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Abstract

Trichoderma reesei is a biotechnically important filamentous fungus used commercially in enzyme production. *T. reesei* is also one of the best known cellulolytic organisms, producing readily and in large quantities a complete set of extracellular cellulases for the degradation of crystalline cellulose. In addition to *T. reesei*, a wide variety of other bacteria and fungi also produce cellulolytic enzymes. Cellulases originating from various organisms and having different characteristics are used industrially in many applications, such as in the textile industry in finishing of denim fabric to impart a stonewashed appearance (biostoning) and in biofinishing of cotton.

In this work *T. reesei* strains producing significant amounts of homologous and heterologous cellulases and having defined cellulase profiles were constructed for specific industrial applications, *i.e*. biostoning and biofinishing of cotton. The production of *T. reesei* endoglucanase II (EGII), cellobiohydrolases I and II (CBHI and CBHII) was improved in separate strains. Strains producing high amounts of EGI and EGII without CBHs or CBHI and CBHII without the main EGs were also constructed. The cellulase genes were expressed under the powerful *T. reesei cbh1* promoter; in a transformant overproducing both CBHI and CBHII, the *cbh2* promoter was also used for *cbh2* expression. The level of endoglucanase activity produced by the EGII-overproducing transformants correlated with the copy number of the *egl2* expression cassette. Production of the major secreted cellulase CBHI was increased up to 1.5-fold and production of CBHII fourfold compared with the parent strain. In transformants overproducing both CBHI and CBHII, production of CBHI was increased up to 1.6-fold and production of CBHII up to 3.4-fold as compared with the host strain and approximately similar amounts of CBHII protein were produced by using the *cbh1* or *cbh2* promoters.

The enzyme preparation with elevated EGII content most clearly improved the biostoning of denim fabric and the biofinishing of cotton fabric. Better depilling and visual appearance were achieved with the enzyme preparation having an elevated CBHII content compared to the wild type preparation in biofinishing of cotton, but the improvement was not as pronounced as in the case of the EGII preparation.

Novel neutral cellulases were demonstrated to have potential in biostoning. The cellulase preparation of the thermophilic fungus *Melanocarpus albomyces* was found to be effective in releasing dye from indigo-dyed denim and to cause low backstaining at neutral pH. *M. albomyces* produces at least three cellulases and these cellulases with an effect on biostoning were purified and the genes encoding them were cloned and sequenced. *Ma* 20 kDa EGV (*Ma* Cel45A) belongs to the glycosyl hydrolase family 45 and the 50 kDa EGI (*Ma* Cel7A) and CBHI (*Ma* Cel7B) to family 7. None of the cellulases harbours a cellulose binding domain. Especially purified *Ma* Cel45A performed well in biostoning. The *Ma* cellulases were produced in *T. reesei* under the *T. reesei cbh1* promoter for biostoning applications. The endoglucanase production levels of *Ma cel45A*- and *cel7A*-transformants were several times higher than those of the parental *M. albomyces* strain. The cellulase preparation produced by the recombinant *Ma cel45A* transformant performed well at neutral pH in the finishing of denim fabric and caused considerably less backstaining than the acid cellulase product of *T. reesei*.

Preface

This work was carried out at Primalco Ltd. Biotec and Röhm Enzyme Finland Oy. I wish to thank Kari Edelmann and Ralf Lundell for providing excellent working facilities. I thank Professor Hannu Saarilahti from the Division of Genetics in Helsinki University for his support during the final stages of my studies. I am grateful to Professor Helena Nevalainen and Professor Georg Gübitz for reviewing this thesis work and for useful comments.

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List of publications

This work is based on the following articles, referred to in the text by the Roman numerals given below. Additional data published in US Patent 5,874,293 (http://www.uspto.gov, Miettinen-Oinonen, A., Elovainio, M. and Suominen, P.: Cellulase composition for treating cellulose-containing textile material, filed January 1997) is also presented.

I. Miettinen-Oinonen, A. and Suominen, P. 2002. Enhanced production of *Trichoderma reesei* endoglucanases and use of the new cellulase preparations in producing the stonewashed effect on denim fabric. Applied and Environmetal Microbiology 68:3956–3964.

II. Miettinen-Oinonen, A., Paloheimo, M., Lantto, R. and Suominen, P. 2004. Enhanced production of cellobiohydrolases in *Trichoderma reesei* and biofinishing of cotton fabric with the new preparations. Submitted for publication in Journal of Biotechnology.

III. Miettinen-Oinonen, A., Londesborough, J., Joutsjoki, V., Lantto, R. and Vehmaanperä, J. 2004. Three cellulases from *Melanocarpus albomyces* with applications in the textile industry. Enzyme and Microbial Technology 34:332–341.

IV. Haakana, H., Miettinen-Oinonen, A., Joutsjoki, V., Mäntylä, A., Suominen, P. and Vehmaanperä, J. 2004. Cloning of cellulase genes from *Melanocarpus albomyces* and their efficient expression in *Trichoderma reesei.* Enzyme and Microbial Technology 34:159–167.

Contents

Abbreviations

ACE	Activator of cellulase expression
BGL	β-glucosidase
CBD	Cellulose-binding domain
CBH	Cellobiohydrolase
CBM	Carbohydrate-binding module
cbh	Gene encoding cellobiohydrolase
Cel	Cellulase
cel	Gene encoding cellulase
CD	Catalytic domain
CMC	Carboxymethyl cellulose
CRE	Carbon catabolite repressor element
DP	Degree of polymerisation
3D-structure	Three-dimensional structure
ECU	Activity against hydroxyethyl cellulose
EG	Endoglucanase
egl	Gene encoding endoglucanase
ER	Endoplasmic reticulum
EST	Expressed sequence tag
FPU	Filter paper-hydrolyzing activity
GH	Glycoside hydrolase
HEC	Hydroxyethyl cellulose
ORF	Open reading frame
PCR	Polymerase chain reaction
pdi	Gene encoding protein disulphide isomerase
pgk	Gene encoding phoshoglycerate kinase
pki	Gene encoding pyruvate kinase
UPR	Unfolded protein response

1. Introduction

The genus *Trichoderma* comprises a group of filamentous ascomycetes that are widely used in industrial applications because of their ability to produce extracellular lignocellulose-degrading hydrolases in large amounts. Enzymes secreted by *Trichoderma* have received widespread industrial interest, leading to commercial applications in the textile industry (Galante *et al.,* 1998a, Cavaco-Paulo and Gübitz, 2003, Nierstrasz and Warmoeskerken, 2003), the food and feed industries (Galante *et al.,* 1998b) and the pulp and paper industry (Buchert *et al.,* 1998). *Trichoderma reesei* has the capacity to secrete enzymes in high yields and this property can be exploited when using *T. reesei* as an industrial host for homologous and heterologous enzyme production (Mäntylä *et al.,* 1998, Penttilä, 1998, Penttilä *et al.,* 2004). However, there is a continuous need to improve enzyme production processes in order to enhance protein yields and improve the production economics.

Cellulolytic enzymes hydrolyze cellulose and are produced by a wide variety of bacteria and fungi, *T. reesei* being one of the best-known cellulolytic organisms. Cellulases are industrially important enzymes (reviewed by Schülein, 2000) with a current market value of about 190 million US $ (Nierstrasz and Warmoeskerken, 2003). In the textile industry cellulase enzymes are tools for the fabric and garment finisher to produce higher value products, as cellulases clean fuzz and prevent formation of pills on the surface of cotton garments. Cellulases can also be used in denim finishing to create a fashionable stonewashed appearance in denim cloths in a process called biostoning. About 10 % of textile finishing of cellulose materials is estimated to be performed by cellulases and approximately 80 % of the 1.8 mrd pairs of denim jeans produced annually are finished with cellulases as an alternative to pumice stones (Buchert and Heikinheimo, 1998). Cellulases from the fungi *T. reesei* and *Humicola insolens* are widely used in the textile industry and depending on the desired effect different types of cellulases are utilized. In the detergent industry cellulases are used to clean cotton garments or to brighten faded coloured garments by removing fuzz (Maurer, 1997). In animal feed cellulases are utilized together with other hydrolases in the degradation of non-starch polysaccharides to improve feed conversion rates (Galante *et al.,* 1998b). The food industry uses cellulases together with other plant cell wall-degrading enzymes in fruit and

vegetable processing (Urlaub, 2002). Enzymatic hydrolysis of biomass to sugars for subsequent ethanol production has also been a major research area during recent years (Himmel *et al.,* 1999). Cellulases have potential in the pulp and paper industry, e.g. in deinking to release ink from fibre surfaces and in improving pulp drainage (Suurnäkki *et al.,* 2004). Within the forest industry cellulases have been shown to be effective in decreasing the energy consumption of mechanical pulping (Pere *et al.,* 2002). Cellulases have been found to increase the alkali solubility of treated pulp and directly alkali soluble cellulose has been obtained with specific cellulase compositions (Vehviläinen *et al.,* 1996, Rahkamo *et al.,* 1996). This property can be utilized in developing new, environmentally benign processes for manufacturing cellulosic articles such as films and fibres. The wide spectrum of industrial uses for cellulases establishes a need for commercial cellulase products containing different cellulase components and functioning optimally in different pH and temperature ranges.

1.1 Enzymatic degradation of cellulose

1.1.1 Cellulose

Cellulose is the main constituent of plants and thus the most abundant biopolymer on earth. Native cellulose is an unbranched homopolysaccharide consisting of D-glucose residues linked by β-1,4-glycosidic bonds to form a linear polymeric chain (Figure 1). The smallest repetitive unit in cellulose is cellobiose, which consists of two glucose units. In nature, cellulose chains have a degree of polymerization (DP) of approximately 10 000 glucose units in wood cellulose and 15 000 in native cotton cellulose (Sjöström, 1981). In crystalline cellulose the chains adhere to each other by hydrogen bonding and van der Waals forces to form highly insoluble structures. In addition to crystalline regions, native cellulose contains less-ordered amorphous or paracrystalline regions (Teeri, 1997 and refs there). Six polymorphs of cellulose (I, II, III_1, III_{11}, IV_1 and IV_{11}) have been documented (reviewed in O'Sullivan, 1997). Cellulose I, or native cellulose, the form found in nature, exists further in two crystalline forms termed celluloses Iα and Iβ. Cellulose II can be obtained from cellulose I by regeneration or merceration. Celluloses III_1 and III_{11} are formed from celluloses I and II by treatment with liquid ammonia. Polymorphs IV_1 and IV_{11} may be prepared by heating celluloses III_1 and III_{11}.

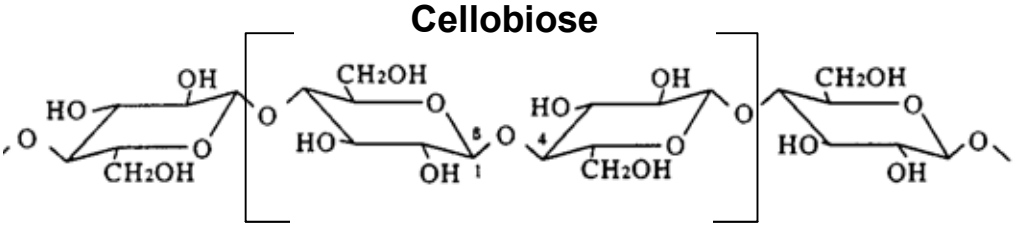


Figure 1. The cellulose chain.

1.1.1.1 Cotton

Cotton fibre originates from the seed hair of plants of the genus *Gossypium*. The fibre appears as a long, irregular, twisted, and flattened tube (Morton and Hearle, 1997). In cross-section, mature fibres have the form shown in Figure 2. The outermost layer is the cuticle, a thin film of fats, pectin and waxes (Trotman, 1993). Beneath this is the primary wall, composed mainly of cellulose in which the fibrils are arranged in a criss-cross pattern. The bulk of the fibre is made up of the secondary wall, composed of cellulose. The secondary wall is differentiated into three discernible zones. In the centre is a narrow collapsed lumen.

In addition to cellulose, raw cotton fibre contains impurities such as oil, waxes, pectins, proteins and simpler related nitrogenous compounds, organic acids, mineral matter, and natural colouring agents (Trotman, 1993). 85–90 % of raw cotton is composed of cellulose.

According to Rouette (2002), one cotton fibre consists of 15 000 microfibrils, and one microfibril contains 400 elementary fibrils. In one elementary fibril 100 cellulose chains are arranged in 6–8 packages. Cotton fibres consist of crystalline fibrils between which amorphous unordered regions are found. Approximately 70 % of the cotton fibre is crystalline (Needles, 1986, Morton and Hearle, 1997). Voids, spaces, and structural irregularities occur in the amorphous areas, whereas the cellulose chains in the crystalline regions are

tightly packed. Penetration of dyestuffs and chemicals occurs more readily in the amorphous regions (Needles, 1986).

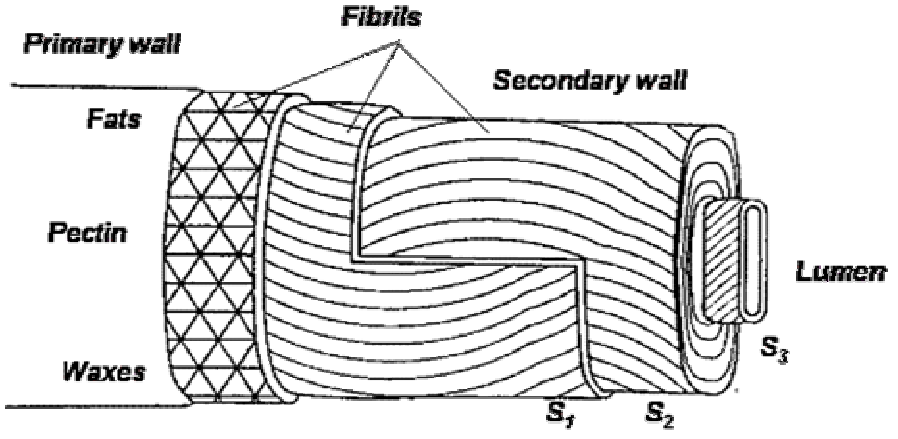


Figure 2. Morphological structure of the cotton fibre, S = secondary wall, S_1–S_3 represent different layers of secondary wall (Morton and Hearle, 1997).

1.1.2 Hydrolysis of cellulose

A wide variety of bacteria and fungi produce cellulolytic enzymes able to hydrolyze cellulose. However, relatively few fungi and bacteria produce high levels of extracellular cellulase capable of solubilizing crystalline cellulose extensively (reviewed in Bhat and Bhat, 1997). Saprophytic filamentous fungi produce three categories of cellulases, endoglucanases (EG, 1,4-β-D-glucan glucanohydrolase; EC 3.2.1.4), cellobiohydrolases (also called exoglucanases, CBH, 1,4-β-D-glucan cellobiohydrolase; EC 3.2.1.91), and β-glucosidases (cellobiase or β-D-glucoside glucohydrolase, BGL; EC 3.2.1.21) in order to degrade insoluble cellulose into glucose. Endoglucanases cleave bonds along the length of the cellulose chains in the middle of the amorphous regions, resulting in a decrease in the DP of the substrate (reviewed in Teeri and Koivula, 1995, Teeri, 1997) (Figure 3). Cellobiohydrolases are processive enzymes, initiating their action from the ends of the cellulose chains. They attack the crystalline parts of the substrate, produce primarily cellobiose, and decrease the DP of the substrate only very slowly. The hydrolysis of the glycosidic bonds occurs by

general acid catalysis with the involvement of two carboxylic amino acids (reviewed in Koivula *et al.,* 1998). The cellobiohydrolases act synergistically with each other and with endoglucanases: *i.e*. mixtures have a higher activity than the sum of the activities of the individual enzymes acting alone. Cellulolytic fungi generally produce two different CBHs. *Trichoderma reesei* CBHI and CBHII have opposite chain-end specificities with regard to liberation of cellobiose from the end of the cellulose glucan chains. *T. reesei* CBHI attacks the reducing end, whereas CBHII acts at the non-reducing end (Barr *et al.,* 1996). Cellobiohydrolase and endoglucanases act together to hydrolyze cellulose to small cello-oligosaccharides. In the final cellulose hydrolysis step β-glucosidases hydrolyze the soluble oligosaccharides and cellobiose to glucose.

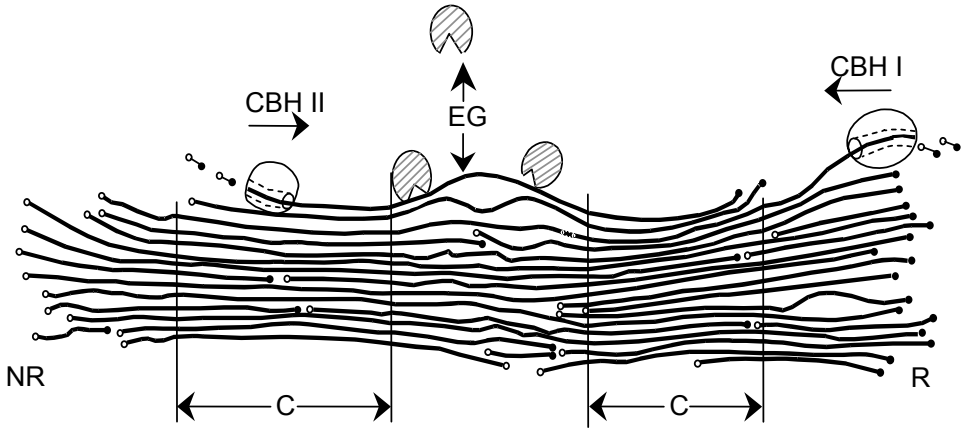


Figure 3. Mechanism of enzymatic hydrolysis of cellulose (Teeri, 1997). The two cellobiohydrolases (CBH) attack the crystalline areas at the opposite chain ends and endoglucanases (EG) in the middle of the more disordered regions of cellulose. The filled circles, denoted R, represent the reducing ends and the open circles, denoted NR, represent the non-reducing ends. C indicates the highly ordered crystalline regions.

Degradation of native cotton cellulose by *T. reesei* EGI and CBHII has been studied by analyzing the insoluble cellulose fragments remaining after enzymatic hydrolysis (Kleman-Leyer *et al.,* 1996). During the incubation EGI alone solubilized the cellulose (increase of weight loss, decrease of DP), but CBHII did not depolymerize the cellulose. A synergistic effect was observed in reducing sugar production. It was concluded that EGI degrades cotton cellulose

by selectively cleaving through the microfibrils at the amorphous sites, whereas CBHII releases soluble sugars from the EGI-degraded cotton cellulose. After cellulase treatment without additional mechanical action, purified EGI, EGII, CBHI and CBHII from *T. reesei* all reduced the molecular weight of powder formed from cotton poplin (Heikinheimo *et al.,* 2003). When mechanical action was combined with the enzyme treatments, only EGII reduced the molecular weight. Rousselle *et al.* (2002) observed changes in molecular weight distribution, weight and strength losses, fibre pore distribution and hydrogen bonding patterns of cotton cellulose after treatment of the cotton fabric with total cellulase of *T. reesei.*

1.2 Fungal cellulolytic enzymes

The cellulase systems of the aerobic fungi *Trichoderma reesei, Trichoderma viride, Penicillium pinophilum, Phanerochaete chrysosporium* (*Sporotrichum pulverulentum*)*, Fusarium solani, Talaromyces emersonii, Trichoderma koningii* and *Rhizopus oryzae* are well characterized (reviewed in Bhat and Bhat, 1997, Murashima *et al.,* 2002). Some thermophilic aerobic fungi (see 1.2.3.) and mesophilic anaerobic fungi (*Neocallimastix frontalis, Piromonas communis, Sphaeromonas communis*) (Bhat and Bhat, 1997) also produce cellulases.

1.2.1 Classification of cellulases

Cellulases belong to the O-glycoside hydrolases (EC 3.2.1.–), which are a widespread group of enzymes hydrolyzing the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. The IUB Enzyme Nomenclature (EC-number) is based on the type of reaction that enzymes catalyze and on their substrate specificity. According to the new classification glycoside hydrolases (GHs) are classified in families based on amino acid sequence similarities (Henrissat, 1991, Henrissat and Bairoch, 1993, Henrissat and Bairoch, 1996, Bourne and Henrissat, 2001). There is a direct relationship between sequence and folding (Henrissat, 1991). There are two major cleavage mechanisms for glycoside hydrolases, leading to overall retention or inversion of the stereochemistry at the cleavage point, and the mechanism appears to be conserved within each family (reviewed in Henrissat and Bairoch, 1996). Thus the catalytic domains of GHs in one family have the

same three-dimensional fold and exhibit the same stereospecificity of hydrolysis; for example retaining in family 5 and inverting in family 6 (Henrissat *et al.,* 1998). There are currently (as in July 2004) 97 families of GHs (URL http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html), with cellulases being found in at least 13 of them. Some 450 cellulase genes (derived from all kinds of microorganisms) exist in public domain databases (Schülein, 2000).

1.2.2 Modular structures of cellulases

Many of the fungal hydrolases are modular proteins, and all of them contain a catalytic domain (CD). In addition to the CD, GHs may contain a carbohydrate-binding module (CBM), which is defined as contiguous amino acid sequence within a carbohydrate-active enzyme with a discreet fold having carbohydrate-binding activity (Bourne and Henrissat, 2001). CBMs were previously classified as cellulose-binding domains (CBD), based on the initial discovery of domains that bind to cellulose (Tomme *et al.,* 1995a, b). The CBMs also form sequence-based families and are numbered with arabic numerals (Bourne and Henrissat, 2001). Currently (as in July 2004) there are 39 families of CBM (http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html), with CBMs of cellulases being found in at least 13 of them.

CBMs in most fungal cellulases are located at either terminus of the catalytic domain and are connected to CD by an often heavily glucosylated linker. The characterized role of the CBM is to mediate binding of the enzyme to the insoluble substrate. The overall binding efficiency of the cellulase is much enhanced by the presence of the CBM and the enhanced binding clearly correlates with better hydrolytic activity towards insoluble cellulose (reviewed in Linder and Teeri, 1997). Removal of the CBM has little influence on activity of cellulases towards soluble subtrates, although their binding properties and catalytic activity towards insoluble cellulose is clearly decreased (reviewed in Linder and Teeri, 1997). For example the presence of CBM in *T. reesei* cellulases is reported to enhance the enzymatic hydrolysis of insoluble isolated cellulose and chemical pulp (Suurnäkki *et al.,* 2000). CBMs allow cellulases to act on crystalline cellulose by destabilising the hydrogen bond structure of cellulose, making the polysaccharide chains more accessible to the catalytic domain (Quentin *et al.,* 2003). Fungal CBMs are also reported to contribute to the non-hydrolytic disruption of cotton fibre.

1.2.3 Thermophilic cellulases

Thermophilic fungi have a growth temperature minimum at or above 20°C and a growth temperature maximum at or above 50°C. Cellulases have been isolated from various thermophilic fungi, e.g. *Chaetomium thermophile, Humicola insolens, Humicola grisea* var. *thermoidea, Myceliophthora thermophila, Talaromyces emersonii* and *Thermoascus aurantiacus* (reviewed in Maheshwari *et al.,* 2000). The identified endoglucanases (30 to 100 kDa) of thermophilic fungi are thermostable, with optimal activity between 55 and 80°C at pH 5.0 to 5.5 and with carbohydrate contents from 2 to 50 % (reviewed in Maheshwari *et al.,* 2000) (Table 1). The exoglucanases (40 to 70 kDa) are reported to have an activity optimum between 50 and 75°C and they are thermostable.

Melanocarpus albomyces is a thermophilic ascomycete with alternative names *Myriococcum albomyces* and *Thielavia albomyces* (Maheshwari *et al.,* 2000). Qureshi *et al*. (1980) reported M*elanocarpus albomyces* to be a cellulolytic fungus, but according to Maheshwari and Kamalam (1985) it is unable to utilize cellulose. According to El-Gindy (1991) and Chung (1971) *Myriococcum albomyces* produces cellulases. *Melanocarpus albomyces* has been reported to produce xylanase (Prabhu and Maheshwari, 1999) and laccase (Kiiskinen *et al.,* 2002) activities.

Table 1. Properties of various endoglucanases of thermophilic fungi (reviewed in Maheshwari et al*., 2000).*

Fungus	**Optimal pH**	**Optimal temp. (°C)**	**Mol mass kDa**	**Carbo-hydrate %**
*Chaetomium thermophile**	5–6	55–60	36–41	n.r.
Humicola grisea var. thermoidea	5	n.r.	63	n.r.
Humicola insolens	5–5.6	50	45–57	16–39
*Myceliophthora thermophila***	4.8	65	100	n.r.
Talaromyces emersonii	5.5–5.8	75–80	35	27.7–50.8
Thermoascus aurantiacus	2.9–4.5	65–76	32–34	1.7–1.8

* Other names *C. thermophilum, C.thermophilium*

** Other names *Sporotrichum thermophilum / thermophile, Chrysosporium thermophilum*

n.r.= Not reported

1.2.4 Structure of fungal cellulases used in cotton finishing

Fungal cellulases from various sources are currently used in denim finishing to impart a stone-washed appearance and in biofinishing (more details in 1.5. and Table 6).

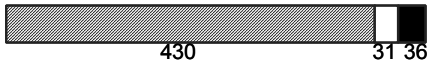
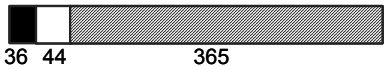
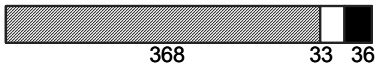
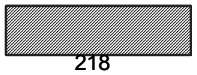
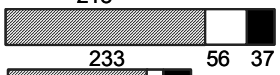
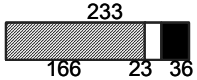
Trichoderma reesei secretes a complete mixture of cellulolytic enzymes for degradation of crystalline cellulose to glucose. It is an asexually reproducing filamentous fungus, isolated from cotton canvas in the Solomon Islands during World War II (Kuhls *et al.,* 1996). *T. reesei* is an asexual clonal line derived from a population of the tropical saprophytic Ascomycete *Hypocrea jecorina* (Kuhls *et al.,* 1996). The cellulolytic system of *T. reesei* is composed of at least eight endoglucanases and two cellobiohydrolases (Table 2). Seven β-glucosidases, which hydrolyze cellobiose to glucose, have been identified (Table 2). *T. reesei* cellulases generally have a modular structure consisting of a cellulose binding module (CBM, previously called CBD) at either end of the polypeptide chain, connected to the catalytic domain by a linker region (Shoemaker *et al.,* 1983, Teeri *et al.,* 1987, Penttilä *et al.,* 1986, Saloheimo *et al.,* 1988, 1994, 1997). *T. reesei* EGIII lacks the CBM and the linker region (Ward *et al.,* 1993, Okada *et al.,* 1998). The most recently identified genes encoding endoglucanases are *cel74a, cel61b* and *cel5b* (Foreman *et al.,* 2003). Cel74a is the first member of GH family 74 to be found in *T. reesei*, and it includes a C-terminal carbohydrate-binding domain of the CBM1 family. Cel61B has no CBM. Cel5B may represent a membrane-bound endoglucanase. The 3D-structures of the catalytic cores of CBHI (Cel7A, Divne *et al.,* 1994), CBHII (Cel6A, Rouvinen *et al.,* 1990), EGI (Cel7B, Kleywegt *et al.,* 1997) and EGIII (Cel12A, Sandgren *et al.,* 2001) and of the cellulose binding domains of CBHI (Cel7A, Kraulis *et al.,* 1989) and EGI (Cel7B, Mattinen *et al.,* 1998) have been solved. The active site of the cellobiohydrolases CBHI and CBHII is located in a tunnel, whereas the endoglucanases EGI and EGIII have the active site in an open cleft. An additional protein, swollenin (encoded by a gene *swo1*), which is assumed to disrupt e.g. the structure of cotton fibres, has been described (Saloheimo *et al.,* 2002b). This protein has an N-terminal fungal type CBM connected by a linker to the expansin-like domain.

The saprophytic thermophilic fungus *Humicola insolens* produces at least six different endoglucanases and two cellobiohydrolases, genes for which have all

been cloned (Dalboge and Heldt-Hansen, 1994, Schülein, 1997, Schülein *et al.,* 1998, Murashima *et al.,* 2000) (Table 3). At least five cellulase genes have been cloned from *Fusarium oxysporium* (Sheppard *et al.,* 1994) (Table 4). Cellulase genes have been cloned from the fungal ascomycetes *Myceliophthora thermophila, Acremonium* sp.*, Macrophomina phaseolina* and *Thielavia terrestris* and from the fungal basidiomycete *Crinipellis scabela* (Schülein *et al.,* 1996, 1998) (Table 4). Two family 45 endoglucanase genes were found in *Acremonium* sp. (Schülein *et al.,* 1998).

3D-structures of *Humicola* EGI (Cel7B, Mackenzie *et al.,* 1998) and EGV core (Cel45, Davies *et al.,* 1995) have been published. The 3D-structure of *Fusarium* EGI has been solved (Sulzenbacher *et al.,* 1996). The crystal structure of the family 45 cellulase (*Ma* 20kDa EG) from *Melanocarpus albomy*ces has recently been determined (Hirvonen and Papageorgiou, 2002, Valjakka and Rouvinen, 2003).

Table 2. Cellulolytic system of T. reesei.

Enzyme	Gene	GenBank™ accession no.	Length[1]	Molecular mass, kDa[11]	Structural organisation[3]	Ref.
CEL7A (CBHI)	*cbh1/cel7a*	P00725[12]	513	59-68		Shoemaker *et al*., 1983, Teeri *et al*., 1983
CEL6A (CBHII)	*cbh2/cel6a*	M16190	471	50-58		Teeri *et al*., 1987
CEL7B (EGI)	*egl1/cel7b*	M15665	459	50-55		Penttilä *et al*., 1986
CEL5A (EGII)	*egl2/cel5a*	M19373	418	48		Saloheimo *et al*., 1988
CEL12A (EGIII)	*egl3/cel12a*	AB003694	234	25		Ward *et al*., 1993, Okada *et al*., 1998
CEL61A (EGIV)	*egl4/cel61a*	Y11113	344	34[2]		Saloheimo *et al*., 1997
CEL45A (EGV)	*egl5/cel45a*	Z33381	242	23[2]		Saloheimo *et al*., 1994
EGVI[4]				95-105		Bower *et al.*, 1998a
CEL74A (EG)[5,8]	*cel74a*	AY281371	838	87[2]	Contains CBM[10]	Foreman *et al*., 2003
CEL61B (EG)[5,8]	*cel61b*	AY281372	249	27[2]	No CBM[10]	Foreman *et al*., 2003
CEL5B (EG)[5,9]	*cel5b*	AY281373	438	47		Foreman *et al*., 2003
CEL3A (BGLI)	*bgl1/cel3a*	U09580	744	75		Barnett *et al.*, 1991, Mach, 1993
CEL1A (BGLII)	*bgl2/cel1a*	AB003110	466	52		Takashima *et al*., 1999, Saloheimo *et al*., 2002a
CEL3B (BGL)[5,6]	*cel3b*	AY281374	874	94[2]		Foreman *et al*., 2003
CEL3C (BGL)[5,7]	*cel3c*	AY281375	833	91[2]		Foreman *et al*., 2003
CEL1B (BGL)[5,7]	*cel1b*	AY281377	484	55[2]		Foreman *et al*., 2003
CEL3D (BGL)[5,7]	*cel3d*	AY281378	700	77[2]		Foreman *et al*., 2003
CEL3E (BGL)[5,6]	*cel3e*	AY281379	765	83[2]		Foreman *et al*., 2003

[1]Length of the protein including the signal peptide, amino acid residues, [2] Molecular mass calculated from the amino acid sequence, [3] = CD, β = linker, ■ = CBM [4] Described on the protein level,

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Table 3. Cellulolytic system of H. insolens.

Enzyme	Amino acid residues			Reference
	Core	Linker	CBM	
CBHI (Cel7)	437	42	36	Dalboge and Heldt-Hansen, 1994
CBHII (Cel6)	366	45	38	Schülein, 1997
EGI (Cel7)	398	15		Schülein, 1997
EGII (Cel5)	303	33	36	Dalboge and Heldt-Hansen, 1994
EGIII (Cel12)	224			Dalboge and Heldt-Hansen, 1994
EGV (Cel45)	213	33	38	Rasmussen *et al.,* 1991
EGVI (Cel6)	346	37	37	Dalboge and Heldt-Hansen, 1994
EG (NCE4)	Total 284			Murashima *et al.,* 2000

Table 4. Cellulases of Acremonium sp., C. scabela, M. thermophila, M. phaseolina, T. terrestris and F. oxysporium.

Organism	Enzyme	Gene	Amino acid residues	Reference
Acremonium sp.	EGV (Cel45)	n.r.	208 core, 36 linker, 35 CBM	Schülein *et al.,* 1998*
	EG	n.r.	295	Schülein *et al.,* 1996*
	EG	n.r.	349	Schülein *et al.,* 1996*
	CBHI (partial)	n.r.	160	Lange *et al.,* 2003*
	CBHI (partial)	n.r.	164	Lange *et al.,* 2003*
C. scabela	EGV (Cel45)	n.r.	204, no linker or CBM	Schülein *et al.,* 1998*
	EG	n.r.	226, no linker or CBM	Schülein *et al.,* 1996*
M. thermophila	EGI (Cel7)	n.r.	436	Schülein *et al.,* 1998*, Osten and Schülein, 1999
	EGV (Cel45)	n.r.	207	Schülein *et al.,* 1998*
	EG	n.r.	225, no linker or CBM	Schülein *et al.,* 1996*
M. phaseolina	EGV (Cel45)	n.r.	203, no linker or CBM	Schülein *et al.,* 1998*
	EGI (Cel5)	*egl1*	333	Wang and Jones, 1995b
	EGII (Cel5)	*egl2*	368, no linker or CBM	Wang and Jones, 1995a
T. terrestris	EGV (Cel45)	n.r.	211 core, 30 linker, 37 CBM	Schülein *et al.,* 1998*
	EG	n.r.	299	Schülein *et al.,* 1996*
F. oxysporium	EGI (Cel7B)	*Cfam1*	427	Sheppard *et al.,* 1994
	EGV (Cel45)	*Kfam1*	312 core, 34 linker, 31 CBM	Sheppard *et al.,* 1994
	n.r.	*Ffam1*	323 core, 32 linker, 31 CBM	Sheppard *et al.,* 1994
	CBHI (Cel7A)	*Cfam2*	441 core, 43 linker, 31 CBM	Sheppard *et al.,* 1994
	EG B	*Bfam1*	392 core, 39 linker, 31 CBM	Sheppard *et al.,* 1994

n.r. = not reported, * Limited information is given in the references.

1.3 Production of cellulases by *T. reesei*

Filamentous fungi are used for the commercial production of various metabolites. The main genera of filamentous fungi used in industry are *Trichoderma* (enzymes), *Aspergillus* (enzymes, organic acids, fermented foods), *Mucor* (enzymes), *Rhizopus* (enzymes), *Penicillium* (antibiotics, cheese) and *Cephalosporium* (antibiotics) (Nevalainen, 2001).

T. reesei has several advantages for industrial-scale production of homologous and heterologous cellulases and proteins in general. *T. reesei* is easy and inexpensive to cultivate and it is currently grown in fermenters up to 230 m^3, which shows that its fermenter technical properties are good and that it is not susceptible to contamination (Penttilä, 1998). *T. reesei* has a secretory machinery with protein modifications typical of eukaryotes (Palamarczyk *et al.,* 1998). *T. reesei* is considered to be a safe production organism, because it is non-pathogenic to healthy humans and does not produce mycotoxins or antibiotics under the conditions used for enzyme production (Nevalainen *et al.,* 1994, Nevalainen and Neethling, 1998). *T. reesei* strains producing cellulases have been evaluated as belonging to Group I (low-risk level) microorganisms (EC Directive 90/219/EEC) (Penttilä *et al.,* 2004). *T. reesei* produces extracellular proteins and cellulases naturally in large quantities. Industrial *T. reesei* strains have been obtained by using classical mutagenesis techniques to enhance the release of extracellular proteins in general, to increase cellulase production levels (reviewed in Mäntylä *et al.,* 1998, Durand *et al.,* 1988a) and to obtain protease-deficient mutants (Mäntylä *et al.,* 1994). *T. reesei* mutant strains have been reported to produce up to 40 g/l proteins in the culture medium in optimized cultivation conditions (Durand *et al.,* 1988b). Of the extracellular proteins produced by *T. reesei* the main part is composed of cellulases. CBHI represents approximately 50 % of all protein secreted by wild-type *T. reesei* (Penttilä, 1998). The proportion of CBHI is about 60 %, CBHII 25 % and of endoglucanases 15 % of the cellulase proteins of *T. reesei* (Gritzali and Brown, 1979). CBHII accounts for the major portion of the conidial-bound cellulases (Stangl *et al.,* 1993). Production of EGI is reported to represent 5 to 10 % of the secreted protein (Penttilä *et al.,* 1987), but EGII is proposed to account for most of the endoglucanase activity produced by *T. reesei* (Suominen *et al.,* 1993).

Successful mutagenesis, screening and process development has led to *T. reesei* strains feasible for commercial cellulase production, but genetically modified strains have further been developed from the classical strains for various applications (Mäntylä *et al.,* 1998). The essential tools needed for construction of industrial production strains by genetic engineering have been developed for *Trichoderma.* These include different mutant strains that are used as hosts for recombinant protein production, transformation techniques with a variety of selection markers (reviewed in Mach and Zeilinger, 1998, Hazell *et al.,* 2000), methods for gene targeting and replacement (Suominen *et al.,* 1993) and a series of expression cassettes and transcription termination sequences (reviewed in Nevalainen, 2001). Recent developments in sequencing of the genome of *T. reesei* (completed, not published, http://www.jgi.doe.gov) and EST sequencing of *T. reesei* (http://trichoderma.iq.usp.br/TrEST.html), as well as the first transciptional profiling experiments with available EST sequences (Chambergo *et al.,* 2002) and the first proteomics methods (Lim *et al*., 2001), will provide means to further improve the efficient protein production system of *Trichoderma* (Penttilä *et al.,* 2004).

1.3.1 Regulation of cellulase gene expression

In *T. reesei* the production of cellulases is controlled at the transcriptional level depending on the available carbon source (Kubicek and Penttilä, 1998). Transcription of the major components of cellulase (CBHI, CBHII, EGI, EGII, EGIII, EGIV, EGV, Cel61B, Cel74A) is induced by cellulose and a variety of disaccharides including lactose, β-cellobiono-1,5-lactone, cellobiose, and sophorose (two β-1,2-linked glucose units) (Kubicek *et al.,* 1993, Saloheimo *et al.,* 1997, Ilmén *et al.,* 1997, Nogawa *et al.,* 2001, Foreman *et al.,* 2003). The best inducing compound known to date is sophorose (reviewed in Mach and Zeilinger, 2003). When glucose, fructose, or glycerol are used as the carbon source, no significant levels of cellulases are produced (Ilmén *et al.,* 1997). In inducing conditions the cellulase genes (*cbh1, cbh2, egl1, egl2, egl4* and *egl5*) have been shown to be expressed coordinately, and expression of the *cbh1* gene has shown to be the highest (Ilmén *et al.,* 1997, Saloheimo *et al.,* 1997). Behaviour of the *egl3* has been studied less, but according to Nogawa *et al.* (2001) it is coordinately transcribed together with the other cellulase genes at least when induced by sophorose. The gene for the putative membrane-bound endoglucanase *cel5b* has different transcriptional regulation as compared to the

other endoglucanases. It is expressed on both glucose and glycerol and is induced only slightly by either cellulose or sophorose (Foreman *et al.,* 2003).

T. reesei cellulase regulation has been analyzed at the molecular level. Cellulase genes are repressed in the presence of glucose by the carbon catabolite repressor CRE1 (Ilmén *et al.,* 1996, Strauss *et al.,* 1995). In addition to CRE1 the transcription factors ACEI and ACEII are known to regulate the cellulase promoters in *T. reesei*. ACEII is known to bind to the *cbh1* promoter (Aro *et al.,* 2001). ACEII is an activator of the main cellulase genes in cellulose-induced cultures (Aro, 2003). ACE1 is also able to bind to the *cbh1* promoter (Saloheimo *et al.,* 2000). Deletion of *ace1* resulted in an increase in the expression of all the main cellulase genes in sophorose- and cellulose-induced cultures, indicating that ACEI acts as a repressor of cellulase expression (Aro *et al.,* 2003). Zeilinger *et al*. (1998) identified a *cbh2* activating element in the 5' regulatory sequences of the *cbh2* gene to which the transcriptional regulator HAP2/3/5 protein complex and an as yet unknown further protein bind and are essential for induction of *cbh2* gene expression (reviewed in Mach and Zeilinger, 2003).

1.3.2 Homologous and heterologous expression

In filamentous fungi the production of heterologous proteins is often limited whereas the production of homologous proteins can be high (Iwashita, 2002). Many fungi can secrete gram per liter amounts of endogenous and heterologous fungal gene products in the culture medium, but attempts to produce high levels of proteins of bacterial, plant and mammalian origin have been less successful (Nevalainen, 2001). The factors limiting the amount of heterologous products synthesized in filamentous fungi have been codon usage, proteolytic processing, protein folding, glycosylation and proteolytic degradation (Nevalainen, 2001). The strategies used for the overproduction of fungal proteins in filamentous fungi have included introduction of multiple copies of the gene of interest, use of the promoters of highly expressed genes, expression at a locus of a highly expressed gene, accomplishing position-independent expression, minimizing the proteolytic degradation and development of improved cultivation media (Verdoes *et al.,* 1995). Additionally especially heterologous gene expression of non-fungal proteins in filamentous fungi has been improved by fusing the

corresponding gene to the 3' end of the homologous gene or a fragment thereof (Gouka *et al.,* 1997, Paloheimo *et al.,* 2003).

T. reesei has excellent capacity to secrete large amounts of proteins. CBHI is the major secreted protein of *T. reesei,* accounting about 50 % of the total secreted proteins (Penttilä, 1998). CBHI is the product of a single gene and thus the *cbh1* promoter is regarded as a strong promoter. According to Ilmén *et al.* (1997) in inducing conditions the steady-state mRNA level of *cbh1* is approximately 1.5 and 3 times more abundant than that of *cbh2* and *egl1* mRNAs, respectively. For these reasons the *cbh1* promoter has been exploited for expressing various homologous hydrolases (reviewed in Mäntylä *et al.,* 1998) and heterologous fungal, bacterial and mammalian proteins (reviewed in Paloheimo *et al.,* 1993, Verdoes *et al.,* 1995, Gouka *et al.,* 1997, Radzio and Kück, 1997, Penttilä, 1998, Bergquist *et al.,* 2002) for various applications. Examples of heterologous products expressed and secreted in *T. reesei* include calf chymosin, antibody Fab fragments and single chain antibodies, interleukin-6, tissue plasminogen activator and heterologous fungal proteins such as laccase, chitinase, acid phosphatase and thermophilic enzymes (reviewed in Penttilä *et al.,* 2004). Production of proteins from unrelated species has been shown to benefit from production as fusions to a well-expressed native protein such as CBHI (Penttilä *et al.,* 2004). Successful production of a bacterial xylanase in *T. reesei* has been demonstrated by altering the codon usage pattern (Te'o *et al.,* 2000).

In *T. reesei,* secreted amounts of heterologous fungal enzymes have reached grams per litre level (Paloheimo *et al.,* 1993), although the levels of some proteins such as lignin peroxidases and certain laccases have remained low (Penttilä *et al.,* 2004). For example *Hormoconis resinae* glucoamylase was expressed at a rather high level (0.7 g/l) in *T. reesei* under the *cbh1* promoter (Joutsjoki *et al.,* 1993). Expression of proteins as a fusion to native protein has also been used in production of heterologous fungal proteins in *T. reesei*. As an example expression of *xyn2,* encoding a family 11 xylanase of the thermophilic fungus *Humicola grisea* var. *thermoidea,* as a fusion to the *cbh1* signal sequence yielded enzymatically active xylanase protein at a level of 0.5 g / l (de Faria *et al.,* 2002). However, fusion of *xyn* to CBHI core-linker resulted in a considerably lower amount of xylanase.

A summary of the published reports on expression of cellulases in *T. reesei* is presented in Table 5. Production of cellulases in *T. reesei* has been performed for various reasons. To improve the production of *T. reesei* EGI in a hypercellulolytic mutant strain, the *egl1* promoter of *T. reesei* was exchanged with the *T. reesei cbh1* promoter and the copy number of the *egl1* gene was increased (Karhunen *et al.,* 1993). The *egl1* cDNA was expressed from the *cbh1* promoter as efficiently as *cbh1* itself. Furthermore, a strain carrying two copies of the *cbh1-egl1* expression cassette produced twice as much EGI as the amount of CBHI produced by the host strain. The level of *egl1*-specific mRNA in the single-copy transformant was about 10-fold higher than that found in the non-transformed host strain, indicating that the *cbh1* promoter is about 10 times stronger than the *egl1* promoter. This apparent discrepancy with the result of 3 times stronger reported by Ilmén *et al.* (1997) could be simply due to the use of different strains and different culture conditions. *T. reesei* EGIII has been overproduced for textile processing. The *egl3* gene product was expressed as the major secreted product (deduced from SDS-PAGE) of *T. reesei* by overexpressing it under the *cbh1* promoter in a quad deletion strain (Δ*cbh1*, Δ*cbh2*, Δ*egl1* and Δ*egl2*) (Bower *et al.,* 1998b). EGIII was produced as glycosylated and unglycosylated enzymes. There was no apparent difference in thermostability, pH stability or in spesific activity of the two glycoforms.

T. reesei cellulases have also been expressed in *T. reesei* as fusions to different tags (purification aids). Karlsson *et al.* (2001) performed homologous expression of *T. reesei* EGIV (Cel61A) as a fusion protein with a histidine tag under the *cbh1* promoter in order to facilitate the purification of EGIV from the other cellulases. The tagged protein was produced in significant amounts (yield at the g / l level not reported) and 120 mg EGIV could be purified from 1 liter of culture filtrate. Collen *et al.* (2002) investigated the effects of fusion tags on partitioning of EGI to the hydrophobic detergent-enriched phase of an aqueous two-phase system to facilitate purification of cellulases. *T. reesei* EGI core fused with a peptide molecule $(WP)_4$ (= $Trp\text{-}Pro)_4$ was expressed under the *cbh1* promoter in *T. reesei*. Production of the fusion protein was lower than that of the endogeneous EGI production and only approximately 15 % of the total EG activity corresponded to the fusion protein $EGI_{core\text{-}P5}(WP)_4$.

Because the *cbh1* promoter is repressed by glucose, promoters active in glucose-containing media have also been screened and tested for cellulase production. A synthetic glucose-containing medium would be beneficial, because it is expected to support synthesis of lower levels of proteases, a condition important to production, and it would allow easier purification of the product (Nakari-Setälä and Penttilä, 1995). Some genes, such as *pgk1* encoding phosphoglycerate kinase and *pki1* encoding pyruvate kinase, active in the presence of glucose have been isolated, but only modest cellulase levels have been obtained by using these promoters (reviewed in Penttilä, 1998). *T. reesei cbh1* and *egl1* cores have been expressed by the promoters of the elongation factor 1α, *tef1*, and the unidentified gene for cDNA1 (Nakari-Setälä and Penttilä, 1995). In glucose-containing medium the cDNA1 promoter gave the highest amounts of CBHI and EGI core, more than 50 mg/l, and accounted for more than half of the total protein secreted by the fungus. Although the expression levels were rather high, they were not comparable to the endogenous cellulase protein levels produced on cellulase-inducing media. Furthermore the amount of total secreted proteins remained low (0.05–0.23 g/l), compared with that obtained with this strain on cellulose medium (1.1–1.8 g/l).

Recently a new promoter (*hex1* gene encoding hexagonal protein of the fungal Woronin body) of *T. reesei* has been described with option for gene expression (Nevalainen *et al.,* 2003). The *hex1* promoter was shown to be highly functional in both glucose medium and under conditions promoting induction of *cbh1*. *Hex1* promoter with a secretion signal sequence was used to express heterologous DsRed1-E1 -reporter gene and DsRed1-E1 message was observed (the amounts of DsRed1-E1 message produced were not reported).

Table 5. Expression of fungal cellulases in T. reesei.

Promoter	Cellulase gene	*T. reesei* host strain	Amount	Reference
Cellulase promoters				
T.r. cbh1	*T.r. egl1*	VTT-D-79125	EGI 1.9 g/l	Harkki *et al.,* 1991
T.r. egl1	*T.r. egl1*	VTT-D-79125	EGI 2.2 g/l, 3x parent	Karhunen *et al.,* 1993
T.r. cbh1	*T.r. cel61A* - His tag fusion	Rut-C30	Not determined	Karlsson *et al.,* 2001
T.r. cbh1	*T.r. egl1*$_{\text{core-P5}}$(WP)$_4$, fusion	Rut-C30	< endogeneous EGI	Collen *et al.,* 2002
T.r. cbh1	*T.r. egl3*	RL-P37	Major secreted product	Bower *et al.,* 1997, 1998b
T.r. endogeneous *cbh1*	*T.r.* endogeneous *cbh1*	*T.r.* strain overproducing *S. cerevisiae* MPD synthase	Amount of secreted proteins 7x wild type, elevated CBHI	Kruszewska *et al.,* 1999
T.r. cbh2	*T.r. cbh2*	QM9414	CBHII 34 μg / l, 2-4x parent	Kubicek-Pranz *et al.,* 1991
Promoters active in the presence of glucose				
T.r. modified *cbh1*	*T.r. egl1* core	QM9414	EGI 2 mg/l	Nakari-Setälä *et al.,* 1993
T.r. pgk1	*T. r. egl1*	QM9414	EG activity below detection limit, no result with Mab EGI	Vanhanen, 1991
T.r. cDNA1	*T. r. egl1 and cbh1* core	QM9414	EGI and CBHI 50 mg/l	Nakari-Setälä *et al.,* 1995
T.r. tef1	*T. r. egl1* core	QM9414	EGI 2 mg/l	Nakari-Setälä *et al.,* 1995

T.r. = T. reesei. The results are from shake flask cultivations.

1.3.3 Secretory pathway

The molecular mechanism of the protein secretion system of filamentous fungi, including *T. reesei*, has recently been studied to an increasing extent in order to further improve the efficiency of protein secretion (Radzio and Kück, 1997, Veldhuisen *et al.,* 1997, Conesa *et al.,* 2001, Saloheimo *et al.* 2004). In fungi, protein folding, glycosylation, disulphide bridge formation, phosphorylation and subunit assembly are performed in the endoplasmic reticulum (ER). ER-related events contribute to the folding of proteins and are believed to be one of the main reasons for low yields of heterologous products (Penttilä *et al.,* 2004). The unfolded protein response (UPR) mechanism detects the presence of unfolded proteins in the ER and induces the synthesis of folding enzymes (reviewed in Conesa *et al.,* 2001). ER-resident chaperones and foldases assist in protein secretion.

The gene *pdi1* encoding protein disulphide isomerase, an ER foldase, has been isolated from *T. reesei* (Saloheimo *et al.,* 1999). The *pdi1* promoter has two potential UPR elements and it was shown that the gene is under the control of the UPR pathway. A *T. reesei hac1* gene encoding the UPR transcriptional factor has been described (Saloheimo *et al.,* 2003a). Saloheimo *et al*. (2003b) observed that concurrently with the induction of the UPR pathway, the genes encoding secreted proteins are rapidly down-regulated in *T. reesei*. This type of regulation can be caused by different secretion inhibitors and by foreign protein expression.

In eukaryotes proteins travel from ER to the Golgi apparatus, where additional modifications (glycosylation) take place. The structure and function of secreted proteins can be modified by glycosylation processes. In general, filamentous fungi produce high mannose type N-glycans and are also capable of effective O-glycosylation (reviewed in Nevalainen, 2001). O-glycosylation has been shown to be essential for secretion of EGI and EGII in *T. reesei* (Kubicek *et al.,* 1987). Kruszewska *et al*. (1999) overexpressed in *T. reesei* a gene encoding *Saccharomyces cerevisiae* mannosylphosphodolichol synthase, which is required for glycan synthesis and is a key enzyme in the O-mannosylation reaction, and were able to increase the level of secreted CBHI (Table 5). This suggests that insufficient glycosylation might limit the overproduction of glycoproteins.

Finally, proteins packed in secretory vesicles are directed to the plasma membrane from where they are secreted. There is evidence that the secretory pathway also involves transport from the ER through the Golgi complex to the plasma membrane in *T. reesei* (Kruszewska *et al.,* 1999). Most studies indicate that protein secretion in filamentous fungi occurs in the apical or subapical hyphal regions (Archer and Peberdy, 1997). However, the *cbh1* mRNA and CBHI of *T. reesei* were both found to localize to all hyphae of a colony (Nykänen *et al.,* 1997). Thus *cbh1* mRNA can be found not only in apical compartments involved in the growth of the hyphae, but also in the old compartments of the colony.

1.4 Cellulases in cotton finishing

Cotton is the most important of all textile fibres. In 2001 about 21 Mt of cotton was produced, which accounts for about 32 % of the world textile fibre production (CIRFS, 2002). Processing of cotton fabric includes preparation, dyeing, printing and finishing (Fig. 4). The purpose of cotton preparation is to remove impurities and prepare the fabric for dyeing and for any other wet processing treatments that follow, such as printing and finishing (Rouette, 2002). Different processing steps are included in cotton preparation: desizing (removing sizing agents), scouring (removing impurities such as pectins and waxes) and bleaching (increasing whiteness). The textile finishing step provides a method whereby deficiences in the textile can be corrected or specific properties can be introduced. The most important cellulosic finishes include crease resistant and stabilizing finishes, soil release and softening finishes, oil and water repellant finishes, biologically protective finishes, and flame retardant finishes (Needles, 1986). Cellulase enzymes have been used as finishing agents in processing of cotton-containing materials since the 1980s (Oslon and Stanley, 1990). Cellulase treatments are carried out in the textile wet processing stage mainly before or after dyeing.

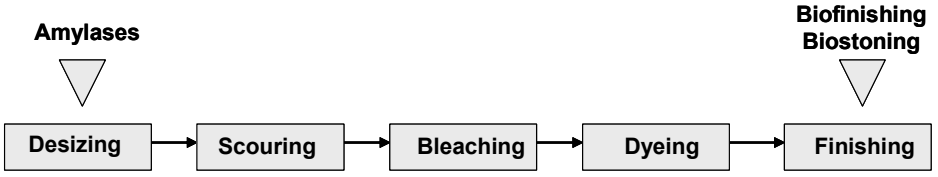


Figure 4. General processing stages of cotton fabric.

1.4.1 Use of cellulases in denim finishing

Denim is cotton twill-weave fabric with a dyed warp and raw white weft. The warp yarn is traditionally dyed with blue indigo. Stone-washed finish of denim refers to indigo-dyed denim with a faded appearance (Rouette, 2002). This is traditionally achieved by using pumice-stones when washing the desized (starch coating removed) articles, so that the dye is washed out partially and unevenly from the fabric. Cellulases have been introduced in the finishing of denim jeans and other denim garments to replace pumice stones and to achieve a washed-out appearance similar to that provided by pumice stones (Olson and Stanley, 1990). The process is called biostoning (Fig. 4). Cellulases attack primarily the surface of the fibre, but leave its interior intact. This mode of action makes cellulases especially suitable for stonewashing of denim garments dyed with indigo-blue, as the dye is located on the surface of the fibre (Tyndall, 1990). Washing the garments with stones partially removes this surface dye by abrasion. Cellulase treatment results in similar effect, as cellulases partially hydrolyze the surface of the fibre including the dye (Kochavi *et al.* 1990). In both cases mechanical action is needed to remove the dye and thus biostoning is usually carried out in e.g. jets or rotating drum washers. There are several advantages of using cellulases instead of stones. The use of cellulases prevents the damage both to the washing machines and to the garments, eliminates the need for disposal of the used stones, improves the quality of the waste-water and eliminates the need for labour-intensive removal of dust from the finished garments (Kochavi *et al.,* 1990). Furthermore the garment load can be increased by as much as 50 % as no stones need to be added to the machine.

Different types of cellulases are used for biostoning. The enzymes differ in a number of ways, one of the differences being pH optimum. Cellulases used for biostoning have traditionally been classified by the pH optimum of the enzyme: neutral cellulases operate in the pH range 6–8, alkaline-neutral cellulases in the pH range 7.5–8 and acid cellulases in the range of pH 4.5–6 (Videbaek *et al.,* 1994, Klahorst *et al.,* 1994). Acid cellulases used in biostoning mainly originate from *T. reesei* and are characterized by highly aggressive action on cotton, resulting in abrasion in a short time and associated with backstaining. Neutral and alkaline cellulases used in biostoning come from a variety of fungi (Table 6) and generally have been characterized by less aggressive action on cotton than

acid cellulases, requiring a longer wash time and resulting in little or no backstaining (Klahorst *et al.,* 1994, Solovjeva *et al*, 1998).

Cellulase preparations from various sources differ in their denim-washing performance and backstaining (Gusakov *et al.,* 1998, 2000a). Gusakov *et al.* (2000a) showed that commercial and laboratory cellulase preparations produced by different fungi (from the genera *Trichoderma, Penicillium, Chaetomium, Humicola*, and others) differed in their abrasive effect (the ability to remove indigo from denim) and no direct correlation was found between the ability to remove indigo and any specific cellulase activity (CMC, endoglucanase, filter paper activity, avicelase). Gusakov *et al.* (2000b) indicated that there is a certain correlation between the washing performance and the quantity of non-polar amino acid residues in the enzyme. Heikinheimo *et al.* (2000) indicated that by selecting the optimum *T. reesei* cellulase profile, the cellulase action can be directed towards the indigo-dyed parts, and further that endoglucanases are the cellulases required for good stone washing effects.

1.4.1.1 Backstaining

During biostoning with cellulases the released indigo dye has a tendency to redeposit on the surface of the denim fabric, thus reducing the desired contrast between white and the indigo-dyed blue yarn. This effect is termed backstaining and it is undesired because of lowered contrast between the blue and white yarn. In early reports backstaining was claimed to be dependent on pH (Kochavi *et al.,* 1990). Further experiments indicated that the nature of the enzyme used has an impact on backstaining, and neutral cellulases were found to cause low backstaining (Klahorst *et al.,* 1994). Indigo-cellulase affinities and enzyme adsorption on the white yarn of the denim fabric have been shown to cause backstaining (Cavaco-Paulo *et al.,* 1998b, Gusakov *et al.,* 1998, 2000b). *T. reesei* cellulases adsorbed onto cotton fabric caused higher indigo staining than comparable amounts of adsorbed *H. insolens* neutral cellulases (Cavaco-Paulo *et al.,* 1998b). Adsorption studies with plain indigo showed that cellulases from *T. reesei* with a higher content of neutral amino acids have a higher affinity for indigo dye than cellulases of *H. insolens* (Campos *et al.,* 2000). The authors also proposed that the non-polar residues present in higher percentages in the cellulases of *H. insolens* play an important role in the agglomeration of indigo

dye particles and probably in the reduction of backstaining. Gusakov *et al*. (1998) showed by using a model microassay system that backstaining by different enzyme preparations is different and suggested that protein adsorption on cotton garment is a crucial parameter causing backstaining. Gusakov *et al.* (2000b) showed with immobilized amino acids that indigo may be bound to non-polar amino acids.

Backstaining can be avoided by adding antiredeposition chemicals during the enzyme washing step and/or by adding a mild bleaching agent or stain removing agents during the rinsing steps (Tyndall, 1990, Yoon *et al.,* 2000). The use of different, particularly *Trichoderma,* cellulase compositions with less specific activity on denim has also been tested (Clarkson *et al.,* 1994a). Use of truncated *T. reesei* EGI and EGII cellulases (EGI and EGII catalytic core) and truncated CBHI (core) with EGIII was found to decrease backstaining while maintaining an equivalent or superior level of abrasion over their un-modified counterparts (Fowler *et al.,* 2001). Protease enzyme added during rinsing or at the end of the cellulase washing step resulted in significant reduction of backstaining and improved contrast (Yoon *et al.,* 2000). Denim that was stonewashed with the addition of lipolytic enzyme during cellulase treatment showed a reduction in the level of backstaining, especially the backstaining of pocket parts (Uyama and Daimon, 2002).

1.4.1.2 Effect of different cellulases in biostoning

The origins of the fungal cellulases used in biostoning and their reported effects are summarized in Table 6. In addition to the examples in Table 6, the use of *T. reesei* cellulases in biostoning has been described in numerous other publications. Cellulases of *T. reesei* are widely used in denim finishing one reason being their low price. Furthermore although *Trichoderma* cellulases result in high backstaining they are preferred because of their high activity on denim material, resulting in a short processing time (Clarkson *et al.,* 1994a). Finishing of denim with different ratios of *T. reesei* EGI and II and CBHI and II has been reported in several publications (Clarkson *et al.,* 1992a, 1992b, 1993, 1994a, b). Heikinheimo *et al.* (2000) showed that purified *T. reesei* EGII is the most effective cellulase for removing colour from denim, producing a good stone washing effect with the lowest hydrolysis level.

Schülein *et al.* (1998) isolated family 45 cellulases from six different fungal sources (Table 6) and found that they could be used for increased abrasion in denim finishing due to their efficient removal of indigo from the surface of cotton. They also isolated three family 7 cellulases from different sources (Table 6) and these cellulases accomplished very little abrasion. Murashima *et al.* (2000) isolated a novel cellulase NCE4 from *Humicola insolens* that can be used for various treatments of cellulose-containing fibres, such as for decolouring denim-dyed cellulose-containing fibres. A cellulase preparation of *Chrysosporium lucknowense* had higher abrasive activity and lower backstaining on denim than the *T. reesei* preparation, when a model microassay was used for testing denim-washing performance (Sinitsyn *et al.,* 2001). Of the four individual cellulase components (two endoglucanases – EG-25 and EG-50, and two cellobiohydrolases – CBH-43 and CBH-55) of *C. lucknowense*, EG-25 was the key enzyme responsible for indigo removal with relatively low backstaining. A cellulase preparation of *Penicillium occitanis* was successfully applied in a biostoning process in industrial scale (Belghith *et al.,* 2001). Cellulases (EGI) derived from *Myceliophthora thermophila* exhibited enhanced enzyme activity in the alkaline pH range and are described to be useful in preventing backstaining, but no examples of denim washing were presented in the patent publication (Osten and Schülein, 1999). In addition to fungal enzymes, bacterial and actinomycete cellulases have also been applied in denim treatment (van Beckhoven *et al.*, 1996, Farrington *et al.,* 2001, van Solingen *et al*., 2001).

Table 6. Fungal cellulases in biostoning applications.

Source	Cellulase	Application pH	Special effect	Reference
T. reesei	No or low CBHI EG:CBH, 5:1 Enriched CBHI EGII (purified) EGIII EGIII-dimer	4.5–5.5	Low strength loss Lint reduction, low strength loss Decreased strength loss Low hydrolysis level Suggestion of reduced strength loss in textile processing	Clarkson *et al.,* 1992a,b Clarkson *et al.,* 1992c, 1994b Clarkson *et al.,* 1993 Heikinheimo and Buchert, 2001 Fowler *et al.,* 2001 Bower *et al.,* 1998b
T. terrestris	EG (299 aa)	5	Almost bleached appearance	Schülein *et al.,* 1996
Acremonium sp.	EG (295 aa)	7	Low temperature optimum, high abrasion level	Schülein *et al.,* 1996
M. thermophila	EG (225 aa) + linker and CBM of *H. insolens* 43 kDa EG	6	Minimum strength loss, high abrasion level	Schülein *et al.,* 1996
H. insolens, M. thermophila, Acremonium sp., *M. phaseolina, T. terrestris, C. scabela*	EGV (Cel45)	n.r.	Good abrasion, fuzz removal	Schülein *et al.,* 1998
H. insolens, F. oxysporium	EGI (Cel7)	n.r.	Low strength loss, little abrasion	Schülein *et al.,* 1998
M. thermophila	EGI and variants	n.r.	Enhanced activity in the alkaline pH range	Osten and Schülein, 1999
H. insolens	EGV + EGI	7	Combination of desizing and stonewashing: abrading and streak-reducing	Lund, 1997
H. insolens	NCE4	7	De-colouring of denim	Murashima *et al.,* 2000
C. lucknowense	Total mixture	6.5 - 8	Similar wash performance to commercial neutral cellulase (Denimax XT / Ultra)	Solovjeva *et al.,* 1998
C. lucknowense	Total mixture EG-25	5	High abrasion on denim, prevention of backstaining	Sinitsyn *et al.,* 2001
P. occitanis		5.5	Denim finishing	Belghith *et al.,* 2001

1.4.2 Use of cellulases in biofinishing

Cellulase treatment for finishing of cellulose-containing textile materials, such as cotton, linen, lyocell and viscose materials, is called biofinishing or biopolishing (Videbaek and Andersen, 1993, Fig. 4). In the biofinishing process, cellulases achieve a controlled surface hydrolysis and the fibre ends protruding from the fabric surface are weakened and subsequently separated from the material with the aid of mechanical action. The benefits of cellulase treatment of cotton fabrics and garments include permanent improvement of depilling, cleared surface structure by reduced fuzz, improved textile softness, improved drapeability, brighter colours of the textile, improved dimensional stability and fashionable wash-down effects (Tyndall, 1992, Pedersen *et al.,* 1992, Kumar *et al.,* 1997, Cavaco-Paulo, 2001, Cortez *et al.,* 2002). One advantage of the biofinishing process is that the treatments can be adapted to run on existing equipment in the textile industry. In most cases the treatments have been carried out on garments and fabrics. Treatment of cotton yarn with a *Trichoderma* EGII was reported to result in decreased hairiness and increased evenness (Pere *et al.,* 2001). When the treated yarn was further knitted to a fabric, a decreased tendency to pilling was observed. Yarn treatment for pilling control may offer an advantage by overcoming the dust problems often encountered with biofinishing of knitted fabrics.

Of the *T. reesei* cellulases the endoglucanases have been shown to play a key role in biofinishing. Several studies have been conducted to evaluate the best cellulase component or cellulase combination for biofinishing with minimal effects on the weight and strength properties of the fabric. Heikinheimo *et al.* (1998) showed that purified *T. reesei* EGI and II caused more strength loss than purified CBHI, but also had positive effects on bending behaviour and pilling properties of cotton twill and poplin fabrics. According to the results of Heikinheimo and Buchert (2001) there are clear differences between individual purified CBHI, CBHII, EGI and EGII and their defined mixtures. Treatment of cotton interlock fabric showed that EGII-based combinations always resulted in good depilling properties. Furthermore, with an EGII: CBHI ratio of 25:75, practically no decrease in strength was observed despite high depilling. Pure CBHI or CBHII did not affect pilling properties, whereas EGI and EGII treatments clearly improved pilling values. Relatively more EGI was required as compared to EGII in order to obtain a similar improvement in pilling resistance.

T. reesei EGIV (Cel61A) has been included in experiments searching for defibrillating activity together with other several *T. reesei* cellulases, but no defibrillating activity was found (Karlsson *et al.,* 2001).

With experimental *T. reesei* cellulase preparations, it was found that high pilling removal is dependent on the fabric type (Miettinen-Oinonen *et al.,* 2001). In all cases, EGII-based cellulase products gave the most positive depilling result. On the other hand removal or decrease of EGII in a cellulase composition resulted in better strength properties of cellulose-containing textile materials as compared with treatment with the whole cellulase mixture of *T. reesei* (Miettinen-Oinonen *et al.,* 1996). Clarkson *et al.* (1993, 1992b) described the use of either CBHI-enriched *T. reesei* or a preparation free of CBHI to obtain reduced strength loss of the fabrics. By using an EG:CBH composition of 5:1 improved feel and appearance was obtained with low strength loss and without production of an excessive amount of lint (Clarkson *et al.,* 1992c, 1994b). Kumar *et al*. (1997) tested *T. reesei* whole cellulase (complete with endo- and exoactivities) and an endo-enriched product, enriched with specific EG components (more precise information on the EG was not given) and with partial removal of CBH components. Whole acid cellulase was found to be best for cotton when a high level of surface polishing was required. The endo-enriched cellulase was useful as a less aggressive cellulase that can help minimize strength loss. According to Lenting and Warmoeskerken (2001) the use of a single type of cellulase can avoid substantial breakdown of crystalline cellulose and therefore minimize tensile strength loss.

Liu *et al*. (2000) investigated three cellulase compositions for the biofinishing of cotton interlock knitted fabric. An acid cellulase complex, an endo-enriched cellulase and a mono-component endoglucanase (the origin of the preparations was not given), which all had similar application pH and temperature ranges, were shown to have different sensitivities to liquor ratio and to mechanical agitation. The effects of process conditions were also studied by Cavaco-Paulo *et al*. (1998a) and Cortez *et al.* (2001), who showed that increasing mechanical agitation favours attack by *Trichoderma* EG-rich cellulase product as compared to CBH-rich or total crude mixture. According to Liu *et al*. (2000) several parameters affect successful biofinishing: pH, temperature, liquor ratio, enzyme concentration, time, mechanical agitation, fabric type and product quality,

cellulase characteristics such as selectivity depending on the machine type, custom needs, and cellulase composition.

In addition to *Trichoderma* cellulases, cellulases originating from e.g. *Humicola insolens* and *Cellulomonas fimi* have been used in treatments of cotton (Lund and Pedersen, 1996, Boisset *et al.,* 1997, Azevedo *et al*., 2000).

1.5 Aims of the present study

The general aim of this study was to construct *T. reesei* cellulase production strains for industrial use for various applications and especially for applications in the textile industry.

The specific goals were:

- to construct different genetically tailored strains producing high levels of *T. reesei* endoglucanase and cellobiohydrolase for specific applications,

- to study the use of the preparations derived from the tailored *T. reesei* strains in biostoning and biofinishing of cotton,

- to identify novel cellulases with cellulolytic activity over a broad pH range, especially at neutral pH, and functioning in biostoning with low backstaining,

- to isolate novel genes encoding the neutral cellulases and transfer them to *T. reesei* for effective production of the cellulases for use in biostoning at neutral pH.

2. Materials and methods

A summary of the materials and methods used in this work is presented in this section. Those described in sections 2.8.1 and 2.8.2 were also published in US Patent 5,874,293. More detailed information is given in the original publications I–IV.

2.1 Strains and plasmids

Escherichia coli strain XL1-Blue was used for propagation of plasmids. pUC19 was used as a vector backbone in plasmid constructions. Plasmids constructed for expression of *T. reesei* and *M. albomyces* cellulases in *T. reesei* are listed in Table 7.

Table 7. Properties of DNA fragments used for transformation of T. reesei for expression of T. reesei (Tr) or M. albomyces (Ma) cellulases.

Plasmid	*T. reesei* promoter / terminator	Expressed gene	Flanking regions*	Marker	Reference
pALK537	*cbh1*/ *cbh1*	*Tr egl2*	*cbh1*	*amdS*	I
pALK496	*cbh1*/ *cbh1*	*Tr cbh1*	*egl1*	*amdS*	II
pALK540	*cbh1*/ *cbh1*	*Tr egl2*	*cbh2*	*ble*	I
pALK543	*cbh2*/ *cbh2*	*Tr cbh2*	*egl2*	*ble*	II
pLAK546	*cbh1*/ *cbh1*	*Tr cbh2*	*egl2*	*ble*	II
pALK1231	*cbh1*/ *cbh1*	*Ma cel45a*	*cbh1*	*amdS*	IV
pALK1235	*cbh1*/ *cbh1*	*Ma cel45a*	*egl1*	*hygB*	IV
pALK1238	*cbh1*/ *cbh1*	*Ma cel7a*	*cbh1*	*amdS*	IV
pALK1240	*cbh1*/ *cbh1*	*Ma cel7a*	*egl1*	*hygB*	IV
pALK1242	*cbh1*/ *cbh1*	*Ma cel7b*	*cbh1*	*amdS*	IV

*5' and 3' regions of the *T. reesei* cellulase gene used to target the expression cassette in the corresponding locus.

Melanocarpus albomyces ALKO4237, *Myceliophthora thermophila* ALKO4179, *Chaetomium thermophilum* ALKO4265 and *Sporotrichum thermophilum* ALKO4125 were used for production of cellulases. A summary of the cellulase compositions of the *T. reesei* strains used and constructed in this work is presented in Table 8.

Table 8. Cellulase compositions of the T. reesei strains constructed and used in this work.

Strain	Cellulase composition*		Reference
	T. reesei* cellulases*	***M. albomyces* cellulases**	
VTT D-79125	CBHI, CBHII, EGI, EGII (hypercellulolytic mutant)	-	Bailey and Nevalainen, 1981
ALKO2221	CBHI, CBHII, EGI, EGII (low protease mutant of VTT D-79125)	-	Mäntylä *et al.,* 1994
ALKO3760	**CBHI+**, CBHII, EGI, EGII	-	II
ALKO3862	**CBHI+**, CBHII, EGI, EGII	-	II
ALKO3761	**CBHI+**, CBHII, EGI-, EGII	-	II
ALKO3798	CBHI, **CBHII+**, EGI, EGII	-	II
ALKO3799	CBHI, **CBHII+**, EGI, EGII	-	II
ALKO3873	CBHI, **CBHII+**, EGI, EGII-	-	II
ALKO4095	**CBHI+**, **CBHII+**, EGI-, EGII- (*cbh1*promoter for *cbh2* expression)	-	II
ALKO4097	**CBHI+**, **CBHII+**, EGI-, EGII- (*cbh2* promoter for *cbh2* expression)	-	II
ALKO2698	CBHI-, CBHII, **EGI+**, EGII	-	Karhunen *et al.,* 1993
ALKO2697	CBHI-, CBHII, **EGI+**, EGII	-	Karhunen *et al.,* 1993
ALKO2656	CBHI-, CBHII, **EGI+**, EGII	-	Karhunen *et al.,* 1993
ALKO3529	CBHI, CBHII, EGI, **EGII+**	-	I
ALKO3530	CBHI-, CBHII, EGI, **EGII+**	-	I
ALKO3574	CBHI-, CBHII, EGI, **EGII+**	-	I
ALKO3528	CBHI-, CBHII-, **EGI+**, **EGII+**	-	I
ALKO3620	CBHI, CBHII, EGI, EGII-	-	Suominen *et al.,* 1993
ALKO4072	CBHI-, CBHII, EGI, EGII-	-	M. Paloheimo, Roal Oy, Finland
A3620/1231/14 and 16	CBHI-, CBHII, EGI, EGII-	**Cel45A+ (EGV+)**	IV
A3620/1235/40 and 49	CBHI, CBHII, EGI-, EGII-	**Cel45A+ (EGV+)**	IV
A3620/1238/42	CBHI-, CBHII, EGI, EGII-	**Cel7A+ (EGI+)**	IV
A3620/1240/32	CBHI, CBHII, EGI-, EGII-	**Cel7A+ (EGI+)**	IV
A3620/1242/13	CBHI-, CBHII, EGI, EGII-	**Cel7B+ (CBHI+)**	IV

* Overproduction is indicated as **+ and in bold** and deletion as - and in gray.

** Listing main cellulase components.

2.2 Media, growth of organims and transformation of *Trichoderma*

Media and cultivation conditions are described in detail in articles I–IV. Transformation of *T. reesei* was carried out as described by Penttilä *et al.* (1987) with the modifications described by Karhunen *et al.* (1993).

2.3 DNA techniques

Standard DNA techniques as described by Sambrook *et al*. (1989) and Maniatis *et al.* (1982) were used. Construction and screening of an *M. albomyces* genomic library, isolation of plasmid and chromosomal DNA, isolation of DNA fragments for cloning, sequencing of DNA, amplification of DNA fragments and genes by PCR and Southern blot analysis are described in detail in articles I-II and IV. The primers used to amplify the genes encoding *M. albomyces* 20 and 50 kDa EGs and 50 kDa CBH are shown in Table 1/IV.

2.4 Enzyme activity assays

Endoglucanase activity was measured at pH 4.8 as the release of reducing sugars from hydroxyethyl cellulose (HEC, ECU-activity) as described by Bailey and Nevalainen (1981). At neutral pH endoglucanase activity was measured using HEC or carboxymethyl cellulose (CMC) as substrate by the same method with the following modifications: 1 % HEC was used at 50^{o}C, pH 7 or 3 % CMC at 50 ^{o}C or 70 ^{o}C (NCU activity), pH 7 in 50 mM Hepes buffer. Activity against barley β-glucan was assayed in the same way as activity against HEC (Bailey and Nevalainen, 1981), replacing HEC by barley β-glucan. Filter paper-hydrolyzing activity (FPU) was measured according to the method by Mandels *et al.* (1976). Cellobiohydrolase I activity was measured as activity against 4-methylumbelliferyl-β-D-lactoside (MUL) according to van Tilbeurgh *et al.* (1988). The total MUL activity represents activities of EGI and CBHI. The MUL (CBHI) activity was measured by inhibiting CBHI in the presence of 5 mM cellobiose and by subtracting the MUL (EGI) activity thus obtained from the total MUL activity. β-Glucosidase activity was measured using 4-nitrophenyl-β-D-glucopyranoside as substrate as described in Bailey and Nevalainen (1981).

2.5 Purification of *M. albomyces* cellulases

Purification of the 20 and 50 kDa EGs and the 50 kDa CBH from *M. albomyces* ALKO4237 is described in article III. Digestion of the purified proteins and separation of the peptides as well as the subsequent protein and peptide sequencing are explained in article III.

2.6 Immunological methods

The presence of the *Trichoderma* EGI, CBHI and CBHII proteins was detected from Western blot filters (Towbin *et al.*, 1979) or from Dot blot filters (Schleicher & Schüll) immunostained using a monoclonal EGI antibody EI-2, CBHI-89 antibody and CBHII antibodies CII-8 and CII-30 (Aho *et al.*, 1991) and the Protoplot Western Blot AP system (Promega). *Melanocarpus* Cel45A, Cel7A and Cel7B-specific polyclonal antibodies were used in a similar manner for detection of the respective proteins. Quantitation of secreted *T. reesei* EGI, CBHI and CBHII was carried out by a double antibody sandwich ELISA (Bühler, 1991) using the monoclonal antibodies EGI-2, CI-258 and CII-8 (Aho *et al.*, 1991) as capture antibodies.

2.7 Biofinishing

Biofinishing of cotton fabric was performed at pH 5 with cellulase preparations of *T. reesei* VTT D-79125, ALKO3529, ALKO2656, ALKO3528, ALKO3760, ALKO3798 and ALKO4097 (Table 8) and the effects were evaluated visually and by determining pilling and weight loss as described in detail in article II.

2.8 Biostoning

Biostoning of denim fabric was performed as described in articles I, III–IV and in sections 2.8.1 and 2.8.2. Cellulase preparations of *T. reesei* VTT D-79125, ALKO3529, ALKO3528, ALKO2656, ALKO3760, ALKO3798, ALKO4097 (Table 8) and Ecostone L (Primalco Ltd. Biotec) were used in biostoning at pH 5. At pH 7 culture supernatants of *M. albomyces* ALKO4237, *M. thermophila* ALKO4179, *S. thermophilum* ALKO4125 and *C. thermophilum* ALKO4265,

purified *M. albomyces* Cel45A, Cel7A and Cel7B cellulases and *T. reesei* recombinant *Ma* Cel45A cellulase preparations were used.

2.8.1 Biostoning with purified *T. reesei* cellulases

Denim fabric was prewashed for 10 min at 60°C with an amylase product Ecostone A 200 (1ml/l, Primalco Ltd. Biotec, Finland). The fabric was cut into 12x12 cm swatches. The colour was measured from the fabric as reflectance values with the Minolta (Osaka, Japan) Chroma Meter 1000 R L*a*b* system. Cellulase treatments were performed in an LP-2 Launder-Ometer (Atlas, USA). About 7 g of denim swatches were loaded into the 1.2 litre container containing 200 ml of 50 mM citrate buffer, pH 5. 10 steel balls were added into each container to help the colour removal. *T. reesei* preparation produced using the hypercellulolytic mutant strain VTT D-79125 (Table 8) and cultivated as in article I was used. 300 ECU per g of fabric was used in each test and purified *T. reesei* CBHI, CBHII, EGI and EGII were added at dose levels of 1 or 2 mg per g of fabric. Cellulases were purified according to Pere *et al.* (1995) and Rahkamo *et al.* (1996) and were obtained from Matti Siika-aho (VTT Biotechnology, Finland). The containers were loaded into a 50 °C Launder-Ometer bath. The Launder-Ometer was run at 42 rpm for 1 or 2 hours. After removing the swatches from the containers they were soaked for 10 min in 200 ml of 10 mM NaOH and rinsed 2x5 min with cold water. The swatches were dried for 1 h at 105 °C and air dried overnight at room temperature. The colour from both sides of the swatches was measured with the Minolta Chroma Meter.

2.8.2 Biostoning with *T. reesei* cellulase preparations with enhanced CBH activity

Denim fabric was treated with cellulase preparations derived from the strains overproducing CBHI (ALKO3760), CBHII (ALKO3798) and both CBHI and CBHII (ALKO4097) and from VTT D-79125 (Table 8). The strains and cultivation conditions for obtaining the cellulase preparations are described in II. The experimental set-up was as in 2.8.1. 3 or 6 mg of the total protein (Lowry *et al.*, 1951) in the cellulase preparations per g of fabric was used in each experiment. The washing times were 1 and 2 hours at 50 °C.

3. Results

3.1 Construction of *T. reesei* strains overproducing EG and CBH (I, II)

Eight different types of *T. reesei* strains producing elevated amounts of homologous EGs or CBHs were constructed. The aim was to construct different tailored high endoglucanase activity or high cellobiohydrolase activity-producing strains for specific industrial applications, *i.e*. for the textile industry. The production of *T. reesei* EGII, CBHI and CBHII was improved in separate strains. Strains producing high levels of EGI and EGII without any cellobiohydrolases (EG-overproducing strains) or CBHI and CBHII without the main endoglucanases EGI and EGII (CBH-overproducing strains) were also constructed. For overexpression of the *egl2, cbh1* or *cbh2* genes in *T. reesei* the powerful promoter of the *cbh1* gene of *T. reesei* was used. The *cbh2* promoter of *T. reesei* was also used in the CBH-overproducing strain for expression of *cbh2.*

3.1.1 EGII- and EG-overproducing strains

Two different plasmids were constructed. The plasmid pALK537 (Fig. 1/I, Table 7) was constructed for expression of the *egl2* gene under the control of the *cbh1* promoter either in the locus of *cbh1* or elsewhere in the genome of *T. reesei* VTT D-79125 depending on the homologous or non-homologous recombination. The plasmid pALK540 (Fig. 2/I, Table 7) was constructed for expression of *egl2* from the *cbh1* promoter and for replacement of the *cbh2* locus of *T. reesei* ALKO2698, an EGI-overproducing CBHI-negative strain and containing one copy of the *egl1* expression cassette (Karhunen *et al.,* 1993). For construction of the plasmids the gene replacement strategy developed by Suominen *et al*. (1993) was used.

The transformants were cultivated on cellulase-inducing medium and were first screened by measuring endoglucanase activity from the culture medium. 61 % of the best endoglucanase producing pALK537-transformants were CBHI negative as analyzed by dot blotting and immunostaining. The transformants producing the highest endoglucanase activity in the culture medium, ALKO3529 (CBHI-

positive) and ALKO3530 and ALKO3574 (CBHI-negative) were analyzed in more detail. Southern hybridization data showed that in ALKO3574 one copy and in ALKO3530 two copies of the transformed pALK537 fragment had replaced the coding region of the *cbh1* gene (Figure 3/I). ALKO3529 contained two copies of the transformed fragment integrated into or close to the *cbh1* locus (Figure 4/I).

The frequency of targeting the pALK540-fragment into the *cbh2* locus was 71 %. Of the pALK540-transformants, ALKO3528 produced the highest endoglucanase activity and was shown by Southern hybridization to contain one full-length copy of the *egl2* expression cassette in the *cbh2* locus (Figure 6/I).

3.1.1.1 Enzyme production

All the transformants and the parent strains VTT D-79125 and ALKO2698 were grown in shake flasks on cellulase-inducing medium. The results of the measurements of cellulase activities and ELISA analysis from the culture medium are shown in Tables 2 and 3/I and in Table 9 (summary). In EGII-transformants one copy of the *egl2* expression cassette increased the endoglucanase and β-glucanase activities about twofold (ALKO3574) and two cassettes (ALKO3529, ALKO3530) about 3-fold compared to the parent strain VTT D-79125. Higher increases in both endoglucanase and β-glucanase activities were detected in the EGII-overproducing strains compared to EGI-overproducing strains (Karhunen *et al.,* 1993), which were used as controls (Table 9). Endoglucanase activity was increased about twofold in the EG-overproducing transformant strain ALKO3528 compared to the parent strain ALKO2698 and fourfold compared to VTT D-79125, the parent of ALKO2698. FPU activity was decreased 60–70 % in the EGII-overproducing strains lacking the *cbh1* gene (ALKO3530, ALKO3574). EGII appears to affect the FPU-activity, since FPU of the CBHI-positive EGII-overproducing strain ALKO3529 was about 10 % higher than that of the parent strain. ALKO3528 does not contain *cbh1* or *cbh2* and thus its FPU-activity was decreased almost to zero.

As compared to the parent strain the amount of secreted EGII protein (evaluated visually in several SDS-PAGE analyses with different dilution series with a known concentration of purified EGII protein as a standard) was increased

2-fold with one expression cassette and 3.2-fold with two expression cassettes, being then approximately 1.3 g/l (Figure 5/I). Although the *cbh1* promoter was used in the *egl2* expression vector, the amount of secreted CBHI as analyzed by ELISA was not significantly changed in the CBHI-positive EGII-overproducing strain ALKO3529 compared to the parent strain (Table 2/I, Table 9). The production of both EGI and CBHII was increased in the EGII-overproducing strains and the lack of *cbh1* further increased the amounts.

Table 9. Summary of the properties and cellulase production levels of the EGII-, EGI- and EG-overproducing strains.

Strain	***cbh1/ cbh2****	***egl2* cassette copy no**	***egl1* cassette copy no**	**HEC nkat/ml**	**β–glu-canase nkat/ml**	**FPU /ml**	**CBHI mg/ml**	**CBHII mg/ml**	**EGI mg/ml**
VTT D-79125	+/+	0	0	1200	9300	5.3	3.7	0.05	0.36
ALKO3529	+/+	2	0	3400	23 000	6.0	3.4	0.07	0.59
ALKO3530	-/+	2	0	3600	25 000	2.1	na	0.08	0.59
ALKO3574	-/+	1	0	2800	19 400	1.7	na	na	0.65
ALKO2697	-/+	0	2**	2600	20 200	1.8	na	na	na
ALKO2698	-/+	0	1**	2300	15 700	1.6	na	na	na
VTT D-79125	+/+	0	0	1300	11 500	4.8	na	na	na
ALKO2698	-/+	0	1**	2600	15 300	1.4	na	na	na
ALKO3528	-/-	0	0	5100	27 800	0.2	na	na	na

*Southern analysis, **Karhunen *et al.* (1993), na = not analyzed.

3.1.2 CBHI-, CBHII- and CBH-overproducing strains

The plasmid pALK496 was constructed for inceasing the copy number of the *cbh1* gene in *T. reesei* and the plasmids pALK546 and pALK543 for expression of the *cbh2* gene from either the *cbh1* or the *cbh2* promoter (Figure 1/II, Table 7). The *T. reesei* strain ALKO2221 was used as a host in the transformations (Table 8).

T. reesei CBHI-overproducing transformants were screened by measuring the increased activity against 4-methylumbelliferyl-β–D-lactoside (MUL). 10 % of the transformants were EGI-negative as detected by Western blotting indicating replacement of the *egl1* gene by the expression cassette. Three transformants producing high MUL (CBHI) activity were analyzed in more detail. According

to Southern hybridizations the strain ALKO3761 contained one full-length copy of the *cbh1* expression cassette replacing the *egl1* gene (Figure 2/II). ALKO3862 and ALKO3760 contained two tandem copies of the *cbh1* expression cassette (in ALKO3760 the second copy had an incomplete pALK496 fragment) in unknown loci (not in *egl1* or *cbh1*) (Figure 2/II).

The *T. reesei* CBHII-overproducing transformants were screened for increased amounts of CBHII by the ELISA method (Bühler, 1991). The genomes of the transformant strains ALKO3873, ALKO3798 and ALKO3799 producing highest amounts of CBHII protein were analyzed by Southern blotting. The *egl2* gene was replaced by one copy of the pALK546 expression fragment in the transformant strain ALKO3873. ALKO3798 and ALKO3799 contained one copy of the transformed fragment integrated in an unknown locus (not *egl2, cbh2,* or *cbh1*). The 3'-end of the transformed fragment could not be detected in the Southern blots of ALKO3798 and ALKO3799 (Figure 3/II). However, on the basis of the ELISA analysis, SDS-PAGE and Western analysis these transformants produced additional complete CBHII and no truncated form of CBHII was detected (Table 2/II, Figure 4/II).

For construction of the strains overproducing both CBHI and CBHII without EGI and EGII (CBH-overproducing strains), the CBHI-overproducing *egl1*-negative strain ALKO3761 was transformed with the expression cassette from either the plasmid pALK543 or pALK546. The strains ALKO4095 (transformed with pALK546, *cbh2* under the *cbh1* promoter) and ALKO4097 (transformed with pALK543, *cbh2* under the *cbh2* promoter) had a single copy of the respective expression cassette replacing the *egl2* gene (Figure 5/II).

3.1.2.1 Enzyme production

The transformants and the host strains were cultivated in shake flasks on cellulase-inducing medium. The results of enzyme production are summarized in Table 10. The amount of EGI, CBHI and CBHII in the culture supernatants of the transformants were quantified by the ELISA method. In CBHI-overproducing transformants the amount of CBHI was increased 1.4–1.5 fold in the two-copy transformants ALKO3760 and ALKO3862 and 1.3-fold in the one-copy transformant ALKO3761 as compared to the host ALKO2221, resulting in

corresponding increase in MUL (CBHI) activity (Table 1/II, Table 10). The amount of CBHII was reduced by 25 % in ALKO3760 and ALKO3862, but increased by 15 % in ALKO3761. Although the *egl1* gene was intact in ALKO3760 and ALKO3862 the amount of EGI was decreased by 30–40 %, which was also detected as lowered endoglucanase activity. The level of total secreted proteins was increased in CBHI-overproducing transformants as compared to the host strain (Table 1/II).

ALKO3798 produced about four times and ALKO3799 and ALKO3873 about three times more CBHII than the host strain ALKO2221 (Table 2/II, Table 10). The amount of CBHI was decreased by 10–20 %. Production of endoglucanase activity was clearly decreased in the EGII-positive transformants ALKO3798 and ALKO3799. Production of endoglucanase activity was decreased by 50 % in the EGII-negative CBHII-overproducing strain ALKO3873.

Table 10. Summary of the properties and cellulase production levels of the CBHI-, CBHII- and CBH-overproducing strains.

Strain	***egl1/ egl2****	***cbh1* cassette copy no**	***cbh2* cassette copy no**	**MUL** (CBHI)**	**FPU/ ml**	**HEC nkat/ml**	**CBHI mg/ml**	**CBHII mg/ml**	**EGI mg/ml**
ALKO2221	+/+	0	0	1	3.6	700	2.2	0.18	0.32
ALKO3760	+/+	2	0	1.4	3.5	530	3.4	0.13	0.22
ALKO3862	+/+	2	0	1.3	3.4	490	3.1	0.13	0.17
ALKO3761	-/+	1	0	1.1	3.0	340	3.0	0.22	0.00
ALKO3798	+/+	0	1	na	3.6	570	1.7	0.70	0.31
ALKO3799	+/+	0	1	na	3.9	560	1.8	0.50	0.28
ALKO3873	+/-	0	1	na	3.0	360	1.9	0.53	0.39
ALKO2221	+/+	0	0	na	3.4	720	2.7	0.25	na
ALKO3761	-/+	1	0	na	2.8	390	3.9	0.23	na
ALKO4097	-/-	1	1	na	2.7	30	4.4	0.78	na
ALKO4095	-/-	1	1	na	2.5	30	3.5	0.85	na

* Southern analysis, ** ALKO2221 produced about 0.3 MUL (CBHI)/ml. The MUL (CBHI) activity of ALKO2221 was adjusted to 1 and the activities produced by the transformants are presented compared to the host strain, na = not analyzed.

Filter paper-hydrolyzing activity, which is mainly affected by cellobiohydrolases but also indicates total hydrolysis of cellulose, was not significantly changed in either CBHI (EGI+) or CBHII (EGII+) -overproducers. However, lack of EGI in

the CBHI-overproducer ALKO3761 or of EGII in the CBHII-overproducer ALKO3873 decreased the FPU activity by 10–15 %.

Production of CBHII by the CBH-overproducers ALKO4095 and ALKO4097 was increased by 3.9 and 3.4 fold, respectively, and thus approximately similar amounts of CBHII protein were produced using the *cbh1* or *cbh2* promoters (Table 3/III, Table 10). In ALKO4095, in which the *cbh1* promoter was used for *cbh2* expression, there was a slight decrease of CBHI.

3.2 Use of *T. reesei* preparations enriched with EGs and CBHs in cotton finishing (I, II)

3.2.1 Use of purified *T. reesei* cellulases in biostoning

The impact of the major *T. reesei* cellulases in biostoning was evaluated by Heikinheimo *et al.* (2000). According to their results, purified EGII was the most effective cellulase among EGI, EGII and CBHI at removing colour from denim. However, clearly higher enzyme dosage of the monocomponent EGII as compared to the whole cellulase preparation was needed to obtain an equal stone-washed effect. In this work the stone-washing effects of the cellulase preparation derived from the *T. reesei* strain VTT D-79125 (Table 8) with the addition of purified *Trichoderma* CBHI, CBHII, EGI and EGII were studied. VTT D-79125 produces all the identified *Trichoderma* cellulases, including the main cellulases CBHI, CBHII, EGI and EGII. These results have been published previously in US Patent 5,874,293.

The results showed that addition of EGII cellulase to the VTT D-79125 preparation increased the lightness, blueness and deltaE of denim fabric most clearly compared to the addition of other purified cellulases (Table 11). High deltaE and lightness indicate good biowashing performance. An increase in L value of about 1 unit can clearly be seen visually. Addition of CBHI or EGI improved the stone-washing effect as compared to the VTT D-79125 preparation but the effect was clearly lower than that obtained with EGII. In order to obtain the same stone-washed effect a double amount of CBHI or EGI was needed as compared to EGII. Addition of CBHII did not improve the stonewashing properties of the pure VTT D-79125 preparation.

Table 11. Colour measurements of the right side of denim fabrics treated with VTT D-79125 cellulase preparation and VTT D-79125 preparation fortified with purified cellulases.

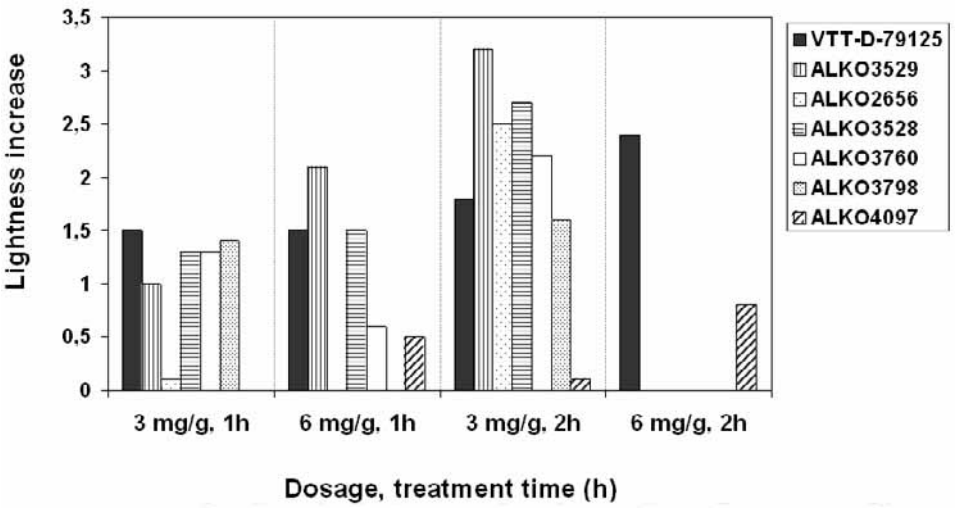
Cellulase added to VTT D-79125 preparation	Dosage mg / g	L	b	deltaE
1 hour				
-*	-	0.8	1.5	1.5
CBHI	1	0.8	2.0	1.5
CBHI	2	1.8	2.0	2.2
CBHII	1	1.1	1.7	1.6
CBHII	2	0.6	1.7	1.3
EGI	1	1.1	2.0	1.9
EGI	2	1.8	2.5	3.0
EGII	1	1.9	3.2	3.2
EGII	2	2.7	2.5	3.8
2 hours				
-*	-	1.2	1.9	1.5
CBHI	1	2.2	2.5	3.1
CBHI	2	1.4	2.5	2.7
CBHII	1	1.0	2.2	1.5
CBHII	2	1.3	2.7	2.6
EGI	1	2.2	2.9	2.2
EGI	2	2.0	3.1	3.5
EGII	1	2.5	3.7	1.9
EGII	2	4.2	3.5	5.2

*Pure VTT D-79125 preparation. The effects of the buffer on colours was deleted from these values. L: lightness unit of the fabric after treatment minus lightness unit of the fabric before the treatment. b: blueness unit of the fabric after treatment minus blueness unit of the fabric before the treatment. deltaE: colour difference in the L*a*b* colour space between the specimen colour and the target colour (target = untreated denim fabric).

3.2.2 Use of *T. reesei* preparations enriched with EGs and CBHs in biostoning (I)

Cellulase preparations derived from the EGII-overproducing strain ALKO3529, the EG-overproducing strain ALKO3528, the CBHI-overproducing strain ALKO3760, the CBHII-overproducing strain ALKO3798 and the CBH-overproducing strain ALKO4097 (Table 8) were compared in biostoning of denim fabric. The strain VTT D-79125 and the EGI-overproducing strain

ALKO2656 (containing three copies of the *egl1* expression cassette, Karhunen *et al.,* 1993) were used as controls. Results of the colour measurements of fabrics are shown in Figure 5. The results with EGII- and EG-overproducers have been presented in article I (Table 4) and with CBHI-, CBHII- and CBH-overproducers in US Patent 5,874,293.



The results are means of two duplicate treatments. The effects of the buffer on colours were deleted from these values. L: lightness unit of the fabric after treatment minus lightness unit of the fabric before the treatment.

Figure 5. Colour measurements of the right side of denim fabrics treated with VTT D-79125, ALKO3529, ALKO2656, ALKO3528, ALKO3760, ALKO3798 and ALKO4097 cellulase preparations.

The results showed that after 1 hour of treatment with the lower dosage almost the same stone-washed effects were obtained with ALKO3528, ALKO3529, ALKO3760 and ALKO3798 as with the strain VTT D-79125. When the dosage was increased to 6 mg/g (treatment time 1 h) or the time to 2 hours, the best effect was achieved with ALKO3529. After the 2 h treatment a slightly better stone-washing effect was also obtained with ALKO2656 and ALKO3528 as compared to the natural enzyme composition (VTT D-79125). The washing effects obtained with ALKO3760 or ALKO3798 were equal to that obtained with VTT D-79125. No clear increase in lightness units was obtained with the CBH-overproducing strain ALKO4097, in which the main endoglucanases had

been removed. Thus, a considerably lower dosage of cellulases from endoglucanase-overproducing strains, especially from the EGII-overproducing strain, was needed to achieve a comparable stone-washing effect as with the cellulases of VTT D-79125.

3.2.3 Use of *T. reesei* preparations enriched with EGs and CBHs in biofinishing (II)

Cellulase preparations derived from the EGII-overproducer ALKO3529, the EG-overproducer ALKO3528, the CBHI-overproducer ALKO3760, the CBHII-overproducer ALKO3798 and the CBH-overproducer ALKO4097 (Table 8) were used in biofinishing of cotton fabric. Cellulase from the strain VTT D-79125 was used as a control. The effects of the different cellulases were evaluated by measuring the weight loss and analyzing the pilling performance and visual appearance (amount of fuzz and pills on the fabric surface) of the fabric. The results showed (Table 4/II) that the best visual appearance and the greatest reduction in pilling tendency was achieved with the EGII-overproducing ALKO3529 preparation when comparing the same dosages of different preparations. Moreover a considerably smaller dosage of this preparation was needed as compared to the other preparations. Furthermore, by using the CBHII-overproducing strain ALKO3798 the visual appearance of the cotton fabric could be improved and the pilling tendency could be reduced as compared to the effects achieved with VTT D-79125 or the CBHI-overproducing strain ALKO3760. Almost equal effects were obtained with ALKO3798 and the EG-overproducing strain ALKO3528.

3.3 Novel neutral cellulases for biostoning (III)

The use of *T. reesei* acid cellulases in biostoning has been limited due to the high backstaining effect and weakening of the fabric. Neutral cellulases available on the market have been less aggressive and have not been affected by the pH increase during denim finishing, but are reported to require a longer wash time than *Trichoderma* cellulases (Klahorst *et al.,* 1994). In this part of the work novel cellulases were screened for the denim finishing application. New cellulases with the following characteristics are still needed:

• active over a broad pH range leading to less need for a buffering system to control pH,
• short reaction time,
• no significant weakening of the fabric,
• good finishing properties, e.g. low backstaining.

When acid cellulases are used, an efficient buffering system is always needed because alkalinity increases during denim washing as the caustic soda used in dyeing is released from the fabric. Incoming water in wet processing is also typically in the neutral range (Solovjeva *et al.,* 1998).

A laboratory scale biostoning assay was used for screening of microorganisms and in conjunction with the purification of cellulases. High lightness increase on the right side of the fabric was used as an indication of good indigo-dye release and stonewashing effect. Backstaining was quantified by measuring the colour values on the reverse side of the fabric, i.e. low lightness and high blueness values indicated high backstaining.

3.3.1 Screening of microorganisms producing neutral cellulases

Various fungal species were screened for identification of extracellular cellulases acting over a broad pH range (pH 5–8, especially at pH 7). The culture supernatants from about 25 fungal strains (Budapest University of Technology and Economics, Hungary / III) were tested for endoglucanase activity at neutral pH (pH 7.0, data not shown). Preparations derived from the thermophilic fungal strains *M. thermophila* ALKO4179, *M. albomyces* ALKO4237, *C. thermophilum* ALKO4265 and *S. thermophilum* ALKO4125 were chosen for biostoning experiments. *S. (Chrysosporium) thermophilum* and *M. thermophila* are the anamorph and teleomorph stages of the same fungus (Maheshwari *et al.,* 2000).

In the biostoning assay in neutral conditions the lightness units were increased on the right side of the denim fabric washed with culture supernatants of *M. albomyces, M. thermophila* and *S. thermophilum,* showing effective release of indigo-dye (Table 3/III, summary in Table 12). Denim fabric treated with the culture supernatant of *C. thermophilum* did not lighten, but the blueness unit was increased. This is probably an indication of cellulase action on denim, but

resulting at the same time in high backstaining. A commercial acid cellulase product of *T. reesei* (Ecostone L, Primalco Ltd. Biotec) did not produce a stonewashing effect at pH 7. Practically no backstaining was measured from the fabrics treated with supernatants of *M. albomyces, M. thermophila* and *S. thermophilum* as compared to *C. thermophilum* or Ecostone L (pH 5.2 and 7) (Table 3/III, Table 12). Because the best stone-washing effect with the lowest backstaining was obtained with the preparation derived from *M. albomyces*, this fungus was chosen for isolation of cellulases for stone-washing in neutral conditions.

According to information in the website www.indexfungorum.org/Names/NAMES.ASP, *M. albomyces* is a fungal ascomycete and its phylogenetic classification is: Incertae sedis, Sordariales, Sordariomycetidae, Ascomycetes. According to Guarro *et al.* (1996) *Melanocarpus* belongs to the non-ostiolate Sordariales and its closest relatives are the genera *Thielavia, Chaeromidium, Boothiella, Corynascus* and *Corynascella.*

Table 12. Colour measurements of denim fabric treated with different culture supernatants.

Source of enzyme	***Treatment pH***	***ECU/g of fabric***	**L_{right}**	**$b_{reverse}$**
-	7	-	2.1	-1.1
M. albomyces	7	200	5.5	2.3
M. thermophila	7	200	4.4	2.2
S. thermophilum	7	200	3.5	1.4
C. thermophilum	7	200	3.3	6.6
Ecostone L	5.2	200	2.0	4.8

L: increase of lightness on the right side, b = increase of blueness on the reverse side.

3.3.2 Purification and properties of three cellulases of *M. albomyces*

Culture supernatant of *M. albomyces* ALKO4237 was fractionated on DEAE Sepharose and the protein pools were tested in biostoning. According to the results two endoglucanases with molecular masses of about 20 and 50 kDa and a third protein with a molecular mass of about 50 kDa, but no endoglucanase

activity, were responsible for the biostoning activity (data not shown). The three cellulases were purified to homogeneity (Tables 1 and 2/III). The 20 kDa endoglucanase crystallized spontaneously when the SP-Sepharose eluates were stored for a few days at 7°C. The washed crystals showed a single band on SDS-PAGE corresponding to a molecular mass of 20 kDa (Fig. 1/III). The 20 kDa endoglucanase was a relatively heat-stable cellulase and had a high pH optimum. At 50°C it exhibited 80 % or more of its maximum activity over the pH range 4–9 and between pH 5.5 and 7.5 the enzyme was more active at 70 °C than at 50 °C (Figure 3/III).

The purified 50 kDa endoglucanase (Figure 1/III) had activity both against hydroxyethyl cellulose and MUL (CBHI). This EG was also active over a broad pH range: at 50 °C a constant activity was observed between pH 4.4 and 7 and at 70 °C the optimum was at pH 6 (Figure 4/III). The third protein was the 50 kDa cellobiohydrolase with a low activity against MUL (CBHI) at pH 5–7 but no measurable endoglucanase activity.

Amino acid sequences of tryptic peptides derived from the 20 kDa EG and the 50 kDa CBH and from CNBr-digested 50 kDa EG (Table 4/III) showed homology towards e.g. *Humicola* and *Fusarium* cellulases.

Table 13. Colour measurements of denim fabric treated at pH 7 with purified Ma 20 and 50 kDa endoglucanases and 50 kDa cellobiohydrolase.

Sample	**Dosage mg protein / g fabric**	**L_{right}**	**$b_{reverse}$**
-	-	2.8	1.6
20 kDa EG	0.18	5.6	4.0
50 kDa EG	0.15	2.6	1.0
50 kDa CBH	0.15	2.7	0.5
20 kDa EG + 50 kDa EG	0.18 + 0.075	5.6	2.5
20 kDa EG + 50 kDa CBH	0.18 + 0.15	4.7	3.0

L: increase of lightness on the right side, b = increase of blueness on the reverse side.

At a protein dosage of 1/80th of that of unfractionated *M. albomyces* culture filtrate, the purified 20 kDa EG resulted in the same degree of lightening in biostoning at neutral pH (Table 5/III, summary in Table 13). Furthermore the purified 20 kDa protein caused less backstaining on the reverse side than the

unfractionated culture filtrate. The 50 kDa EG or 50 kDa CBH did not alone release indigo-dye (Table 6/III, Table 13). However, both enzymes decreased backstaining on the reverse side when used in combination with 20 kDa endoglucanase.

3.4 Cloning of neutral cellulase genes of *M. albomyces* and their expression in *T. reesei* (IV)

The genes coding for the three cellulases of *M. albomyces* ALKO4237 which were effective in biostoning at neutral pH (III) were subsequently cloned, sequenced and expressed in *T. reesei* for use of the cellulase preparations produced by the transformants in biostoning of denim.

3.4.1 Cloning and characterisation of the genes

Degenerated primers (Table 1/IV) based on the peptide sequences (Table 4/III) of the purified proteins were used to amplify the genes coding for the 20 kDa and 50 kDa EGs and the 50 kDa CBH of *M. albomyces*. The amplified fragments obtained encoded the majority (in the case of 20 kDa EG) or one (50 kDa EG, 50 kDa CBH) of the cellulase-derived peptides.

The genomic library of *M. albomyces* was screened with each of the amplified fragments and the positive clones were identified. The 849 bp open reading frame (ORF) of 20 kDa EG codes for 235 amino acids, is disrupted by two predicted introns and predicts a protein of 25 kDa for the full-length preprotein and 22.9 kDa for the mature protein. The 1364 bp ORF of 50 kDa EG codes for 428 amino acids, has one intron and predicts a molecular weight of 46.8 kDa for the full-length protein and 44.8 kDa for the mature protein. The 1735 bp ORF of 50 kDa CBH codes for 452 amino acids, has five introns and predicts a molecular weight of 49.9 kDa for the full-length protein and 47.6 kDa for the mature protein. All the peptides sequenced from each of the purified cellulases (III) were found in the corresponding predicted protein sequences. The cloned cellulases lack a consensus cellulose binding domain and its associated linker.

The 20 kDa EG belongs to the family 45 of GH (Henrissat *et al.,* 1998) and was named *Ma* Cel45A-cellulase (EGV). The 50 kDa EG and 50 kDa CBH belong to the family 7 of GH and were named *Ma* Cel7A (EGI) and *Ma* Cel7B (CBHI), respectively. *Ma* Cel45A-cellulase is similar to *Thielavia terrestris* endoglucanase (76% identity in a 234 amino acid overlap) and *H. insolens* endoglucanase V (76 % identity in a 235 amino acid overlap) (Table 2/IV, Fig. 6). The proposed active site aspartates are at positions 10 and 120 of the mature protein (Fig. 6). The *Ma* Cel7A protein is similar to *Humicola grisea* endoglucanase I (about 73 % identity in a 416 aa overlap) and the *Ma* Cel7B to *H. grisea* cellobiohydrolase (78 % identity in a 449 aa overlap) (Table 2/IV).

3.4.2 Heterologous production in *T. reesei*

Plasmids pALK1231 and pALK1235 were constructed for expression of the *Ma cel45A* (*egl5*) gene in the *cbh1* and *egl1* loci of *T. reesei*, respectively, plasmids pALK1238 and pALK1240 for expression of the *Ma cel7A* (*egl1*) gene in the *cbh1* and *egl1* loci and plasmid pALK1242 for expression of the *Ma cel7B* (*cbh1*) gene in the *cbh1* locus (Fig. 1/IV, Table 7). The genes were expressed from the *cbh1* promoter of *T. reesei* individually in an EGII-negative *T. reesei* host ALKO3620. *T. reesei* EGII has been shown to weaken the strength properties of cotton fabrics significantly more than CBHI or EGI (Miettinen-Oinonen *et al.,* 1996, Heikinheimo *et al.,* 1998). Because of this *T. reesei* deficient in EGII was used as a host in transformations. The replacement frequencies, detected from the transformants by dot blotting and immunostaining, of the *cbh1* locus varied between 24 and 80 % and of the *egl1* locus between 15 and 23 % depending on the plasmid. Seven transformants were studied in more detail.

The genome analysis (Southern hybridization) of Cel45A transformants showed that in the CBHI-negative transformants ALKO3620/1231/14 and ALKO3620/1231/16 the *cbh1* gene of *T. reesei* was replaced by one copy of the expression cassette – *amd*S marker fragment of pALK1231 and in the EGI-negative transformants ALKO3620/1235/40 and ALKO3620/1235/49 the *egl1* gene was replaced by one copy of the expression cassette – *hygB* marker fragment of pALK1235. Thus, the first two strains are unable to produce *T. reesei* CBHI and EGII. The latter two strains are unable to produce EGI and EGII.

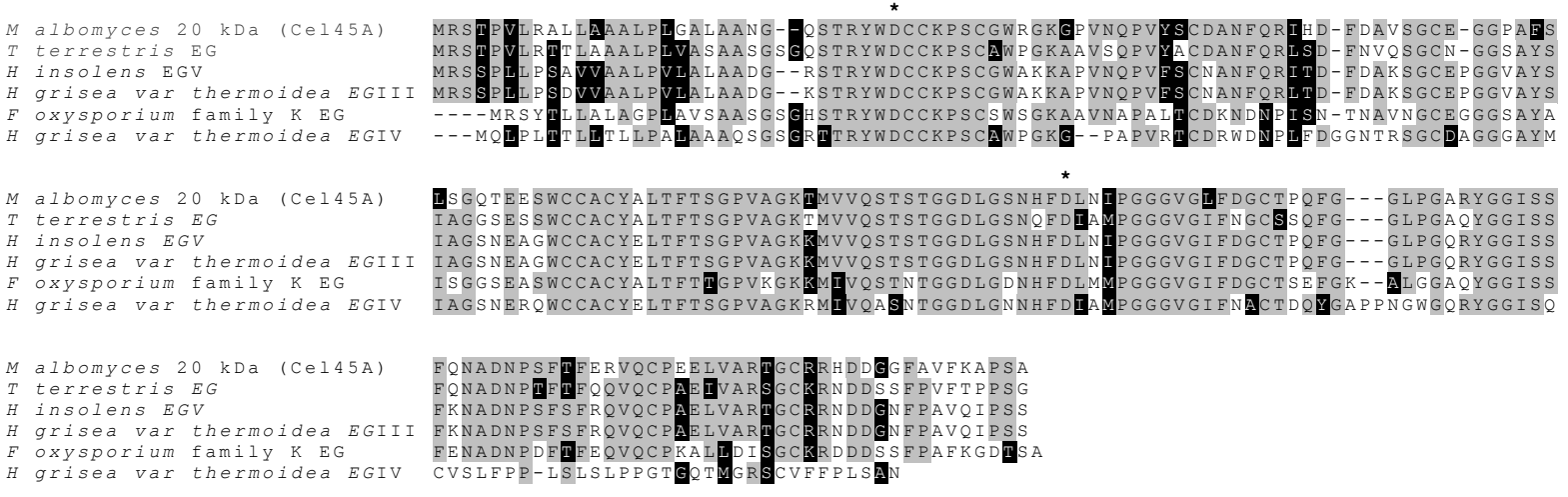


Figure 6. Alignment of the M. albomyces Cel45A with homologous endoglucanases. Identical residues are depicted in grey and similar residues in a black background. The complete genus names are shown in Table 2/IV. Only the catalytic domains are shown. The catalytic active site aspartates are indicated with stars. Previously unpublished.

Cel45A (EGV) transformants were cultivated on cellulase-inducing medium. The pH at the end of the cultivation was about 3. In these conditions Cel45A could be detected only by Western blotting with Cel45A-specific polyclonal antibody and in samples extracted from the mycelium. When the pH of the cultivation medium was maintained above 4 by using 5% KH_2PO_4, Cel45A could be detected from the culture medium by Western blotting and the size was the same as the size of the purified protein (Fig. 2/IV). Single copy replacement transformants of Cel45A produced several times higher endoglucanase activity levels as compared to the parent strain *M. albomyces* ALKO4237 (Table 3/IV). The Cel45A-transformants produced 2100–2500 NCU/ml (activity against carboxyethyl cellulose, pH 7) at 70°C. The integration site of the expression cassette had no significant effect on the activity levels.

Proteolytic degradation of the secreted *Ma* Cel45A in the *T. reesei* culture at pH < 4 was tested by mixing purified Cel45A into the culture supernatant of *T. reesei* ALKO3620 (pH about 3) and incubating at 30 °C. The Cel45A band intensity detected by Western blotting decreased with time and disappeared after 60 min (Fig. 3/IV). When an aspartic protease inhibitor Pepstatin A alone or together with a serine and cysteine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added into the culture supernatant of *T. reesei,* no degradation of Cel45A was observed (Fig. 3/IV). When PMSF was added into the culture supernatant alone, degradation of Cel45A still occurred. Thus the probable cause of *Ma* Cel45 degradation was an aspartic protease.

One copy of the pALK1238 expression fragment containing the *Ma cel7A* (*egl1*) gene had replaced the *cbh1* gene in *T. reesei* transformant ALKO3620/1238/42 and one copy of the pALK1240 expression fragment containing *cel7A* had replaced the *egl1* gene in ALKO3620/1240/32. The activity against CMC at pH 7, 70° produced by ALKO3620/1238/42 in cellulase-inducing conditions (pH 4) was higher (6000 NCU/ml) than that produced by ALKO3620/1240/32 (3500 NCU/ml) (Table 3/IV). Thus, a higher production level was obtained when the expression cassette had integrated into the *cbh1* locus compared to the *egl1* locus. ALKO3620/1240/32 produces Cel7A (EGI) protein with the same size as the purified native protein (Fig. 2/IV).

In transformant ALKO3620/1242/13 the expression fragment of pALK1242 containing the *Ma cel7B* (*cbh1*) gene had replaced *cbh1* of *T. reesei.* The

transformant produced slightly higher activity against MUL (CBHI) than the control strain ALKO4072 in which the *cbh1* and *egl2* genes had been deleted (Table 3/IV). The transformant produced *Ma* Cel7B (CBHI) protein with the same size as the purified native protein (Fig. 2/IV).

3.4.3 Use of the heterologous *Ma* Cel45A in biostoning

The purified *Ma* Cel45A (EGV) alone was able to effectively impart a stonewashed appearance to denim fabric (Tables 5–6/III, Table 13). Therefore endoglucanase preparations produced by the Cel45A transformants were tested in biostoning in neutral conditions. Preparations produced by the transformants ALKO3620/1231/14 and ALKO3620/1231/16 combined (CBHI-, EGII-) and ALKO3620/1235/49 (EGI-, EGII-) increased the release of indigo dye from the right side of the denim fabric, resulting in a lightening of 6.1 (L_{right}) with 400 ECU/g dosage after two hours treatment (Table 4/IV, summary in Table 14). At pH 7 the stone-washing effect of heterologous *Ma* Cel45A preparations was significantly better and the backstaining was clearly lower compared to the effects obtained with the acid cellulase product Ecostone L at pH 5.

Table 14. Colour measurements of denim fabric treated with T. reesei cel45A-transformants and Ecostone L (Primalco Ltd. Biotec).

Preparation	***Treatment pH***	***ECU/g of fabric***	$\mathbf{L_{right}}$	$\mathbf{b_{reverse}}$
Buffer only	7	-	2.6	0.2
ALKO3620/1235/49	7	100	4.7	2.5
	7	400	6.1	3.6
ALKO3620/1231/14+16	7	100	4.4	1.5
	7	400	6.1	2.4
Ecostone L	5.2	200	2.0	4.8

L: increase of lightness on the right side, b = increase of blueness on the reverse side.

4. Discussion

4.1 Homologous production of cellulases by *T. reesei*

The biotechnically important filamentous fungus *T. reesei* was exploited as a host in efficient production of homologous cellulases. Different tailored strains were constructed in order to obtain specific industrial cellulase preparations for commercial applications in biostoning and biofinishing. In total, eight strains producing homologous *T. reesei* cellulases with different profiles were constructed.

Production of the native natural endoglucanases and cellobiohydrolases of *T. reesei* was improved individually by using the *Trichoderma cbh1* promoter and by adding extra copy numbers of the *egl2*, *cbh1* or *cbh2* genes. As a result high EGII, CBHI or CBHII activity-producing strains with variable backgrounds of other cellulases were obtained. In shake flask cultivations in inducing conditions the best EGII-overproducing strain ALKO3529 produced EGII protein 3.2 times more than the parent strain, whereas the best CBHI-overproducing strain ALKO3760 produced 1.5-fold more CBHI and the best CBHII-overproducing strain 3.9-fold more CBHII than the parent strain (Table 15). Previously, the highest reported overproduction level of homologous cellulase in *T. reesei* has been 2.2 g EGI/l, which is three times higher than in the parent (Karhunen *et al.,* 1993).

Table 15. Amounts of cellulases overproduced by the best T. reesei strains overproducing homologous cellulases individually under the cbh1 promoter.

Strain	Cellulase composition*	Amount
ALKO3529	CBHI, CBHII, EGI, **EGII**$^{+}$	1.3 g EGII / l, 3.2x parent
ALKO3760	**CBHI**$^{+}$, CBHII, EGI, EGII	3.4 g CBHI / l, 1.5x parent
ALKO3798	CBHI, **CBHII**$^{+}$, EGI, EGII	0.7 g CBHII / l, 3.9x parent

*Overproduction is indicated as +, of the main cellulases

Factors reported to affect overproduction of proteins in filamentous fungi include promoter efficiency, copy number of the expression cassette and integration site (Verdoes *et al.,* 1995, Archer and Peberdy, 1997, Nevalainen *et al.,* 2004). In this work the *cbh1* promoter was used for homologous cellulase

expression in *T. reesei.* The site of integration was not observed to have a significant effect on the CBHII production levels of the CBHII-overproducer transformants or on the EGII production levels of the EGII-overproducer strains. However, copy number of the expression cassette did have an effect on the production levels, depending on the expressed gene. In the EGII-overproducing strains the increase in the production of EGII protein and endoglucanase activity followed the copy number of the *egl2* expression cassette (total up to three *cbh1* promoters). Simultaneous inactivation of *cbh1* did not increase significantly the EGII production or the endoglucanase activity levels in the studied EGII-overproducing strains. In the case of CBHI-overproduction some limiting factors appeared already with one additional copy of the *cbh1* expression cassette (a total of two *cbh1* promoters), since one additional copy of the *cbh1* expression cassette increased the production of CBHI protein and MUL (CBHI) activity only about 1.3 fold and two copies about 1.5 fold compared to the parent. In the strains used as hosts in this work, CBHI accounted for 70–80% of the secreted cellulases (Table 2/I, Table 1/II). Thus, although CBHI is the major secreted protein of *T. reesei* its production could be further increased, but the increase was not linear according to the copy number of the expression cassette. It is also notable that there was an increase in the amount of total secreted protein in the CBHI-overproducer strain, indicating that *T. reesei* has a capacity for increased overall protein production levels. Interestingly, in the CBHII-overproducing strains one copy of CBHII under the *cbh1* promoter increased production of CBHII more than twofold (Table 15). Karhunen *et al.* (1993) suggested that the third copy of the *cbh1* promoter would be enough to titrate out regulatory proteins or other essential transcription factors in overproduction of EGI. In *Aspergillus niger,* copy number-dependent improvement in the levels of secreted glucoamylase has been observed with up to about 20 copies (Archer and Peberdy, 1997).

The overproduction of different endogeneous cellulases (other cellulases present) had effects on the cellulase activities and on the production levels of the other main cellulases. FPU activity is mainly produced by cellobiohydrolases and is also an indication of total hydrolysis of cellulose. EGII was found to affect the FPU activity by increasing it about 10% in the EGII-overproducer (*cbh1* gene present, ALKO3529) compared to the parent strain. Increase in FPU activity was also observed in fermenter culture supernatant of the CBHII-

overproducing transformant ALKO3799 (data not shown). No increase in FPU activity was observed in CBHI-overproducing strains.

As expected the deletion of *egl1* or *egl2* genes decreased (50 %) the endoglucanase activity in CBHI- or CBHII-overproducing transformants. However, endoglucanase activity was also decreased (20–30 %) in the EGII-positive CBHII-overproducers and the EGI-positive CBHI-overproducers. The amounts of EGI and CBHII were decreased in EGI-positive CBHI-overproducing transformants. In CBHII-overproducing transformants the amount of secreted CBHI was clearly decreased (both EGII+ and - background). In one EGII-overproducing strain (CBHI-positive) a very slight reduction in the amount of secreted CBHI was observed. These might be indications of mutual cellulase regulation or possibly of a secretion stress. Saloheimo *et al.* (2003b) recently showed that in *T. reesei* the genes encoding secreted proteins are rapidly downregulated concurrently with induction of the UPR pathway. However, our data suggests that overproduction of individual *T. reesei* cellulases can be performed successfully without the possible interference of UPR. As an example EGII was overproduced according to the copy number of the expression cassette and no significant reduction in the amounts of other main cellulases was detected. However, UPR might have some role, because the amount of proteins overproduced was not always linear, e.g. when overproducing CBHI, and the amounts of the other main cellulases may decrease as described above.

One additional copy of the *egl2* expression cassette in the *cbh*1 locus increased the endoglucanase activity by 2.3 fold compared to the parent strain, whereas one additional copy of the *egl1* gene expressed under the *cbh1* promoter in the *cbh*1 locus increased the endoglucanase activity by 1.9 fold as shown by Karhunen *et al.* (1993). By further increasing the copy number of *egl2* a greater increase in endoglucanase activity was obtained as compared with the corresponding copy number of the *egl1* expression cassette. Thus it can be concluded that EGII has a major impact on the endoglucanase activity measured as activity against HEC. This is consistent with the higher specific activity of EGII than of EGI on HEC and β-glucan (Suurnäkki *et al.,* 2000) and with the results obtained with cellulase deletion strains of *T. reesei* (Suominen *et al.,* 1993).

In addition to the strains overproducing EGII, CBHI and CBHII separately, strains overproducing the main endoglucanases EGI and EGII without

cellobiohydrolases and strains overproducing CBHI and CBHII without the main EGs were constructed. Endoglucanases without any cellobiohydrolases can be produced with the strain ALKO3528 which produces, as expected, endoglucanase activity fourfold higher than the parent strain. In culture supernatant of the CBH-overproducers some endoglucanase activity was detected due to the minor EGs still present (Table 3/II). In the CBH-overproducing transformants the host strain ALKO3761 contained two copies of the *cbh1* promoter and when *cbh2* was expressed in this background with either *cbh1* or *cbh2* promoters similar increases in CBHII were obtained. However, the simultaneous use of the *cbh1* promoter for both *cbh1* and *cbh2* expression in one strain decreased the amount of secreted CBHI as compared to the use of the *cbh2* promoter, suggesting limitations in the *cbh1* promoter.

4.2 Novel neutral cellulases

There is a continuing need for new cellulases with a variety of characteristics for use in different industrial applications and conditions such as in treating textiles. Cellulases acting over broad pH ranges with near-neutral optima and causing low backstaining would be preferable in biostoning. In the present work, new cellulases for finishing of cotton-containing denim articles were screened, isolated and characterized and the genes encoding them were cloned and sequenced.

Enzymatic washing results of denim cannot be predicted simply by measuring the cellulase activities in the preparations (Gusakov *et al.,* 2000b). In this work the laboratory-scale biostoning method was successfully used in screening and purification of novel neutral cellulases for finishing of denim fabric. When preparations produced by the strains *Melanocarpus albomyces, Myceliophthora thermophila, Chaetomium thermophilum* and *Sporotrichum thermophilum* were compared, the preparation of *M. albomyces* was found to be the most effective in releasing indigo dye from denim and to cause lowest backstaining. Backstaining was earlier claimed to depend on pH, with lower redeposition at pH 7 than at pH 5 (Tyndall, 1990), but further studies have indicated that the nature of the enzyme has an impact on backstaining (Klahorst *et al.,* 1994, Gusakov *et al.,* 1998). Our studies clearly showed differences in performance and especially in backstaining between the strains (Table 12). The culture supernatant of *C.*

thermophilum caused extremely high backstaining at pH 7, contrary to the culture supernatants of *M. albomyces, M. thermophila* or *S. thermophilum,* confirming the hypothesis that backstaining is dependent on the origin of the cellulase preparation and the functional properties of cellulases rather than on the pH of the treatment solution.

M. albomyces produces at least three cellulases with an effect on the biostoning process, and all three were purified. The purified enzymes were 20 kDa endoglucanase (Cel45A / EGV), 50 kDa endoglucanase (Cel7A / EGI) and 50 kDa cellobiohydrolase (Cel7B / CBHI). Low hydrolysis of MUL was the only enzyme activity found for Cel7B. As this protein had sequence similarities to *H. grisea* cellobiohydrolase I it was named cellobiohydrolase. The isolated Cel45A and Cel7A endoglucanases are stable enzymes that exhibit endoglucanase activity over a wide range of pH values and at high temperatures. Especially Cel45A is a relatively heat stable enzyme. These properties increase the application potential of the endoglucanases in many conditions and applications, such as in household detergents in which cellulases should act at higher pH values (pH 7–12) and temperatures (40–90 ^{o}C).

The genes encoding the three isolated cellulases of *M. albomyces* were cloned and sequenced. The protein sequences deduced from the gene sequences showed significant sequence similarity with other fungal cellulases (Table 2/IV, Fig. 6), but lacked a consensus CBM and a linker region. *Ma* Cel45A (EGV) is similar to *T. terrestris* endoglucanase and *H. insolens* EGV (Cel45A). *Ma* Cel7A (EGI) is similar to *H. grisea* EGI and *Ma* Cel7B (CBHI) to *H. grisea* CBH.

According to Schülein *et al*. (1996), when *T. terrestris* EG was used in denim finishing (pH 5) an almost bleached appearance could be obtained. In addition to cellulases of *T. terrestris* and *T. reesei,* cellulases from various fungal sources have been reported to be applicable to denim finishing at different pH values (Table 6). Different finishing effects have been achieved depending on the enzyme. The cellulases (Table 6) for which the sequence is available and the cellulases of *M. albomyces* were compared in the predicted phylogenetic tree (Fig. 7). In addition to *Ma* Cel45, high abrasion of denim was reported to be achieved with EGV of *H. insolens* (Schülein *et al.,* 1998, pH 7), EG of *T. terrestris* (pH 5) and *Acremonium* sp. (Schülein *et al.,* 1996, pH 7) and with *M. thermophila* EG attached to *H. insolens* EGV CBM and linker (Schülein *et al.,*

1996, pH 6). These cellulases appear to be clustered closer to each other than to the cellulases with reported effects of little (*H. insolens* EGI and *F. oxysporium* EGI, Schülein *et al.,* 1998) or no (*M. albomyces* Cel7A and Cel7B, this work, *T. reesei* CBHI, Heikinheimo *et al.,* 2000) abrasion. Interestingly, although high abrasion is obtained at pH 5 with *T. reesei* EGII (Heikinheimo *et al.,* 2000, this work), it seems to be very distant from *M. albomyces* Cel45A working efficiently at pH 7 and also at pH 5 (data not shown). However, the backstaining levels are significantly higher with *T. reesei* cellulases as compared to *Ma* Cel45A (Table 4/IV, Table 14). *H. insolens* EGI and *F. oxysporium* EGI have been claimed to prevent backstaining in denim wash (Schülein *et al.,* 1998; no results were presented). This was shown in the case of *Ma* Cel7A. *Ma* Cel7A prevents backstaining when used together with *Ma* Cel45A (Table 13). *Ma* Cel7A, *H. insolens* EGI and *F. oxysporium* EGI appear to be situated close to each other in the predicted phylogenetic tree (Fig. 7).

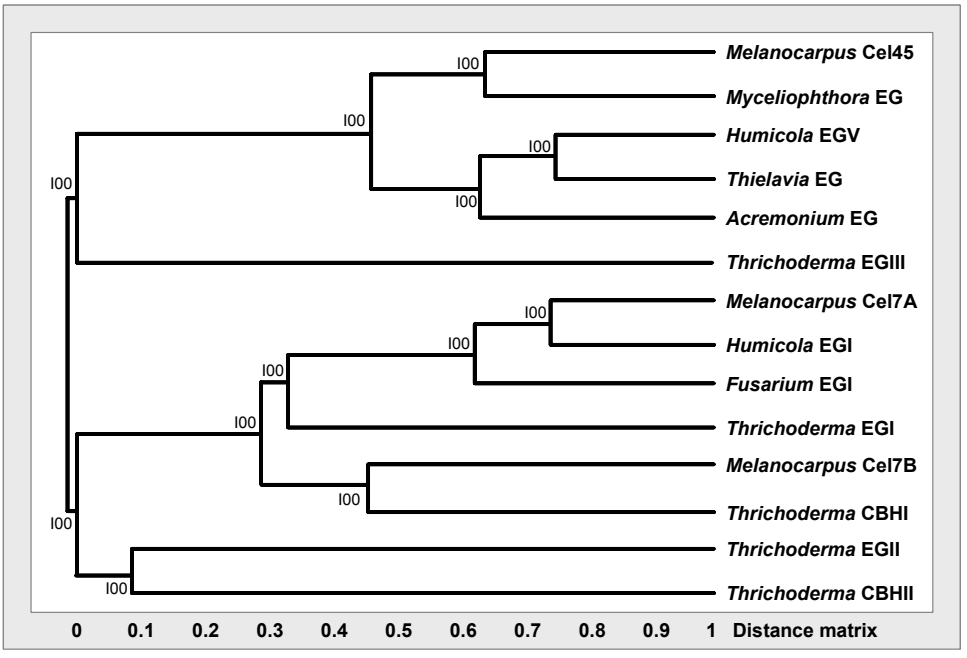


Figure 7. The predicted phylogenetic tree (with bootstrap values) of cellulases tested in biostoning. The tree was made with the GeneBee – Molecular Biology Server with the TreeTop – Phylogenetic Tree prediction program. Cluster algorithm.

In this work a rather labour-intensive screening method was used to look for novel cellulases for biostoning. If more novel cellulases with similar performance are desired, these could be searched by looking for genes which are homologous with the genes encoding the cellulases of *M. albomyces* (especially Cel45A) or closely related genes based on phylogenetic analysis. The cellulases thus identified could then be tested in the application.

4.3 Heterologous production of cellulases by *T. reesei*

The cellulase production capacity of *M. albomyces* is far too low for efficient and cost-effective industrial-scale production. Therefore *T. reesei* was chosen as a potential host for production of Cel45A (EGV), Cel7A (EGI) and Cel7B (CBHI) of *M. albomyces*. In this work five strains producing heterologous *M. albomyces* cellulases with different profiles were constructed. *M. albomyces* cellulases were produced specifically for biostoning at neutral pH. *Trichoderma*'s own cellulases can have undesired effects (backstaining, strength loss) in biostoning (Klahorst *et al.,* 1994, Heikinheimo and Buchert, 2001), especially if the pH of the washing liquor is decreased below 6–7. Cellulases were expressed under the *T. reesei cbh1* promoter in *T. reesei* hosts lacking EGII and either EGI or CBHI and single copy transformants produced commercially significant levels of *M. albomyces* cellulases even in shake flask cultures: approximately 0.3–0.8 g Cel45A/l and 1–1.5 g Cel7A/l were produced. The proteolytic degradation, caused by aspartic protease, of recombinant *Ma* Cel45A had to be prevented by maintaining the pH of the cultivation medium above 4. Proteolytic degradation has been one reason for low yields of heterologous products in filamentous fungi (Nevalainen, 2001). The characteristics of the recombinant *Ma* Cel45A preparations were suitable for biostoning in neutral conditions. Published secreted yields of fungal enzymes heterologously expressed in *T. reesei* from the *cbh1* promoter have varied from no detectable protein to a few grams depending on the source of the gene (Paloheimo *et al.,* 1993). For example expression of *Aspergillus niger* acid phosphatase yielded 0.5 g/l in shake flasks (Miettinen-Oinonen *et al.,* 1997), *Hormoconis resinae* glucoamylase 0.7 g/l (Joutsjoki *et al.,* 1993) and *Phlebia radiata* laccase about 3 mg/l (Saloheimo and Niku-Paavola, 1991). A few published reports exist on the expression of genes from thermophilic fungi or bacteria in *T. reesei* using the *cbh1* promoter (reviewed in Penttilä *et al.,* 2004). The *xyn2* gene encoding

xylanase from the thermophilic fungus *Humicola grisea* var. *thermoidea* was fused to the CBHI secretion signal and the XYNII enzyme was shown to be correctly processed in the *T. reesei* host (de Faria *et al.,* 2002). The highest level of XYNII production was about 0.5 g/l, approximately similar to the production of *Ma* Cel45A in *T. reesei* under the *cbh1* promoter (no fusion). Expression of *M. albomyces* laccase in *T. reesei* under the *cbh1* promoter resulted in 0.2 g laccase/l (Kiiskinen *et al.*, 2004). Thus, the amount of *Ma* Cel7A produced by *T. reesei* (1–1.5 g/l) is the highest level of heterologously expressed fungal protein hitherto reported. Production economics of *T. reesei* strains producing *M. albomyces* cellulases may be further improved by adding more copies of the respective gene, by using other mutant strains as hosts, by using complex industrial media and appropriate fermenter cultivation strategies and by random mutagenesis and selection of the production strains.

4.4 Cotton finishing

One of the most important objectives of biostoning and biofinishing processes is to carry out the treatment processes cost-effectively. Furthermore, it is essential to achieve a good appearance of the textile while preserving the strength properties. Cellulases derived from *T. reesei* are commonly employed in textile treatments and investigations have been made of the effects of different cellulase ratios in the treatments (Clarkson *et al.,* 1992a, b, c, 1993, 1994b, Kumar *et al.,* 1997, Heikinheimo *et al.,* 1998, Heikinheimo and Buchert, 2001, Table 6).

In this work the role of the main cellulases of *T. reesei* in textile applications was further elucidated in order to identify suitable cellulase combinations for industrial biofinishing and biostoning. Each of the purified main cellulases was added to the normal overall cellulase composition of *T. reesei*. When the cellulase composition with an elevated content of EGII was used for treating cotton fabric and denim fabric, improved biofinishing effect and stone-washed appearance were obtained as compared to the total cellulase composition or compositions containing elevated amounts of EGI, CBHI or CBHII. These results are consistent with those studies in which purified EGII was found to be the most effective of the *T. reesei* main cellulases in biostoning and biofinishing (Heikinheimo *et al.,* 1998, Heikinheimo *et al.,* 2000, Heikinheimo and Buchert, 2001). The phenomenon was confirmed by comparing cellulase preparations

derived from *T. reesei* strains producing enhanced EGI, EGII, both EGI and EGII, CBHI, CBHII or both CBHI and CBHII in biostoning and biofinishing (I, II). Cellulase preparation derived from the EGII-overproducing strain ALKO3529 improved the stone-washing effect and biofinishing performance compared to its parent strain VTT D-79125 or to the other overproducing strains, when the same enzyme (protein) dosage and time were used (Table 4/II, Fig. 5). Furthermore, the same stone-washing effect could be obtained, with considerably lower enzyme dosage and time, when using the EGII-enriched preparation than when using the preparation produced by the parental strain. Thus, it is possible to achieve improved stone-washing and biofinishing effects by utilizing the production strain producing an increased relative amount of EGII in the total cellulase mixture. Preparations produced by the EGII-overproducer strains described in this work have also been used together with different experimental *T. reesei* cellulase preparations for biofinishing of different types of fabrics (Miettinen-Oinonen *et al.,* 2001). EGII-based preparations (background CBHI+/-) gave the highest pilling removal. The improvement was not dependent on the ratios of CBH and EG, but an increased level of EGII appeared to be responsible for the improvement.

Use of the EGII-enriched preparation in denim finishing and biofinishing allows shorter processing times, i.e. the EGII-preparation acts more rapidly than the other tested preparations. This would mean more time- and cost-effective treatment procedures and savings in equipment as well as treatment facilities. Another advantage may be reduction of the amount of enzyme required in the treatment solution leading to savings in enzyme costs.

Endoglucanases are known to be key enzymes in biostoning and biofinishing applications (Kumar *et al.,* 1997, Heikinheimo *et al.,* 1998, Heikinheimo *et al.,* 2000, Liu *et al.,* 2000), but strength and weight losses have been negative impacts often associated with endoglucanase treatments (Heikinheimo and Buchert, 2001). Attempts to minimize the strength losses caused by *T. reesei* cellulases have been made by altering the ratios of EG and CBH in the cellulase preparation (Clarkson *et al.* 1992a, b, 1993). Although a cellulase preparation with increased levels of especially EGII is the most promising in biostoning and biofinishing applications, improved performance in biofinishing was also observed with the preparation from the CBHII-overproducing strain as compared to the wild type (Table IV/II). However, no difference in weight loss of the

fabric was observed when EGII- and CBHII-enriched preparations were compared at the same depilling level and dosage. The improved performance attained with the CBHII-preparation might be explained by the synergistic action of cellulases (Heikinheimo and Buchert, 2001) or possibly by the low β-glucanase activity of CBHII (Henriksson *et al.,* 1995).

T. reesei cellulases are useful in denim finishing to impart a certain type of stonewashed appearance, but they have disadvantages such as a tendency to promote backstaining and weakening of fabrics. Cellulases of *M. albomyces* were shown to have a good capability to impart stonewashed appearance in denim. Of the isolated *M. albomyces* cellulases Cel45A (EGV) was mainly responsible for the good stone-washing effect of *M. albomyces* ALKO4237 growth medium, with reasonably low backstaining. *Ma* Cel45A is also an excellent cellulase for biostoning applications because of its broad operational pH and temperature ranges. The superiority of *Ma* cellulases over *T. reesei* cellulases with regard to backstaining was clearly demonstrated (Tables 3/III, 4/IV, 12, 14). Cellulase preparation containing heterologous *Ma* Cel45A performed well in biostoning at neutral pH and an equal degree of abrasion and equally low backstaining were obtained as with the purified *Ma* Cel45A.

M. albomyces cellulases differ from the cellulases of *T. reesei* and *H. insolens* (except EGI/Cel7A), commonly used in denim finishing, as they do not contain a CBM. Cellulases from which the native CBM have been deleted, both bacterial (*Cellumonas fimi)* and fungal (*H. insolens)*, generally decrease indigo staining levels and cause less backstaining than do the intact enzymes (Andreaus *et al.,* 2000). Fowler *et al.* (2001) used *T. reesei* EGI and EGII catalytic core and CBHI core with EGIII (no CBD) and reported decreased backstaining. Cavaco-Paulo *et al.* (1998b) suggested that the prevention of backstaining during stone-washing requires an enzyme with very little affinity for indigo dye and reduced binding of the cellulase protein to the cotton cellulose. Thus the absence of a CBM might play a role in the low backstaining properties of the *Ma* cellulases. Cellulases of *T. reesei* cause higher indigo staining than those of *H. insolens* (Cavaco-Paulo *et al.,* 1998b). Campos *et al.* (2000) showed that cellulases of *T. reesei* have more affinity for plain indigo dye than the cellulases of *H. insolens.* The affinity of *M. albomyces* cellulases for indigo dye remains to be clarified. Gusakov *et al.* (2000b) suggested that certain cellulases may have hydrophobic domains (clusters of closely located non-polar residues) on their surface, and that these

may bind indigo and thus act as emulsifiers helping the dye to float out of cellulose fibres into the bulk solution. This might be one explanation for the observed phenomenon of reduced backstaining levels obtained when *Ma* Cel45A was used together with *Ma* Cel7A or *Ma* Cel7B.

4.5 Future perspectives

Industrially useful *T. reesei* strains producing significant amounts of homologous and heterologous cellulases and having defined cellulase profiles were constructed. Some recent results of the molecular mechanisms of cellulase gene regulation and protein secretion in *T. reesei* could possibly be utilized to increase the production levels of cellulases further. As an example the overexpression of positively acting regulatory factors might be a tool to circumvent the possible titration of transcriptional factors or regulatory proteins in enhancing production of *T. reesei* EGs and CBHs with the aid of the *cbh1* promoter, leading to still more cost-effective levels of cellulase production. Deletion of the negative regulatory factor ACEI and overexpression of the positive regulatory factor ACEII of cellulase promoters could represent one possible way to increase the production of cellulases in *T. reesei* (Aro, 2003). However, deletion of *ace1* or overproduction of *ace2* did not significantly improve cellulase production levels of *T. reesei* ALKO2221 in industrially feasible medium (M. Paloheimo and J. Vehmaanperä, Roal Oy, personal communication). The cloning of genes involved in the secretion processes in *Trichoderma* has been started, with the aim of improved secretion. Thereby, the overall production levels of cellulases may possibly also be increased.

Backstaining is not a desired effect in denim wash. *Ma* Cel7A and Cel7B were able to decrease the backstaining level in denim finishing when used along with *Ma* Cel45A. This phenomenon requires further clarification. Better understanding of the mechanism behind reduction or prevention of backstaining by certain cellulases or cellulase mixtures will enable the design of optimal cellulase compositions having the desired denim finishing effect with high abrasion and no backstaining.

Other potential uses of the new cellulase preparations also need to be elucidated. Strains producing enhanced *T. reesei* EGII or EGI and EGII or the *Ma* Cel45A

(EGV) or Cel7A (EGI) activity can possibly be used for more economical production of β-glucanase for degradation of β-glucan in feed in order to improve the quality of feed. The suitability of the cellulases in efficient conversion of biomass to sugars that can be fermented to ethanol should also be investigated. Cellobiohydrolases are the key components of the redesigned, highly synergistic cellulase mixtures required for such processes (Teeri, 1997). Therefore the newly described CBH preparations as well as possibly the novel cellulases of *M. albomyces* could be used in applications in which total hydrolysis of cellulose is needed. *M. albomyces* cellulases could also be exploited in detergent applications, if they are found to be compatible with chemical components present in detergents. In addition to detergents, deinking is also a potential application due to the broad pH range of the cellulases and their ability to function in the alkaline pH range.

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Enhanced Production of *Trichoderma reesei* Endoglucanases and Use of the New Cellulase Preparations in Producing the Stonewashed Effect on Denim Fabric

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***Trichoderma reesei* strains were constructed for production of elevated amounts of endoglucanase II (EGII) with or without cellobiohydrolase I (CBHI). The endoglucanase activity produced by the EGII transformants correlated with the copy number of the *egl2* expression cassette. One copy of the *egl2* expression cassette in which the *egl2* was under the *cbh1* promoter increased production of endoglucanase activity 2.3-fold, and two copies increased production about 3-fold above that of the parent strain. When the enzyme with elevated EGII content was used, an improved stonewashing effect on denim fabric was achieved. A *T. reesei* strain producing high amounts of EGI and -II activities without CBHI and -II was constructed by replacing the *cbh2* locus with the coding region of the *egl2* gene in the EGI-overproducing CBHI-negative strain. Production of endoglucanase activity by the EG-transformant strain was increased fourfold above that of the host strain. The filter paper-degrading activity of the endoglucanase-overproducing strain was lowered to below detection, presumably because of the lack of cellobiohydrolases.**

The filamentous fungus *Trichoderma reesei* is known as an efficient producer of cellulases. The cellulolytic system of *T. reesei* is composed of two cellobiohydrolases (CBHI and CBHII) and at least five endoglucanases (EGI, EGII, EGIII, EGIV, and EGV) (19, 20). Lack of EGII (originally called EGIII [18]) production reduces the endoglucanase activity in the culture supernatant by as much as 55%, whereas lack of EGI reduces it by only 25% (21). Thus, EGII is proposed to account for most of the endoglucanase activity produced by *T. reesei* (21). *T. reesei* EGI represents 5 to 10% of the secreted protein (16). The production of EGI has been improved in *T. reesei* by placing the *egl1* gene under the control of the strong promoter of the *Trichoderma* CBHI (*cbh1*) gene and by increasing the copy number of the *egl1* gene (8).

Cellulases are used widely in the textile industry in treatments of cellulose-containing textile materials during their manufacture and finishing (5). The most well-known application is the use of cellulases in biostoning. Biostoning of fabric means the use of cellulases in place of, or in addition to, the use of pumice stones for the treatment of denim fabric to impart a stonewashed effect. Heikinheimo et al. (7) showed that *T. reesei*-purified cellulase EGII was the most effective at removing color from denim, producing a good stonewashing effect with the lowest hydrolysis level. Endoglucanases are important also for degradation of β-glucan in feed. Degradation of β-glucan lowers the viscosity of the intestinal contents and this improves the quality of the feed (3).

In this study we have constructed *T. reesei* strains that produce elevated amounts of endoglucanase activity. The aim of our work was to construct different tailored high endoglucanase activity-producing strains for specific applications. We have improved the production of the EGII enzyme in *T. reesei* and we have constructed a *T. reesei* strain that produces high amounts of EGI and -II without any cellobiohydrolases. Cellulase preparations derived from these *T. reesei* overproduction strains were tested on the biostoning application.

MATERIALS AND METHODS

Microbial strains and plasmids. *Escherichia coli* strain XL1-Blue (Stratagene) was used for propagation of plasmids. *T. reesei* strains VTT-D-79125 (2) and ALKO2698 (8) were used as recipients for transformations (Table 1). *T. reesei* VTT-D-79125 is a high cellulase activity-producing mutant strain that contains all the identified *Trichoderma* cellulases, including the main cellulases EGI, EGII, CBHI, and CBHII. ALKO2698 is an EGI-overproducing, *cbh1*-negative strain in which the *cbh1* locus of VTT-D-79125 has been replaced by one copy of an *egl1* expression cassette. In the expression cassette, *egl1* is under the control of the *cbh1* promoter. ALKO2697 (8), used for comparison, is an EGI-overproducing, CBHI-negative strain in which the *cbh1* gene of VTT-D-79125 is replaced by two copies of the *egl1* expression cassette (Table 1). A cellulase preparation derived from ALKO2656 (8) was used as a control in biostoning experiments. ALKO2656 is a high EGI activity-producing strain which contains three copies of *egl1* in the place of *cbh1* (Table 1).

Plasmids were constructed by using pUC19 as a vector backbone, using standard recombinant DNA techniques. The pALK537 and pALK540 plasmids were constructed for EGII overproduction from the strong *cbh1* promoter (Fig. 1 and 2). pALK537 and pALK540 can be used to target the expression cassette into the *cbh1* and *cbh2* loci, respectively, by homologous recombination. The precise fusion between the *cbh1* promoter and *egl2* cDNA was done with PCR. The *Sac*II site in the *cbh1* promoter was included in the 5′ primer, and the *Hpa*I site of the *egl2* cDNA (229 nucleotides downstream from the N terminus of the *egl2* gene) was included in the 3′ primer. The fusion and the PCR fragment were sequenced to ensure that no mistakes had occurred in the PCR amplification. The plasmid pALK537 contains a 2.2-kb *T. reesei* cellobiohydrolase (*cbh1*) promoter and a 0.7-kb *Ava*II fragment of the *cbh1* terminator region (starting 113 bp before the stop codon of *cbh1*). A 1.4-kb *Bam*HI-*Eco*RI *cbh1* 3′ fragment was used together with the promoter to target the expression cassette to the *cbh1* locus (21). A 3.1-kb *Spe*I-*Xba*I fragment containing the *Aspergillus nidulans* acetamidase gene from the plasmid p3SR2 (9) was used in the pALK537 plasmid as a marker.

The plasmid pALK540 contains the *cbh1* promoter and terminator and *egl2* cDNA as in plasmid pALK537. Resistance to phleomycin was used for selecting the transformants. A 3.3-kb *Xba*I-*Bgl*II fragment containing the *Streptoalloteichus*

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TABLE 1. *T. reesei* strains used as recipients for transformations and as comparison in cultivations

Strain	*cbh1*	*egl1* expression cassette copy no.
High cellulase-producing mutant strain		
VTT-D-79125	+	0
EGI-overproducing strains		
ALKO2698	−	1
ALKO2697	−	2
ALKO2656	−	3

hindustanus phleomycin gene (*ble*) from the plasmid pAN8-1 (14) was used in pALK540. In addition, pALK540 contains a 3.4-kb *Xho*I-*Pvu*II *cbh2* 5′ fragment (starting 1.4 kb upstream from the *cbh2* gene) and a 1.6-kb *Xba*I-*Bgl*II *cbh2* 3′ fragment (starting 1.1 kb downstream from the *cbh2* gene). *cbh2* 5′ and 3′ fragments were used to target the *egl2* expression cassette to the *cbh2* locus of ALKO2698.

Inquires concerning the availability of the *Trichoderma* strains, plasmids, and antisera can be forwarded to Roal Oy, Rajamäki, Finland.

Growth of organisms. *E. coli* strains were grown at 37°C overnight in L broth (13) supplemented with 50 μg of ampicillin/ml when needed. Potato dextrose (PD; Difco) agar slants were used for growing the *Trichoderma* strains. The plates and media for *Trichoderma* transformations with acetamide selection were essentially as those described by Penttilä et al. (16). MnR medium (per liter, 2.5 g of glucose, 2.5 g of yeast extract, 0.3 g of potassium phthalate, and 15 g of agar) was used in *Trichoderma* transformations with phleomycin selection. Liquid cultures of *T. reesei* were started from conidiospores grown on PD agar. A lactose-based complex medium was used for liquid cultivations (21). Cultivations were carried out at 30°C and 250 rpm for 7 days. Mycelia for isolation of the chromosomal DNA from the *Trichoderma* transformants were grown in shake flasks for 2 days (30°C, 250 rpm) on *Trichoderma* minimal medium (16) supplemented with 0.2% proteose peptone.

DNA techniques. DNA manipulations were performed by standard techniques (13). Plasmid DNA from *E. coli* was isolated by using Qiagen columns (Qiagen GmbH) according to the supplier's instructions. DNA fragments for cloning or transformations were isolated from low-melting-point agarose gels (FMC Bioproducts) by the freeze-thaw phenol method (4). Chromosomal DNA was isolated from *T. reesei* by using the method of Raeder and Broda (17). For Southern blot analysis the DNA was transferred from agarose gels to nylon membranes by using a VacuGene XL apparatus (Pharmacia). The labeling of the probes with digoxigenin and the hybridization of the filters were performed according to the procedures of Boehringer Mannheim.

The PCRs were performed by using a Techne thermal cycler PHC-2 (Techne Ltd.) in 100-μl volumes. The reaction mixture contained a 0.2 mM concentration of each deoxynucleoside triphosphate (Pharmacia), 20 to 50 pmol of each primer, and 10 ng of plasmid template in 1× buffer supplied by Boehringer. The protocol used was the following: 96°C for 10 min before adding *Taq* DNA polymerase (2 U; Boehringer) and 100 μl of paraffin oil, denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min for 30 cycles. The PCR fragments were purified by using a Mermaid kit (Bio 101 Inc.) according to the supplier's instructions. The ends of the fragments were filled by using DNA polymerase I Klenow fragment.

Sequencing of the fusion between the *cbh1* promoter and *egl2* cDNA was carried out by means of pUC/M13 and extension primers using a *Taq* DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) and an automated sequencer (model 373A; Applied Biosystems).

Transformation of *Trichoderma*. Transformation of *T. reesei* was carried out by protoplast transformation as described by Penttilä et al. (16) with the modifications described by Karhunen et al. (8). In transformations where phleomycin was used as a selection marker, aliquots of the transformed protoplasts were plated onto the surface of MnR plates osmotically stabilized with 0.44 M sucrose and incubated for 6 h at 30°C prior to the addition of 5 ml of molten MnR (0.6% agar) as an overlay containing 300 μg of phleomycin (Cayla)/ml. The transformants were purified on selective MnR medium supplemented with 50 μg of phleomycin/ml through single spores before transfer to MnR slants containing 50 μg of phleomycin/ml for three generations and after that to PD slants. The acetamidase transformants were purified on selective acetamide-CsCl medium through single spores before transfer to PD slants.

Enzyme activity and protein assays. The cellulase activities were measured from the culture supernatant as the release of reducing sugars from hydroxyethylcellulose (HEC; Fluka Chemie AG) using 2,4-dinitrosalicylic acid, as described by Bailey and Nevalainen (2) and from filter paper according to the method reported by Mandels et al. (12). Activity against barley β-glucan was measured the same way as activity against HEC, replacing HEC by barley β-glucan (Biocon Biochemicals Ltd.) in the assay. The β-glucosidase activity was measured using 4-nitrophenyl-β-D-glucopyranoside (Merck) as a substrate as described by Bailey and Nevalainen (2). Protein concentrations were determined from the trichloroacetic acid-precipitated *T. reesei* culture media by the method of Lowry et al. (11), using bovine serum albumin as the standard.

SDS-PAGE and immunological methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (10). For Western blot analysis, purified transformants were grown

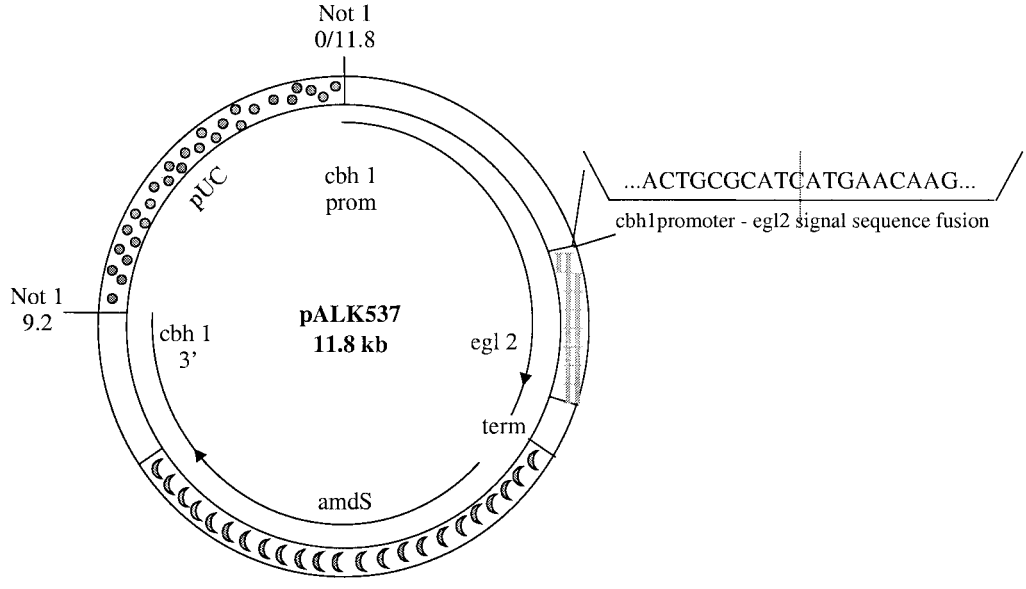

FIG. 1. Restriction map of the plasmid pALK537. The *egl2* cDNA is exactly joined to the *cbh1* promoter. A 9.2-kb *Not*I fragment was isolated from the plasmid for transformation.

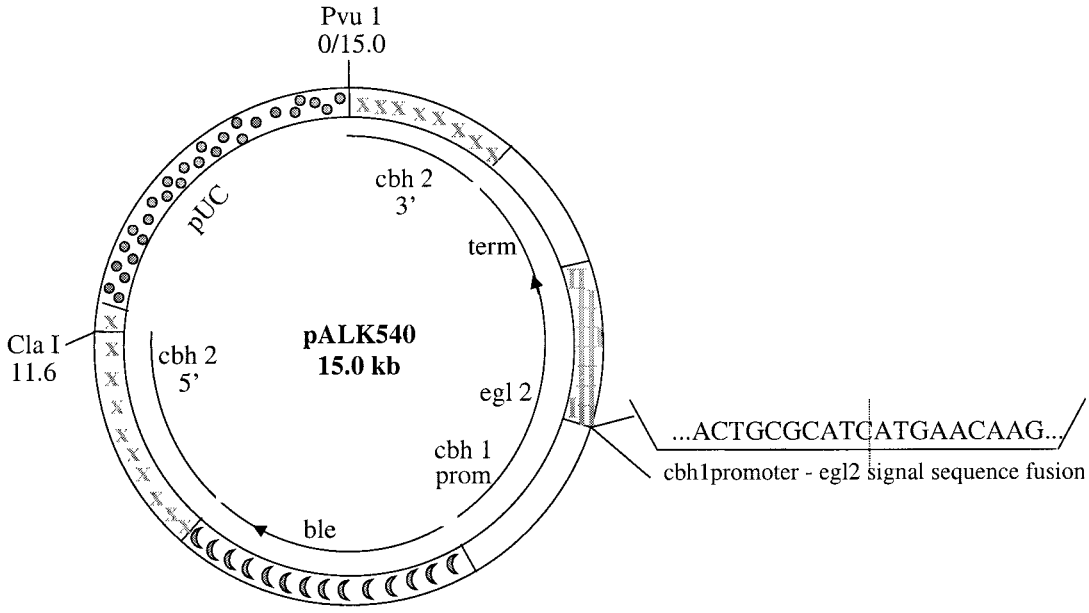

FIG. 2. Restriction map of the plasmid pALK540. An 11.6-kb *Cla*I-*Pvu*I fragment was isolated from the plasmid for transformation.

in 96-well Millititer filtration plates (Millipore Corp.) at 30°C for 7 days. The presence of the CBHII protein was detected by SDS-PAGE followed by Western blotting (23) and immunostaining using monoclonal CII-8 antibody (1) and the ProtoBlot Western blotting AP system (Promega). Dot blot analysis was done with a Minifold Micro-Sample Filtration Manifold (Schleicher & Schull) according to the manufacturer's instructions. Visualization of the CBHI protein was done using the monoclonal mouse antibody CBHI MAb 89 (1) and immunostaining as described above. Quantitation of secreted EGI was carried out by a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) (6), using the monoclonal anti-EG antibody EI-2 (1) as capture antibody. For quantitation of CBHI and CBHII by ELISA, monoclonal antibodies CI-258 and CII-8, respectively, (1) were used as capture antibodies.

Biostoning. The color of the desized denim fabric was measured as reflectance values with the Minolta Croma Meter 1000R using the L*a*b* system (illuminant D65) before and after enzyme treatments. In this system, *L* is the measure of black and white, *a* is the measure of red and green, and *b* is the measure of yellow and blue. Cellulase treatments were performed in an LP-2 Launder-Ometer (Atlas). A denim swatch of about 7 g was loaded into a 1.2-liter container that contained 200 ml of 0.05 M citrate buffer (pH 5), and 10 steel balls were added. Cellulase preparations from strains VTT-D-79125, ALKO3529, ALKO3528, and ALKO2656 were used for biostoning. Three and 6 mg of the total protein in cellulase preparations per g of fabric was used in each experiment for 1 and 2 h at 50°C. After cellulase treatment the swatches were soaked for 10 min in 0.01 M NaOH, rinsed with water, and dried.

RESULTS

EGII overproduction. (i) Transformation of *T. reesei* For overexpression of the *egl2* gene in *T. reesei*, the powerful promoter of the *cbh1* gene of *T. reesei* was used. The plasmid pALK537 (Fig. 1) was constructed as described in Materials and Methods for expression of the *egl2* gene under the control of the *cbh1* promoter either in the place of *cbh1* or elsewhere in the genome of *T. reesei* strain VTT-D-79125, depending on homologous or nonhomologous recombination.

For construction of EGII-overproducing CBHI-positive and CBHI-negative strains, the 9.2-kb *Not*I linear fragment of pALK537 containing the *egl2* expression cassette (Fig. 1) was released from the vector backbone and transformed to *T. reesei* strain VTT-D-79125. The transformation frequency was 20 transformants per μg of DNA.

A total of 119 purified transformants were cultivated in shake flasks on cellulase-inducing medium, and the endoglucanase activity (activity against HEC) was measured from the culture medium of the transformants. The presence of CBHI protein in the culture medium was detected by dot blotting and immunostaining from the 23 best endoglucanase producers. Of these transformants, 61% proved to be CBHI negative, which indicates that in these transformants the expression cassette had replaced the *cbh1* gene.

(ii) DNA analysis of transformants. The transformants producing the best endoglucanase activity, ALKO3529 (CBHI positive) and ALKO3530 (CBHI negative), and a transformant strain, ALKO3574, thought to contain one copy of the *egl2* expression cassette, were analyzed by Southern blotting to evaluate the copy number of *egl2* and the integration of the expression cassettes into the genome.

The ALKO3530 and ALKO3574 strains that did not secrete CBHI according to dot blot analysis were shown by Southern blotting to lack the chromosomal *cbh1* gene. The transformants showed no hybridization when the coding region of the *cbh1* gene was used as a probe (Fig. 3A). The integration of the expression cassette in these transformants was further studied by Southern blotting using the 9.2-kb *Not*I fragment of the plasmid pALK537 as a probe. According to the blot analysis (Fig. 3B and C), one copy of the transformed pALK537 fragment replaced the coding region of the *cbh1* gene in the ALKO3574 strain, generating a *Xho*I fragment of about 13 kb. The ALKO3530 strain had two vector fragments (a *Xho*I fragment of about 20 kb) replacing the *cbh1* locus. The 4.7-kb band present in the transformants and VTT-D-79125 is from the wild-type *egl2* locus. The 9.4-kb band present in VTT-D-79125 is from the wild-type *cbh1* locus. The results were confirmed by

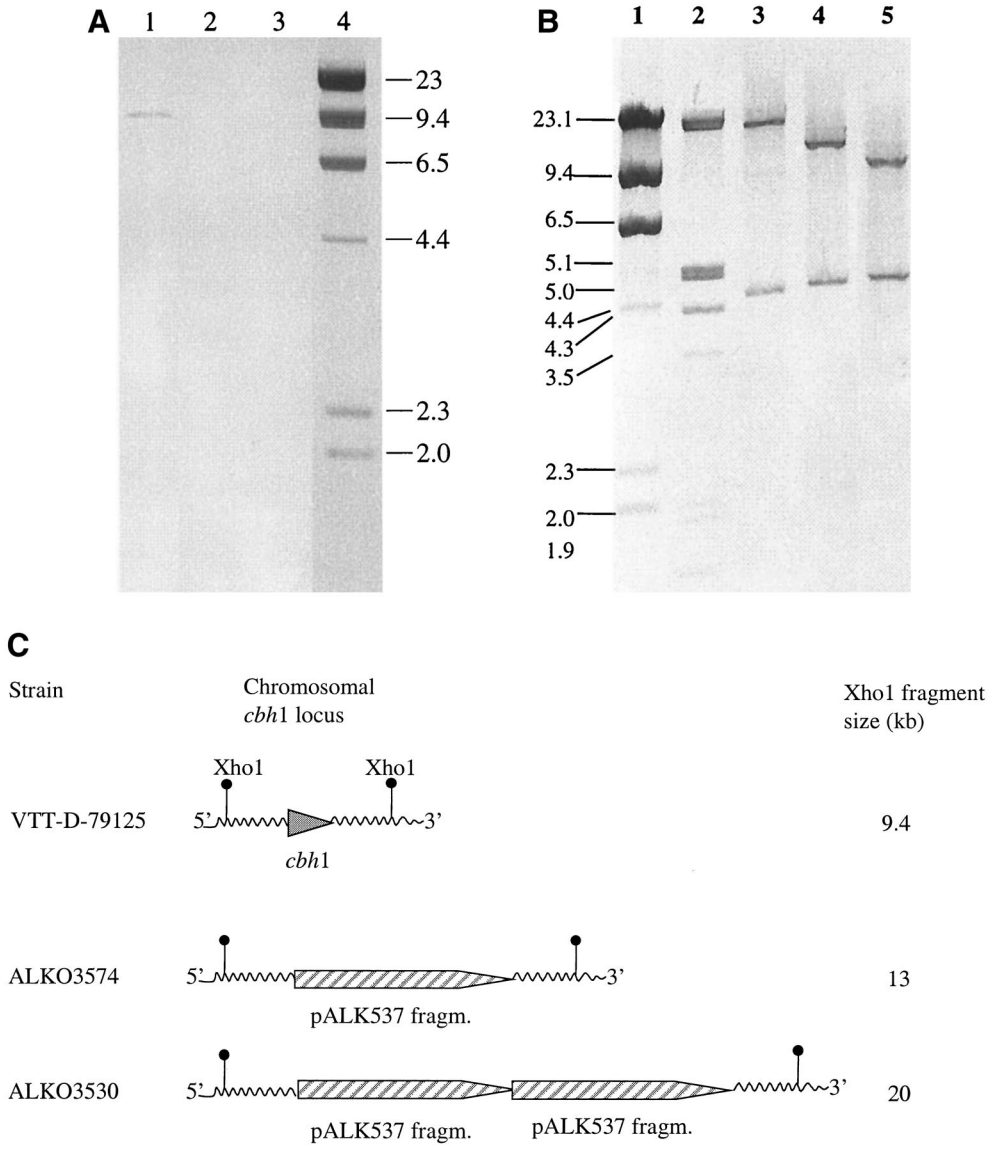

FIG. 3. Southern analysis of transformants ALKO3530 and ALKO3574 in the which *cbh1* locus has been replaced with the 9.2-kb *Not*I fragment from pALK537. (A) Genomic DNA was digested with *Xho*I. Hybridization was done with a *cbh1* probe. Lane 1, VTT-D-79125; lane 2, ALKO3530; lane 3, ALKO3574; lane 4, molecular weight marker λ*Hin*dIII. (B) Genomic DNA was digested with *Xho*I. Hybridization was performed with the 9.2-kb *Not*I fragment used for the transformations. Lanes 1 and 2, molecular weight markers λ*Hin*dIII and λ*Eco*RI-*Hin*dIII; lane 3, ALKO3530; lane 4, ALKO3574; lane 5, VTT-D-79125. (C) Schematic presentation of the organization of the *cbh1* chromosomal locus in the host strain and the transformants, showing the *Xho*I cleavage site.

hybridizing the chromosomal DNA digested with different restriction enzymes with *egl2* and *amdS* probes (data not shown).

Probing of the *Xho*I-digested genomic DNA of the CBHI-positive ALKO3529 strain with the *cbh1* probe resulted in one band of more than 20 kb while the host strain VTT-79125 gave a 9.4-kb band (Fig. 4A). Thus, the coding region of the *cbh1* gene is still present in the ALKO3529 strain. This result suggests that the pALK537 expression cassette has integrated at the *cbh1* locus or close to the *cbh1* locus, because the band recognized by the *cbh1* probe has increased in size. Chromosomal DNA of ALKO3529 was further digested with *Pvu*I and probed with *egl2*. Since the pALK537 fragment contains one *Pvu*I site (in the *amdS* gene), a tandem copy of the pALK537 fragment in the genome would generate a band of 9.2 kb and one band of unknown size. One copy of the pALK537 fragment would generate one band of unknown size. In Southern hybridization with *egl2*, ALKO3529 gave bands of about 7, 8, and 9 kb (Fig. 4B). The 6- to 7-kb band present in ALKO3529 and in VTT-D-79125 is from the wild-type *egl2* locus. Thus, it can be concluded that ALKO3529 contains two copies of the transformed vector fragment integrated into the *cbh1* locus or close to the *cbh1* locus. The results were confirmed by hybridizing the chromosomal DNA digested with different restriction enzymes with *amdS* and pALK537 fragment probes (data not shown).

(iii) Enzyme production of ALKO3529, ALKO3530 and

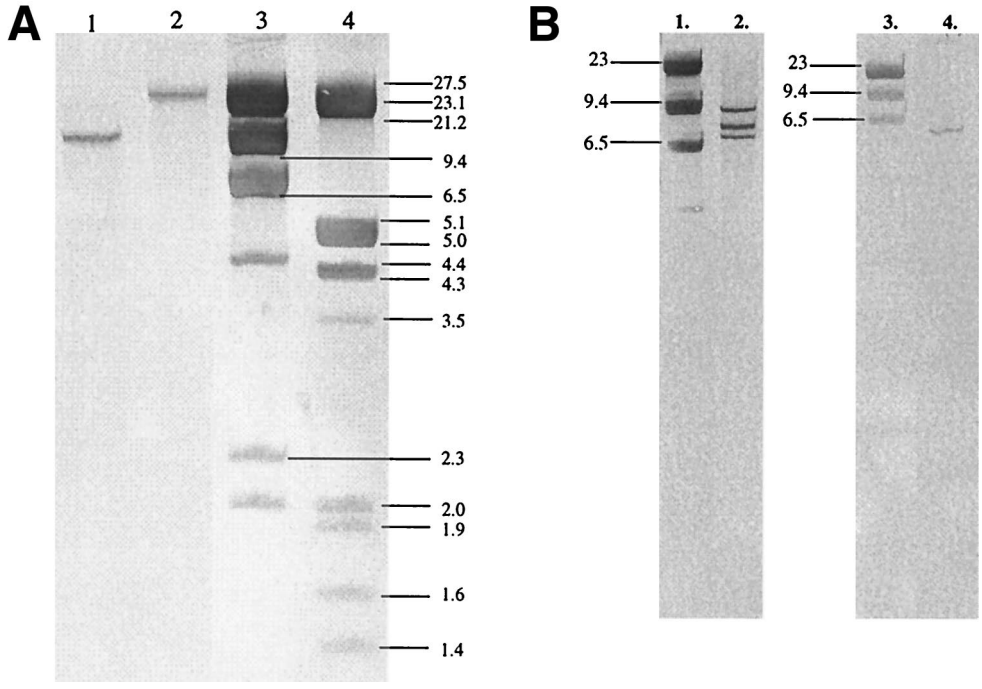

FIG. 4. Southern analysis of the transformant ALKO3529 and the host strain VTT-D-79125. (A) Genomic DNA was digested with *Xho*I. Hybridization was done with a *cbh1* probe. Lane 1, VTT-D-79125; lane 2, ALKO3529; lanes 3 and 4, molecular weight markers λ*Hin*dIII and λ*Eco*RI-*Hin*dIII. (B) Genomic DNA was digested with *Pvu*I. Hybridization was done with an *egl2* probe. Lane 1, molecular weight marker λ*Hin*dIII; lane 2, ALKO3529; lane 3, molecular weight marker λ*Hin*dIII; lane 4, VTT-D-79125.

ALKO3574 transformant strains. The EGII transformant strains ALKO3529, ALKO3530, and ALKO3574 as well as the parent strain VTT-D-79125 were grown in shake flasks on cellulase-inducing medium. For comparison, the CBHI-negative EGI-overproducing strains ALKO2698, ALKO2697, and ALKO2656 containing one, two, and three *egl1* expression cassettes, respectively (8) were also grown in the same cultivations. The results of the measurement of different cellulase activities and ELISA analyses from the culture medium are shown in Table 2.

The endoglucanase (activity against HEC) and β-glucanase activities produced by the studied EGII transformants correlate to the copy number of the *egl2* expression cassette. One copy of the *egl2* expression cassette increased the endoglucanase activity 2.3-fold (ALKO3574), and two cassettes (ALKO3529, ALKO3530) increased the activity about 3-fold compared to the VTT-D-79125 strain (Table 2). The β-glucan-hydrolyzing activity was 2.1 times higher in ALKO3574 and 2.5 to 2.7 times higher in ALKO3529 and ALKO3530 than in the VTT-D-79125 host strain. Higher increases in both endoglucanase and β-glucanase activities could be detected in the EGII-overproducing strains compared to EGI-overproducing strains. One additional copy of the *egl1* gene expressed under the *cbh1* promoter increased the endoglucanase activity by 1.9-fold (ALKO2698), and two copies increased it by 2.2-fold (ALKO2697) (Table 2). The same effect can be seen with β-glucanase: higher β-glucanase activity could be obtained with an additional copy of *egl2* (2.1 times) than with an additional copy of *egl1* (1.7 times).

The filter paper-hydrolyzing activity (FPU), which is mainly

TABLE 2. Production of cellulases by the host strain VTT-D-79125, transformants ALKO3529, ALKO3574, and ALKO3530, and *T. reesei* EGI-overproducing strains ALKO2697, ALKO2698, and ALKO2656[a]

Strain	*cbh1* Southern analysis result	*egl2* cassette copy no.	Secreted protein (mg/ml)	Endoglucanase (HEC) (nkat/ml)	β-glucanase (nkat/ml)	FPU/ml	β-glucosidase (nkat/ml)	CBHI (mg/ml)	CBHII (mg/ml)	EGI (mg/ml)
VTT-D-79125	+	0	9.2	1,200 ± 0	9,300 ± 500	5.3 ± 0.1	211 ± 10	3.7	0.051	0.361
ALKO3529	+	2	10.1	3,400 ± 100	23,000 ± 2,000	6.0 ± 0.7	239 ± 5	3.4	0.069	0.587
ALKO3530	−	2	8.7	3,600 ± 100	25,000 ± 1,000	2.1 ± 0.2	320 ± 5	NA[b]	0.085	0.590
ALKO3574	−	1	7.4	2,800 ± 50	19,400 ± 1,000	1.7 ± 0.2	281 ± 12	NA	NA	0.650
ALKO2697	−	0	9.2	2,600 ± 100	20,200 ± 500	1.8 ± 0.1	NA	NA	NA	NA
ALKO2698	−	0	7.6	2,300 ± 50	15,700 ± 500	1.6 ± 0	200 ± 5	NA	NA	NA
ALKO2656	−	0	9.1	2,700 ± 500	20,200 ± 3,000	1.4 ± 0.4	231 ± 5	NA	NA	NA

[a] Strains were grown for 7 days in cellulase-inducing medium; the results are the average from three flasks. The standard errors are shown.
[b] NA, not analyzed.

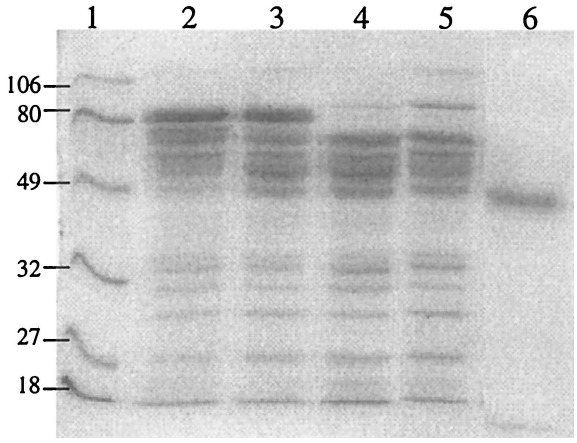

FIG. 5. SDS-PAGE of the samples from the culture supernatants of host strain VTT-D-79125 and the EGII transformants ALKO3529, ALKO3530, and ALKO3574. A total of 20 μg of total secreted protein was loaded in each lane. Lane 1, VTT-D-79125; lane 2, ALKO3529; lane 3, ALKO3530; lane 4, ALKO3574; lane 5, 3 μg of purified EGII protein.

affected by cellobiohydrolases, was decreased 60% in ALKO3530 (two *egl2* expression cassettes) and 67% in ALKO3574 (one *egl2* cassette), which lack the *cbh1* gene. FPU activity of the CBHI-positive ALKO3529 strain was about 10% higher than in the VTT-D-79125 parent strain.

Production of β-glucosidase activity was not significantly changed in transformants ALKO3529 and ALKO3574 compared to the VTT-D-79125 parent strain. In ALKO3530 production of β-glucosidase was 1.5-fold higher than in the host strain.

The amount of the secreted EGII protein was roughly evaluated by eye in several SDS-PAGE analyses with a known concentration of purified EGII protein as a standard (Fig. 5). Production of EGII in ALKO3529 and ALKO3530 was about 1.3 mg/ml, in ALKO3574 it was about 0.8 mg/ml, and in VTT-D-79125 it was about 0.4 mg/ml. Thus, one *egl2* expression cassette increases the amount of EGII protein by 2-fold and two cassettes increases it up to about 3.2-fold.

The amounts of CBHI, CBHII, and EGI were analyzed by ELISA from the same culture supernatants from which enzyme activities were analyzed (Table 2). The amount of secreted CBHI was almost the same in the CBHI-positive EGII overproducer ALKO3529 (3.4 mg/ml) as in the VTT-D-79125 parental strain (3.7 mg/ml). Surprisingly, there was an increase in the production of EGI: 1.6-fold by the ALKO3529 and ALKO3530 strains and 1.8-fold by the ALKO3574 strain. The production of CBHII was enhanced in the ALKO3529 and ALKO3530 strains. The lack of *cbh1* seemed to increase the amount of CBHII more, resulting in 1.7-fold more in ALKO3530 and 1.3-fold more in ALKO3529.

EGI and -II overproduction without CBHI and -II in *T. reesei*. (i) Transformation of *T. reesei* and replacement of *cbh2*. The plasmid pALK540 (Fig. 2) was constructed as described in Materials and Methods for replacement of the *cbh2* locus of *T. reesei* ALKO2698 (EGI overproducer, CBHI negative) with the *egl2* expression cassette.

For construction of the strain overproducing EGI and EGII without CBHI and CBHII, the 11.6-kb *Cla*I-*Pvu*I linear fragment of pALK540 containing the *egl2* expression cassette was released from the vector backbone and transformed to *T. reesei* strain ALKO2698 (Fig. 2). The transformation frequency varied from 9 to 42 transformants per μg of DNA. The purified transformants were grown on microtiter plates for detection of the CBHII protein by Western blotting and immunostaining. Twenty-two out of 31 tested transformants were CBHII negative, suggesting that the frequency for targeting of the expression cassette into the *cbh2* locus was 71%. The CBHII-negative transformants were grown in shake flask cultivations on cellulase-inducing medium to measure the endoglucanase activity in the culture medium. Strain ALKO3528 produced the highest endoglucanase activity.

(ii) DNA analysis of strain ALKO3528. The absence of the chromosomal *cbh2* gene from strain ALKO3528 was shown by Southern blot analysis. No hybridization to the chromosomal DNA of ALKO3528 was obtained when probed with the coding region of the *cbh2* gene (Fig. 6A). The 2.4- and 2.0-kb bands present in *Pst*I-digested chromosomal DNA of the ALKO2698 host strain are from the wild-type *cbh2* locus.

The integration of the expression cassette in ALKO3528 was analyzed further by hybridization of the genomic DNA digested with appropriate restriction enzymes to *egl2* and phleomycin probes as well as to the 11.6-kb transformation fragment of the plasmid pALK540. The hybridization patterns with the *egl2* probe are shown in Fig. 6B and C. Probing of the *Bgl*II-digested genomic DNA of strain ALKO3528 resulted in two bands of 6.6 and about 15 kb. The 6.6-kb band present in *Bgl*II-digested chromosomal DNA of the ALKO3528 transformant and ALKO2698 host strain is from the wild-type *egl2* locus. The 15-kb band indicates that one copy of a transforming vector had integrated to the genome of strain ALKO3528. Hybridization of the *Xba*I-*Sma*I-digested chromosomal DNA of ALKO3528 with the *egl2* probe gave bands of 9.2 and 3.1 kb. The 9.2-kb band is from the wild-type *egl2* locus and this hybridization was seen also to ALKO2698 DNA. Hybridization of the *egl2* gene to the 3.1-kb band is an indication of an intact *cbh1* promoter-*egl*2 fusion. Thus, strain ALKO3528 contains one full-length copy of the *egl2* expression cassette in the *cbh2* locus.

(iii) Enzyme production of the ALKO3528 transformant strain and the host strains. EG-overproducing strain ALKO3528 (CBHI and -II negative) and the parent strains ALKO2698 (EGI overproducer) and VTT-D-79125 (parent for ALKO2698) were grown on cellulase-inducing medium for measurement of cellulase activities (Table 3). The endoglucanase activity (measured against HEC) was increased about twofold in strain ALKO3528 above that in the parent strain ALKO2698 and by fourfold above that in VTT-D-79125. The production of β-glucanase activity in ALKO3528 was increased 1.8-fold above that in strain ALKO2698. The filter paper-hydrolyzing activity of strain ALKO3528 was lowered to almost zero because of the lack of the CBHI and CBHII proteins.

Use of endoglucanase preparations in biostoning. Cellulase preparations derived from the endoglucanase-overproducing *T. reesei* strains ALKO3529 and ALKO3528 were used to impart a stonewashed appearance to denims. The parent strain VTT-D-79125 and the EGI-overproducing strain ALKO2656

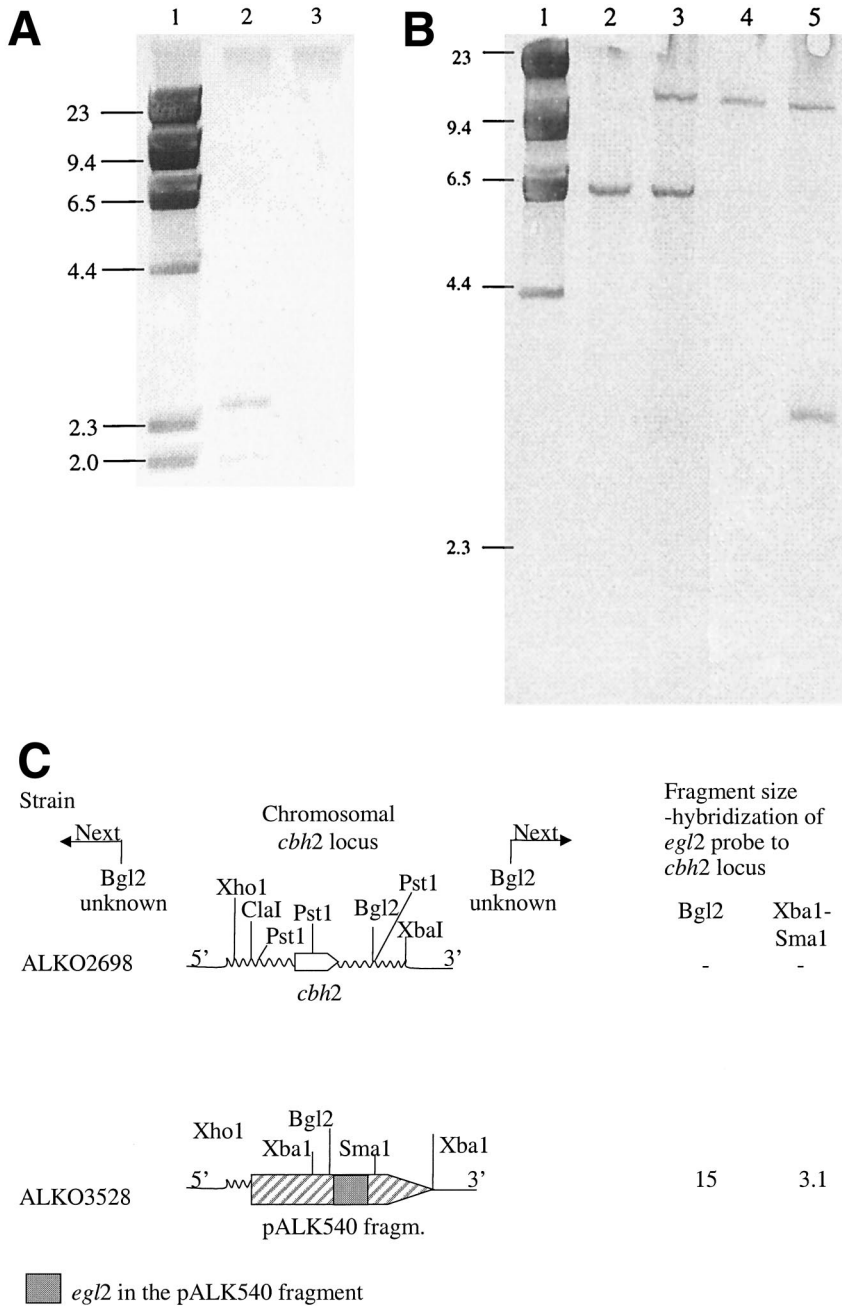

FIG. 6. Southern analysis of the transformant ALKO3528 and the host strain ALKO2698. (A) Genomic DNA was digested with *Pst*I. Hybridization was done with a *cbh2* probe. Lane 1, molecular weight marker λ*Hin*dIII; lane 2, ALKO2698; lane 3, ALKO3528. (B) Genomic DNA was digested with *Bgl*II or *Xba*I-*Sma*I. Hybridization was done with an *egl2* probe. Lane 1, molecular weight marker λ*Hin*dIII; lane 2, ALKO2698 digested with *Bgl*II; lane 3, ALKO3528 digested with *Bgl*II; lane 4, ALKO2698 digested with *Xba*I-*Sma*I; lane 5, ALKO3528 digested with *Xba*I-*Sma*I. (C) Schematic presentation of the organization of the *cbh2* locus in the host strain and the ALKO3528 transformant strain showing different cleavage sites and the fragment sizes from the *cbh2* locus when probing with the *egl2* probe.

were used as controls. Results from the color measurements are shown in Table 4.

Results show that after 1 h of treatment with a 3-mg/g dosage, the stonewashing effects (measured in lightness units) with the EGII-overproducing strain ALKO3529 and the EG-overproducing strain ALKO3528 were almost equal with that of VTT-D-79125. No clear increase in lightness units was obtained with the EGI-overproducing strain ALKO2656. With a 6-mg/g dosage, after 1 h of treatment ALKO3529 showed the highest increase in lightness units, compared to VTT-D-79125 or ALKO3528. After 2 h of treatment with the 3-mg/g dosage, the best stonewashing effect (measured as lightness) was obtained with the ALKO3529 preparation. ALKO3528 was slightly better than ALKO2656. A considerably higher dosage

TABLE 3. Production of cellulases by the host strains VTT-D-79125 and ALKO2698 and by the transformant ALKO3528

Strain	Secreted protein (mg/ml)	Endoglucanase (HEC) (nkat/ml)	β-glucanase (nkat/ml)	FPU/ml	Southern analysis	
					cbh1	*cbh2*
VTT-D-79125	9.0	1,300 ± 50	11,500 ± 1,000	4.8 ± 0.8	+	+
ALKO2698	7.9	2,600 ± 100	15,300 ± 1,000	1.4 ± 0.1	−	+
ALKO3528	8.5	5,100 ± 400	27,800 ± 2,000	0.2 ± 0.1	−	−

of cellulases from VTT-D-79125 was needed to achieve a comparable stonewashing effect as with the cellulases of the endoglucanase-producing strains.

DISCUSSION

The production of endoglucanase enzymes has been improved in the biotechnically important filamentous fungus *T. reesei*. By using the strong *Trichoderma cbh1* promoter and by adding copy numbers of *egl2*, high EGII activity-producing strains were obtained. One additional copy of the *egl2* gene expressed under the *cbh1* promoter in the *cbh1* locus increased the endoglucanase activity (activity against HEC) by 2.3-fold, while one additional copy of the *egl1* gene expressed under the *cbh1* promoter in the *cbh1* locus increased the endoglucanase activity by 1.9-fold above that of the parent strain. The same effect could be observed by increasing the copy numbers further: two copies of *egl2* increased the endoglucanase activity by 3-fold, while two copies of *egl1* increased the endoglucanase activity by 2.2-fold. Thus, it can be concluded that EGII has a major impact on the endoglucanase activity measured as activity against HEC. This is in agreement with the results obtained with cellulase-deletion strains of *T. reesei* (21). This is also consistent with the specific activities on HEC and on β-glucan: the specific activities of EGII are higher than those of EGI (22). The integration place of the expression cassette had no effect on the endoglucanase activity levels of the two-copy transformants: in ALKO3530 the expression cassettes had replaced the *cbh1* locus and in ALKO3529 they had integrated close to the *cbh1* locus. EGII also had an effect on the filter paper activity, increasing it by about 10% in the EGII transformant (*cbh1* gene present) above that of the parent strain.

By replacing the *cbh1* locus with one copy of the *egl1* gene under the *cbh1* promoter (8) and by replacing the *cbh2* locus with one copy of the *egl2* gene under the *cbh1* promoter, we have been able to construct a *T. reesei* strain that produces high amounts of pure EGI and -II without any contamination by CBHI or -II. In ALKO3528, the production of endoglucanase activity was increased fourfold above that of the VTT-D-79125 parent strain.

Cellulase preparations derived from the high EGII activity-producing strain ALKO3529 proved to improve the stonewashing effect above that of its parent strain VTT-D-79125 when the same enzyme dosage was used. The same stonewashing effect could be obtained with a considerably lower enzyme dosage when using the EGII cellulase preparation derived from the EGII-overproducing strain than when using the parental strain. Heikinheimo et al. (7) have shown that purified EGII is the most effective of the main cellulases at removing color from denim fabric. Thus, by increasing the relative amount of EGII in the cellulase mixture, an improved stonewashing effect can be obtained. Cellulase enzymes are used in the textile industry for biostoning and also for finishing of cellulosic fibers. Cellulase preparations produced by strains ALKO3529 and ALKO3530 have been tested in cotton finishing (15). The cellulase mixture obtained with the EGII-overproducing strain ALKO3529 proved to reduce pilling with low strength and weight losses on cotton knit fabric. The cellulase preparation of strain ALKO3530 resulted in improved depilling, but at the same time caused relatively high weight loss.

β-Glucanase is an important activity in the degradation of β-glucan in feed. The β-glucanase activity was improved in the EG-overproducing strains. In addition to textile applications, these new preparations can possibly be used for more economical production of β-glucanase for modification of feed.

TABLE 4. Color measurements of denim fabrics treated with VTT-D-79125, ALKO2656, ALKO3529, and ALKO3528 cellulase preparations[a]

Preparation and strain	Dosage (mg of total protein/g of fabric)	*L*, right side of the swatch
1 h		
ALKO3529	3	1.0
	6	2.1
ALKO3528	3	1.3
	6	1.5
VTT-D-79125	3	1.5
	6	1.5
ALKO2656	3	0.1
2 h		
ALKO3529	3	3.2
ALKO3528	3	2.7
VTT-D-79125	3	1.8
	6	2.4
ALKO2656	3	2.5

[a] The fabrics were treated for 1 and 2 h with doses of 3 and 6 mg of total protein, as explained in Materials and Methods. The results are the average of two parallel treatments. *L*, lightness unit of the fabric after treatment minus lightness unit of the fabric before the treatment.

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APPENDIX II

Enhanced production of cellobiohydrolases in *Trichoderma reesei* and biofinishing of cotton fabric with the new preparations

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Abstract

In the search for suitable cellulase combinations for industrial biofinishing of cotton, five different types of *Trichoderma reesei* strains were constructed for elevated cellobiohydrolase production: CBHI overproducers with and without endoglucanase I (EGI), CBHII overproducers with and without endoglucanase II (EGII) and strains overproducing both CBHI and CBHII without the major endoglucanases I and II. One additional copy of *cbh1* gene increased production of CBHI protein 1.3-fold, and two copies 1.5-fold according to ELISA (enzyme-linked immunosorbent assay). The level of total secreted proteins was increased in CBHI transformants as compared to the host strain. One copy of the *cbh2* expression cassette in which the *cbh2* was expressed from the *cbh1* promoter increased production of CBHII protein 3–4-fold when compared to the host

strain. *T. reesei* strains producing elevated amounts of both CBHI and CBHII without EGI and EGII were constructed by replacing the *egl1* locus with the coding region of the *cbh1* gene and the *egl2* locus with the coding region of *cbh2*. The *cbh1* was expressed from its own promoter and the *cbh2* gene using either the *cbh1* or *cbh2* promoter. Production of CBHI by the CBH-transformants was increased up to 1.6-fold and production of CBHII up to 3.4-fold as compared with the host strain. Approximately similar amounts of CBHII protein were produced by using *cbh1* or *cbh2* promoters. When the enzyme preparation with elevated CBHII content was used in biofinishing of cotton, better depilling and visual appearance were achieved than with the wild type preparation; however the improvement was not as pronounced as with preparations with elevated levels of endoglucanases.

Key words: cellobiohydrolase, *T. reesei*, overproduction, biofinishing

1. Introduction

Trichoderma reesei is a biotechnically important filamentous fungus known to produce high amounts of cellulases. *T. reesei* produces at least nine different endoglucanases (EG) and two different cellobiohydrolases (CBH) (Shoemaker *et al.*, 1983, Teeri *et al.*, 1987, Penttilä *et al.*, 1986, Saloheimo *et al.*, 1988, 1994, 1997, Ward *et al.*, 1993, Bower *et al.*, 1998, Foreman *et al.*, 2003). In cellulase-inducing conditions the proportion of cellobiohydrolases of the extracellular proteins of *T. reesei* is 80–85% (Gritzali and Brown, 1979). The major cellobiohydrolase component of *T. reesei* is CBHI, which accounts for 50–60% of the total secreted protein. It is encoded by a single gene, and thus the promoter of the cellobiohydrolase 1 (*cbh1*) gene is regarded as a strong promoter. The high production capacity of *T. reesei* as well as the strong *cbh1* promoter have been exploited for expressing various homologous and heterologous proteins for industrial applications (reviewed in Mäntylä *et al*., 1998 and in Penttilä, 1998).

Cellulases act synergistically in the hydrolysis of crystalline cellulose (reviewed by Teeri, 1997). The endoglucanases hydrolyse the cellulose chains internally, providing new chain ends for cellobiohydrolases. The cellobiohydrolases hydrolyse the cellulose processively from the ends to create mainly cellobiose.

CBHII splits cellobiose from the non-reducing and CBHI from the reducing ends of cellulose chains. The complementary activities of endoglucanases and cellobiohydrolases lead to synergy, i.e. enhancement of activity over the added activities of the individual enzymes.

Cellulases of *T. reesei* are widely used in several industrial applications, for example in the food and feed (Galante *et al*., 1998a) and pulp and paper industries (Buchert *et al.*, 1998) as well as in the textile industry in biostoning and biofinishing applications (Galante *et al*., 1998b). In biofinishing, individual loose fibre ends that protrude from the textile surface are removed from cellulose-containing textile materials, such as cotton fabrics. The key benefits are permanent improvement of depilling, cleared surface structure by reduced fuzz, improved textile handling (softness), brighter colours of the textile and improved dimensional stability and moisture absorbability. Endoglucanases are known to be important in obtaining biofinishing effects of cotton materials (Kumar *et al.*, 1997, Liu *et al.*, 2000), and e.g. according to Miettinen-Oinonen *et al.* (2001) EGII-based cellulase products gave the most positive depilling result. On the other hand strength and weight losses of the fabric have been problems associated with cellulase and especially endoglucanase treatments (Heikinheimo and Buchert, 2001). It has been proposed that cellobiohydrolases may have applications in specific fiber modifications and various surface treatments of paper and textiles, since unlike endoglucanases, they do not significantly affect fiber strength (reviewed by Teeri, 1997). No improvement in pilling resistance of cotton fabric was obtained by single purified *T. reesei* CBHI or CBHII (Heikinheimo and Buchert, 2001).

The aim of this study was to search for *Trichoderma* cellulase combinations that could be used for industrial biofinishing of cotton with minimal weight and strength losses. In the present work various *T. reesei* strains producing elevated amounts of either CBHI or CBHII enzymes were constructed by increasing the copy number of the respective gene and by expressing the cellobiohydrolase 2 (*cbh2*) gene from the *cbh1* promoter. We also improved the production of both CBHI and CBHII enzymes in one strain. The cellulase production profiles of the strains were analysed and the utility of the preparations derived from the new strains was tested in biofinishing of cotton. The results obtained in the application test were compared to those obtained with preparations produced by previously constructed *T. reesei* EG-overproducing strains.

2. Materials and methods

2.1. Microbial strains and plasmids

Escherichia coli strain XL1-Blue (Stratagene, USA) was used for propagation of plasmids. *T. reesei* strain ALKO2221 was used as a recipient for transformations. ALKO2221 is a low-protease mutant strain (Mäntylä *et al.*, 1994) of the high cellulase-producing mutant strain VTT-D-79125 (Bailey and Nevalainen, 1981). *T. reesei* ALKO3529 and ALKO3528 (Miettinen-Oinonen and Suominen, 2002) were used as controls in biofinishing experiments. ALKO3529 has two additional copies of the *T. reesei* endoglucanase 2 gene and produces elevated levels of EGII. ALKO3528 is an EGI- and EGII-overproducing strain from which the *cbh1* and *cbh2* genes have been deleted. Plasmids were constructed using pUC19 (EMBL Database Accession No L09137) as a vector backbone. The plasmid pALK496 contains a *T. reesei cbh1* promoter region of 2.2 kb, a 1.6 kb *cbh1* gene and a *cbh1* terminator region of 0.7 kb (Fig. 1). A 1.8 kb *Sca*I-*Stu*I *egl1* 5' fragment was used together with a 1.6 kb *Bam*HI-*Xho*I *egl1* 3' fragment to target the expression cassette to the *egl1* locus by homologous recombination. A 3.1 kb *Spe*I-*Xba*I fragment containing the *Aspergillus nidulans* acetamidase gene from the plasmid p3SR2 (Kelly and Hynes, 1985) was used as a marker. The plasmid pALK543 contains a 4.7 kb *Sph*I-*Sac*II *cbh*2 fragment of *T. reesei* containing a 2.5 kb *cbh2* promoter, a 1.5 kb *cbh2* gene and a 0.7 kb *cbh2* terminator region (Fig. 1). The plasmid pALK546 contains a *T. reesei* 2.2 kb *cbh1* promoter, a 2.2 kb *cbh2* gene (coding region of 1.5kb) and a 0.7 kb *Ava*II fragment of the *cbh1* terminator region (Fig. 1). The precise fusion between the *cbh1* promoter and *cbh2* signal sequence was synthetized by PCR. In both pALK543 and pALK546 an *egl2* 5' flanking region (1.4 kb *Xho*I-*Sac*I) was used together with an *egl2* 3' flanking region (1.6 kb *Avr*II-*Sma*I) to target the expression cassette to the *egl2* locus. Resistance to phleomycin was used for selecting the transformants. A 3.3 kb *Xba*I-*Bgl*II fragment containing the *Streptoalloteichus hindustanus* phleomycin gene (*ble*) from the plasmid pAN8-1 (Mattern *et al.*, 1987) was used in pALK543 and pALK546. The expression cassettes, an 11.6 kb *Not*I fragment from pALK496, an 11.2 kb *Bam*HI fragment from pALK543 and an 11.8 kb *Eco*RI-*Bam*HI fragment from pALK546 were isolated from the vector backbones for *T. reesei* transformations.

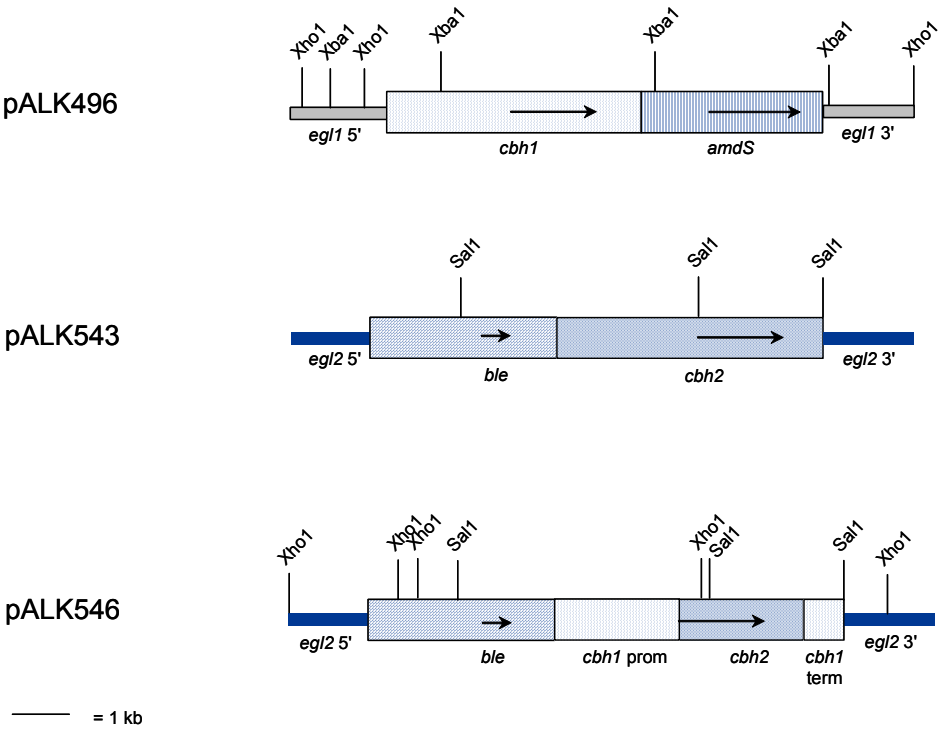

Figure 1. Schematic presentation of the expression cassettes isolated from the plasmids pALK496, pALK543 and pALK546 used for transformations of T. reesei ALKO2221 and ALKO3761 for elevated production of T. reesei CBHI and CBHII. Only the relevant restriction sites are shown. The arrows show the coding regions of genes.

2.2. Growth of organisms and transformation of Trichoderma

E. coli strains were grown at 37°C overnight in L-broth (Sambrook *et al.,* 1989) supplemented with 50 µg/ml ampicillin. Potato dextrose agar slants (PD, Difco, USA) were used for growing the *Trichoderma* strains. Transformation of *T. reesei* was carried out using protoplasts as described by Penttilä *et al.* (1987) with the modifications described in Karhunen *et al.* (1993). The plates and media for *Trichoderma* transformations with acetamide selection were as in Penttilä *et al.* (1987). The acetamidase transformants were purified on selective acetamide-CsCl medium through single spores before transferring to PD slants. In transformations in which phleomycin was used as a selection marker, aliquots

of the transformed protoplasts were plated onto the surface of MnR plates (per litre 2.5 g glucose, 2.5 g yeast extract, 0.3 g K-phtalate and 15 g agar, pH 7.0) osmotically stabilized with 0.44 M sucrose and incubated for 6 h at 30°C, prior to the addition of 5 ml of molten MnR (0.6% agar) as an overlay containing 300 μg/ml of phleomycin (Cayla, France). The transformants were purified on selective MnR-medium supplemented with 50 μg/ml of phleomycin through single spores and grown on MnR-slants containing 50 μg/ml phleomycin for three generations prior to transfer to PD slants. A lactose-based complex medium was used for liquid cultivations (Suominen *et al.*, 1993). Cultivations were carried out at 30°C and 250 rpm for seven days. Mycelia for isolation of the chromosomal DNA were grown in shake flasks for two days (30°C, 250 rpm) on *Trichoderma* minimal medium (Penttilä *et al.*, 1987) supplemented with 0.2% proteose peptone.

2.3. DNA techniques

DNA manipulations were carried out by standard techniques (Sambrook *et al.,*1989). The PCR was carried out using a Techne thermal cycler PHC-2 (Techne Ltd., UK). Sequencing of the fusion between the *cbh1* promoter and *cbh2* gene was carried out by means of internal primers using a *Taq* DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems, USA) and an automated sequencer (model 373A; Applied Biosystems). Plasmid DNA from *E. coli* was isolated using Qiagen columns (Qiagen GmbH, Germany). DNA fragments for cloning were isolated from agarose gels by Qiagen's QIAEX gel extraction protocol (Qiagen GmbH). DNA fragments for transformations were isolated from low-melting-point agarose gels (FMC Bioproducts, USA) by the freeze-thaw phenol method (Benson, 1984). DNA was isolated from *T. reesei* using the method of Raeder and Broda (1985). For Southern blot analysis the DNA was transferred from agarose gels to nylon membranes using a VacuGene™ XL apparatus (Pharmacia). The labeling of the probes with digoxigenin and hybridization of the filters were performed according to the procedures of Roche.

2.4. Enzyme activity and protein assays

The cellulase activities were measured from the culture supernatants. Cellobiohydrolase I activity was measured as activity against 4-methylumbelliferyl-β-D-lactoside (MUL) according to van Tilbeurgh *et al.* (1988). The

total MUL activity represents activities of EGI and CBHI. The MUL (CBHI) activity was measured by inhibiting the CBHI in the presence of 5 mM cellobiose and by subtracting the MUL (EGI) activity thus obtained from the total MUL activity. Filter paper hydrolyzing activity (FPU) was measured according to Mandels *et al.* (1976). Endoglucanase activity was measured as the release of reducing sugars from hydroxyethylcellulose (HEC, Fluka; Bailey and Nevalainen, 1981). The resulting reducing sugars were assayed spectrophotometrically using a 2,4-dinitrosalicylic acid reagent. Protein concentrations were determined from the *T. reesei* culture media by the method of Lowry *et al.* (1951) after TCA precipitation. In the ELISA method the concentration of enzyme protein was determined with a protein assay kit (Bio-Rad, Richmond, Calif., USA; Bühler, 1991).

2.5. SDS-PAGE and immunological methods

The presence of the EGI and CBHII proteins was detected from Western blot filters (Towbin *et al.*, 1979) immunostained using a monoclonal EGI antibody EI-2 and CBHII antibodies CII-8 and CII-30 (Aho *et al.,* 1991) and the ProtoBlot Western Blot AP system (Promega, USA). Quantitation of secreted EGI, CBHI and CBHII was carried out by a double antibody sandwich ELISA (enzyme-linked immunosorbent assay, Bühler, 1991) using the monoclonal antibodies EI-2, CI-258 and CII-8 (Aho *et al.*, 1991) as capture antibodies.

2.6. Biofinishing of cotton

100% cotton woven fabric (Pirkanmaan Uusi Värjäämö Oy, Finland) was treated with cellulase preparations of VTT-D-79125, ALKO3529, ALKO3528, ALKO3760, ALKO3798 and ALKO4097 strains. The fabric was desized for 10 min at 60^{o}C with Ecostone A 200 (1ml/l, AB Enzymes Oy, Finland). 20 g of fabric was treated in an LP-2 Launder-Ometer (Atlas, USA) in 300 ml of 0.05 M Na-citrate buffer pH 5 at 50^{o}C for 1h. 2 and 6 mg of protein in the cellulase preparations per gram of fabric were used in each experiment. After rinsing with alkali and water, the fabrics were dried in a tumble drier. The effects of the cellulase treatments were evaluated by visual appearance and pilling and by measuring weight loss of the treated fabrics. Visual appearance was evaluated by a panel consisting of five persons. The fabrics were ranked on a score from 1 to 5, with a score of 5 representing a clean surface with no fuzz or pills and a clear

fabric texture. The Martindale Rubbing method (SFS-4328) was used for evaluation of pilling. Pilling was evaluated on a score from 1 to 5, in comparison to standard fabrics, by a panel after 200 cycles of abrasion (1 = many pills, 5 = no pills). Weight loss was defined as percentage from weight of the fabric before and after conditioning in an atmosphere of 21+/-2^{o}C and 50+/-%RH.

3. Results

3.1. CBHI overproduction

3.1.1. Construction of strains

The plasmid pALK496 (Fig. 1) was constructed and transformed to *T. reesei* ALKO2221 to increase the copy number of the *cbh1* gene. The purified transformants were cultivated in shake flasks on cellulase-inducing medium and the enzyme activities were measured from the culture supernatants. *T. reesei* CBHI-overproducing transformants were screened by measuring the increased MUL (CBHI) activity. The presence or absence of EGI protein was detected by Western blotting from the transformants. 10% of the transformants were EGI-negative, indicating replacement of *egl1* gene by the expression cassette. The transformants producing the highest activity against MUL, ALKO3760 and ALKO3862 (EGI-positive) and ALKO3761 (EGI-negative), were analyzed by Southern blotting to evaluate the copy number and integration site of the expression cassette in the genome. The genomic DNAs were digested with several restriction enzymes and probed with fragments containing *egl1*, *amdS* and the pALK496 expression cassette. ALKO3761 showed no hybridization when the *egl1* probe was used, showing deletion of the *egl1* gene (Fig. 2a). The expected 3.7 kb *Hind*III fragment was obtained from the host strain ALKO2221 and the EGI-positive strains ALKO3760 and ALKO3862, showing that the native *egl1* locus was intact. According to the hybridization patterns obtained the ALKO3761 strain contains one full-length copy of the *cbh1* expression cassette replacing the *egl1* gene (Fig. 2b). ALKO3862 contains two tandem copies of the *cbh1* expression cassette and ALKO3760 two tandem copies (the second copy has an incomplete pALK496 fragment) of the *cbh1* expression cassette. The integration site of the *cbh1* expression cassette in ALKO3760 and ALKO3862 is unknown, but it is not in the *egl1* or *cbh1* loci, because bands deriving from the native *egl1* and *cbh1* loci were detected in the hybridizations (Fig. 2).

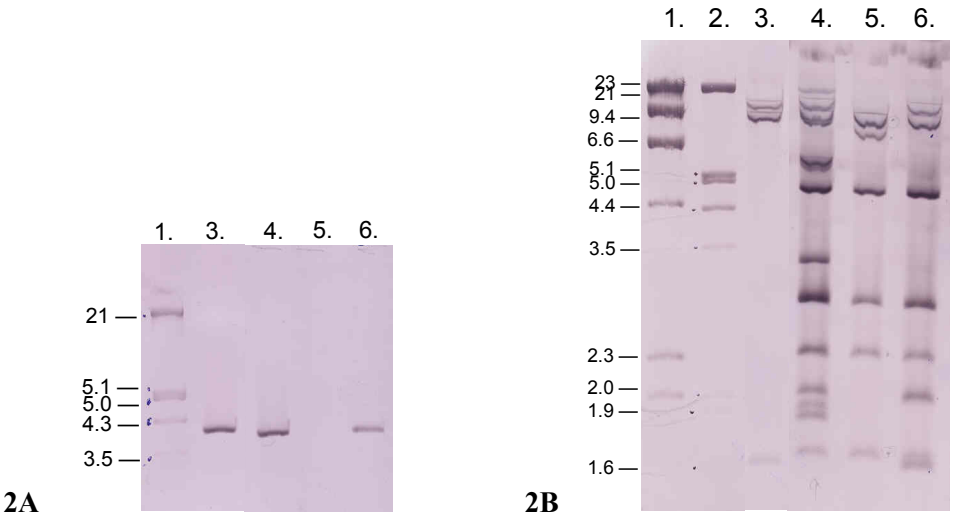

Figure 2. Southern analysis of the genomes of the transformants ALKO3760, ALKO3761, ALKO3862 and the host strain ALKO2221. Genomic DNAs were digested with HindIII (2A) and XbaI (2B) and hybridized with an egl1 probe (2a) and with a 10.3 kb XhoI fragment of pALK496 (2b). Lanes 1 and 2: Molecular weight markers, Lane 3: ALKO2221, Lane 4: ALKO3760, Lane 5: ALKO3761, Lane 6: ALKO3862.

The 3.7 kb HindIII fragment in 2A derives from the native egl1 locus. The about 12 kb band (2B) is from the native egl1 locus (ALKO2221, ALKO3760, ALKO3862) and bands of 9 kb and 1.7 kb are from the native cbh1 locus (all the strains). The 4.5 kb, the about 3 kb and the 2.3 kb bands detectable in all the transformants show the presence of the cbh1 expression cassette, the amdS fragment and the egl1 5' fragment of pALK496. Replacement of the egl1 locus by the expression cassette results in a 7.0 kb fragment from the egl1 3' side of pALK496 as in ALKO3761. The intensity of the 4.5 kb band in ALKO3862 and ALKO3760 is stronger than that of the corresponding 4.5 kb band of ALKO3761, suggesting a higher copy number of the expression cassette. The band of 2.0 kb in ALKO3862 and in ALKO3760 confirms the presence of two tandem copies of the pALK496 fragment. The 1.6 kb fragment in ALKO3862 is from the egl3' end of pALK496. In ALKO3760 the bands of 6, 3.3, 2.1 and 1.9 kb derive from an incomplete insertion of the fragment transformed.

3.1.2. Enzyme production by the CBHI-transformants

The amount of CBHI was increased 1.5-fold in ALKO3760, 1.4-fold in ALKO3862 and 1.3-fold in ALKO3761 as compared to the host strain ALKO2221, resulting in 1.1–1.4-fold increases in MUL (CBHI) activity (Table 1). In addition, changes in the amounts of CBHII and EGI were observed. The amount of CBHII was reduced by 25% in ALKO3760 and ALKO3862, but in the EGI-negative transformant ALKO3761 the amount of CBHII was increased by 15%. Surprisingly, the amount of EGI was decreased by 30% and 40% in ALKO3760 and ALKO3862, although the *egl1* gene appeared to be intact in the genomes of the transformants. The level of the secreted proteins produced by all the transformants was increased compared to the host strain (Table 1). The filter paper-hydrolyzing activity (FPU) was not significantly changed in the transformants ALKO3760 and ALKO3862. The activity towards filter paper in ALKO3761, which lacks the *egl1* gene, was about 15% lower compared to the host strain. Although FPU is mainly affected by cellobiohydrolases it measures total hydrolysis of cellulose, and thus the lack of EGI in the preparation was expected to decrease the FPU activity. In the strain ALKO3761 HEC activity was about 50% lower as compared to the host strain. Endoglucanase activity was also decreased in the ALKO3760 and ALKO3862 transformants by 25 and 30% which is at least partly explained by the lowered amount of EGI as shown by ELISA.

Table 1. Production of cellulases by the host strain ALKO2221 and by the transformants ALKO3760, ALKO3862 and ALKO3761.

Strain	*egl1* Southern analysis	*cbh1* cassette copy no.	Secreted protein mg/ml	MUL (CBHI)x ALKO 2221*	FPU/ ml	Endo-glucanase (HEC) nkat/ml	ELISA CBHI mg/ml	CBHII mg/ml	EGI mg/ml
ALKO2221	+	0	6.8	1	3.6±0.3	700	2.2±0.1	0.18±0.01	0.32±0.01
ALKO3760	+	2	7.6	1.4	3.5±0.3	530	3.4±0.2	0.13±0	0.22±0
ALKO3862	+	2	7.6	1.3	3.4±0.1	490	3.1±0.1	0.13±0.01	0.17±0.01
ALKO3761	-	1	7.2	1.1	3.0±0	340	3.0±0.2	0.22±0.04	0.00

* ALKO2221 produced about 0.3 MUL(CBHI)/ml. The MUL(CBHI) activity of ALKO2221 was adjusted to 1 and the activities produced by the transformants are presented compared to the host strain. Strains were grown for seven days in cellulase-inducing medium; the results are averages from three replicate flasks.

3.2. CBHII overproduction

3.2.1. Construction of strains

The expression cassette of pALK546 (Fig. 1) was transformed to *T. reesei* ALKO2221 for expressing *cbh2* from the *cbh1* promoter. The transformants were screened for increased level of CBHII by using the ELISA method. The transformant strains ALKO3873, ALKO3798 and ALKO3799 producing increased levels of CBHII protein were analyzed by Southern blots to evaluate the copy number of the *cbh2* expression cassette and the presence or absence of the *egl2* gene. Genomic DNAs isolated from the transformants were digested with several restriction enzymes and probed with fragments containing *egl2*, *cbh2, ble* and the pALK546 expression cassette. Probing of *Xho*I-digested genomic DNAs with the *egl2* probe confirmed the lack of the *egl2* gene in ALKO3873 (no hybridization, data not shown). The *egl*2 locus was intact and the expected 4.9 kb band was hybridized from genomic digests of ALKO3798, ALKO3799 and the host strain ALKO2221 (data not shown). According to further analysis using pALK546 (Fig. 3), *cbh2* and *ble* fragments (not shown) as probes it was shown that the *egl2* gene was replaced by one copy of the pALK546 expression fragment in the transformant strain ALKO3873. ALKO3798 and ALKO3799 contained one copy of the transformed fragment integrated in an unknown location (not *egl2, cbh2,* or *cbh1* loci). The 3'-end part of the transformed fragment could not be detected in the Southern blots of ALKO3798 and ALKO3799 (Fig. 3). However, based on the ELISA analysis the transformants contained an additional complete *cbh2*, because the monoclonal antibody CII-8 used recognizes only the full-length CBHII protein (Aho *et al*., 1991). Furthermore, according to SDS-PAGE and Western blotting with CII-8 (Fig. 4) and CII-30 (data not shown) antibodies (Aho *et al*., 1991), ALKO3798 and ALKO3799 produced increased amounts of full-length CBHII as compared to ALKO2221 and no truncated forms of CBHII were detected.

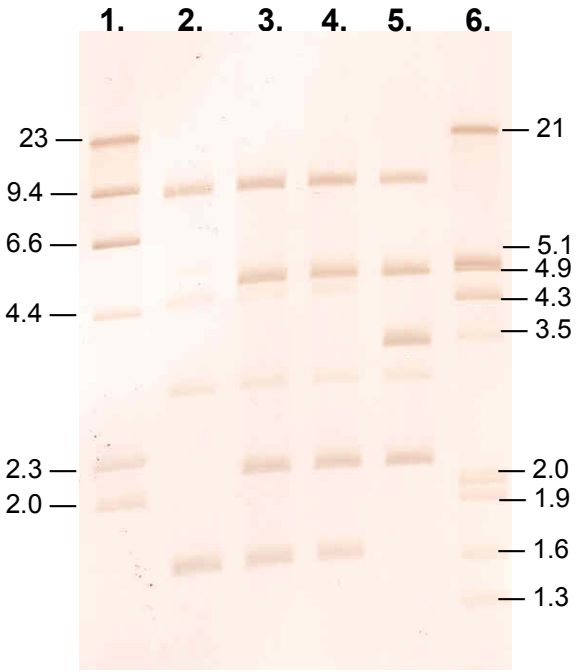

Figure 3. Southern analysis of the transformants overproducing CBHII. Genomic DNAs were digested with XhoI and hybridized with an 11.8 kb EcoRI-BamHI fragment of pALK546. Lane 1: Molecular weight markers, Lane 2: ALKO2221, Lane 3: ALKO3798, Lane 4: ALKO3799, Lane 5: ALKO3873, Lane 6: Molecular weight markers.

The bands originating from the native cbh1 and cbh2 loci are 9.1 and 9.8 kb and run as one band (all the strains). The 1.6, 4.4 and 3.2 bands originate from the native egl2 locus. The bands of 4.9 kb (from the ble-cbh1 promoter region), 3.4 (from cbh2 - egl2 3') and 2.3 kb (from egl2 5' - ble) derive from an intact expression cassette (Fig. 1).

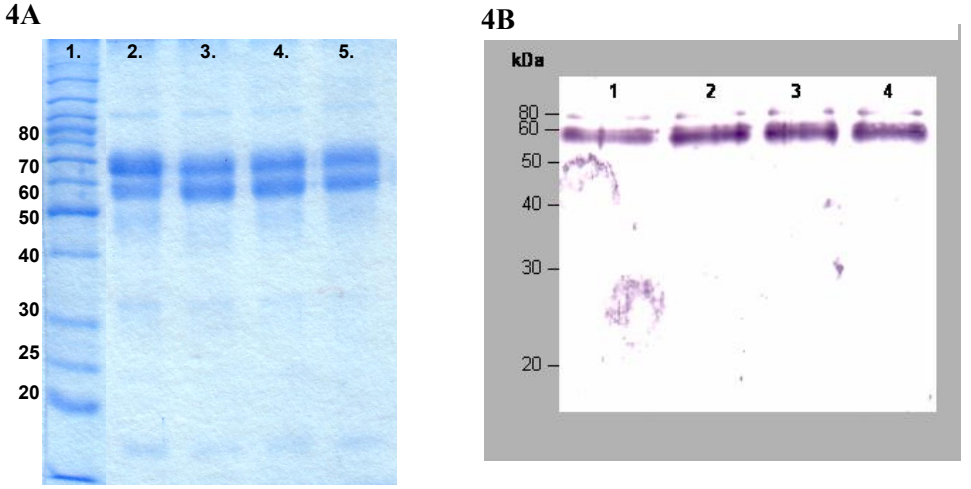

Figure 4. SDS-PAGE (A) and Western blot (B) of the samples from the culture supernatants of the host strain ALKO2221 and the CBHII transformants ALKO3873, ALKO3798 and ALKO3799. The size of CBHI is 59–68 kDa and of CBHII 50–58 kDa.

A. Lane 1: Molecular weight markers, kDa, Lane 2: ALKO2221, Lane 3: ALKO3873, Lane 4: ALKO3798, Lane 5: ALKO3799. 1 µl of culture supernatant was loaded to each lane.

B. Lane 1: ALKO2221, Lane 2: ALKO3873, Lane 3: ALKO3798, Lane 4: ALKO3799. 3 µl 1:1000 diluted culture supernatant was loaded to each lane. CII-8 monoclonal antibody was used.

3.2.2. Enzyme production of CBHII-transformants

ALKO3798 (EGII-positive) produced the highest amount of CBHII protein, which was about four-fold compared to that of the host strain (Table 2). ALKO3873 (EGII-negative) and ALKO3799 (EGII-positive) produced about three times more CBHII protein compared to the host strain ALKO2221 (Table 2). The transformant strains ALKO3798 and ALKO3799 produced about 20% and ALKO3873 about 10% less CBHI protein as compared to the host strain ALKO2221 (Table 2). The production of EGI was slightly enhanced in the EGII-negative ALKO3873 strain compared with ALKO2221. Production of endoglucanase activity was decreased by 20% in the EGII-positive transformants

ALKO3798 and ALKO3799. Lack of the *egl*2 gene in the ALKO3873 strain caused a 50% decrease in production of endoglucanase activity. Lack of EGII decreased the FPU activity by 15% compared to the host strain.

Table 2. Production of cellulases by the host strain ALKO2221 and by the transformants ALKO3797, ALKO3798 and ALKO3873.

Strain	*egl2* Southern analysis	*cbh2* cassette copy no.	Secreted protein mg/ml	FPU/ml	Endogluca-nase (HEC) nkat/ml	ELISA CBHI mg/ml	CBHII mg/ml	EGI mg/ml
ALKO2221	+	0	6.8	3.6±0.3	700	2.2±0.1	0.18±0.01	0.32±0.01
ALKO3798	+	1	6.1	3.6±0.2	570	1.7±0.1	0.70±0.07	0.31±0.05
ALKO3799	+	1	6.5	3.9±0.1	560	1.8±0.1	0.50±0.05	0.28±0.01
ALKO3873	-	1	6.8	3.0±0.6	360	1.9±0.4	0.53±0.17	0.39±0.10

Strains were grown for seven days in cellulase-inducing medium; the results are averages from three replicate flasks.

3.3. Combined CBHI and CBHII overproduction

3.3.1. Construction of strains

For construction of the strains overproducing both CBHI and CBHII without EGI and EGII (CBH-transformants), the *T. reesei* CBHI-overproducing *egl1*-negative strain ALKO3761 (Table 1) was transformed with the *cbh2* expression cassette either from the plasmid pALK543 or pALK546. The transformants were first screened by Southern blotting for the deletion of *egl2* and the chosen one-copy strains were further characterized by analyzing the amounts of cellulases from the culture supernatants. 57% of the pALK546 transformants and 44% of the pALK543 transformants were *egl2*-negative according to Southern hybridizations. The strains ALKO4095 (transformed with pALK546) and ALKO4097 (transformed with pALK543) had a single copy of the respective expression cassette replacing the *egl2* gene. The *egl2* probe did not hybridize to the *Xho*I digested genomic DNAs of the transformants ALKO4095 and ALKO4097, whereas the expected band of 4.9 kb originating from the native *egl2* gene was obtained from the host strains ALKO2221 and ALKO3761 (data not shown). The Southern blots of the *Sal*I digested genomic DNAs of ALKO4095 and ALKO4097-transformants probed with the pALK546 and pALK543 fragments are shown in Fig. 5.

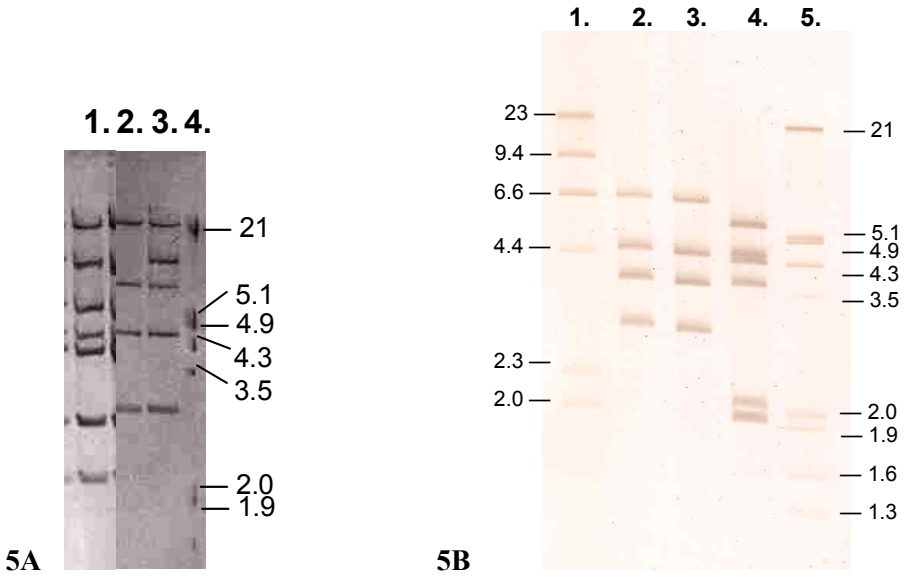

Figure 5. Southern analysis of the transformants ALKO4095 and ALKO4097. Genomic DNAs were digested with SalI and hybridized with an 11.8 kb EcoRI-BamHI fragment of pALK546 (5A) and with an 11.2 kb BamHI fragment of pALK543 (5B).

A. Lane 1: ALKO4095, Lane 2: ALKO2221, Lane 3: ALKO3761, Lane 4: Molecular weight marker.

B. Lanes 1 and 5: Molecular weight marker, Lane 2: ALKO2221, Lane 3: ALKO3761, Lane 4: ALKO4097.

The bands of about 6 and 2.1 kb in ALKO4095 and ALKO4097 originate from fragments that react to 5' and 3' regions of the egl2 locus from the sites included in pALK546 and pALK543 showing replacement. A. The 4.5 kb band is from the native cbh2, the band of about 20 kb is from the native cbh1 and the band of about 10 kb is from the pALK496 integrated in the genome of ALKO3761. The 4.3kb and 2.6 kb bands are from the expression cassette and the ble marker region in the pALK546 fragment. The 6.6 and 2.8 kb bands in ALK03761 and ALKO2221 are from the native egl2. B. The bands of 3.7 and 4.5 kb originate from the native cbh2 locus. The 4.3 kb and 1.9 kb bands are from the expression cassette and the ble marker in the pALK543. The 6.6 and 2.8 kb bands in ALKO2221 and ALK03761 are from the wild type egl2.

3.3.2. Enzyme production by the CBH-transformants

The production of CBHII was 3.4-fold higher in the ALKO4097 strain (*cbh2* promoter in the *cbh2* expression cassette) compared to the parent strain ALKO3761 (Table 3). The production of CBHI was also slightly enhanced in this strain. A clear increase, 3.9-fold, in CBHII production was also observed in the strain ALKO4095 (*cbh1* promoter used in the *cbh2* expression cassette), but there was a slight decrease in the level of CBHI (Table 3). The filter paper activity was not significantly altered in either of the transformants compared to the host strain ALKO3761. Less than 10% of the endoglucanase activity was left in both CBH-overproducing strains as compared to the host ALKO3761 and only about 4% as compared with the original parent strain ALKO2221.

Table 3. Production of cellulases by the host strains ALKO2221 and ALKO3761 ($CBHI^+$, EGI^-) and by the transformants ALKO4097 and ALKO4095.

Strain	Southern analysis		Promoter in *cbh2* cassette	Secreted protein mg/ml	FPU/ml	Endoglucanase (HEC) nkat/ml	ELISA CBHI mg/ml	CBHII mg/ml
	egl1	*egl2*						
ALKO2221	+	+	-	6.1±0.4	3.4±0.3	720±90	2.7±1.1	0.25±0.06
ALKO3761	-	+	-	6.6±0.4	2.8±0.4	390±40	3.9±2.1	0.23±0.03
ALKO4097	-	-	*cbh2*	7.8±1.8	2.7±0.9	30±10	4.4±1.8	0.78±0.3
ALKO4095	-	-	*cbh1*	7.5±1.7	2.5±0.9	30±15	3.5±1.8	0.85±0.2

Strains were grown for seven days in cellulase-inducing medium; the results are averages from three replicate flasks.

3.4. Use of cellobiohydrolase preparations in biofinishing

Cellulase preparations derived from the cellobiohydrolase-overproducing strains ALKO3760 ($CBHI^+$), ALKO3798 ($CBHII^+$) and ALKO4097 ($CBHI^+$, $CBHII^+$, EGI^-, $EGII^-$) were used in biofinishing of cotton fabric. The original parent VTT-D-79125 strain was used for comparison, and the EGII-overproducing strain ALKO3529 ($EGII^+$) and the EG-overproducing strain ALKO3528 ($CBHI^-$, $CBHII^-$, EGI^+, $EGII^+$) as positive controls. In comparison to the VTT-D-79125 or ALKO3760-preparations better visual appearance, higher weight loss and greater depilling was achieved with the same enzyme dosage of cellulase

preparation of the CBHII-overproducing ALKO3798 strain indicating better fuzz removal (Table 4). Only slightly better depilling and visual appearance was achieved with the cellulase preparation of the CBHI-overproducing strain ALKO3760 as compared to the VTT-D-79125 strain with the same dosage. However, the best visual appearance and depilling was obtained with the EG-strains ALKO3529 and ALKO3528. The weight loss obtained with the CBHII-strain ALKO3798 was not lower but equal to that obtained with the EGII-strain ALKO3529 with the same dosage and depilling level. The cellulase preparation derived from the CBH-overproducing strain ALKO4097 did not significantly improve the visual appearance or pilling of the cotton fabric.

Table 4. Weight loss, visual appearance and pilling of the fabrics treated in a Launder-Ometer with VTT-D-79125, ALKO3760, ALKO3798, ALKO4097, ALKO3528 and ALKO3529 cellulase preparations.

Preparation and strain	Dosage (mg protein/g of fabric)	Pilling	Visual appearance	Weight loss %
Buffer alone	-	1	1	0
VTT-D-79125 (wild type)	2	2.3	2.3	0.8
	6	3.6	2.7	2.2
ALKO3760 ($CBHI^+$)	2	2.3	3.1	2.1
	6	3.8	3.4	4.2
ALKO3798 ($CBHII^+$)	2	3.1	3.3	3.1
	6	4.3	3.4	4.9
ALKO4097 ($CBHI^+$,	2	1.2	1.5	0
$CBHII^+$, EGI^-, $EGII^-$)	6	2.2	2.5	1.0
ALKO3529 ($EGII^+$)	2	4.3	3.9	3.1
	6	4.4	3.8	4.8
ALKO3528 ($CBHI^-$,	2	3.5	3.7	0.7
$CBHII^-$, EGI^+, $EGII^+$)	6	4.3	3.4	2.0

Visual appearance: 1 = fuzzy surface, 5 = clean surface
Pilling: 1 = many pills, 5 = no pills

4. Discussion

CBHI is the major secreted protein of *T. reesei*. Despite this we were able to further increase the amount of CBHI by increasing the copy number of the *cbh1* gene. There was also an increase in the amount of total secreted proteins.

Compared to the host strain, production of the CBHI protein increased 1.3-fold in a one-copy transformant ALKO3761 and 1.5-fold in the best transformant ALKO3760 with two additional *cbh1* copies. The MUL-activities in the culture supernatants increased correspondingly. When the *cbh1* promoter was used for overexpression of *egl1* in the *cbh1* locus two copies of the expression cassette roughly doubled the EGI amount, while no further increase was observed with the third copy (Karhunen *et al.,* 1993). Now, it seems that in the case of CBHI itself, at least by using ALKO2221 strain as a host strain, some limiting factors in transcription and / or post-transcriptional stages appear already with one additional copy of the *cbh1* expression cassette.

The amount of secreted CBHII protein could be increased from three- to fourfold with an additional copy of the *cbh2* gene under the *cbh1* promoter. Multicopy transformants in filamentous fungi have resulted in higher protein production levels, but there was considerable variation in the production levels of transformants with comparable numbers of gene copies, suggesting that besides the number of gene copies, the site of integration may also affect the expression of genes introduced (Verdoes *et al.*, 1995). The site of integration did not have much effect on the CBHII production levels. The result was the same with duplicate transformants (data not shown). The slight difference observed, the EGI+ transformants (integration to an unknown locus) producing somewhat better levels of CBHII than the EGI- transformant (integration to the *egl1* locus), may have been due to the different enzymatic background (EGI+/-). The amount of CBHI protein produced by the CBHII-transformants was lower than by the host strain. This may be due to e.g. limitation in some transcription factors of the *cbh1* promoter controlling the expression of both of the genes.

Deletion of the *egl2* gene clearly and expectedly decreased the endoglucanase activity in ALKO3873. This activity was also decreased (but less) in the EGII-positive CBHII-overproducing transformants and the EGI-positive CBHI-overproducing transformants. This, and the decreased production of CBHI of CBHII transformants, might be indications of mutual cellulase regulation or possibly of a secretion stress. It has been shown that in *T. reesei* the genes encoding secreted proteins are rapidly downregulated concurrently with induction of the UPR (unfolded protein response) pathway (Saloheimo *et al.*, 2003).

In the transformants overproducing both CBHI and CBHII, the host strain ALKO3761 carried two copies of the *cbh1* promoter, one in front of the native *cbh1* gene and one in the *cbh1* expression cassette transformed to the strain. In this background a similar increase in the secreted amount of CBHII was obtained when *cbh2* was expressed with either *cbh1* or *cbh2* promoters. The simultaneous use of the *cbh1* promoter for both *cbh1* and *cbh2* expression in one strain (in total three copies of the promoter) had an effect on the amount of secreted CBHI (as in the case of strains overproducing CBHI or CBHII individually), since slightly less CBHI was secreted in ALKO4095 than in ALKO4097 or the host ALKO3761. Thus the capacity of the *cbh1* promoter appears to approach some limitation in ALKO4095.

We have described here the results from a few transformants, but in addition to these CBHI- CBHII- and CBH-overproducers, duplicate transformants were isolated producing similar activities and cellulase protein levels, confirming the results obtained.

Endoglucanases are known to be important in obtaining biofinishing effects of cotton-containing textile material. Purified CBHI and CBHII alone or in combination have not been reported to affect the pilling properties of cotton fabric, whereas fabrics treated with EGI and especially EGII had improved pilling values (Heikinheimo and Buchert, 2001). By increasing the relative amount of EGII in the cellulase mixture improved depilling with lower strength and weight losses of cotton knitted fabric were obtained (Miettinen-Oinonen *et al.*, 2001). We have also shown that by increasing the amount of CBHII (ALKO3798) in a wild-type background, significantly improved pilling properties and better visual appearance of cotton fabric can be achieved than with the wild type preparation. However, the effects achieved with EGII-enriched (ALKO3529) preparation were still better. Interestingly, no difference in weight loss of the fabric was observed when EGII- and CBHII-enriched preparations were compared at the same depilling level and dosage, although endoglucanases rather than cellobiohydrolases have previously been associated with strength loss (Heikinheimo and Buchert, 2001). Because the CBHII-overproducing strain contains all the native cellulase genes, one explanation for the improved effect could be synergistic effects of cellulases. Heikinheimo and Buchert (2001) demonstrated slight synergism between purified CBHII and EGI on cotton when weight loss and pilling properties were analyzed. CBHII has also

been shown to cause small changes in the degree of polymerization of barley β−glucan, whereas CBHI has no β-glucanase activity (Henriksson *et al.*, 1995). This activity of CBHII might have an effect in biofinishing, because our results further show that by increasing the amount of CBHI in the cellulase preparation (ALKO3760) the pilling and performance of cotton was not improved as compared to the wild type strain.

Only a slightly positive effect on depilling and visual appearance of cotton was obtained with the preparation produced by the CBH-transformant ALKO4097 compared to buffer alone. This effect was only seen at the highest dosages used, suggesting that the effect is most probably due to the endoglucanase activity in the preparation caused by the minor endoglucanases still present. Based on the biofinishing results with the CBHII-overproducing strain, CBHII may also contribute to this effect. The cellulase preparation produced by the strain ALKO4097 has been used to improve the dimensional stability of cotton fabrics (Cortez *et al.* 2002). The CBH preparation improved the dimensional stability compared to a control treated only with buffer, but the improvement after one wash was consistently greater for the EG preparation than for the CBH. However, strength losses were lower for the CBH preparation than for the EG preparation.

In a summary, even though positive effects on pilling and visual appearance were detected, cellulase preparations with increased levels of endoglucanase(s) have more promise in biofinishing than preparations with increased levels of cellobiohydrolase(s). However, the newly described preparations can possibly be used in applications in which total hydrolysis of cellulose is needed, such as in enzymatic hydrolysis of biomass to sugars. Increase in filter paper activity was seen in fermentor culture supernatants of for example the CBHII-transformant ALKO3799 (data not shown). According to Teeri (1997), cellobiohydrolases are the key components of the redesigned, highly synergistic cellulase mixtures required for enzymatic conversion of biomass to sugars that can be fermented to ethanol for future sources of fuels and chemicals.

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Three cellulases from *Melanocarpus albomyces* for textile treatment at neutral pH

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Abstract

Culture supernatants from strains of *Melanocarpus albomyces*, *Myceliophthora thermophila*, *Chaetomium thermophilum*, and *Sporotrichum thermophilum* were tested for their ability to release dye in neutral pH conditions from indigo-dyed cotton-containing fabric in biostoning applications. The supernatants from *M. albomyces* worked well in biostoning, with low backstaining. Three cellulases were purified to homogeneity from the culture medium of this species. Two of the cellulases were endoglucanases with apparent molecular masses of 20 and 50 kDa. The 20 kDa endoglucanase was a relatively heat-stable cellulase with high pH optimum. The partially purified enzyme crystallized spontaneously at pH 4.0 and 7 °C. The 50 kDa endoglucanase also had activity against 4-methylumbelliferyl-β-D-lactoside (MUL) and was active over a wide range of pH values. The third purified cellulase was the 50 kDa cellobiohydrolase with low MUL activity at acidic pH and detectable activity towards filter paper and acid swollen Solca Floc-cellulose, but no endoglucanase activity. The purified 20 kDa endoglucanase performed well in biostoning of denim fabric at neutral pH. Addition of the purified 50 kDa endoglucanase or the 50 kDa cellobiohydrolase to the 20 kDa endoglucanase decreased backstaining in biostoning.

Keywords: *Melanocarpus albomyces*; Textile treatment; Neutral cellulase

1. Introduction

Endoglucanases (EG), cellobiohydrolases (CBH) and β-glucosidases are three types of cellulolytic enzymes, which act synergistically to hydrolyze cellulose [1]. Endoglucanases hydrolyze cellulose chains internally providing new chain ends for cellobiohydrolases. Cellobiohydrolases hydrolyze cellulose processively from the ends to create mainly cellobiose. β-Glucosidases hydrolyse cellobiose to glucose.

Cellulases are used widely in the textile industry for biostoning and biofinishing of cellulosic fibers [2–6]. The best-known of these applications is in the so-called biostoning of denim garments to impart a stone-washed appearance and improved softness to the fabric. In biostoning cellulases weaken the surface fibers and mechanical action applied in the treatment removes the weakened fibers that contain the indigo dye. Since indigo dye is on the surface of the denim fabric, removal of the surface fibers by cellulases reveals the white yarn with a faded effect desired by many customers. Cellulases from the fungi *Trichoderma reesei* and *Humicola insolens* [7] are widely used in the textile industry to treat cotton-containing denim materials [8–10]. EGII is the most effective of the *T. reesei* cellulases at removing color from denim, producing a good stone-washed effect with a low hydrolysis level [11].

The use of acid cellulases, such as cellulases produced by *Trichoderma*, in biostoning is limited by the backstaining (redeposition of released dye in the fabric) and weakening of fabrics they cause. To avoid backstaining anti-redeposition chemicals or mild bleaching agents are often added during washing or rinsing steps. The protease added during rinsing or at the end of the acid cellulase washing step resulted in significant reduction of backstaining [12]. Neutral cellulases, with activities in the pH range 6–8, are less aggressive

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against cotton than acid cellulases, and do not compromise the strength of the fabric as readily as acid cellulases [13]. Neutral cellulases have a broad pH profile and are not affected by the pH increase that occurs during denim washing due to alkalinity released from the garment [3]. However, neutral cellulases generally require a longer wash time than do the acid cellulases [13]. Thus, in the textile industry, there is a need for novel cellulases that are active at neutral and alkaline pH values, have short reacting time, do not compromise the strength of fabric and have good denim finishing properties, including low backstaining.

In this paper, we describe screening of fungal species for identification of neutral cellulases useful in biostoning. Because the enzyme washing performance does not correlate well with cellulase activity against carboxymethylcellulose or filter paper [14], the ability of the cellulase preparations to cause successful biostoning was used directly in screening and in subsequent purifications. The purification and characterization of three novel cellulases of a fungal ascomycete *Melanocarpus albomyces* and their utility in biostoning are reported. On the basis of their properties and sequence we call these 20 kDa endoglucanase (*Ma* Cel45A), 50 kDa endoglucanase (*Ma* Cel7A) and 50 kDa cellobiohydrolase (*Ma* Cel7B) [15].

2. Materials and methods

2.1. Strains, media and growth conditions

M. thermophila ALKO4179 (CBS 689.95), *M. albomyces* ALKO4237 (CBS 685.95), *C. thermophilum* ALKO4265 (CBS 730.95) and *S. thermophilum* ALKO4125 (CBS 688.95) strains were used for production of cellulases. *M. albomyces* ALKO4237 was grown for biostoning tests at 45 °C for 3 days in a rotatory shaker (250 rpm) in 500 ml of the mineral medium [16]. *M. albomyces* ALKO4237 was cultivated in a 700 l fermentation for 3 days (45 °C, pH 6.5) to obtain starting material for protein purification. The medium contained per liter: 20 g Solka Floc, 15 g corn steep powder, 15 g $(NH_4)_2SO_4$, 15 g KH_2PO_4, 590 mg $MgSO_4{\cdot}7H_2O$, 600 mg $CaCl_2$, 1 g Struktol SB (Schill & Seilacher, Germany), 5 mg $FeSO_4{\cdot}7H_2O$, 2 mg $MnSO_4{\cdot}H_2O$, 1 mg $ZnSO_4{\cdot}7H_2O$, and 5 mg $CoSO_4{\cdot}7H_2O$. The final culture was filtered through a rotary vacuum filter, concentrated by ultrafiltration to 30 kg and sterilized by using a Seitz sheet filter (40 cm × 40 cm). Concentrated growth medium was stored at −20 °C.

M. thermophila ALKO4179, *S. thermophilum* ALKO4125 and *C. thermophilum* ALKO4265 were cultivated for biostoning tests in 500 ml of medium: 1 l contained 6 g Solka Floc, 6 g distiller's spent grain, 3 g oat spelt xylan, 2 g $CaCO_3$, 1.5 g soybean meal, 1.5 g $(NH_4)_2HPO_4$, 1 g barley bran, 0.5 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.5 g NaCl, 0.5 ml trace element solution 1 (1 l contains: 1.6 g $MnSO_4$, 3.45 g $ZnSO_4{\cdot}7H_2O$ and 2 g $CoCl_4{\cdot}6H_2O$) and 0.5 ml trace element solution 2 (1 l contains: 5 g $FeSO_4{\cdot}7H_2O$ and two drops of concentrated H_2SO_4); pH 6.5. Cultures were incubated at 45 °C for 3 days in a rotatory shaker (250 rpm). Culture filtrates were concentrated about 10-fold in an Amicon concentrator using a cut-off of 30 kDa.

2.2. Indigo dye release (biostoning)

Indigo dyed cotton-containing denim fabric (Lauffenmuehl, Germany) was desized (removal of size from the fabric) for 10 min at 60 °C with 1 ml/l Ecostone A 200 (Primalco Ltd., Finland) in a drum-washer. The fabric was cut into 12 cm × 12 cm swatches. The color from both sides of the fabric swatches was measured as reflectance values with a Minolta (Osaka, Japan) Chroma Meter CM 1000R L *a*b* system (illuminant D65). Cellulase treatments were performed in a LP-2 Launder-Ometer (Atlas, USA). Seven grams of denim swatches were loaded into the 1.2 l container that contained 200 ml of 50 mM citrate/phosphate buffer (pH 7.0) or 50 mM citrate buffer (pH 5.2). Sixty microliters of 10% (v/v) Berol 08 (Berol Nobel AS, Sweden) was added as a surfactant when indicated. Ten steel balls (diameter 0.6 cm) were added to each container to help remove fiber. Finally, the cellulase solutions were added to the container (Tables 3, 5 and 6). Ecostone L, a commercial acid cellulase product (Primalco Ltd.), was used as a control. The Launder-Ometer was run at 42 rpm, 50 °C, 2 h. The swatches were soaked for 10 min in 10 mM NaOH, rinsed with cold water, and dried. The color from both sides of the swatches was measured with the Minolta Chroma Meter.

2.3. Enzyme activity assays

The endoglucanase activity was measured as the release of reducing sugars from hydroxyethylcellulose (HEC, Fluka) as previously described [17] except that pH 7.0 was used. The method of Bailey and Nevalainen [17] was modified as follows: enzyme sample (100 μl) was added at 50 °C to 900 μl of 1% HEC (w/v) in 50 mM Hepes/NaOH pH 7.0. After 10 min the reaction was stopped by adding of 1.5 ml of dinitrosalicylic acid (DNS) reagent containing 120 μM glucose. Enough enzyme was used to cause a change in A_{540} between 0.08 and 0.12. A calibration curve was made for each batch of DNS/glucose reagent by replacing the enzyme sample with 100 μl of 0–10 mM glucose. Activities are reported as ECUs (nkat/ml), where 1 ECU produces 1 nmol of reducing equivalent per second under these conditions. For cellulase activity determinations, amorphic Solca Floc cellulose was used as the substrate. For preparation of the amorphic form, 50 g of solid Solca Floc cellulose was mixed with 800 ml of cold 85% (w/v) *ortho*-phosphoric acid. The mixture was homogenized with a household food processor and allowed to swell for 2 h in a cold room (7 °C). Acid-swollen cellulose was washed four times with distilled ice-cold water by using Whatman 1 filter paper in a Büchner-funnel, and resuspended into 4 l of 1% (w/v) Na_2CO_3. The mixture was

incubated over night with magnetic stirring in a cold room. The cellulosic material was washed with distilled water by vacuum filtering through cotton cloth at room temperature until the pH of the water used in washing remained constant (about pH 5). Cellulase activity determinations used 3% (w/v) Solca Floc cellulose and were based on the release of reducing sugars from amorphic Solca Floc cellulose [17]. Activity against 4-methylumbelliferyl-β-D-lactoside (MUL) was assayed as in [18]. One MUL is the amount of activity that liberates 1 nmol of methylumbelliferone per second. The filter paper activity was measured according to [19].

2.4. Purification of the 20 kDa endoglucanase from M. albomyces ALKO4237

All procedures were performed at about 7 °C, except that DEAE Sepharose FF™ chromatography was at about 20 °C. Concentrated growth medium (4500 ml) of *M. albomyces* ALKO4237 was thawed, adjusted to 1 mM EDTA, and fractionated with $(NH_4)_2SO_4$. The proteins precipitated between 17 and 42 g of $(NH_4)_2SO_4$ per 100 ml of concentrate were dissolved in 900 ml of 25 mM Tris–HCl, pH 7.2, containing 0.25 mM EDTA to give the "42% Precipitate" (Table 1). The solution was brought to 1 mM phenylmethyl-sulphonylfluoride (PMSF) and 10 μg/ml Pepstatin A by adding 9 ml of methanol containing 100 mM PMSF and 1 mg/ml Pepstatin A. It was then diluted with water to a conductivity of 4 mS/cm and adjusted with 1 M NaOH to pH 8.0. The resulting solution (45 l) was pumped at 150 ml/min through a 6.3-l column of DEAE-Sepharose FF™. Bound proteins were eluted at 110 ml/min with a linear gradient from 0 to 0.5 M NaCl in 20 l of 25 mM Tris–HCl pH 7.7 containing 0.25 mM EDTA. Two peaks of neutral ECU activity eluted and were pooled as follows: DEAE-Pool A at conductivities of 13–19 mS/cm and DEAE-Pool B at 22–28 mS/cm. Proteins in DEAE-Pool A were precipitated with $(NH_4)_2SO_4$ (45 g per 100 ml) and dissolved in 170 ml of 25 mM Pipes/KOH, pH 6.0/1 mM EDTA. One half of this material was desalted on a 600 ml column of G25-Sephadex equilibrated with 25 mM Na acetate, pH 4.0/0.5 mM EDTA. The protein eluate (167 ml) was adjusted to pH 4.1 by addition of 2.5 ml of 10% (w/v) acetic acid and centrifuged. The clear supernatant was applied at 2.5 ml/min to a 4.5 × 31 cm column of SP-Sepharose™ equilibrated with 25 mM Na acetate, pH 4.0/0.5 mM EDTA. The column was developed at 1.3 ml/min with a linear gradient from 0 to 0.4 M NaCl in 3400 ml of the same buffer. Most of the endoglucanase activity eluted at about 0.2 M NaCl. When active fractions (15 ml) were stored at 7 °C, a crystalline precipitate formed in them and contained nearly all of the endoglucanase activity. Fractions in which crystallization was slow were seeded with 30 μl of suspension from adjacent fractions. After 2–3 days the crystals were collected by centrifugation, suspended in 30 ml of 25 mM Pipes pH 6.0/1 mM EDTA and immediately re-centrifuged. About 40% of the HEC-activity dissolved. The washed crystals were then dissolved in 18 ml of 25 mM Tris, pH 7.2/0.25 mM EDTA.

2.5. Purification of a 50 kDa endoglucanase and a 50 kDa cellobiohydrolase from M. albomyces ALKO4237

Concentrated growth medium of *M. albomyces* ALKO4237 was purified through DEAE-Sepharose FF™, and the fraction corresponding to DEAE-Pool B (Table 2) was concentrated by adding 45 g $(NH_4)_2SO_4$, collecting the precipitated proteins, and dissolving them in 25 mM Pipes, pH 6.0/0.25 mM EDTA. This material was stored at −25 °C. Thawed material (228 ml) was desalted in two runs on a 5.0 cm × 29 cm column of G25 Sephadex equilibrated with 25 mM Na acetate, pH 4.0/0.5 mM EDTA. A large precipitate formed in the desalted material and was removed by centrifugation and discarded. The clear supernatant (11 g of protein; "Input to SP-Sepharose" in Table 2) was run at 40 ml/h into a 4.5 cm × 33 cm column of SP-Sepharose equilibrated with 25 mM Na acetate, pH 4.0/0.5 mM EDTA. After applying the enzyme the column was washed with a 140 ml of the same buffer and then developed with a gradient of 0–0.25 M NaCl in 1300 ml of the same buffer. Under these conditions (high protein load), over 70% of the applied activity towards HEC at pH 7.0 eluted in a sharp peak (SP-Pool A in Table 2) before the NaCl gradient, and at an enzyme concentration up to three-fold higher than that of the applied enzyme. A second major protein peak eluted

Table 1
Purification of the 20 kDa cellulase

Fraction	Volume (ml)	Protein (mg/ml)	Activity (ECU/ml)	Total activity (ECU × 10^{-3})	Specific activity (ECU/mg)
Concentrated growth medium	4500	65	1160	5220	18
42% Precipitate	900	250	4420	3980	18
DEAE-Pool A	3450	8.4	357	1230	43
SP-Sepharose Pool	225	4.7	1300	292 (×2)	270
Total crystals	48	6.3	5600	269 (×2)	890
Washed crystals	18	8.9	8670	156 (×2)	980

Half of the DEAE-Pool A was applied to the SP-Sepharose column (DEAE-Pool B was used to purify the 50 kDa cellulase; see Table 2). Protein was calculated from the A_{280} assuming an $E^{1\,mg/ml}280$ of 1.53 for the crystalline fractions and 1.4 for earlier fractions. The row "Total crystals" shows the sum of the material that dissolved in 30 ml of Pipes, pH 6.0 and the remaining crystals that then dissolved in Tris pH 7.2, i.e. "Washed crystals".

Table 2
Purification of the 50 kDa endoglucanase

Fraction	Volume (ml)	Protein (mg/ml)	Activity (ECU/ml)	Total activity (ECU × 10^{-3})	Specific activity (ECU/mg)
DEAE-Pool B (concentrated)	228	114	3290	524	29
Input to SP-Sepharose	248	45	1830	453	41
SP-Pool A	60	18	3730	224	200
Phenyl-Sepharose-Pool A	141	1.9	878	124	465
Concentrated 50 kDa endoglucanase	11.0	22	10050	111	450

The enzyme was purified from a DEAE-Pool B made in the same way as in Table 1. Most of SP-Pool A was applied to the Phenyl-Sepharose column (SP-Pool B was used to purify the 50 kDa cellobiohydrolase; see text). Protein was calculated from the A_{280} assuming an $E^{1\,mg/ml}280$ of 1.29 for pure 50 kDa endoglucanase and 1.4 for earlier fractions.

at about 0.2 M NaCl and contained only a small amount of enzyme activity (SP-Pool B in Table 2). SP-Pool A (58 ml) was mixed with 12 ml of 0.25 M Pipes, pH 6.0, and 10.5 g of $(NH_4)_2SO_4$. The mixture was loaded at 90 ml/h to a 3.2 cm × 26 cm column of Phenyl-Sepharose equilibrated with 25 mM Pipes/KOH, pH 6.0 containing 15% (w/v) $(NH_4)_2SO_4$. The column was developed with a linear gradient from 15 to 0% $(NH_4)_2SO_4$ in 1040 ml of 25 mM Pipes/KOH, pH 6.0 and 15 ml fractions collected. Protein without activity eluted between 8.5 and 6.5% $(NH_4)_2SO_4$ followed by a larger peak of active protein at the end of the gradient (Phenyl-Sepharose-Pool A). Fractions from this peak all had a single, 50 kDa, band on SDS–PAGE. Protein was precipitated from this pooled material by addition of $(NH_4)_2SO_4$ (45 g/100 ml) and dissolved in 25 mM Pipes/KOH, pH 6.0/0.25 mM EDTA to give the "Concentrated 50 kDa" endoglucanase (Table 2). The low activity protein peak from SP-Sepharose (SP-Pool B above) was fractionated on Phenyl-Sepharose in a similar manner and a large peak of protein with no activity towards HEC eluted at about 7% (w/v) $(NH_4)_2SO_4$, before a small peak of active protein at the end of the gradient. The inactive fractions all contained a single, 50 kDa band on SDS–PAGE. This material was pooled, concentrated by precipitation with $(NH_4)_2SO_4$ and named 50 kDa cellobiohydrolase.

2.6. Endoglycosidase treatment

Purified 50 kDa endoglucanase was deglycosylated with endoglycosidase H_f of Boehringer Mannheim (Germany) according to the supplier's instructions.

2.7. Digestion of the proteins and separation of the peptides

The purified proteins were alkylated with 4-vinylpyridine and digested either with sequencing grade modified trypsin or endoproteinase LysC as described previously [20]. The 50 kDa endoglucanase was, prior to enzymatic digestion, chemically cleaved with a 1000 × molar amount of cyanogen bromide to the estimated molar amount of protein in 70% trifluoroacetic acid. The enzymatically generated peptides were then separated by reversed phase chromatography and collected [20].

2.8. Protein and peptide sequencing

The alkylated proteins and selected purified peptides were subjected to N-terminal sequence analysis using a Procise 494A sequencer (Perkin-Elmer Applied Biosystems Division, Foster City, CA, USA).

3. Results

3.1. Indigo dye release in neutral conditions

After testing culture supernatants from about 25 fungal strains for endoglucanase activity at neutral pH (pH 7.0, data not shown), cellulase preparations derived from the strains *M. thermophila* ALKO4179, *M. albomyces* ALKO4237, *C. thermophilum* ALKO4265 and *S. thermophilum* ALKO4125 were selected for biostoning tests. To compare the final look of the denim fabrics after washing at pH 7.0 with different cellulase preparations, the color from both sides of the fabrics was measured (Table 3). The lightness and blueness units are clearly increased on the right side of the garments washed with preparations of *M. thermophila* ALKO4179, *M. albomyces* ALKO4237 and *S. thermophilum* ALKO4125, showing a good stone-washed effect. The best stone-washing effect could be obtained with the preparation of *M. albomyces* ALKO4237: L_{right} was the highest in *M. albomyces* ALKO4237 treated fabrics when the same ECU activities were used. The blueness unit was also increased on the right side of the fabric washed with the *C. thermophilum* ALKO4265 preparation but there was no (with Berol) or a small (without Berol) increase in the lightness unit. This is probably because the enzyme does work at this pH but at the same time causes a lot of backstaining. There was no stone-washing effect at pH 7.0 on the fabric with the commercial acid cellulase product, Ecostone L.

Backstaining on the reverse side of the fabric is used as an indication of the degree of backstaining on the right side of the fabric. To quantify the level of backstaining, the color was measured on the reverse side of the fabric. High backstaining is shown by low or negative $L_{reverse}$-value and high $b_{reverse}$-value. When the ECU amounts are the same, there was practically no backstaining in the fabrics treated with the

Table 3
Color measurements of denim fabrics treated with different culture supernatants

Source of enzyme	ECU/g of fabric	Berol 08	Right side of fabric		Reverse side of fabric	
			L_{right}	b_{right}	$L_{reverse}$	$b_{reverse}$
pH 7[a]						
Buffer alone	−	−	2.1	0.5	1.7	−1.1
	−	+	2.3	0.8	1.5	0.1
ALKO4237	200	−	5.5	3.1	1.8	2.3
M. albomyces	200	+	6.4	3.3	2.4	1.7
	400	+	7.7	3.8	2.5	1.8
ALKO4179	200	−	4.4	3.2	1.4	2.2
M. thermophila	200	+	5.5	2.4	2.8	1.9
	400	+	4.6	2.8	2.2	1.5
ALKO4125	200	−	3.5	2.6	1.6	1.4
S. thermophilum	200	+	4.0	2.7	2.3	1.5
ALKO4265	200	−	3.3	3.3	−5.7	6.6
C. thermophilum	200	+	2.2	3.6	−4.9	6.6
Ecostone L	200	−	1.4	0.9	0.3	1.4
	400	−	1.4	0.8	−0.1	1.7
	200	+	1.6	0.7	0	1.7
	400	+	1.6	0.9	−1.9	2.2
pH 5.2[b]						
Ecostone L	200	−	2.0	2.1	−4.0	4.8
	200	+	2.0	2.3	2.7	4.3
	400	+	3.2	2.8	2.6	4.8

The Launder-Ometer experiments were done at pH 7.0 and 5.2.
L: lightness unit of the fabric after the treatment minus lightness unit of the fabric before the treatment, *b*: blueness unit of the fabric after the treatment minus blueness unit of the fabric before the treatment.
[a] Launder-Ometer experiments were done at pH 7.0. The ECU activities were measured at pH 7.0.
[b] Launder-Ometer experiments were done at pH 5.2. The ECU activity was measured at pH 4.8.

M. thermophila ALKO4179, *M. albomyces* ALKO4237 and *S. thermophilum* ALKO4125 preparations when compared to the fabrics treated with *C. thermophilum* ALKO4265 or Ecostone L (pH 5.2 and pH 7) preparations (Table 3). Almost the same stone-washing effect can be achieved with the *M. thermophila* ALKO4179, *M. albomyces* ALKO4237 and *S. thermophilum* ALKO4125 cellulase preparations in the absence and presence of the helping agent Berol. Based on the good stone-washing effect with low backstaining *M. albomyces* ALKO4237 was chosen for isolation of cellulases for stone-washing in neutral conditions.

3.2. Endoglucanase assay of the M. albomyces ALKO4237 cellulases

In the endoglucanase assay described by Bailey and Nevalainen [17], the amount of enzyme is found that produces in 10 min about 0.6 mM reducing equivalents from 1% (w/v) hydroxyethylcellulose (HEC). This causes a color production with dinitrosalicylic acid (DNS) of about 0.25 absorption units at 540 nm. With the 20 kDa endoglucanase described below, we found that the linear phase of the reaction at pH 7.0 is over well before production of 0.6 mM reducing equivalents. Further, when the assay is calibrated with glucose as reducing equivalent, the first 0.2 mM of glucose appears to be consumed in a non-color producing reaction. We therefore modified the Bailey and Nevalainen [17] system by changing the pH to 7.0 and using DNS reagent containing enough glucose so that also the first reducing equivalents formed from HEC caused color production. Enough enzyme was used to produce a change in A_{540} of about 0.1, corresponding to about 0.17 mM reducing equivalent formed from 1% (w/v) HEC in 10 min. Under these conditions, the average rate over 10 min was about 70% of the initial rate and the system could be used to quantitate the 20 and 50 kDa endoglucanases of *M. albomyces* ALKO4237.

3.3. Purification of the 20 kDa endoglucanase

Because biostoning performance does not correlate well with activity in cellulase assays, we first fractionated the culture supernatants from *M. albomyces* ALKO4237 on DEAE Sepharose and other chromatographic media and tested the various protein pools in the Launder-Ometer. The results (not shown) indicated that two endoglucanases with molecular masses of about 20 and 50 kDa and a third protein with molecular mass of 50 kDa, but no activity towards HEC, were mainly responsible for the biostoning activity of *M. albomyces*. These proteins were eventually

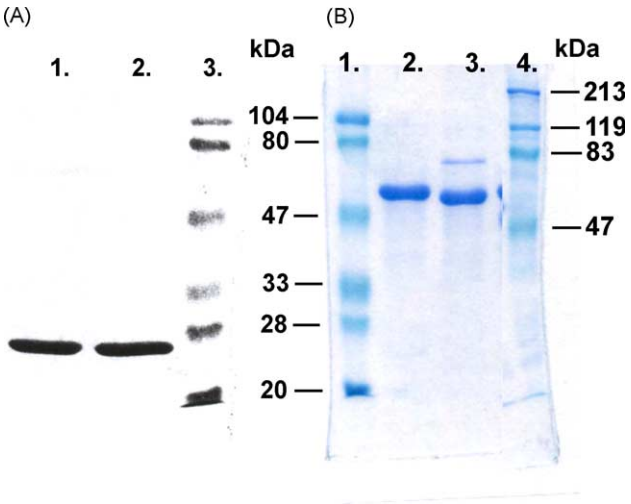

Fig. 1. SDS–PAGE of crystals of the 20 kDa endoglucanase, and of the purified and endoglycosidase H_f-treated 50 kDa endoglucanase. (A) Lane 1: washed crystals of 20 kDa endoglucanase, lane 2: total crystals of 20 kDa endoglucanase, lane 3: molecular weight markers. (B) Lane 1: molecular weight markers, lane 2: purified 50 kDa endoglucanase, lane 3: purified 50 kDa endoglucanase, endo H_f, lane 4: molecular weight markers.

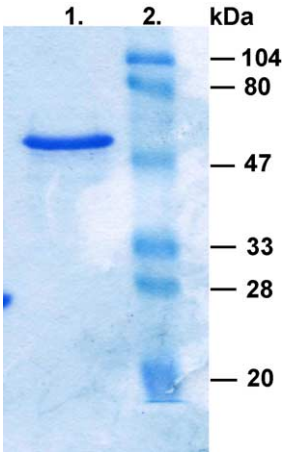

Fig. 2. SDS–PAGE of purified the 50 kDa cellobiohydrolase. Lane 1: purified 50 kDa cellobiohydrolase. Lane 2: molecular weight markers.

purified to homogeneity as described in Section 2. The 20 kDa endoglucanase was purified from the first pool of proteins eluted from DEAE-Sepharose that gave promising Launder-Ometer results (Table 1). The enzyme crystallized spontaneously when the SP-Sepharose eluates were stored for a few days at 7 °C. When these crystals were harvested and quickly extracted with 25 mM Pipes, pH 6.0/1 mM EDTA, only about 40% of the enzyme activity dissolved. The remainder dissolved (to a higher protein concentration) in 25 mM Tris–HCl, pH 7.2/0.25 mM EDTA. The specific activity (980 ECU/mg protein; Table 1) of this washed crystalline material was about 10% higher than that (890) of the total crystalline material. However, both the material extracted into Pipes, pH 6.0 and the washed crystals dissolved in Tris, pH 7.2 showed only a single band on SDS–PAGE corresponding to a molecular mass of 20 kDa (Fig. 1). The protein concentration of purified 20 kDa was determined by the ultraviolet method of Scopes [21]. The A_{205}/A_{280} ratio was 20.0 and the $E^{1\,mg/ml}280$ was 1.53.

3.4. Purification of the 50 kDa endoglucanase and 50 kDa cellobiohydrolase

When the second activity peak eluted from DEAE (DEAE-Pool B) was fractionated on SP-Sepharose or Phenyl Sepharose, we repeatedly obtained a peak of activity against HEC in which the major band seen by SDS-PAGE was at 50 kDa. On SP-Sepharose at pH 4.0 this peak was weakly bound and eluted at the start of the NaCl gradient. It was followed by an overlapping protein peak with no activity against HEC but also containing a major 50 kDa SDS–PAGE band. These two 50 kDa proteins were not resolved on SDS–PAGE, even when samples were mixed and run in the same slot. On Phenyl Sepharose at pH 6.0 the order of elution was reversed, with the active 50 kDa protein eluting immediately after the HEC-inactive 50 kDa protein. By combining the two purification procedures, pure samples of the active (later named 50 kDa endoglucanase based on its properties) and "inactive" (later named 50 kDa cellobiohydrolase based on its properties and sequence) proteins were obtained (Figs. 1 and 2). They had different amino acid sequences, so that 50 kDa cellobiohydrolase was not an inactivated form of 50 kDa endoglucanase. The method of Scopes [21] gave A_{205}/A_{280} ratios of 24.8 and 25.2 and values for $E^{1\,mg/ml}280$ of 1.29 and 1.26 for 50 kDa endoglucanase and 50 kDa cellobiohydrolase, respectively. To facilitate purification of larger amounts of the pure enzymes for laundry tests, the SP-Sepharose column was overloaded. Under these conditions the 50 kDa endoglucanase eluted before the NaCl gradient but at a higher enzyme concentration than in the material applied to the column (see Table 2). Evidently, the 50 kDa endoglucanase was first bound to the column and then displaced by more strongly binding proteins.

3.5. Properties of the purified 20 kDa endoglucanase

The 20 kDa endoglucanase was relatively heat stable. When it was heated at 7 μg/ml in 25 mM Tris–HCl (pH 7.2 at room temperature) containing 0.2 mM EDTA and then cooled and assayed at pH 7.0 and 50 °C, 52 and 35%, respectively, of the endoglucanase activity remained after 30 or 60 min at 100 °C and 70% after 60 min at 80 °C. It had an apparent optimum temperature of 70 °C during 10 min assays at pH 7.0 (Fig. 3a). At 50 °C and with 60 min reaction times (i.e. approaching commercial conditions) the 20 kDa endoglucanase exhibited 80% or more of its maximum activity throughout the pH range 4–9, and nearly 50% at pH

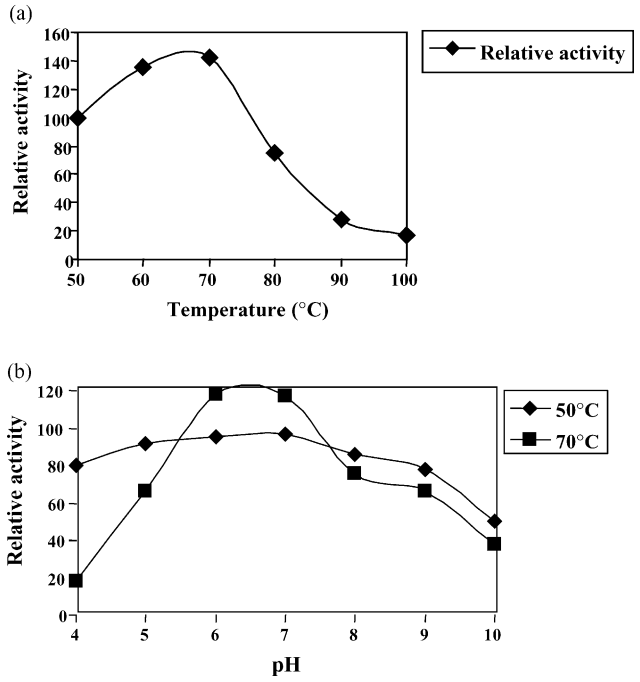

Fig. 3. (a) The temperature dependence of the endoglucanase activity of the 20 kDa endoglucanase at pH 7.0 with a reaction time of 10 min. (b) The pH dependence of the endoglucanase activity of the 20 kDa endoglucanase at 50 and 70 °C with a reaction time of 60 min.

10 (Fig. 3b). In these 60 min assays between pH 5.5 and 7.5 the enzyme was more active at 70 °C than at 50 °C and between pH 8 and 10 it was only slightly less active at 70 °C than at 50 °C. No activity of 20 kDa endoglucanase was detectable for 4-methylumbelliferyl-β-D-lactoside (MUL), a characteristic substrate of cellobiohydrolase I.

3.6. Properties of the purified 50 kDa endoglucanase

Pure 50 kDa endoglucanase had activity both against hydroxyethylcellulose and 4-methylumbelliferyl-β-D-lactoside (1500 ECU/ mg and 210 MUL/mg). The apparent molecular mass of 50 kDa endoglucanase determined by SDS–PAGE decreased by about 2–5 kDa when the protein was treated with endoglycosidase H_f, indicating that the enzyme contains carbohydrate removable by this endoglycosidase (Fig. 1). The 50 kDa-endoglucanase had an apparent optimum temperature between 65 and 70 °C at pH 7.0 and with 60 min reaction times (Fig. 4a). Even with this long reaction time it still exhibited at 75 °C 50% of the activity observed at 50 °C. The pH optimum was broad at 50 °C: with 60 min reaction times essentially constant activity was observed between pH 4.4 and 7 (Fig. 4b). At 70 °C there was an optimum at pH 6. Between pH 5 and 7, the activity was three-fold or more greater at 70 °C than at 50 °C. However, at pH 4.4 and pH values above 8 the activity was greater at 50 °C than at 70 °C suggesting that the stability of the enzyme decreases at 70 °C out side the pH range 5–7.5.

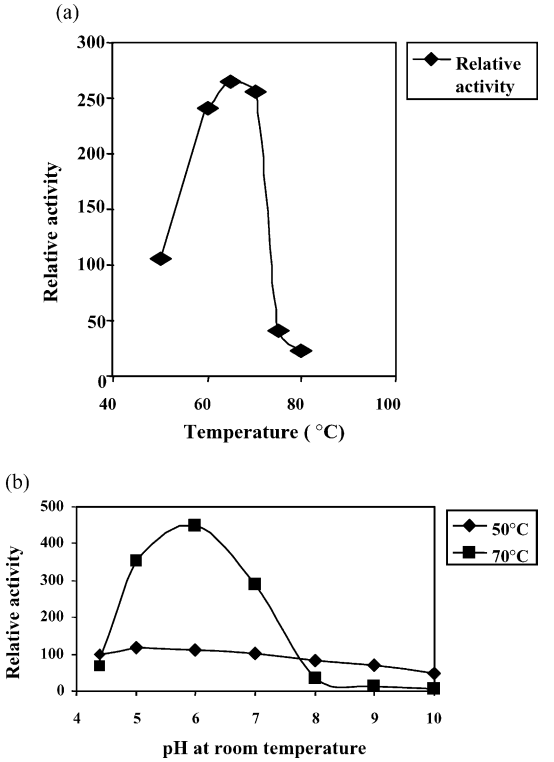

Fig. 4. (a) The temperature dependence of the endoglucanase activity of the 50 kDa endoglucanase at pH 7.0 with a reaction time of 60 min. (b) The pH dependence of the endoglucanase activity of the 50 kDa endoglucanase at 50 and 70 °C with a reaction time of 60 min.

3.7. Properties of the 50 kDa cellobiohydrolase

No endoglucanase activity could be measured for the 50 kDa cellobiohydrolase with hydroxyethylcellulose at pH 5 and 7. At pHs 5 and 7 the 50 kDa cellobiohydrolase had a low cellobiohydrolase I activity (not more than 2 MUL/mg). The activity of 50 kDa cellobiohydrolase towards filter paper at pH 4.8 was under the detection limit. Fifty kiloDaltons cellobiohydrolase released reducing sugars from acid swollen, amorphic Solca Floc-cellulose at pH 5 and 7. However, reproducible determination of this activity was not possible due to difficulties in preparing homogeneous substrate.

3.8. Peptide analyses

Amino acid sequences of tryptic peptides derived from the 20 kDa endoglucanase and 50 kDa cellobiohydrolase were analyzed and are shown in Table 4. The 50 kDa endoglucanase was unusually resistant to tryptic digestion, indicating that it has an unusually stable structure. However, it was cleaved by treatment with cyanogenbromide, and the resulting fragments could then be digested with trypsin or with lysylendopeptidase C. Sequences of the peptides so obtained are shown in Table 4. The partial sequences of these cellulases showed homology towards, e.g. *Humicola*

Table 4
N-Terminal amino acid sequences of tryptic peptides from the 20 kDa endoglucanase and 50 kDa cellobiohydrolase and of tryptic peptides from CNBr-digested 50 kDa endoglucanase

Peptide number #	Sequence
20 kDa EG	
429, N-terminal	ANGQSTRYWDCCKPSCGWRGKGPVNQPVYS
430	YGGISSR
439	QECDSFPEPLKPGCQWR
9	RHDDGGFA
14	YWDCCKP
16	GKGPVNQPVYSCDANFQR
17	VQCPEELVAR
28	DWFQNADNPSFTFER
30	TMVVQSTSTGGDLGSNHFDLNIPGGGVGLF
50 kDa EG	
534	vGNPDFYGK
535	FGPIGSTY
631	LSQYFIQDGeRK
632	FTVVSRFEENK
636	HEYGTNVGSRFYLMNGPDK
50 kDa CBH	
507	VYLLDETEHR
509	xxLNPGGAYYGT
563	MsEGAECEYDGVCDKDG
565	NPYRVxITDYYGNS
603	DPTGARSELNPGGAYYGTGYxDAQ
605	xxVPDYhQHGVda
611	LPxGMNSALYLSEMDPTGARSELNP
612	VEPSPEVTYSNLRxGEIxgxF
619	DGCGWNPYRVvITtDYYnN
620	LPCGMxSALY
621	ADGCQPRTNYIVLDdLIHPxxQ

Uncertain residues are shown in lower case.

and *Fusarium* cellulases. Cloning of the genes encoding 20 and 50 kDa endoglucanases and 50 kDa cellobiohydrolase as well as more detailed comparison of the cellulases with their homologous counterparts are presented in [15].

3.9. Use of the purified cellulases in biostoning

The purified 20 kDa endoglucanase was tested in biostoning at neutral pH without the addition of other enzymes (Table 5). The unfractionated *M. albomyces* ALKO4237 growth medium resulted in lightening, L_{right} of 6.1 with the 20 mg/g concentration. Pure 20 kDa endoglucanase resulted in the same degree of lightening ($L_{\text{right}} = 6.0$) at the 1/80th the protein dosage. Further, there was less backstaining onto the reverse side face of the fabric: L_{reverse} of 20 kDa was −0.5 compared to −2.9 of the unfractionated growth medium and b_{reverse} was 3.6 compared to 5.5. Fabric treated with 20 kDa endoglucanase had also an agreeable soft texture which was felt by hand.

Fifty kiloDaltons endoglucanase and 50 kDa cellobiohydrolase did not by themselves increase the release of indigo dye from the outer face of the denim (i.e. L_{right} did not increase) at the used dosages (Table 6). But 50 kDa endoglucanase repeatedly decreased the backstaining of dye onto the inner face of the denim (L_{reverse} became more positive and b_{reverse} became smaller) when used together with 20 kDa endoglucanase. Fifty kiloDaltons cellobiohydrolase decreased backstaining when used alone and when used in combination with 20 kDa endoglucanase. However, it decreased the effect of 20 kDa endoglucanase on the outside face, and it did not decrease backstaining caused by 20 kDa endoglucanase so well as did 50 kDa endoglucanase.

Table 5
Indigo dye release by the purified 20 kDa endoglucanase at pH 7

Sample	Dosage mg protein/g fabric	L_{right}	L_{reverse}	b_{reverse}
Buffer alone	–	3.6	0.5	0.6
Whole medium	20	6.1	−2.9	5.5
Purified 20 kDa	0.07	5.3	−0.4	2.9
Purified 20 kDa	0.25	6.0	−0.5	3.6
Purified 20 kDa	0.72	8.9	−1.1	4.7

The whole medium indicates the unfractionated *M. albomyces* ALKO4237 concentrated growth medium.
L: lightness unit of the fabric after the treatment minus lightness unit of the fabric before the treatment, *b*: blueness unit of the fabric after the treatment minus blueness unit of the fabric before the treatment.

Table 6
Indigo dye release by the purified 20 kDa endoglucanase, the 50 kDa endoglucanase and the 50 kDa cellobiohydrolase at pH 7

Sample	Dosage mg protein/g fabric	L_{right}	L_{reverse}	b_{reverse}
Buffer alone	–	2.8	−0.6	1.6
20 kDa endoglucanase	0.18	5.6	−1.0	4.0
	0.09	4.8	−1.5	3.3
50 kDa endoglucanase	0.15	2.6	−0.3	1.0
	0.075	3.0	0.4	1.3
50 kDa cellobiohydrolase	0.31	2.8	1.3	0.8
	0.15	2.7	1.5	0.5
20 kDa endoglucanase + 50 kDa endoglucanase	0.18 + 0.075	5.6	0.3	2.5
	0.09 + 0.075	5.1	0.3	2.5
20 kDa endoglucanase + 50 kDa cellobiohydrolase	0.18 + 0.15	4.7	0.0	3.0

L: lightness unit of the fabric after the treatment minus lightness unit of the fabric before the treatment, *b*: blueness unit of the fabric after the treatment minus blueness unit of the fabric before the treatment.

4. Discussion

The Launder-Ometer method was successfully used in screening and purification of novel cellulases for biostoning of denim fabric in neutral conditions (pH 7). Cellulase preparations produced by the strains *M. albomyces*, *M. thermophila*, *C. thermophilum* and *S. thermophilum* were compared in biostoning. The cellulase preparation from *M.*

albomyces was the most effective in releasing dye from the indigo dyed denim fabric. Moreover, backstaining was low. Backstaining has been claimed to be dependent on pH with lower redeposition at pH 7 than at pH 5 [3,22]. Backstaining has been attributed to the affinity of indigo for cellulases and the strong ability of cellulases to bind to cotton cellulose [23]. Studies with commercial cellulases showed that indigo had different affinities for cellulase proteins from different fungal origins. The acid cellulases from *T. reesei* have a higher affinity for indigo dye than neutral cellulases of *H. insolens* [24]. According to our studies backstaining at pH 7 is dependent on the origin of the cellulase preparation. The culture supernatant of *C. thermophilum* caused extremely high backstaining at pH 7 contrary to the culture supernatants of *M. albomyces*, *M. thermophila* or *S. thermophilum* (Table 3). Because of the intensive backstaining the wash effect (high L_{right}) was not clearly detected in denim fabrics washed with the culture supernatant of *C. thermophilum*.

M. albomyces produces at least three different cellulases. In this work we report the isolation of pure cellulases from *M. albomyces*: two endoglucanases and one cellobiohydrolase. The only clear enzyme activity that we found for preparations of the 50 kDa cellobiohydrolase purified from *M. albomyces* was relatively low hydrolysis of the cellooligosaccharide MUL. Because the protein also has sequence similarities to *Humicola grisea* cellobiohydrolase I [15], we have named it 50 kDa cellobiohydrolase.

The 20 kDa endoglucanase can be used over a wide range of pH and at temperatures up to at least 70 °C. The 20 kDa endoglucanase proved to be suitable for applications in which it may be exposed to elevated temperatures, since it is a relatively heat stable enzyme. The 50 kDa endoglucanase is also a stable enzyme that exhibits endoglucanase activity over a wide range of pH values and at high temperatures. Because of these properties both of the endoglucanases are suitable for use in many conditions. For example in compositions of household detergents it is required that cellulases act at higher pHs (pH 7–10) and in home washings a temperature of 60 °C is common but even 90 °C can be used. These cellulases can possibly also be used in the deinking of waste papers, where action of an enzyme at pHs of 7–9 is needed.

The 20 kDa endoglucanase crystallized spontaneously in the SP-Sepharose eluates in Na-acetate, pH 4.0. The spontaneous crystallization facilitated the preparation of high quality crystals. The preliminary crystallographic analysis of the 20 kDa endoglucanase has been presented [25]. In this study crystallization medium containing PEG 4000, sodium acetate and potassium acetate pH 4.6 and microseeding techniques were used to obtain crystals instead of spontaneous crystallization. Spontaneous crystallization can be a disadvantage during production, particularly at lower pH values, which are preferred in large scale cultivations to minimize contamination risks. On the other hand, when more pure and concentrated commercial formulations are desired, the 20 kDa endoglucanase appears to be an excellent candidate because of its easy crystallization. The largest industrial application is the crystallization of glucose isomerase of *Streptomyces rubiginosus*. This crystallization process is presently used in full industrial scale, and the enzyme is used to produce high fructose corn syrup in millions of tons annually [26].

The 20 kDa endoglucanase seemed to be mainly responsible for the good stone-washing effect of *M. albomyces* ALKO4237 growth medium with reasonably low backstaining. Even lower backstaining resulted when the 20 kDa endoglucanase was used together with the 50 kDa endoglucanase. Despite the fact that with the used protein amounts only low enzyme activity could be found for the 50 kDa cellobiohydrolase, this protein reduced backstaining, when used alone or in combination with the 20 kDa endoglucanase. Evidence has been presented that non-polar amino acid residues on the surface of some cellulase molecules may be responsible for binding released indigo dye, thus acting as emulsifier helping the dye to float out of the cellulose fibers [14].

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Cloning of cellulase genes from *Melanocarpus albomyces* and their efficient expression in *Trichoderma reesei*

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Abstract

In our previous study, three purified cellulases of *Melanocarpus albomyces* proved to be effective in biostoning application at neutral pH [Enzyme Microb. Technol., accepted for publication]. We cloned and sequenced three genes of *M. albomyces*, which encode a 20 kDa and two 50-kDa polypeptides. The 20-kDa protein (Cel45A) and one of the 50-kDa proteins (Cel7A) are endoglucanases of the glycosyl hydrolase families 45 and 7, respectively. The other 50-kDa protein (Cel7B) is a family 7 cellobiohydrolase. None of the cellulases harbors a cellulose binding domain (CBD). These genes were expressed in *Trichoderma reesei* under the control of the *T. reesei cbh1* promoter and the proteins detected in the culture medium. The endoglucanase production levels of the *cel45A*- and *cel7A*-transformants were several times higher than those of the parental *M. albomyces* strain. The sizes of Cel45A, Cel7A and Cel7B proteins produced by the transformants were the same as the sizes of the corresponding proteins purified from *M. albomyces*. Cellulase preparations produced by the *cel45A* transformants performed well at neutral pH in stone-washing of denim fabric and caused considerably less backstaining as compared to the acid cellulase product of *T. reesei*.

Keywords: Cloning; Endoglucanase; Heterologous expression; Biostoning

1. Introduction

Cellulases catalyze the hydrolysis of cellulose and may be either cellobiohydrolases (CBH) or endoglucanases (EG). CBHs hydrolyze crystalline cellulose initiating their action from the ends of the chains and produce primarily cellobiose. EGs catalyze hydrolysis internally in the cellulose chain and attack the amorphous regions in cellulose. Most cellulases have a catalytic domain linked by an extended linker region to one or more cellulose-binding domains (CBDs). Cellulases may be classified into glycoside hydrolase families based on the structure and function of the catalytic core [2]. Cellulases are used in detergents and treatments of cellulose-containing textile materials during their manufacture and finishing [3–5], e.g. biostoning to impart a stone-washed effect on indigo-dyed denim fabric. Cellulases derived from fungi such as *Trichoderma* and *Humicola* species are commonly used in textile applications, and most are acid cellulases active in the range of pH 4.5–6. Cellulases active at higher pH (6–10) are especially useful in the textile industry in biostoning [6] and in detergents [7]. Some neutral and alkaline cellulases have been identified and corresponding genes cloned [8,9], e.g. the seven different cellulases representing five families of glycoside hydrolases, namely 5, 6, 7, 12 and 45 from *Humicola insolens* [8].

We have previously purified and characterized three neutral cellulases (pH optima in the range of 6–8), namely a 20- and 50-kDa endoglucanase and a 50-kDa cellobiohydrolase from the fungal ascomycete *Melanocarpus albomyces* [1]. The cellulases produced by *M. albomyces* worked well in biostoning, with low backstaining (redeposition of released indigo-dye in the fabric). The overall aim of our work is to improve biostoning processes by reducing backstaining, by shortening wash time and by identifying cellulases that act at broad pH range with less need for an efficient buffering system to control the pH. In this paper we report the cloning and sequencing of three genes encoding the neutral

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cellulases of *M. albomyces*. We have expressed the cloned neutral cellulase genes of *M. albomyces* at high levels in a heterologous host *Trichoderma reesei* under the control of the strong cellobiohydrolase 1 promoter of the host fungus. This made possible the use of the cellulase preparations produced by the transformants in biostoning application at neutral pH. A preliminary report of this work has been published in Haakana et al. [10].

2. Materials and methods

2.1. Strains, media and transformation of Trichoderma

M. albomyces (ALKO4237, CBS 685.95) was used as the donor for the cellulase genes. For isolation of chromosomal DNA [11] *M. albomyces* was grown in shake flasks for 3 days in potato dextrose medium (Difco, USA) at 42 °C and 250 rpm. *Escherichia coli* XL1-Blue (Stratagene, USA) was used for propagation of plasmids. *E. coli* XL1-Blue MRA (P2) (Stratagene) was used as the host for a genomic library of *M. albomyces*. *T. reesei* ALKO3620 (the endoglucanase 2 gene has been deleted [12]) was used as a host in the transformations. Transformations of *Trichoderma* were performed as previously described [13] with modifications [14]. The plates and media for transformation of *Trichoderma* through selection for *amdS* or *hygB* were as previously described [13,15]. The transformants were purified through subculture of single conidia. *T. reesei* ALKO4072, which is a Δ*cbh1* Δ*egl2* strain (Marja Paloheimo, Roal Oy, Finland, unpublished), was used as a control for the *T. reesei* EGII- and CBHI-negative Cel7B transformants. *T. reesei* transformants were grown on a lactose-based complex medium [12] except that 5% (w/v) KH_2PO_4 was used instead of 1.5% (w/v). Cultures were incubated in shake flasks for 7 days (30 °C, 250 rpm).

2.2. Construction and screening of genomic library

Chromosomal DNA of *M. albomyces* was isolated [11], partially digested with *Sau*3A, and treated with calf intestine alkaline phosphatase. DNA ranging from 5 to 15 kb in size was ligated into Lambda DASH^{R}II with Gigapack II according to the manufacturer's instructions (Stratagene) and transformed into *E. coli* XL1-Blue MRA (P2)-cells. The plates were incubated at 37 °C overnight, and plaques transferred onto a nylon filter (Hybond, Amersham Biosciences, USA). Purified PCR fragments, carrying a portion of the *cel45A*-, *cel7A*- or *cel7B*-cellulase gene, were labeled with digoxigenin (Roche Applied Science, USA). Positive clones were picked and transferred to SM [16] buffer/chloroform, and purified again in a second round of screening.

2.3. General molecular techniques

Standard DNA techniques as described in Sambrook et al. [16] were used. Enzymes for the DNA manipulations were purchased from Roche (Switzerland) and New England Biolabs (USA). The *cel45A*-, *cel7A*- and *cel7B*-cellulase genes were amplified by PCR. The degenerate primers (Table 1) used to amplify the genes were based on previ-

Table 1
Degenerate primers based on the peptide sequences to amplify the genes encoding the 20- and 50-kDa endoglucanases and the 50-kDa cellobiohydrolase of *M. albomyces* by PCR

Cellulase	Peptide	Primer
		*Eco*RI
20 K EG (Cel45A)	#429	5'-ATA GAA TTC TA(C/T) TGG GA(C/T) TG(C/T) TG(C/T) AA(A/G) CC
		Y W D C C K P
		*Eco*RI
	#28-rev	5' -ATA GAA TTC TT (A/G) TC (A/C/G/T)GC (A/G)TT (C/T)TG (A/G)AA CCA
		N D A N Q F W
		5' -GA(C/T) GA(A/G) AC(A/C/G/T) GA(A/G) CA(C/T) (A/C)G
50 K EG (Cel7A)	#507	D E T E H R
		5' -TA (A/C/G/T)GC (A/C/G/T)CC (A/C/G/T)CC (A/C/G/T)GG (A/G)TT
	#509-rev	Y A G G P N
		5'-AA(A/G) CA(C/T) GA(A/G) TA(C/T) GG(A/C/G/T) AC
50 K CBH (Cel7B)	#636	K H E Y G T
		5'-CC (A/G)TA (A/G)AA (A/G)TC (A/C/G/T)GG (A/G)TT
	#534-rev	G Y F D P N

ously described peptides [1]. The reaction mixture contained 0.1 mM of each dNTP, 0.5 μg of each primer (Table 1) and 1–1.5 μg of chromosomal DNA in 100 μl reaction volume in the buffer supplied by Finnzymes Oy (Finland). The amplification protocol was: denaturation at 95 °C (5 min) was followed by addition of DyNazyme (2 units, Finnzymes Oy), and 30 cycles at 95 °C (1 min), 56 °C (1 min) for *cel45A* and *cel7A* or 48 °C (1 min) for *cel7B*, and 72 °C (1 min) with a terminal 72 °C incubation of 8 min. PCR products were purified from agarose gels by the Magic PCR Preps (Promega) and subcloned into pBluescript II SK+ (Stratagene). Plasmid DNA was isolated by Magic minipreps (Promega). Amplified fragments and the genomic genes were sequenced with ABI kits (Applied Biosystems, USA) based on fluorescence-labeled T3 and T7 primers or, with the sequence-specific primers. The Taq cycle sequencing protocol was used with the fluorescence-labeled dideoxynucleotides according to the supplier's instructions. Sequencing reactions were analyzed on an ABI373A sequencer. Sequences were characterized by using the Genetics Computer Group Sequence Analysis Software Package, version 7.2, and the EMBOSS (European Molecular Biology Open Software Suite) program package, version 2.4.1.

2.4. Plasmids

Plasmids pALK1231 and pALK1235 (Fig. 1) were constructed for expression of the *M. albomyces cel45A* cellulase gene. Plasmids pALK1231 and pALK1235 contained identical expression cassette regions: A 0.9 kb *cel45A* fragment beginning with an ATG-codon, the 2.2 kb *T. reesei cbh1* promoter, and the 0.7 kb *Ava*II fragment of *T. reesei cbh1* terminator [14]. By using PCR, the nucleotides (10 bp) missing from the *cbh1* promoter were added resulting in an exact promoter fusion to the first ATG of the *cel45A* cellulase gene. pALK1231 also contains a 1.7 kb *Bam*HI–*Eco*RI 3′-fragment of *cbh1* as sequence homology for targeting integration to the *cbh1* locus of *T. reesei* and the *amd*S gene in a 3.1 kb *Spe*I–*Xba*I fragment [17] for selection of transformants. pALK1235 contains 1.8 kb *Sca*I–*Stu*I *egl1* 5′ and 1.6 kb *Sca*I–*Xho*I *egl1* 3′-fragments of *T. reesei* that target integration to the *egl1* locus, and a 2.2 kb *Not*I–*Pvu*II fragment [15] that confers resistance to hygromycin B. pALK1238 and pALK1240 (Fig. 1) were constructed for expression of *M. albomyces cel7A* gene and are analogous to pALK1231 and pALK1235, respectively, except that the gene coding for *cel45A* was replaced by a 1.7 kb fragment of the *cel7A* cellulase gene beginning with the ATG-codon. pALK1242 (Fig. 1) was constructed for expression of *M. albomyces cel7B* gene and is analogous to pALK1231 except that the *cel45A* gene was replaced by a 2.5 kb *cel7B*-cellulase gene. The *Not*I site in the *cel7B* sequence was inactivated by a silent point mutation with PCR. The plasmids were constructed using pUC19 (EMBL database accession no. L09137) as the basic vector.

2.5. Enzyme activity and protein assays

Enzyme activities are reported as nkat/ml. EG activity was measured in culture supernatant as the release of re-

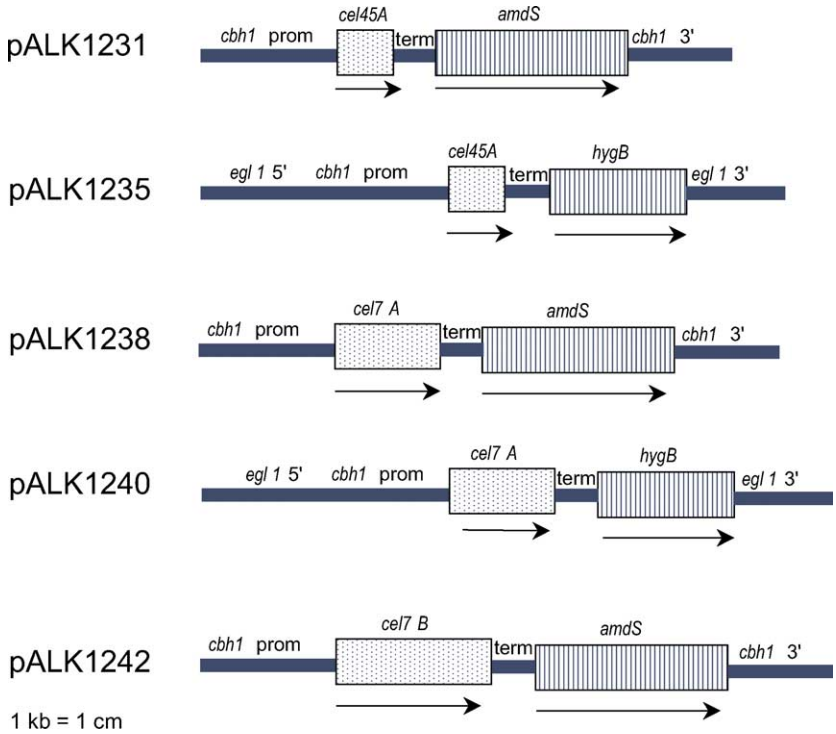

Fig. 1. Schematic presentation of fragments used for transformations of *T. reesei* ALKO3620 for expression of *M. albomyces cel45A*, *cel7A* and *cel7B*.

ducing sugars from hydroxyethylcellulose (HEC) or carboxymethylcellulose (CMC) using 2,4-dinitrosalicylic acid [18] with the following modifications: 1% (w/v) hydroxyethylcellulose was used at 50 °C (ECU activity, endoglucanase activity unit), pH 7 or 3% (w/v) carboxymethylcellulose at 50 and 70 °C (NCU activity, neutral cellulase activity unit), pH 7 in 50 mM Hepes buffer. Background activity resulting from *Trichoderma* cellulases was inactivated by incubating the culture supernatant at 70 °C for 20 min before measuring ECU activity at 50 °C, pH 7. Cellobiohydrolase I activity (MUL-CBHI) was measured by using 4-methyl-umberriferyl-β-D-lactoside (MUL) [19,20]. The total MUL activity represents activities of endoglucanase I and cellobiohydrolase I. In the presence of 5 mM cellobiose the CBHI is inhibited and EGI activity can be measured directly. MUL-CBHI activity is calculated by subtracting MUL activity in the presence of cellobiose from the total MUL activity. Protein concentration was determined from TCA-precipitated *T. reesei* culture media by the method of Lowry et al. [21], using bovine serum albumin as the standard.

2.6. Immunological methods

Culture supernatants were blotted onto nitrocellulose filters (Schleicher & Schüll, Germany [22,23]). Protein samples were applied to 10% SDS–PAGE [24]. CBHI and EGI of *T. reesei* were detected by immunostaining with the Protoplot, Western blot AP system (Promega) with the CBHI-specific monoclonal antibody CI-258 and the EGI-specific monoclonal antibody EI-2, respectively [25]. Cel45A, Cel7A and Cel7B-specific polyclonal antibodies were produced in rabbits (Diabor Ltd., Oulu, Finland) and used in a similar manner.

2.7. Biostoning

Cellulase treatments of denim cloth were performed in a LP-2 Launder-Ometer (Atlas, USA). A denim swatch of 7 g was loaded into 200 ml of 50 mM Na-citrate–phosphate buffer, pH 7 or 50 mM Na-citrate buffer, pH 5.2, in a 1.2 l container, after which 10 steel balls (diameter 0.6 cm) were added. Cellulase preparations from transformants ALKO3620/1235/49, ALKO3620/1231/14 and ALKO3620/1231/16, which all express Cel45A, were used for biostoning. One hundred and 400 ECU (pH 7) of cellulase preparation per gram of fabric was used for 1 and 2 h at 50 °C. Two hundred ECU (pH 4.8) of acid cellulase product Ecostone L (Primalco Ltd. Biotec, Finland) per gram of fabric was used as a control. After cellulase treatment the swatches were soaked for 10 min in 10 mM NaOH, rinsed with water and dried. The stone-washing effect was evaluated by measuring the color of the fabric as reflectance values with the Minolta (Japan) Croma Meter 1000R using the $L^*a^*b^*$ system (illuminant D65).

2.8. Nucleotide sequence accession numbers

Nucleotide sequences of the *M. albomyces* CBS 685.95 *cel45A*, *cel7A* and *cel7B* genes have been submitted to EMBL database under accession numbers AJ515703, AJ515704 and AJ515705, respectively.

3. Results

3.1. Cloning and characterization of the M. albomyces cel45A-gene

The amplified 600-bp fragment encoded the majority of the 20-kDa cellulase-derived peptides [1]. Four positive clones were found after screening the genomic library of *M. albomyces* with the PCR fragment derived from the 20-kDa endoglucanase. The PCR fragment used as a probe corresponds to nucleotides 188–769 in the *cel45A* gene sequence. The 936-bp fragment encompasses an open reading frame (ORF) of 849-bp coding for 235 amino acids disrupted by two predicted introns. The putative signal peptide processing site is after A21, and the N-terminus of the mature protein begins with A22. The ORF predicts a protein with a molecular weight of 25 kDa for the full-length preprotein, and 22.9 kDa for the mature protein which is consistent with the results obtained with the purified protein [1]. All peptide sequences also occurred in the predicted protein sequence.

The 20-kDa endoglucanase of *M. albomyces* belongs to the family 45 of glycosyl hydrolases [2] and was named *Ma* Cel45A-cellulase (accession no. AJ515703). *Ma* Cel45A-cellulase has sequence similar to that of the *Thielavia terrestris* endoglucanase (AR094310, 76% identity in a 234 amino acid overlap) and the *H. insolens* endoglucanase V (A23635, 76% identity in a 235 amino acid overlap) (Table 2). Unlike the other two enzymes, the *M. albomyces* Cel45A-cellulase lacks consensus cellulose binding domain and its associated linker. The proposed active site aspartates are at positions 10 and 120 of the mature protein.

3.2. Cloning and characterization of the M. albomyces cel7A-gene

The amplified 160-bp fragment encoded a peptide similar to *H. grisea* endoglucanase I that corresponded to peptide no. 612 [1] from the purified 50-kDa endoglucanase. Ten positive clones were found after screening the genomic library of *M. albomyces*. *Ma cel7A* (accession no. AJ515704) contains an ORF of 1364 bp in length, interrupted by one intron. The ORF codes for 428 amino acids. The predicted full-length protein has a molecular weight of 46.8 kDa, and the mature protein after signal peptide cleavage 44.8 kDa. All the Cel7A peptides sequenced [1] were found in the predicted protein sequence. The PCR fragment used as a

Table 2
Comparison of the *M. albomyces* cellulases with their homologous counterparts. The alignment was performed using the Water programme of the EMBOSS programme package (gap penalty = 10.0, extend penalty = 0.5)

Organism	Enzyme	Length[a]	Alignment[b]	Similarity[c] (%)	Identity (%)	Accession no.
M. albomyces	Cel45A	235*	235/235	100	100	AJ515703
Thielavia terrestris	Endoglucanase	299	236/234	87.7	76.3	AR094310
Humicola insolens	EGV	306	236/235	86.4	75.8	A23635
Humicola grisea var *thermoidea*	EGIII	305	236/235	86.9	75.4	AB003107
Fusarium oxysporum	Family K endoglucanase	376	235/235	76.8	63.3	L29381
H. grisea var *thermoidea*	EGIV	227*	195/193	73.3	58.5	AB003108
Scopulariopsis brevicaulis	EGI	229*	228/221	70.2	55.3	JC7308
Trichoderma reesei	EGV	242	164/218	35.3	23.1	Z33381
M. albomyces	Cel7A	428*	428/428	100	100	AJ515704
H. grisea var *thermoidea*	EGI	435*	419/416	85.0	72.8	D63516
H. insolens	EGI	435*	419/416	83.8	71.4	AR012244
Myceliophthora thermophila	EGI	456*	446/427	82.1	71.3	AR071934
F. oxysporum var *lycopersici*	EGI	429*	413/414	74.8	59.1	AF292710
F. oxyspoium	Family C endoglucanase	429*	415/414	75.5	59.9	L29378
T. reesei	EGI	459	392/414	57.8	45.1	P07981
M. albomyces	Cel7B	452*	452/452	100	100	AJ515705
H. grisea var *thermoidea*	CBH I.2	451*	448/449	88.2	78.0	AF123441
Thermoascus aurantiacus	CBH	456*	453/450	68.1	51.9	AF421954
Alternaria alternata	Exoglucanase	423*	422/450	69.8	53.5	AF176571
F. oxysporium	Family C cellobiohydrolase	514	451/450	66.9	50.4	L29379
T. reesei	CBHI	513	446/449	66.5	50.0	P00725

[a] Length of the protein, including the signal peptide. Proteins marked with asterisk (*) lack a consensus cellulose binding domain (CBD).
[b] Length of the sequences used in the alignment (comparison sequence/*M. albomyces* sequence).
[c] The EBLOSUM62 was used for the substitution matrix.

probe corresponds to the nucleotides 620–780 in the *cel7A* gene. The *Ma* Cel7A protein lacks a consensus cellulose binding domain, is similar to *H. grisea* endoglucanase I (Table 2), and belongs to the glycosyl hydrolase family 7 [2].

3.3. Cloning and characterization of the M. albomyces cel7B-gene

Several bands were detected by agarose gel electrophoresis after PCR amplification. One of several fragments was 700 bp in length, the size of the *H. grisea* cellobiohydrolase I gene. This fragment encoded peptide #636 from the 50 kDa cellobiohydrolase [1]. Three positive clones were found after screening the genomic library of *M. albomyces* with the PCR fragment derived from the 50 kDa cellobiohydrolase. *Ma cel7B* (accession no. AJ515705) contains an ORF of 1735 bp in length interrupted by five introns. The PCR fragment used as a probe corresponds to nucleotides 508–1195 of the *cel7B* gene. The ORF codes for 452 amino acids. The predicted full-length protein has a molecular weight of 49.9 kDa and the mature protein after signal peptide cleavage is 47.6 kDa. All the Cel7B peptides sequenced [1] are found in the predicted protein sequence. The *Ma* Cel7B protein lacks consensus cellulose binding domain, is similar to *H. grisea* cellobiohydrolase I (Table 2), and belongs to glycosyl hydrolase family 7 [2].

3.4. Production of the M. albomyces Cel45A endoglucanase in T. reesei

About 80% of the ALKO3620/pALK1231 transformants were CBHI-negative and about 15% of the ALKO3620/1235 transformants were EGI-negative. *T. reesei* CBHI-negative transformants ALKO3620/1231/14 and ALKO3620/1231/16 and the EGI-negative transformants ALKO3620/1235/40 and ALKO3620/1235/49 were evaluated in detail. The *cbh1* gene of *T. reesei* was replaced by one copy of the expression cassette—*amd*S marker fragment of pALK1231 in ALKO3620/1231/14 and ALKO3620/1231/16, and the *egl1* gene was replaced by one copy of the expression cassette—HygB marker fragment of pALK1235 in ALKO3620/1235/40 and ALKO3620/1235/49 (Southern blot, data not shown).

When the transformant strains were grown in shake flasks on cellulase-inducing medium containing 1.5% KH_2PO_4, Cel45A could be detected only by Western blotting with Cel45A—specific polyclonal antibody in samples extracted from the mycelium. The low pH (about 3) of the culture medium at the end of the cultivation was suspected to cause degradation of the neutral cellulase, and therefore the KH_2PO_4 concentration was increased to 5% (w/v). In these cultivations the pH remained above 4, and Cel45A could be detected in the culture supernatant by Western blots. The size of the protein produced by the transformants was the same as that of the native protein (Fig. 2).

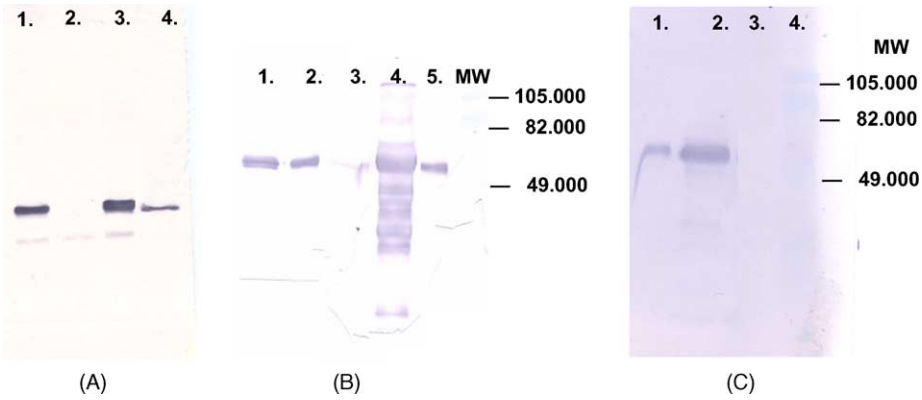

Fig. 2. Western blot of the (A) *cel45A* transformants ALKO3620/1235/40 (*egl1* replacement) and ALKO3620/1231/16 (*cbh1* replacement) and the host strain ALKO3620 with Cel45A-cellulase specific polyclonal antibody, (B) *cel7A* transformant ALKO3620/1240/32 and the host strain ALKO3620 with Cel7A-spesific polyclonal antibody, (C) *cel7B* transformant ALKO3620/1242/13 and the host strain ALKO3620 with Cel7B-spesific polyclonal antibody. (A) Lane 1: 10 μg protein from the culture supernatant of ALKO3620/1235/40; Lane 2: 10 μg protein from the culture supernatant of ALKO3620; Lane 3: 10 μg protein from the culture supernatant of ALKO3620/1231/16; Lane 4: 100 ng of Cel45A-endoglucanase purified from *M. albomyces*. (B) Lane 1: 400 ng protein from the culture supernatant of ALKO3620/1240/32; Lane 2: 100 ng Cel7A-endoglucanase purified from *M. albomyces*; Lane 3: 25 μg protein from the culture supernatant of ALKO3620; Lane 4: 20 μg protein from the culture supernatant of ALKO3620/1240/32; Lane 5: 50 ng Cel7A-endoglucanase purified from *M. albomyces*. (C) Lane 1: 60 ng Cel7B-cellobiohydrolase purified from *M. albomyces*; Lane 2: 10 μg protein from the culture supernatant of ALKO3620/1242/13; Lane 3: 10 μg protein from the culture supernatant of ALKO3620; Lane 4: molecular weight marker.

We tested hypothesis that secreted Cel45A is degraded in the *T. reesei* culture, if the pH is <4 by mixing purified Cel45A into the culture supernatant of *T. reesei* ALKO3620 to a final concentration of 0.1 mg/ml. The mixture was incubated at 30 °C, with samples corresponding to 0.2 μg of Cel45A removed every 15 min and analyzed by Western blot. Band intensity decreases with time and disappears after 60 min of incubation (Fig. 3, data on samples taken after 45 and 60 min are not shown).

In order to elucidate the role of proteolytic enzymes in degradation of the recombinant Cel45A, three parallel mixtures of purified Cel45A (0.1 mg/ml final concentration) and the culture supernatant of *T. reesei* ALKO3620 were made. One of the preparations was supplemented with a final concentration of 1 mM phenylmethylsulfonyl fluoride (PMSF), which is a serine and cysteine protease inhibitor. Another mixture was supplemented with a final concentration of 10 μg/ml Pepstatin A, which is an aspartic protease inhibitor.

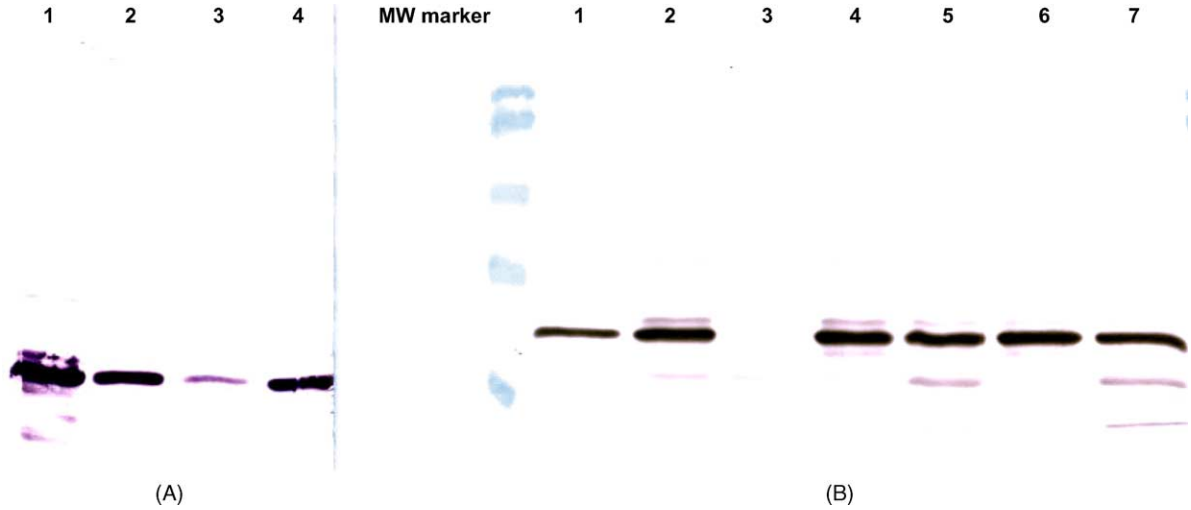

Fig. 3. (A) Time-course of the degradation of Cel45A-endoglucanase in *T. reesei* growth medium during 30 °C incubation. Samples corresponding to 0.2 μg of Cel45A-cellulase from a mixture of Cel45-endoglucanase and *T. reesei* ALKO3620 culture supernatant were subjected to Western blot analysis. The first sample (Lane 1) was taken before the start of a 30 °C incubation period and the subsequent ones after 15 and 30 min (Lanes 2 and 3). Lane 4: 50 ng of purified Cel45A-endoglucanase. (B) Effect of protease inhibitors on the stability of *M. albomyces* Cel45A endoglucanase in *T. reesei* growth medium. Mixtures of the Cel45A endoglucanase and *T. reesei* ALKO3620 culture supernatant at pH 3 were supplemented with protease inhibitors PMSF (1 mM) and Pepstatin A (10 μg/ml). Samples were subjected to Western blot analysis before the start and after the 2 h incubation at 30 °C. Lane 1: 50 ng of purified Cel45A; Lanes 2, 3: sample of the Cel45A and *T. reesei* culture supernatant supplemented with PMSF before (Lane 2) and after (Lane 3); Lanes 4, 5: sample of the Cel45A and *T. reesei* culture supernatant supplemented with Pepstatin A before (Lane 4) and after (Lane 5); Lanes 6, 7: sample of the Cel45A and *T. reesei* culture supernatant supplemented with both PMSF and Pepstatin A before (Lane 6) and after (Lane 7).

Table 3
Production of cellulase activities by *M. albomyces* strain ALKO4237, the *Trichoderma* host strain ALKO3620 (Δ*egl2*) and by the one-copy *T. reesei cel45A*-, *cel7A*- and *cel7B*-transformants with replacement of *cbh1* or *egl1* gene

Strain	CBHI+/−, EGI+/−	Protein (g/l)	NCU (ml)		ECU (ml)		MUL (ml)	
			70 °C, pH 7	50 °C, pH 7	50 °C, pH 7	50 °C, inactive[a], pH 7	Total[b] 50 °C pH 5	CBHI[b] 50 °C, pH 5
Cel45A								
ALKO4237 (the donor)		n.a.	n.a.	n.a.	100[c]	n.a.	n.a.	n.a.
ALKO3620		6.1	48	670	290	16	n.a.	n.a.
A3620/1231/14	CBHI−	4.9	2400	1700	510	100	n.a.	n.a.
A3620/1231/16	CBHI−	4.9	2560	2000	540	100	n.a.	n.a.
ALKO3620		7.7	50	820	340	30	n.a.	n.a.
A3620/1235/40	EGI−	7.4	2400	870	130	90	n.a.	n.a.
A3620/1235/49	EGI−	7.2	2100	820	140	90	n.a.	n.a.
Cel7A								
A3620/1238/42	CBHI−	5.4	6000	4700	n.a.	n.a.	n.a.	n.a.
A3620/1240/32	EGI−	4.4	3500	1700	n.a.	n.a.	n.a.	n.a.
Cel7B								
ALKO3620		4.4	n.a.	n.a.	n.a.	n.a.	42	30.0
ALKO4072	CBHI−	4.3	n.a.	n.a.	n.a.	n.a	16	1.1
A3620/1242/13	CBHI−	3.7	n.a.	n.a.	n.a.	n.a.	16	2.6

Strains were grown for 7 days in cellulase-inducing medium; the results are the average from two flasks; NCU, activity against carboxymethylcellulose; ECU, activity against hydroxyethylcellulose; CBHI cellobiohydrolase I; EGI endoglucanase I. n.a., not analyzed.

[a] Background activity resulting from *Trichoderma* cellulases was inactivated by incubating the culture supernatant at 70 °C for 20 min before measuring the ECU activity at 50 °C and pH 7.

[b] Total MUL (activity with 4-methylumbelliferyl-β-D-lactoside) activity represents activities of endoglucanase I and cellobiohydrolase I. In the presence of 5 mM cellobiose, CBHI activity is inhibited. MUL-CBHI activity is calculated by subtracting MUL activity in the presence of cellobiose from the total MUL activity.

[c] Also contains Cel7A and Cel7B cellulases and other cellulase activities, cultivation was done as described in [1].

The third mixture was supplemented with both of these. The protease responsible for Cel45A degradation is inhibited by Pepstatin A, but not by PMSF (Fig. 3).

Single-copy replacement transformants ALKO3620/1231/14, ALKO3629/1231/16, ALKO3620/1235/49 and ALKO 3620/1235/40 produced 2100–2500 NCU/ml at 70 °C (Table 3). The integration site of the expression cassette, either *cbh1* or *egl1* locus, had no significant effect on the activity levels. The background *T. reesei* endoglucanases contributed to endoglucanase activities (NCU/ml, ECU/ml) measured at 50 °C (pH 7). *T. reesei* endoglucanase activity was lower in the EGI-negative transformants ALKO3620/1235/40 and ALKO3620/1235/49 than in the CBHI-negative transformants ALKO3620/1231/14 and ALKO3620/1231/16. *T. reesei* endoglucanase activity is almost totally heat inactivated at 70 °C after 20 min. *T. reesei* transformants produced substantial amounts of relatively heat stable Cel45A-endoglucanase. The endoglucanase production level of transformants was several times higher than that of the parental strain *M. albomyces* ALKO4237 (Table 3).

3.5. Production of the M. albomyces Cel7A endoglucanase in T. reesei

About 38% of the ALKO3620/pALK1238 transformants were CBHI-negative and about 23% of the ALKO3620/pALK1240 transformants were EGI-negative. The *cbh1* gene of *T. reesei* was replaced by one copy of the pALK1238 expression fragment in ALKO3620/1238/42 and the *egl1* gene was replaced by one copy of the pALK1240 expression fragment in ALKO3620/1240/32 (data not shown).

In culture supernatants ALKO3620/1238/42 produced 4700 NCU/ml at 50 °C, and 6000 NCU/ml at 70 °C (Table 3). ALKO3620/1240/32 produced 1700 NCU/ml at 50 °C and 3500 NCU/ml at 70 °C. The size of the Cel7A protein produced by the ALKO3629/1240/32 transformant was the same as that of purified Cel7A (Fig. 2).

3.6. Production of the M. albomyces Cel7B cellobiohydrolase in T. reesei

Twenty-four percent of the transformants were CBHI-negative. The transformant A3620/1242/13 does not produce CBHI and carries the transformed pALK1242 fragment in place of the *cbh1* gene (data not shown). The Cel7B protein produced by the ALKO3620/1240/13 transformant was the same size as that of the purified Cel7B-cellulase (Fig. 2). The cellulase activity produced by the transformants was measured as MUL activity (Table 3). There was a slight increase in the MUL-CBHI activity at 50 °C in the *cbh1*-negative transformant ALKO3620/1240/13 relative to the Δ*cbh1* Δ*egl2* control strain ALKO4072.

Table 4
Colour measurements of denim fabrics treated with cellulase preparations produced by the *cel45A*-transformants ALKO3620/1235/49 and ALKO3620/1231/14+16. Ecostone L was used as a control

Preparation	Dosage (ECU/g)	Right side		Reverse side	
		L	*b*	*L*	*b*
1 h					
Buffer only	–	2.3	0.6	1.3	1.5
ALKO3620/1235/49 (EGII- and EGI-negative)	100	3.8	1.6	1.6	0.9
	400	4.0	2.3	1.8	1.8
ALKO3620/1231/14+16 (EGII- and CBHI-negative)	100	3.8	1.4	2.8	0
	400	3.7	2.2	2.1	1.6
2 h					
Buffer only	–	2.6	1.7	0.9	0.2
ALKO3620/1235/49	100	4.7	2.7	0.2	2.5
	400	6.1	3.8	0.4	3.6
ALKO3620/1231/14+16	100	4.4	2.4	1.0	1.5
	400	6.1	3.2	1.4	2.4
Ecostone L	200[a]	2.0	2.1	−4.0	4.8

The fabrics were treated for 1 and 2 h with dosages of 100, 200 and 400 ECU/g of fabric as explained in Section 2. The results are the average of two parallel treatments. *L*, lightness unit of the fabric after treatment minus lightness unit of the fabric before the treatment; *b*, blueness unit of the fabric after treatment minus blueness unit of the fabric before the treatment.

[a] Experiment was done at pH 5.2 and the ECU activity was measured at pH 4.8.

3.7. Use of the recombinant Cel45A endoglucanase in biostoning

Cel45A endoglucanase preparations produced by transformants ALKO3620/1231/14 and ALKO3620/1231/16 (CBHI−, EGII−) combined and ALKO3620/1235/49 (EGI−, EGII−) were tested in biostoning at neutral pH (Table 4). Both preparations increased the release of indigo dye from the outer surface of the denim fabric, i.e. L_{right} and b_{right} increased (increase in b_{right} is probably due to low backstaining). The effects improved with treatment time, and after 2-h treatment the L_{right} and b_{right} increased with increasing enzyme dosage. Both preparations resulted in lightening of 6.1 (L_{right}) with 400 ECU/g dosage after 2 h of treatment. Backstaining was slightly lower (higher $L_{reverse}$, lower $b_{reverse}$) with ALKO3620/1231/14+16 (CBHI−, EGII−) cellulase preparation than with the ALKO3620/1235/49 (EGI−, EGII−) preparation. Cel45 preparations caused considerably better stone-washing effect and less backstaining than the acid cellulase product (Ecostone L) of *T. reesei*.

4. Discussion

We report for the first time cloning of cellulases from *M. albomyces*. Three neutral cellulase genes of *M. albomyces* were cloned, sequenced and expressed in *T. reesei*. The cellulases show significant sequence similarity with other neutral cellulases (Table 2), but lack a consensus CBD and a linker region. Cellulases of *T. reesei* and *H. insolens* are commonly used for stone-washing of denim fabric and most of their cellulases contain CBD. The cellulase group of *M. albomyces* seems to have a unique feature, because none of the identified major cellulases harbors a consensus CBD. All the *M. albomyces* cellulases, but especially Cel45A, have excellent stone-washing capability, although they lack CBD. The CBD in *T. reesei* cellulases enhances the enzymatic hydrolysis of insoluble isolated cellulose and chemical pulp [26]. The absence of a CBD might play a role in the low backstaining of the Cel45A-endoglucanase. Cavaco-Paulo et al. [27] have suggested that the prevention of backstaining during stone-washing requires an enzyme with very little affinity for indigo dye and a reduced binding of the cellulase protein to the cotton cellulose. Cellulases from which the CBD had been deleted, both bacterial (*Cellumonas fimi*) and fungal (*H. insolens*), generally decrease indigo staining levels and cause less backstaining than do the intact enzymes [28].

M. albomyces does not secrete proteins efficiently enough for commercial large-scale enzyme production. Heterologous expression of *M. albomyces* cellulases in *T. reesei* one-copy replacement transformants lacking some of the native cellulases produced adequate levels of the *M. albomyces* proteins for large scale application. The background activities caused by *Trichoderma*'s own cellulases can have undesired effects in biostoning, such as backstaining or strength properties of fabric [29]. Therefore transformants deficient in some of the native cellulases were constructed. The expression results indicated that an aspartic protease inhibitor is required to protect Cel45A from proteolytic degradation in *T. reesei* growth medium, in which the pH goes down below pH 4 during cultivation. In order to prevent the proteolytic degradation of recombinant Cel45A in the growth

medium of *T. reesei* without the use of proteolytic inhibitors, the cultivation medium must be composed to sustain the pH above 4 till the end of cultivation.

Cellulase preparation derived from the Cel45A-producing *Trichoderma* strain performed well on biostoning at neutral pH. The same degree of stone-washing with low backstaining could be obtained as with the purified *M. albomyces* Cel45A (20-kDa) endoglucanase [1]. The single-copy transformants are already commercially important. The production level is high enough and the characteristics of the enzyme preparation are suitable for the application, which makes it feasible to use these strains in industrial scale production for the application. The economics of the process can further be improved by expressing the enzymes in another mutant strains, e.g. in protease mutant strains, by using multicopy transformants and complex industrial growth medium. In addition to uses in textile industry, other potential uses for the novel cellulase preparations are in the detergent industry, e.g. color clarification, soil removal and improvement of the fabric-care properties by reducing harshness of the textiles, and in the pulp and paper industry, e.g. deinking of newspapers and magazines.

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Title

Trichoderma reesei strains for production of cellulases for the textile industry

Abstract

Trichoderma reesei is a biotechnically important filamentous fungus used commercially in enzyme production. *T. reesei* is also one of the best known cellulolytic organisms, producing readily and in large quantities a complete set of extracellular cellulases for the degradation of crystalline cellulose. In addition to *T. reesei*, a wide variety of other bacteria and fungi also produce cellulolytic enzymes. Cellulases originating from various organisms and having different characteristics are used industrially in many applications, such as in the textile industry in finishing of denim fabric to impart a stonewashed appearance (biostoning) and in biofinishing of cotton.

In this work *T. reesei* strains producing significant amounts of homologous and heterologous cellulases and having defined cellulase profiles were constructed for specific industrial applications, *i.e.* biostoning and biofinishing of cotton. The production of *T. reesei* endoglucanase II (EGII), cellobiohydrolases I and II (CBHI and CBHII) was improved in separate strains. Strains producing high amounts of EGI and EGII without CBHs or CBHI and CBHII without the main EGs were also constructed. The cellulase genes were expressed under the powerful *T. reesei cbh1* promoter; in a transformant overproducing both CBHI and CBHII, the *cbh2* promoter was also used for *cbh2* expression. The level of endoglucanase activity produced by the EGII-overproducing transformants correlated with the copy number of the *egl2* expression cassette. Production of the major secreted cellulase CBHI was increased up to 1.5-fold and production of CBHII fourfold compared with the parent strain. In transformants overproducing both CBHI and CBHII, production of CBHI was increased up to 1.6-fold and production of CBHII up to 3.4-fold as compared with the host strain and approximately similar amounts of CBHII protein were produced by using the *cbh1* or *cbh2* promoters.

The enzyme preparation with elevated EGII content most clearly improved the biostoning of denim fabric and the biofinishing of cotton fabric. Better depilling and visual appearance were achieved with the enzyme preparation having an elevated CBHII content compared to the wild type preparation in biofinishing of cotton, but the improvement was not as pronounced as in the case of the EGII preparation.

Novel neutral cellulases were demonstrated to have potential in biostoning. The cellulase preparation of the thermophilic fungus *Melanocarpus albomyces* was found to be effective in releasing dye from indigo-dyed denim and to cause low backstaining at neutral pH. *M. albomyces* produces at least three cellulases and these cellulases with an effect on biostoning were purified and the genes encoding them were cloned and sequenced. *Ma* 20 kDa EGV (*Ma* Cel45A) belongs to the glycosyl hydrolase family 45 and the 50 kDa EGI (*Ma* Cel7A) and CBHI (*Ma* Cel7B) to family 7. None of the cellulases harbours a cellulose binding domain. Especially purified *Ma* Cel45A performed well in biostoning. The *Ma* cellulases were produced in *T. reesei* under the *T. reesei cbh1* promoter for biostoning applications. The endoglucanase production levels of *Ma cel45A*- and *cel7A*-transformants were several times higher than those of the parental *M. albomyces* strain. The cellulase preparation produced by the recombinant *Ma cel45A* transformant performed well at neutral pH in the finishing of denim fabric and caused considerably less backstaining than the acid cellulase product of *T. reesei*.

Keywords

cellulase, cloning, *Trichoderma reesei, Melanocarpus albomyces,* homologous and heterologous gene expression, biostoning, biofinishing, textile industry

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