APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 2002, p. 3956–3964 Vol. 68, No. 8
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.8.3956–3964.2002

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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Received 22 January 2002/Accepted 7 May 2002

***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

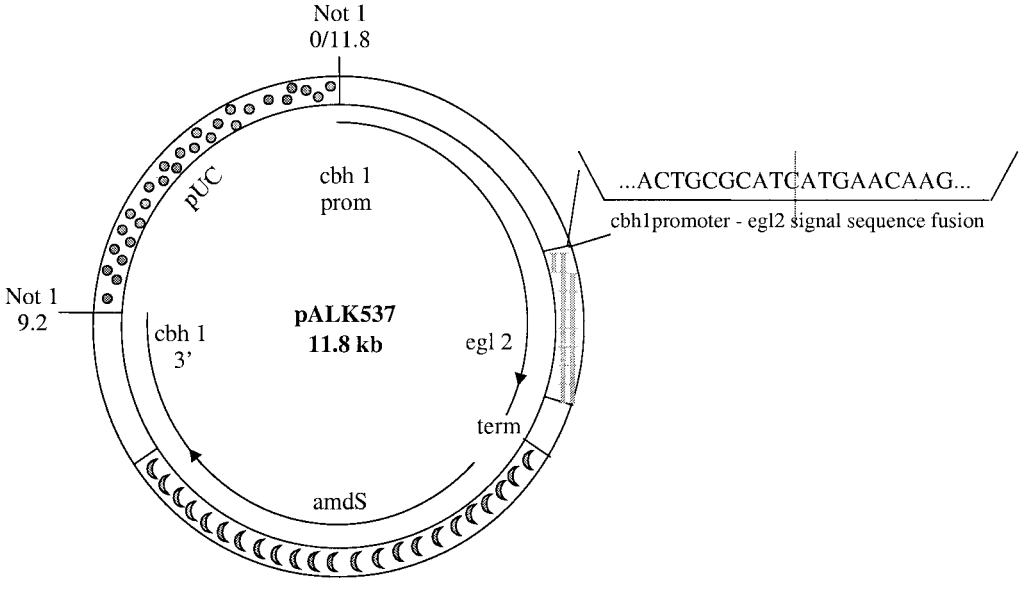

FIG. 1. Restriction map of the plasmid pALK537. The *egl2* cDNA is exactly joined to the *cbh1* promoter. A 9.2-kb *Not*I fragment was isolated from the plasmid for transformation.

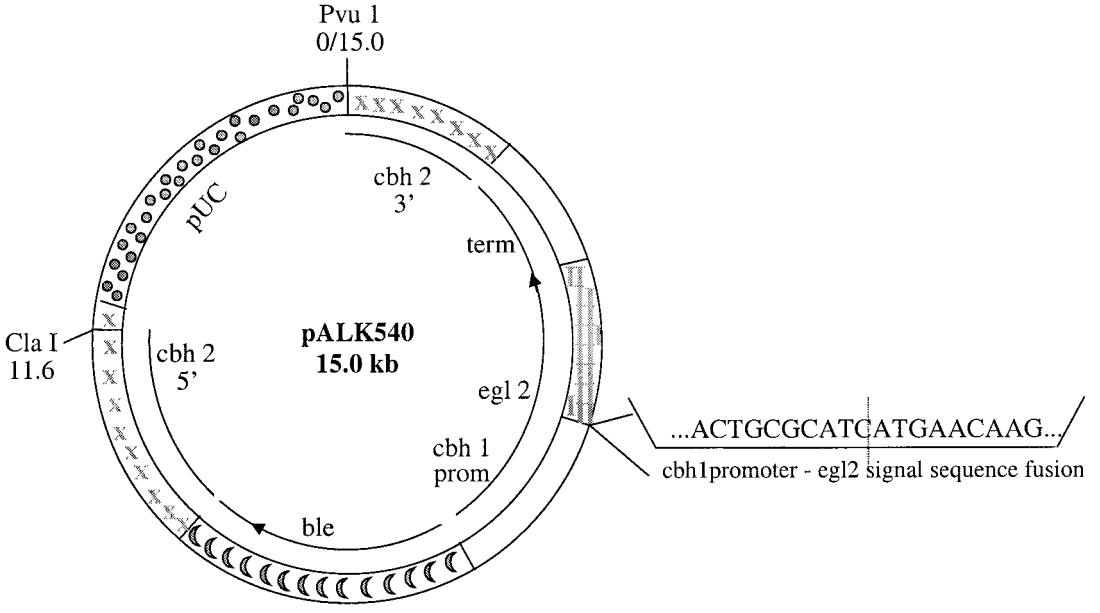

FIG. 2. Restriction map of the plasmid pALK540. An 11.6-kb *Cla*I-*Pvu*I fragment was isolated from the plasmid for transformation.

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

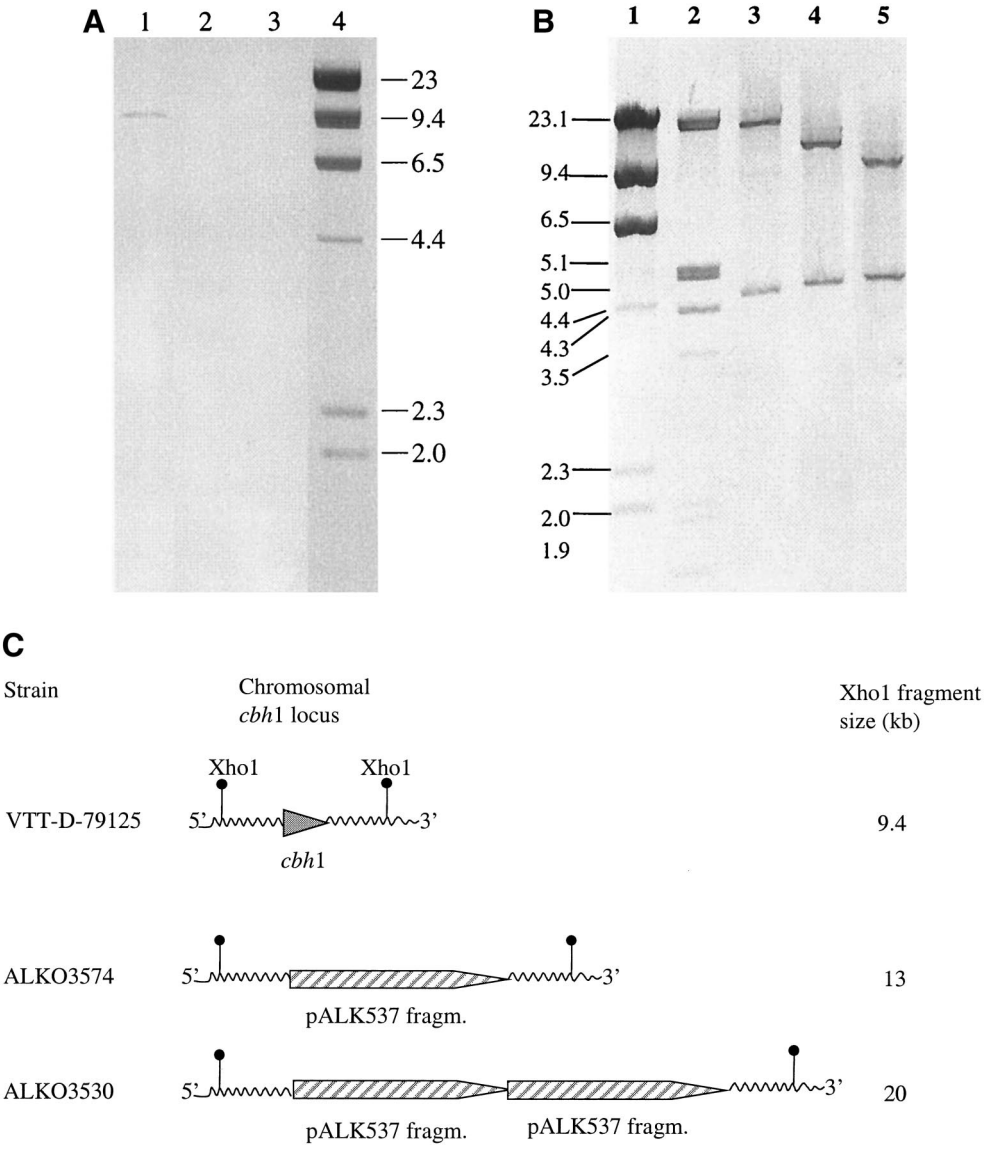

FIG. 3. Southern analysis of transformants ALKO3530 and ALKO3574 in the which *cbh1* locus has been replaced with the 9.2-kb *Not*I fragment from pALK537. (A) Genomic DNA was digested with *Xho*I. Hybridization was done with a *cbh1* probe. Lane 1, VTT-D-79125; lane 2, ALKO3530; lane 3, ALKO3574; lane 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

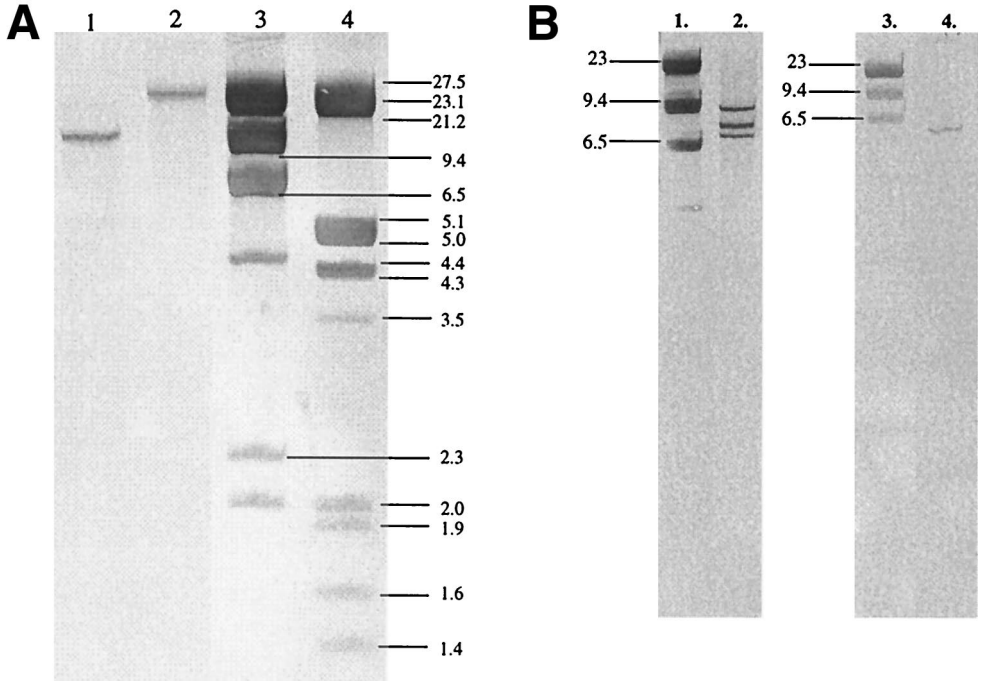

FIG. 4. Southern analysis of the transformant ALKO3529 and the host strain VTT-D-79125. (A) Genomic DNA was digested with *Xho*I. Hybridization was done with a *cbh1* probe. Lane 1, VTT-D-79125; lane 2, ALKO3529; lanes 3 and 4, molecular weight markers λ*Hin*dIII and λ*Eco*RI-*Hin*dIII. (B) Genomic DNA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

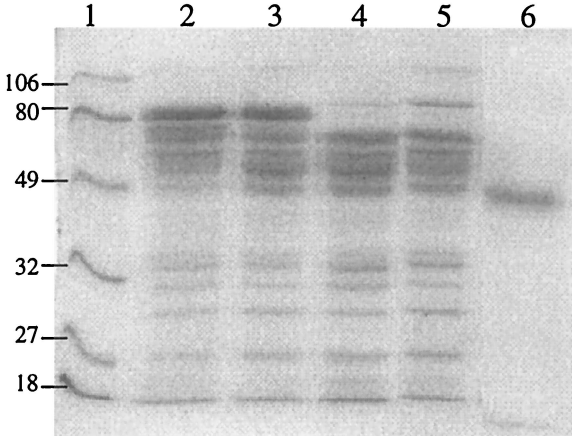

FIG. 5. SDS-PAGE of the samples from the culture supernatants of host strain VTT-D-79125 and the EGII transformants ALKO3529, ALKO3530, and ALKO3574. A total of 20 μg of total secreted protein was loaded in each lane. Lane 1, VTT-D-79125; lane 2, ALKO3529; lane 3, ALKO3530; lane 4, ALKO3574; lane 5,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

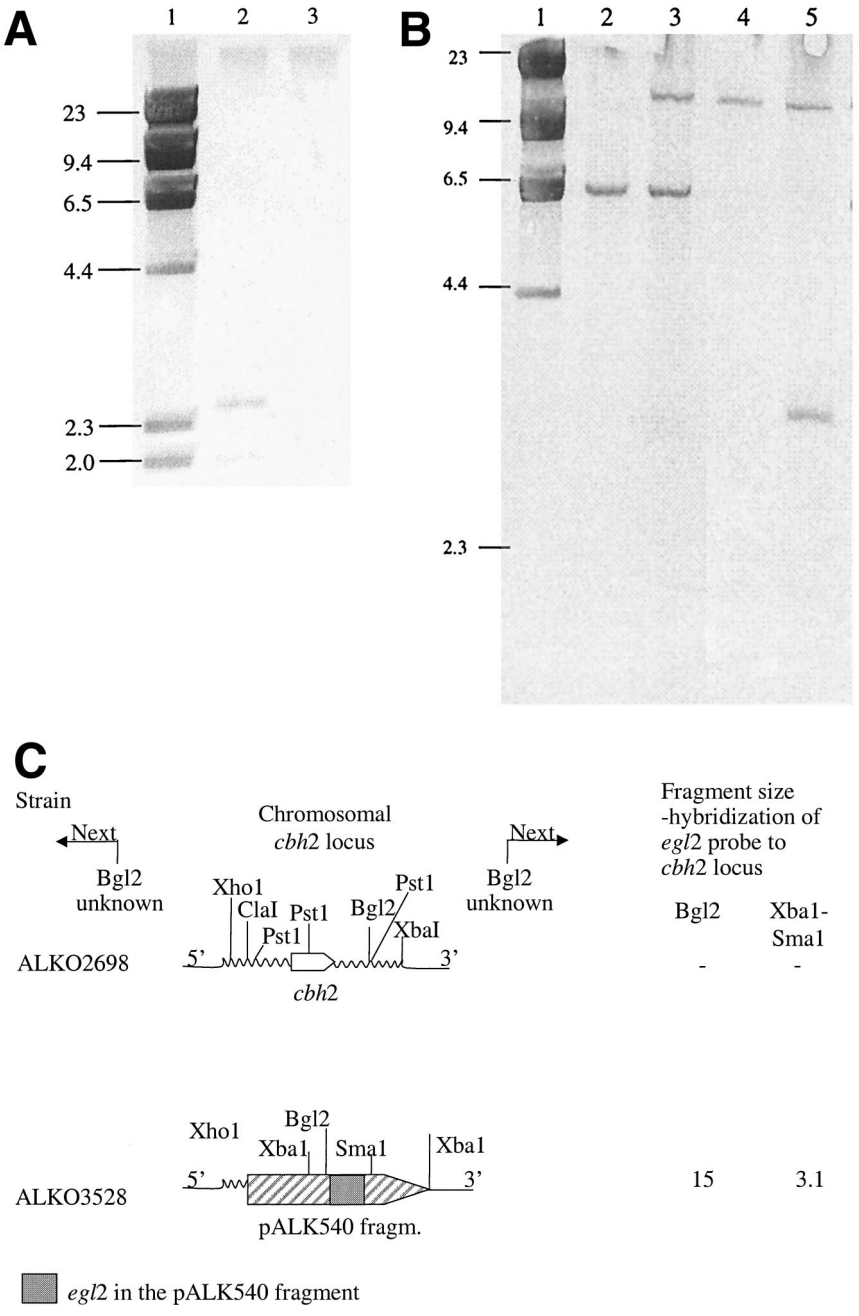

FIG. 6. Southern analysis of the transformant ALKO3528 and the host strain ALKO2698. (A) Genomic DNA was digested with *Pst*I. Hybridization was done with a *cbh2* probe. Lane 1, molecular weight marker λ*Hin*dIII; lane 2, ALKO2698; lane 3, ALKO3528. (B) Genomic DNA was digested with *Bgl*II or *Xba*I-*Sma*I. 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.

ACKNOWLEDGMENTS

We thank Rolf Bühler for ELISA measurements and Outi Könönen for skillful technical assistance. John Londesborough, Marja Paloheimo, and Jari Vehmaanperä are thanked for valuable comments on the manuscript.

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