suggests that contrary to the former theory, the cells were capable of turning their metabolism from glucose consumption to cellulose utilization in such a sudden manner. A possible explanation for the phenomenon would be that the relatively low activity of approx 0.2 FPU/mL (Figure 6 A) and 0.3 FPU/mL (Figure 6 B) found in the exhausted broth of the glucose grown cultures in fact corresponded to relatively high net cellulase contents (approx 400 FPU and 600 FPU, respectively), supposedly enough to initiate the intensive degradation of the added cellulose immediately. To accept this conclusion, the growth triggered by the addition of the medium components alone without parallel addition of SF at t = 47 h in Figure 6 B needs an explanation, since according to the latest concept the medium at this point has not been depleted for nutrients but for carbon. A perfect explanation is available from Kubicek [131], who recognized that peptone - present among the added nutrients - actually serves as a readily metabolizable carbon source for *T. reesei* which is consumed prior to cellulose if both are present in the medium, while low levels of cellulases are formed.

To be able to make a quantitative evaluation of growth and enzyme production in such two-stage cultures as the consequence of cellulose addition, it would be necessary to avoid any coincidences of response signals that may occur from adding any substance along the SF. In this view, the cultivation was repeated in a manner that the nutrients required to support growth on the second batch of carbon source were incorporated in the initial medium, which thus contained a doubled level of nutrients at the beginning of the cultivation (Figure 7).

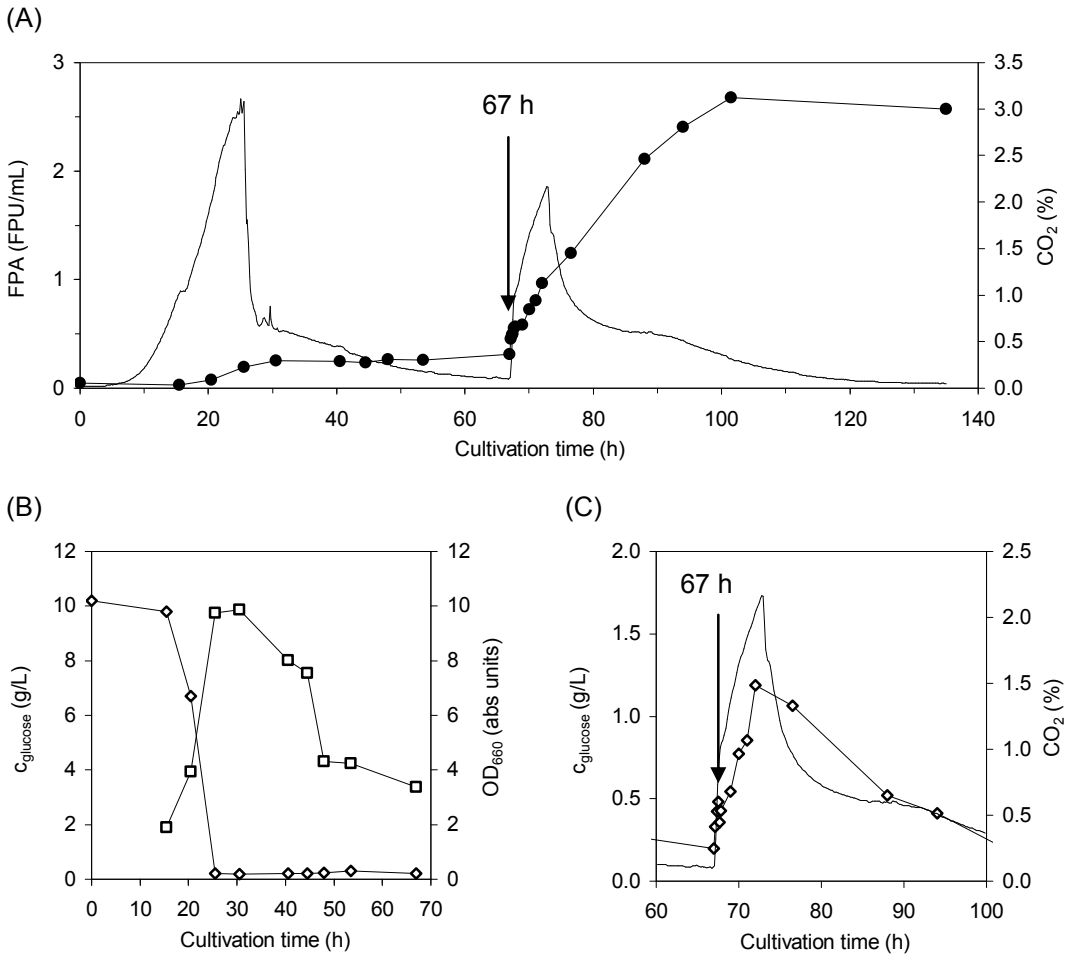

Figure 7 CO_2 concentration in the outlet gas (—), produced FPA (—●—), glucose concentration determined by HPLC (—◊—), and OD_{660} (—□—) plotted *vs* cultivation time during cellulase fermentation by *T. reesei* in a 3-L laboratory bioreactor in Mandels medium (28°C, pH 5.0). The initial growth medium contained 10 g/L of glucose as the carbon source and a doubled amount of nutrients. At t = 67 h SF was added to a final concentration of 10 g/L.

The initial exponential growth phase on glucose lasted until about 26 h with a specific growth rate as determined from the logarithm of the CO_2 evolution rate (CER) of 0.22 1/h. After 25.4 h, glucose was exhausted from the medium. Although CER rapidly decreased, there was a residual CO_2 evolution, which only gradually approached zero. This was accompanied by a reduction in OD_{660} that may reflect the degradation of biomass. From these observations one can conclude that even though the cells stopped growing they remained metabolically active. The FPA remained constant after the depletion of glucose at a level of 0.3 FPU/mL. At t = 67 h SF was added, which caused an immediate increase in CER. After approx 6 h of continuous increase there was a sharp decrease in CER at t = 73 h, which interestingly did not coincide with complete depletion of glucose from the medium (Figure 7 C).

The initial rather rapid increase of CER found in the second stage of two-phase batch cultivation was somewhat unexpected and therefore it was decided to compare this value to the initial rates of glucose and cellobiose formation in *in-vitro* hydrolysis experiments. The enzyme loadings were chosen to represent the actual enzyme to substrate ratio relevant to the point of cellulose addition (24.7 FPU/g cellulose) and the point of maximum CER (91.9 FPU/g cellulose). For the home-produced enzyme the formation rate of glucose was 0.18 g/(L·h) at the lower enzyme loading and 0.5 g/(L·h) for the higher enzyme loading. The formation rate of cellobiose was approx 0.33 g/(L·h) in the first case and < 0.2 g/(L·h) in the second case. Using a commercial cellulase preparation similar values were found for glucose, but slightly higher values for cellobiose (data not presented). With a typical yield of CO_2 on sugar of 0.4 (C-mol/C-mol) one can estimate that the sugar formation rate at the lower enzyme dosage would give a CER of 0.014 mol CO_2 /h for a 2-L culture. This corresponds to a CO_2 level in the outlet gas of approx 1.1%, which is in good agreement with the value measured within 0.5 h after addition of cellulose. Calculations for the higher enzyme loading indicate that formed sugars would give a CER of 0.018 mol CO_2 / h that corresponds to a CO_2 level in the outlet gas of 1.4%, which is much lower than the actual value observed (2.2%).

The maximum cellulase activity obtained in this study was 2.6 FPU/mL, which coincided with the depletion of glucose and occurred at about t = 100 h. The yield obtained on SF is in good agreement with previously reported data for the same strain (*e.g.,* Persson *et al.* [30] quote a yield of 233 FPU/g of substrate in a batch culture of *T. reesei* Rut C-30). However, one should not forget that the culture subjected to pulse-addition of SF had been pre-grown on 10 g/L glucose, which - if taken into account in the calculations - would mean a much lower yield (in FPU/g SF: 130 *vs* 260) for the two-stage culture.

The main point of making a two-stage culture was to enable the quantitative study of the behavior of a non-induced culture after the addition of an inducing substrate. However, separating an initial biomass formation on glucose (or other monosaccharides) from cellulase production with cellulose as substrate may have advantages also from a process point of view. By using a two-stage process a basal cellulase activity can be obtained on soluble substrates, often favored to solid substrates to various reasons (*e.g.,* lower viscosity, better aeration, better mass transfer properties, lower energy demand), which would allow the utilization of the added cellulose to commence rather quickly. In contrast, a simple batch process starting directly from cellulose may initially be rather slow due to the low hydrolysis rate (Figure 8).

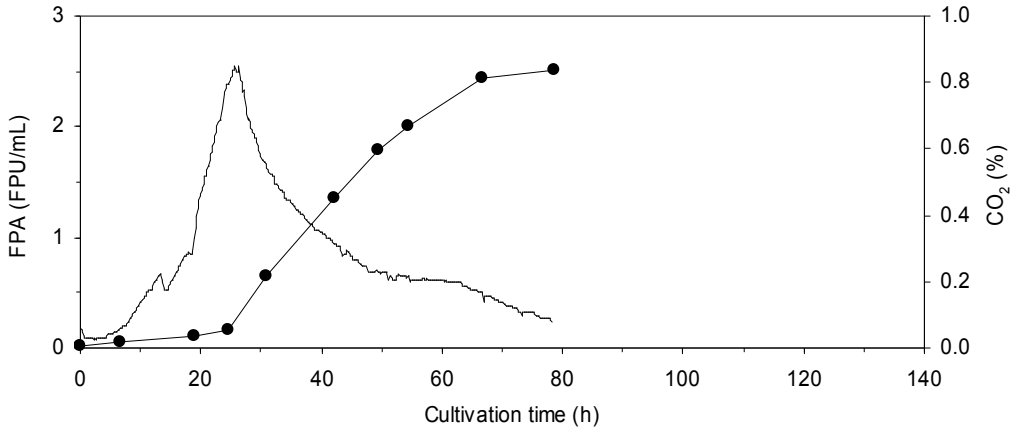

Figure 8 CO_2 concentration in the outlet gas (—) and produced FPA (—●—) plotted *vs* cultivation time during cellulase fermentation by *T. reesei* in a 3-L laboratory bioreactor in Mandels medium (28°C, pH 5.0) on 10 g/L SF.

4.1.4 Effect of medium nitrogen content

The most often used medium for *Trichoderma* cultivation (also used through the course of this work) is that designed by Mandels and Weber [100] containing ammonium-sulfate and urea as the source of nitrogen, together with other minerals and organic additives typically found in media formulated for fungi as buffering agents, nutrients, and trace elements (section 3.3.2). The medium does however not contain vitamins, amino acids, or other growth factors and is therefore relatively cheap. Nevertheless, a large-scale fermentation process – such as the production of cellulases is predicted to become on the long run - may require an even cheaper production medium in order to remain competitive. This is especially important if one considers that some 50% of the expenses involved in the production of glucose from cellulosic materials is attributable to the enzyme cost alone with a major portion of it attributable to the cost of the raw materials (*i.e.,* the substrate and the production medium) used for the cultivation of the cellulase producing organism [132,133]. In this view, medium optimization might be a key tool to produce cheaper enzymes thus improving the economics of cellulase-assisted processes [134]

A favorable medium for cellulase production should provide an optimal nutrient supply that fully covers the need of the cultured organism but – for economical considerations - does not overdose any compound. Because nitrogen is quantitatively the second most important medium component for cell growth and in enzyme production is a key component also for the excreted product (an average protein contains some 16% (wt/wt) of nitrogen), sufficient N supply is of crucial importance during *Trichoderma* cultivation.

As a rough guide, in a common, carbon limited fermentation process performed under optimal conditions the total biomass production equals to approx 50% (wt/wt) of the net available carbon source and about 10% (wt/wt) of the produced biomass is nitrogen [Gunnar Lidén, Lund University, personal communication]. Accordingly, on 10 g/L carbon source approx 5 g/L of biomass is produced and to enable this biomass production the availability of at least 0.5 g/L of N is necessary. This nitrogen level, defined as the estimated stoichiometric nitrogen need of the cultured organism (N_{est}) through this study, can be ensured by using 2.36 g/L of $(NH_4)_2SO_4$ as the sole nitrogen source in the production medium. Surprisingly, even the original recipe by Mandels and Weber [100] proposing a sum N of 0.44 g/L in the form of urea and $(NH_4)_2SO_4$ does not ensure a sufficient N level to this assumption. At the same time it is important to note that complex organic nitrogen sources (0.75 g/L of proteose peptone and 0.25 g/L of yeast extract) present in both the original recipe and the modified protocols were not taken into account in the previous calculation and will neither be considered during the evaluation of results.

To check the effect of medium nitrogen content on cellulase production by *T. reesei*, shake flask cultivations were carried out on 10 g/L SF using various N concentrations in the range of 0.2 to 2.0 times to that of N_{est} corresponding to N = 0.1-1.0 g/L (Figure 9). If added purely as ammonium-sulfate this N supply can be ensured by the addition of 0.47-4.72 g/L of $(NH_4)_2SO_4$ with 2.36 g/L of $(NH_4)_2SO_4$ corresponding to N_{est}. The reason for studying such a relatively wide range of N concentration was the highly approximative nature of the calculations involved in the definition of N_{est} taken as a reference N level to be scattered around by those of the experimental series. Fermentation performance was primarily monitored through observing the secretion of cellulase enzymes by FPA measurement. However, pH profile corresponding tightly to culture growth was just as convenient to map the process (Figure 9).

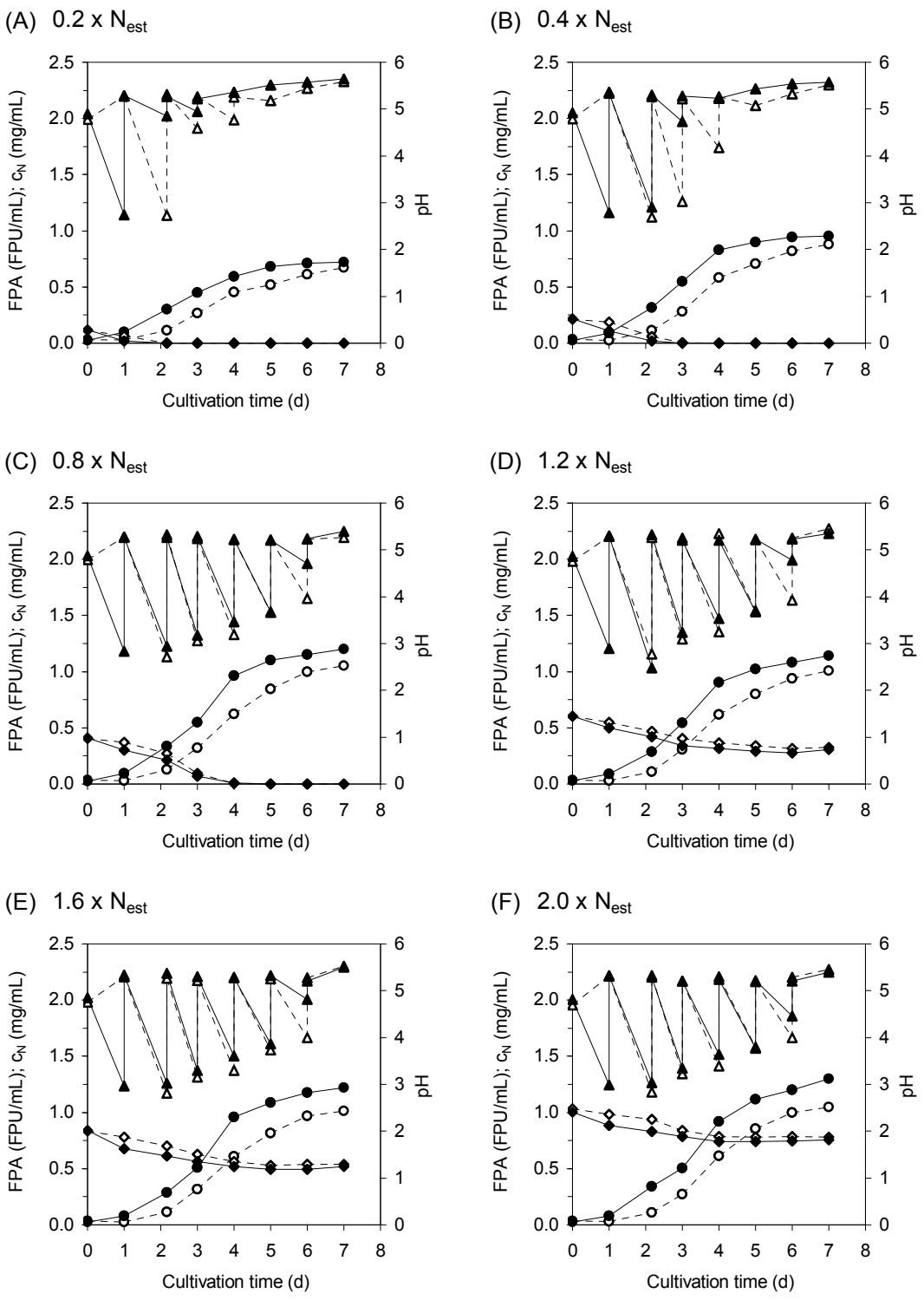

Figure 9 Medium nitrogen content (---◊---, —♦—), pH with manual adjustment (---Δ---, —▲—), and produced FPA (---○---, —●—) obtained during the cultivation of *T. reesei* in shake flasks (pH 5.0, 28°C) initiated by glucose grown (open symbols, dashed line) and SF grown (closed symbols, solid line) inoculum cultures, respectively. The initial nitrogen content of the modified Mandels medium containing 10 g/L of SF as the carbon source was set at (A): 0.2, (B): 0.4, (C): 0.8, (D): 1.2 , (E): 1.6, and (F): 2.0 times to that of N_{est}. For 10 g/L of SF N_{est} was defined as 0.5 g/L and supplied as 2.36 g/L of $(NH4)_2SO_4$.

In cultures initiated by glucose grown inoculum the secretion of cellulases and growth-associated drop in pH resulting from ammonia consumption were both recognized with 1 d delay compared to cultivations initiated by SF grown inoculum. The lack of a similar pH drop observed after 3 to 6 d of cultivation clearly indicated the termination of culture growth, however, approx 5% of the total produced cellulase was secreted after this point. Exceptions are the cultivations heavily limited by a shortage in N (Figure 9 A and B) with a much larger portion of cellulases (at N = 0.1 g/L, 60% and 38%; at N = 0.2 g/L, 68% and 43%, for cultures inoculated by glucose grown and SF grown inoculum, respectively) appearing in the broth after that point.

Secretion of enzymes, in part, occurred later than net nitrogen uptake too. This phenomenon was especially pronounced in nitrogen limited cultivations, where a large portion of total cellulase product (at N = 0.1 g/L: 83% and 58% (Figure 9 A), at N = 0.2 g/L: 68% and 42% (Figure 9 B), at N = 0.4 g/L: 41% and 19% (Figure 9 C), for cultures initiated by glucose grown and SF grown inoculum cultures, respectively) appeared in the medium after all available nitrogen had been consumed.

Obtained enzyme activities did not increase significantly above N = 0.4 g/L, which is lower by 20% than N_{est} and by 10% than the effective N supply recommended by Mandels and Weber [100]. It is important to note that at N = 0.4 g/L (Figure 9 C) medium nitrogen apparently run zero in 4 d of cultivation, which is somewhat contradictory that observed in cultures with obvious overloading of N (Figure 9 D, E, and F) where only an approximate amount of 0.3 g/L of nitrogen was consumed as judged from the differences between applied and residual N concentrations. These data suggest the necessity of a more careful optimization for N in the range of 0.3 to 0.5 g/L. Because the improvement in the produced FPA in FPU/mL from 0.88 to 1.05 and from 0.95 to 1.3 obtained in cultivations initiated by glucose grown and SF grown inoculum cultures, respectively, was far not proportional to the corresponding increase in the N content form 0.4 g/L (Figure 9 C) to 1 g/L (Figure 9 F) the option of using an increased N supply is to be strongly considered with a critical view on cost factors.

4.1.5 Effect of cultivation pH

During the cultivation of *T. reesei* in Mandels medium the pH tends to turn acidic due to the consumption of ammonia and subsequent liberation of protons from the added ammonium-sulfate by the metabolically active cells [100]. It is important to note that this drop in pH does not hamper the culture growth especially in the initial phase of the fermentation [135,136] as far as it does not go extreme (below pH 3 or so), while secretion of the produced enzymes is often facilitated by a pH that is even higher than the culture is usually controlled at during the production phase (pH 6 *vs* pH 4 to pH 5), which is especially so concerning the β-glucosidase [137,138]. This observation created the idea to use a fitting pH profiling strategy instead of using a constant pH all along the cultivation [139].

In shake flasks pH control is usually limited to the incorporation of buffering salts in the production medium (such as KH_2PO_4 in Mandels medium), which is often unable to keep the pH in the desired range. Periodic addition of controlling agents when severe fluctuations in pH occur may be an accessory solution, but due to the difficulties of pH adjustment in shake flasks under sterile conditions the frequency of the manual intervention often remains unsatisfactory. Alternatively, the addition of a compelling buffer system to the production medium may be a reasonable choice for pH control [140].

Among the tested organic acid buffer systems (Table 4) those containing maleic acid were the less vulnerable to microbial uptake as confirmed by HPLC analysis of samples taken frequently during the cultivation (data not shown). The fact that citric acid and succinic acid was consumed by the cultured cells may be a reason for the poor pH stability (*i.e.,* dramatic pH shift to alkaline) observed in the corresponding cultivations. In contrast, maleic acid was not taken up by *T. reesei* and therefore could be used in a relatively low concentration (0.1 M) for an effective pH control. While the final FPA (approx 1.3 FPU/mL) was similar, the β-glucosidase activity obtained by the end of the cultivation (7 d) was significantly higher when using Tris-maleate (1.5 IU/mL) instead of Na-maleate (1.1 IU/mL). In this view the use of 0.1 M Tris-maleate was considered as the best choice for pH control, which was successfully applied in experimental shake-flask cultivations (section 4.1.2).

Table 4 The applicability of various buffer systems (0.1 M) during the cultivation of *T. reesei* in shake flasks (30°C) on 10 g/L SF in Mandels medium as evaluated on the basis of obtained activities and pH stability through 7 d of cultivation.

Buffer system	FPA	β-glucosidase	pH stability
Na-citrate, pH 5.0	poor	poor	poor
Na-succinate, pH 5.0	very good	very good	poor
Na-maleate, pH 6.0	good	very good	very good
Tris-maleate, pH 6.0	good	very good	very good

The pH is easier and more effective to control in a bioreactor equipped with an automatic system to check and adjust the pH found in the vessel. With a smart and efficient pH control strategy the performance of enzyme production can improve significantly (*cf.* the shake flask and bioreactor cultivations reported in section 4.1.6 as an example).

4.1.6 Alternative fermentation substrates

For economical end environmental considerations referred to repeatedly in this study, it would be reasonable to obtain cellulase production on cellulosic materials that are preferably generated as wastes or byproducts in agricultural and industrial production, or collected as wastes from municipal sources. As a major consumer of plant biomass, the pulp and paper industry contributes largely to the organic wastes produced and dumped by the world population and therefore the utilization of used paper material (*e.g.,* OCC) as a feedstock for *T. reesei* cultivation may represent not only a cost-effective but also an environmentally conscious way to produce cellulases [137, 141].

The OCC substrate proved to be a suitable carbon source for cellulase production by *T. reesei*. Using a substrate concentration that corresponded to 10 g/L of cellulose the maximum FPA obtained in shake flasks was 1.17 FPU/mL, which could be improved to 2.27 FPU/mL under similar cultivation conditions in a laboratory bioreactor (Figure 10). Corresponding yields were (in FPU/g cellulose): 117 and 227, respectively. The enormous difference between the performance of cellulase production in shake flask and a laboratory bioreactor (the maximum FPA obtained in shake flasks after roughly 7 d was achieved in approx 40 h in the 30-L fermentation unit) can be explained as the consequence of several factors the bioreactor culture benefited from such as the higher DO level at the applied 1 bar overpressure [83], the more efficient mass transfer due to efficient mixing [142], and the more effective pH control available in the vessel (see section 4.1.5 for considerations in concern).

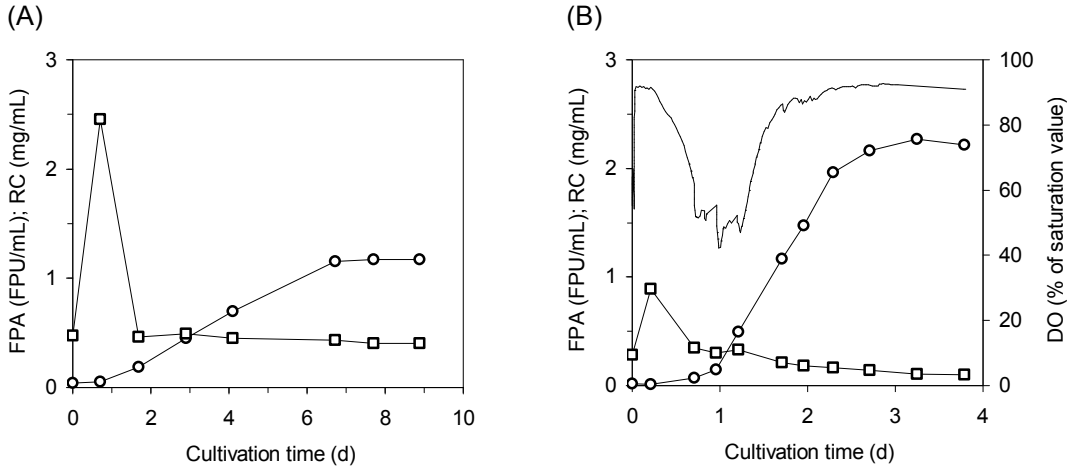

Figure 10 Cellulase enzyme production by *T. reesei* on OCC corresponding to 10 g/L of cellulose in Mandels medium (28°C) in shake flasks (A) and a 30-L bioreactor (B) as the cultivation vessels. Produced FPA (—○—), concentration of reducing carbohydrates (—□—), and dissolved oxygen (solid line, no labelling) plotted *vs* cultivation time.

For applied research curiosities, in selected experiments the liquid phase of the fermentation broth was made up with diluted vinasses instead of water in order to check the applicability of this attractive residue - gained after the production of ethanol or yeast on molasses - as a medium supplement. Two batches of vinasses were tested and compared, one obtained from a distillery (Győr, Hungary), the other obtained from a yeast factory (Budafok, Hungary).

When using 10 g/L (DW) of vinasses in the production medium as the sole nutrient source (*i.e.*, no Mandels nutrients were applied), the fermentation performance was comparable to that obtained by the control prepared in Mandels medium without vinasses. Maximum cellulase activities in the experimental cultivations were in the range of 1.2-1.4 FPU/mL, with better result achieved when using vinasses from yeast manufacture; presumably, this residue was less "exhausted" than that left behind after spirit manufacture.

During the evaluation of results one should consider that this highly viscous material although very rich in minerals, did not contain residual carbohydrates in significant amounts and therefore could not be considered as a supplementary fermentation substrate like it was the case for the other tested liquid supplement: WOCS hydrolyzate (section 4.1.2).

4.2 Characterization of Cel 45A, Cel7A, and Cel7B from *M. albomyces*

Prior to their introduction in various industrial processes, the hydrolytic behavior of novel enzymes has to be carefully characterized. The substrates used in the hydrolysis experiments were chosen to represent two extremities in crystallinity (the portion of the crystalline part in Avicel is 47% while PASC is considered to be 100% amorphous), known as a highly determinative physical parameter to influence the enzymatic degradability of cellulose. Due to the co-existence of crystalline and amorphous regions in natural celluloses and consequently in technical cellulosic materials too, the hydrolytic potential of novel enzymes with different characteristics (*i.e.,* with different ability to attack the various parts of the cellulose macromolecule) was of interest also in mixtures. Because of their different affinity towards the various substrates used in the large selection of available cellulase assays [111,125,126], it was more exact to dose the enzymes in the hydrolysis experiments as a fixed amount of protein than as a fixed level of cellulase activity exhibited by the same protein. This was especially so when the two types of enzymes were applied in controlled mixtures at a constant total dosage with the objective to examine their synergistic action.

4.2.1 The enzyme preparations

Expression of the *M. albomyces* cellulases in *T. reesei* deficient in its major native cellulases (ΔCBHI, ΔCBHII, ΔEGI, ΔEGII) left still the possibility of contamination by the minor endogenous cellulases (EGIII, EGIV and EGV) produced by the host. These were, however, substantially removed by the applied purification method as confirmed by SDS-PAGE of the purified preparations (Figure 11).

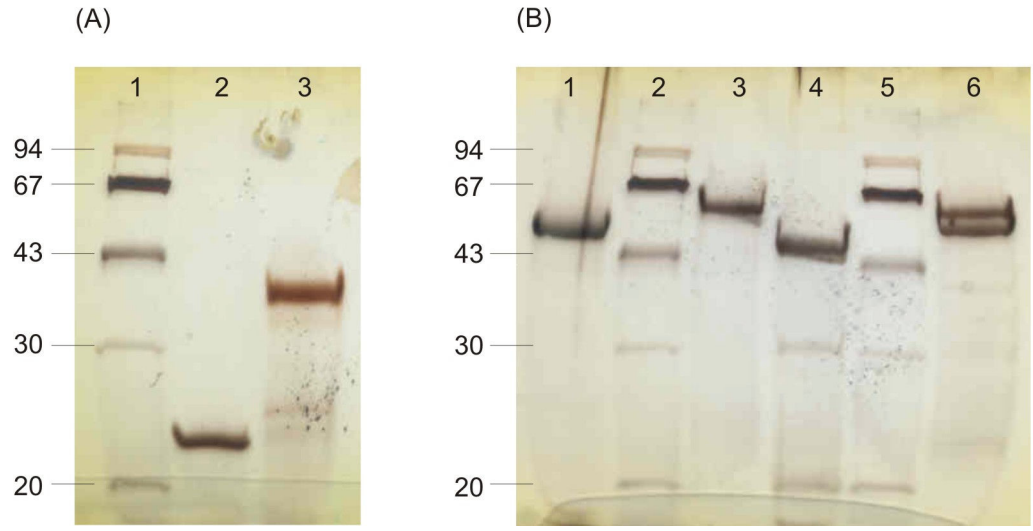

Figure 11 SDS-PAGE of the purified *Melanocarpus* enzymes. (A): Lane 1, LMW; Lane 2, Cel45A; Lane 3, Cel45A(CBM). (B): Lane 1, Cel7A; Lane 2, LMW; Lane 3, Cel7A(CBM); Lane 4, Cel7B; Lane 5, LMW; Lane 6, Cel7B(CBM). Protein loadings were 2 µg for the Cel45A and Cel45A(CBM), 5 µg for the Cel7A and Cel7A(CBM), 10 µg for the Cel7B and Cel7B(CBM) preparations, and 5 µg for the LMW. The molecular weights of the standards (in kDa) are marked at the side.

Supposedly, the more heat-sensitive EGIII was irreversibly denaturated already by the heat treatment, while the residual more thermostable EGV and possible traces of EGIII were basically eliminated by the chromatography steps. EGIV is a non-significant cellulase with very low specific activity [143], which may be present only in traces and is hardly detectable by its activity even when it is intact. The molecular weights gained for each protein by the SDS-PAGE (Figure 11) are in good correspondence with the calculated molecular mass of the proteins (Table 5).

Table 5 Calculated molecular mass, protein content and hydrolytic activity against CMC and HEC of the purified *M. albomyces* enzymes produced by *T. reesei* hosts.

Enzyme preparation	Molecular mass	Protein content	CMC activity		HEC activity	
	Da	mg/mL	nkat/mL	nkat/mg	nkat/mL	nkat/mg
Cel45A	22,913	3.67	3,123	851	145	39.5
Cel45A(CBM)	29,592	3.85	2,540	660	120	31.3
Cel7A	44,833	3.25	4,723	1,453	648	199
Cel7A(CBM)	50,017	3.64	5,175	1,422	749	206
Cel7B	47,552	3.02	12.9	4.3	3.4	1.1
Cel7B(CBM)	53,782	3.53	52.9	15.0	7.7	2.2

Protein contents and hydrolytic activities against CMC and HEC of the purified enzymes are shown in Table 5. The Cel45A and Cel7A proteins had a remarkable hydrolytic activity against CMC and HEC, which is typical for EGs. The specific activity of Cel7A was higher than that of Cel45A against both substrates, but on molar basis the order was reversed against CMC. As expected, Cel7B was much less active against CMC and HEC than the EGs. On the other hand, Cel7B had some very low activity against 4-methyl-umberriferyl-β-*D*-lactoside that was almost totally inhibited by 0.005 M cellobiose (data not presented), which is a typical action of CBHs [144].

4.2.2 Comparative hydrolysis studies using individual enzymes

The investigated CBH, in general, had higher affinity than the studied EGs towards crystalline cellulose (Figure 12), whereas in the case of amorphous substrate (Figure 13) the order was reversed. One single exception to this rule was the relation between Cel45A(CBM) and Cel7B(CBM) during their action on Avicel (*cf.* Figure 13 B and F). Concerning the EGs the Cel45A was more active than the Cel7A on Avicel, whereas on PASC a reverse order was observed in their action when the same protein amounts were dosed of each. Since the molecular mass of Cel7A is roughly the double to that of Cel45A (Table 5), on molar basis the efficiency of Cel45A was superior to that of Cel7A on both substrates. These observations do not clearly correspond to the activities of these enzymes against CMC and HEC, which confirms that the mode of action and consequently the hydrolytic potential represented by the same enzyme may largely differ depending on the nature of the substrate to be degraded. The substantial improvement in the hydrolysis performance when using enzymes to carry the CBM instead of those lacking it, underlined the essential role suggested to CBM in literature [94,95,96]. The effect of the CBM was greater (not in absolute value but in percentage) when Avicel was hydrolyzed - especially when it was done by the EGs - as a possible consequence of its CBH origin. On PASC the improvement in the hydrolysis performance attributable to the presence of CBM was similar (approx 40% in 24 h of reaction) for all the three enzymes.

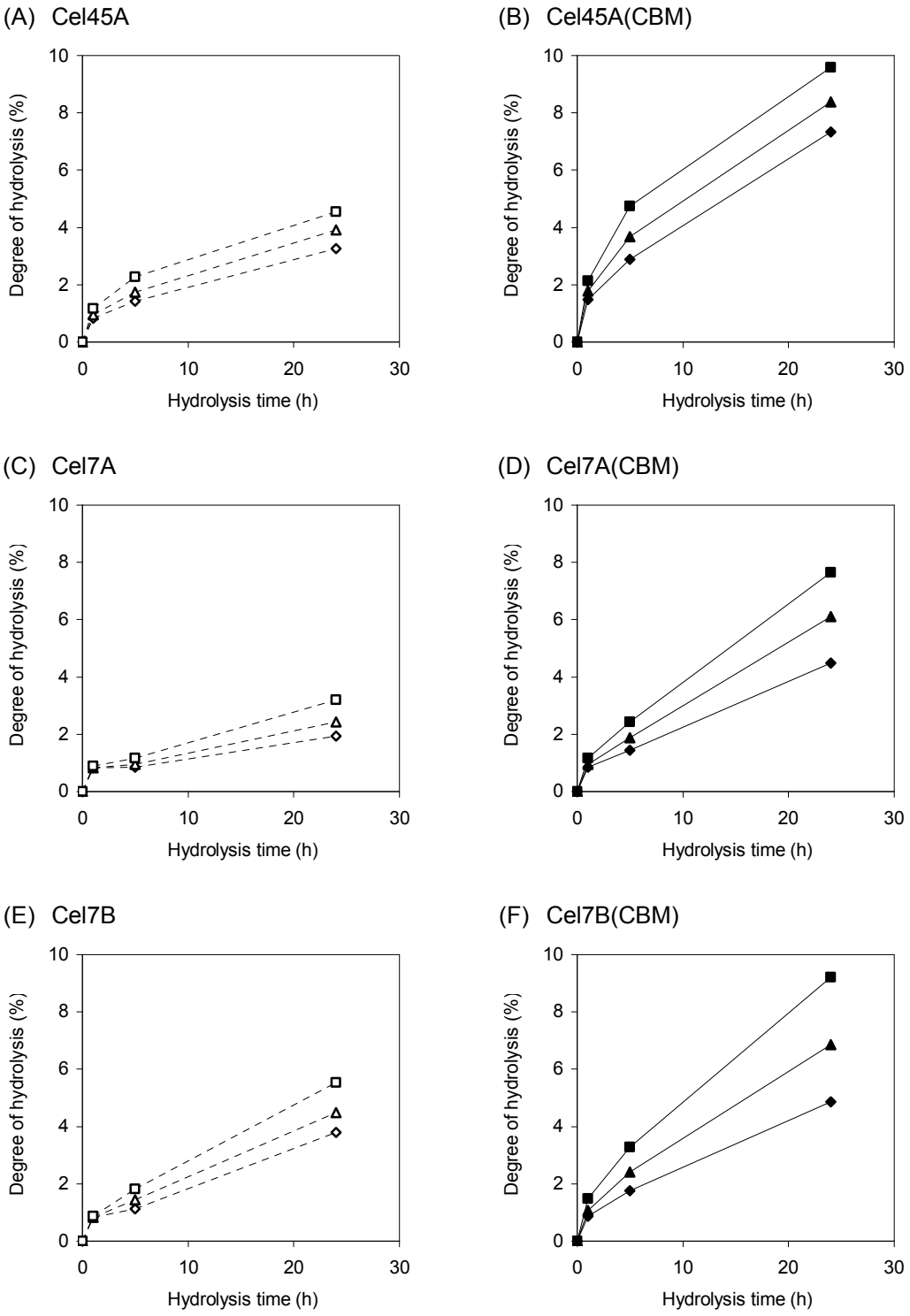

Figure 12 Hydrolysis performance (degree of hydrolysis *vs* hydrolysis time) obtained on 10 g/L crystalline cellulose (Avicel) with the *M. albomyces* cellulases lacking (A, C, E) and carrying (B, D, F) an exogenous CBM. The enzyme dosage was 5 (---◊---, —♦—), 10 (---Δ---, —▲—), and 20 (---□---,—■—) mg/g protein per substrate, respectively. (T=50°C, pH=6.0)

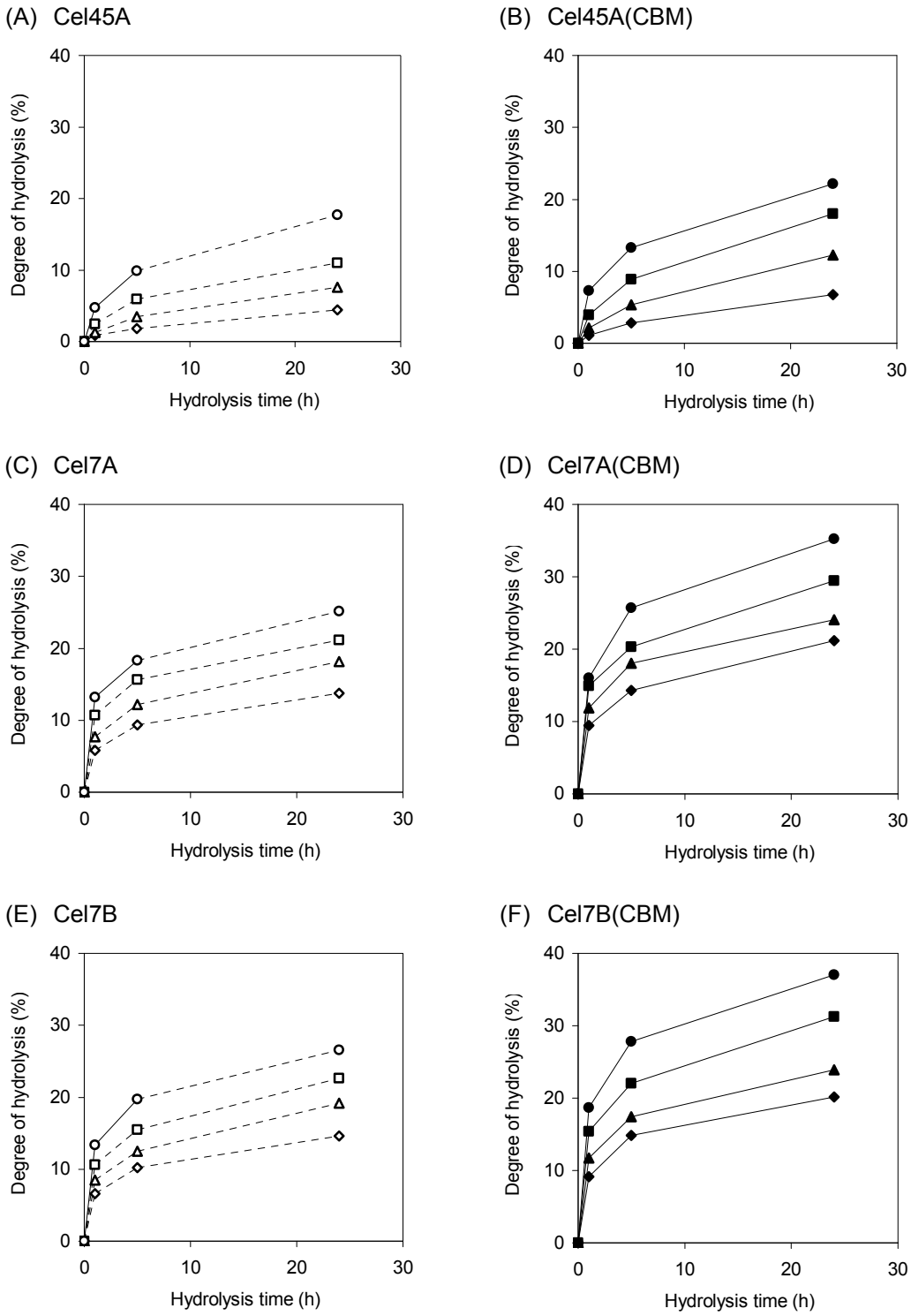

Figure 13 Hydrolysis performance (degree of hydrolysis *vs* hydrolysis time) obtained on 10 g/L amorphous cellulose (PASC) with the *M. albomyces* cellulases lacking (A, C, E) and carrying (B, D, F) an exogenous CBM. The enzyme dosage was 0.5 (---◊---, —♦—), 1 (---Δ---, —▲—), 2 (---□---, —■—), and 5 (---○---, —●—) mg/g protein per substrate, respectively. (T=50°C, pH=6.0)

Comparison of Cel7B and Cel7B(CBM) to the corresponding cellulase products from *T. reesei* (the CBHI/Cel7A catalytic core and the CBHI/Cel7A complete enzyme, respectively) revealed that the novel CBH from *M. albomyces* was less active on crystalline cellulose than the industrially relevant enzyme from *T. reesei* under the applied conditions (Figure 14 A). This was however not the case for the amorphous substrate that was more accessible to the enzymatic attack by the novel cellulase (Figure 14 B). The influence of CBM on *M. albomyces* CBH and *T. reesei* CBH was clearly different depending on the nature of the substrate to be hydrolyzed; on crystalline cellulose (Figure 14 A) it had a greater effect on the *T. reesei* enzyme, while on amorphous cellulose (Figure 14 B) a reverse order was observed.

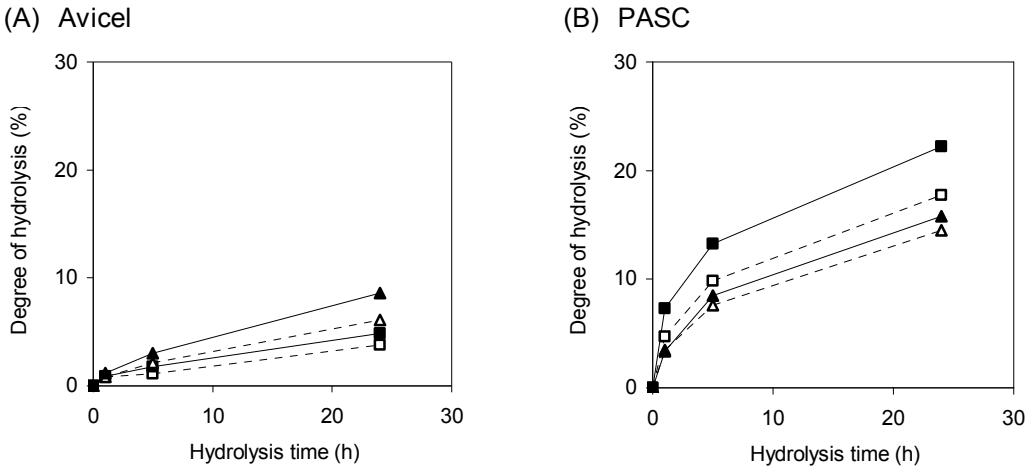

Figure 14 Hydrolysis performance (degree of hydrolysis *vs* hydrolysis time) obtained on 10 g/L Avicel (A) and PASC (B) with the *M. albomyces* CBH (Cel7B) lacking (---□---) and carrying (—■—) an exogenous CBM, and the *T. reesei* main CBH (CBHI/Cel7A) lacking (---Δ---) and carrying (—▲—) its endogenous CBM. The enzyme dosage was 5 mg/g protein per substrate. (T=50°C, pH=6.0)

In general, the crystalline substrate was more recalcitrant to enzyme attack than the amorphous substrate (*c.f.* Figure 12 and Figure 13) and consequently, it required a much higher enzyme load for an equal level of degradation. Numerically speaking, not even a ten times higher protein dosage (5 mg/g) could guarantee a similar degree of hydrolysis on Avicel to that achieved with a ten times lower dosage (0.5 mg/g) on PASC, except for the CBH, that acted comparably, especially without the CBM. When the enzymes were applied in the same dosage (5 mg/g) the degree of hydrolysis obtained on PASC was 5 to 13 times higher than that achieved on Avicel by the 24th hour of the hydrolysis. The fact that Avicel was more resistant than PACS to the enzymatic attack by the studied EGs is not surprising, rather it is in the case of the investigated CBH acting similarly (*i.e.,* it was more active on the amorphous substrate than on the crystalline substrate), which does not correspond to the typical behavior of a CBH [145,146]. An unlikely explanation for this extraordinary behavior of the novel CBH could be that the enzyme preparations contained some background activity due to minor contamination by EGs (most likely EGV) native to the *T. reesei* host. This argument is however to be rejected because the activities against HEC and CMC of the Cel7B and Cel7B(CBM) preparations were higher at pH 6.0 than at pH 5.0 (data not presented), which does not fit to the characteristics of EGV. Neither the SDS-PAGE analysis of purified enzymes suggested the presence of contaminating proteins in considerable amounts. A more reliable explanation for the phenomenon is that the novel CBH might have some endoglucanase-like side activity and consequently some limited ability to attack the HEC or CMC substrates used in the EG

assays (Table 5). Cel7B was, clearly different from Cel7A in its product pattern for glucose and cellobiose during the hydrolysis of PASC and was more similar to *T. reesei* CBHI (Figure 15 A), but in terms of the release of oligomeric sugars it had some more similarity to the product profile of the EG Cel7A (Figure 15 B).

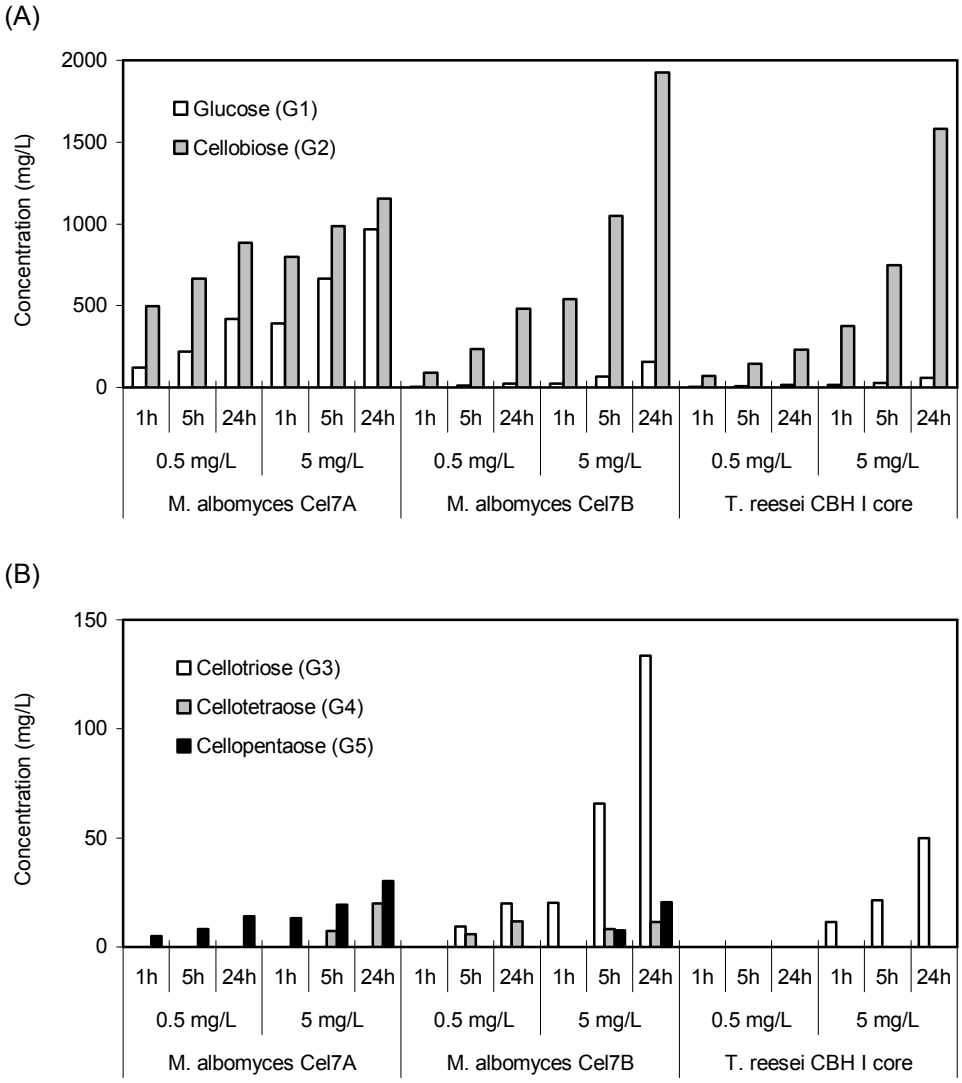

Figure 15 Product profiles obtained after 1 h, 5 h, and 24 h of hydrolysis (T=50°C, pH=6.0) of 10 g/L PASC by *M. albomyces* Cel7A, Cel7B, and *T. reesei* CBHI core with two different enzyme loadings: 0.5 mg/g and 5 mg/g protein per substrate. The concentration of liberated G1-G2 (A) and G3-G5 (B) carbohydrates is plotted *vs* hydrolysis time.

4.2.3 Comparative hydrolysis studies using enzyme mixtures

The concerted action of Cel7A and Cel7B applied together in different ratios in a constant total protein dosage (5 mg/g) followed the general rule of endo-exo synergism described by several authors [147,148,149]. However, it did not fit some other statements like that of Henrissat *et al.* [150] and Nidetzky *et al.* [151] reporting that synergism is present only in the case of crystalline cellulose but is absent in the case of soluble cellulose derivatives, or the theory of Srisodsuk [152] declaring that synergism requires the presence of the CBM. In fact, the synergistic interaction between the studied enzymes was more evident on PASC than on Avicel and even though it was more emphasized for enzymes harboring the CBM, it was also clearly detected for the native enzymes not carrying a CBM (Figure 16).

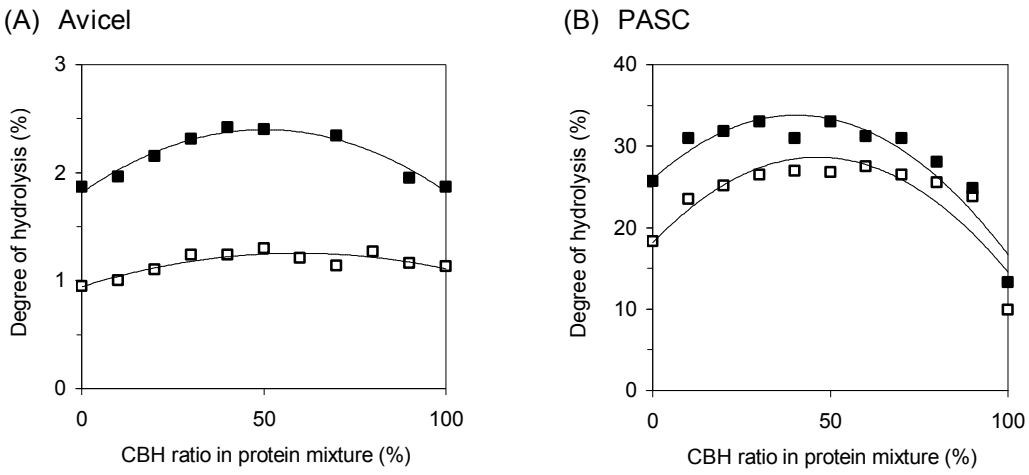

Figure 16 Concerted action of the *M. albomyces* Cel7A and Cel7B added in a total dosage of 5 mg/g protein per substrate (10 g/L). Hydrolysis degree of Avicel (A) and PASC (B) by mixtures of enzymes lacking (---□---) and carrying (—■—) an exogenous CBM is plotted *vs* the applied ratio of the two type of enzymes (the CBH to the total) in the protein mixtures. (T=50°C, pH=6.0, incubation time=5h)

It is mportant to note, however, that the degree of hydrolysis obtained on the more recalcitrant substrate remained too low (less than 3%) to draw up clear consequences, especially if one considers that in the obtained conversion regimes it was the easily accessible amorphous portion of Avicel that was the most likely to come through the enzymatic action keeping the real crystalline structure intact.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The present study reports investigations on *(i)* the production of cellulose degrading enzymes by *Trichoderma reesei* most commonly employed to produce cellulases for commercial purpose at industrial-scale, and *(ii)* the hydrolytic properties of three recently discovered cellulases from *Melanocarpus albomyces* ideally suited for various applications but not yet discovered by the relevant industries.

The contribution of the present report to the existing knowledge on cellulose degrading enzymes can be summarized briefly as follows.

1. A two-stage process for the cultivation of *T. reesei* Rut C-30 using glucose as the carbon source in the first stage and cellulose in the second stage is suitable to study the secretion of cellulases by non-induced cells as the response to pulse addition of their natural substrate. In this manner valuable information on the dynamics and enzyme production by the cultured cells can be obtained. *T. reesei* Rut C-30 is able to switch its metabolism from glucose consumption to cellulose utilization in an immediate manner. This ability, taking into account the numerous advantages that soluble substrates have over solid substrates, can be well exploited in process development.
2. Fermentation performance of *T. reesei* Rut C-30, as evaluated via produced cellulase activity, is substantially (43%) improved when the cells used to initiate growth in the production medium are pre-grown on an inducing substrate (Solka Floc), instead of using a non-inducing substrate (glucose).
3. Nitrogen content of the Mandels medium is approx 10% higher than the nitrogen uptake by the cultivated *T. reesei* Rut C-30.
4. Hydrolyzed hemicellulose obtained after wet-oxidation of corn stover is suitable to supplement the medium of cellulase production of *T. reesei* Rut C-30. In particular, the hydrolyzate derived from wet-oxidation performed at 185°C for 5 min under alkaline conditions can improve fermentation performance by 10% over the control. The use of 0.1 M Tris-maleate is convenient to control the culture at pH 6.0.
5. Old corrugated cardboard (used paper material) is a suitable substrate for cellulase enzyme production by *T. reesei* Rut C-30. Diluted vinasses can be used as the sole nutrient source in such experimental cultivations to replace Mandels nutrients.
6. The studied cellulases from *M. albomyces* are less active on crystalline cellulose than on amorphous cellulose. The studied cellobiohydrolase has greater activity than the endoglucanases against crystalline substrate, whereas in the case of amorphous substrate the order is reversed. Evidence of synergism can be seen when the enzymes are applied in mixtures. Addition of an exogenous cellulose binding module to the enzymes originally lacking it improves their hydrolytic potential; it has a greater effect on the endoglucanases than the cellobiohydrolase, especially against crystalline substrate. The tested cellobiohydrolase was more effective on amorphous cellulose than the corresponding enzyme from *T. reesei* already at pH 6.0, 50°C. Due to their increased thermostability and tolerance of non-physiological pH, novel enzymes are expected to perform even better at elevated T and pH.

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