



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 9/40, 1/00	A1	(11) International Publication Number: WO 92/17572 (43) International Publication Date: 15 October 1992 (15.10.92)
(21) International Application Number: PCT/US92/02629 (22) International Filing Date: 30 March 1992 (30.03.92) (30) Priority data: 678,865 29 March 1991 (29.03.91) US 770,049 4 October 1991 (04.10.91) US (60) Parent Applications or Grants (63) Related by Continuation US 678,865 (CIP) Filed on 29 March 1991 (29.03.91) US 770,049 (CIP) Filed on 4 October 1991 (04.10.91) (71) Applicant (for all designated States except US): GENENCOR INTERNATIONAL, INC. [US/US]; 180 Komball Way, South San Francisco, CA 94080 (US).		(72) Inventors; and (75) Inventors/Applicants (for US only) : CLARKSON, Kathleen, A. [US/US]; 53 28th Street, San Francisco, CA 94110 (US). LARENAS, Edward [US/US]; 352 Caprino Way, San Carlos, CA 94070 (US). WEISS, Geoffrey, L. [US/US]; 457 Buena Vista, San Francisco, CA 94117 (US). (74) Agent: DILLANHUNTY, T., Gene; Burns, Doane, Swecker & Mathis, George Mason Building, Washington and Prince Streets, P.O. Box 1404, Alexandria, VA 22313-1404 (US). (81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US. Published <i>With international search report.</i>
(54) Title: METHODS FOR TREATING COTTON-CONTAINING FABRICS WITH CELLULASE (57) Abstract Disclosed are improved methods for treating cotton-containing fabrics as well as the fabrics produced from these methods. In particular, the disclosed methods are directed to contacting cotton-containing fabrics with a cellulase solution containing a fungal cellulase composition which is substantially free of all CBH I type cellulase components. Cotton-containing fabrics so treated possess decreased strength loss as compared to fabrics treated with a cellulase solution containing a complete cellulase composition.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland	KR	Republic of Korea	SE	Sweden
CI	Côte d'Ivoire	LI	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
DE	Germany	MC	Monaco	TG	Togo
DK	Denmark			US	United States of America

- 1 -

METHODS FOR TREATING COTTON-CONTAINING FABRICS WITH CELLULASE

BACKGROUND OF THE INVENTION

5 1. Field of the Invention.

The present invention is directed to improved methods for treating cotton-containing fabrics with cellulase as well as the fabrics produced from these methods. In particular, the improved methods of the
10 present invention are directed to contacting cotton-containing fabrics with an aqueous solution containing a fungal cellulase composition substantially free of all CBH I type cellulase components. When the cotton-containing fabric is
15 treated with such solutions, the resulting fabric possesses the expected enhancements in, for example, feel, appearance, and/or softening, etc., as compared to the fabric prior to treatment and the fabric also possesses decreased strength loss as
20 compared to the fabric treated with a cellulase composition containing CBH I type cellulase components.

2. State of the Art.

During or shortly after their manufacture,
25 cotton-containing fabrics can be treated with cellulase in order to impart desirable properties to the fabric. For example, in the textile industry, cellulase has been used to improve the feel and/or

- 2 -

appearance of cotton-containing fabrics, to remove surface fibers from cotton-containing knits, for imparting a stone washed appearance to cotton-containing denims and the like.

5 In particular, Japanese Patent Application
Nos. 58-36217 and 58-54032 as well as Ohishi et al.,
"Reformation of Cotton Fabric by Cellulase" and JTN
December 1988 journal article "What's New -- Weight
10 each disclose that treatment of cotton-containing
fabrics with cellulase results in an improved feel
for the fabric. It is generally believed that this
cellulase treatment removes cotton fuzzing and/or
surface fibers which reduces the weight of the
15 fabric. The combination of these effects imparts
improved feel to the fabric, i.e., the fabric feels
more like silk.

 Additionally, it was heretofore known in the
art to treat cotton-containing knitted fabrics with
20 a cellulase solution under agitation and cascading
conditions, for example, by use of a jet, for the
purpose of removing broken fibers and threads common
to these knitted fabrics. When so treated, buffers
are generally not employed because they are believed
25 to adversely affect dye shading with selected dyes.

 It was still further heretofore known in the
art to treat cotton-containing woven fabrics with a
cellulase solution under agitation and cascading
conditions. When so treated, the cotton-containing
30 woven fabric possesses improved feel and appearance
as compared to the fabric prior to treatment.

- 3 -

5 Lastly, it was also heretofore known that the treatment of cotton-containing dyed denim with cellulase solutions under agitating and cascading conditions, i.e., in a rotary drum washing machine, would impart a "stone washed" appearance to the denim.

10 A common problem associated with the treatment of such cotton-containing fabrics with a cellulase solution is that the treated fabrics exhibit significant strength loss as compared to the untreated fabric. Strength loss arises because the cellulase hydrolyzes cellulose (β -1,4-glucan linkages) which, in turn, can result in a breakdown of a portion of the cotton polymer. As more and more cotton polymers are disrupted (broken down), the tensile strength of the fabric is reduced.

20 Because methods involving agitation and cascading of cellulase solutions over cotton woven fabrics require shorter reaction times, these methods are believed to provide cotton-containing woven fabrics of reduced strength loss as compared to cellulase treatment methods not involving agitation and cascading. In any event, such methods still nevertheless result in significant strength loss.

30 Accordingly, it would be particularly desirable to modify such cellulase treatment methods so as to provide reduced strength loss while still achieving the desired enhancements in the treated cotton-containing fabric arising from treatment with

- 4 -

cellulase as compared to the fabric prior to treatment.

5 Additionally, because fungal sources of cellulase are known to secrete very large quantities of cellulase and further because fermentation procedures for such fungal sources as well as isolation and purification procedures for isolating the cellulase are well known in the art, it would be particularly advantageous to use such fungal
10 cellulases in the methods for improving feel and/or appearance.

SUMMARY OF THE INVENTION

15 The present invention is directed to the discovery that heretofore known methods for treating cotton-containing fabrics with fungal cellulases can be improved by employing a fungal cellulase composition which is substantially free of all CBH I type components. Surprisingly, it has been found
20 that EG type components are capable of imparting enhancements to the treated fabric with regard to feel, appearance, softness, color enhancement, and/or stone washed appearance as compared to the fabric before treatment with such a cellulase
25 composition. Additionally, it has been found that it is the CBH I type components in combination with the EG type components which account for a sizable portion of the strength loss in the treated fabric.

30 In view of the above, in one of its method aspects, the present invention is directed to an improved method for the treatment of cotton-

- 5 -

containing fabrics with a fungal cellulase composition wherein said improvement comprises employing a fungal cellulase composition which is substantially free of all CBH I type components. In
5 a preferred embodiment, the fungal cellulase composition is free of all CBH I type and all CBH II type components. In still another preferred embodiment, the fungal cellulase composition comprises at least about 10 weight percent and
10 preferably at least about 20 weight percent of EG type components based on the total weight of protein in the cellulase composition.

In another of its method aspects, the present invention is directed to an improved method for the
15 treatment of cotton-containing fabrics with an aqueous fungal cellulase solution wherein said method is conducted with agitation under conditions so as to produce a cascading effect of the cellulase solution over the fabric wherein said improvement
20 comprises employing a fungal cellulase composition which is substantially free of all CBH I type components. In a preferred embodiment, the fungal cellulase composition is free of all CBH I type components and all CBH II type components. In still
25 another preferred embodiment, the fungal cellulase composition comprises at least about 10 weight percent and preferably at least about 20 weight percent of EG type components based on the total weight of protein in the cellulase composition.

30 Cotton-containing fabrics treated by the methods of this invention have the expected enhancement(s) as compared to the fabric prior to

- 6 -

5 treatment while exhibiting reduced strength loss as compared to the fabric treated with a fungal cellulase composition which contains CBH I type cellulase components. The reduced strength loss evidences that methods of this invention are strength loss resistant.

10 In its composition aspects, the present invention is directed to a cotton-containing fabric treated in the methods of this invention as defined above.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is an outline of the construction of pΔCBHIpyr4.

15 FIG. 2 illustrates deletion of the T. reesei gene by integration of the larger EcoRI fragment from pΔCBHIpyr4 at the cbh1 locus on one of the T. reesei chromosomes.

20 FIG. 3 is an autoradiograph of DNA from T. reesei strain GC69 transformed with EcoRI digested pΔCBHIpyr4 after Southern blot analysis using a ³²P labelled pΔCBHIpyr4 as the probe. The sizes of molecular weight markers are shown in kilobase pairs to the left of the Figure.

25 FIG. 4 is an autoradiograph of DNA from a T. reesei strain GC69 transformed with EcoRI digested pΔCBHIpyr4 using a ³²P labelled pIntCBHI as the probe. The sizes of molecular weight markers are shown in kilobase pairs to the left of the Figure.

- 7 -

FIG. 5 is an isoelectric focusing gel displaying the proteins secreted by the wild type and by transformed strains of T. reesei. Specifically, in FIG.5, Lane A of the isoelectric focusing gel employs partially purified CBHI from T. reesei; Lane B employs a wild type T. reesei : Lane C employs protein from a T. reesei strain with the cbh1 gene deleted; and Lane D employs protein from a T. reesei strain with the cbh1 and cbh2 genes deleted. In FIG. 5, the right hand side of the figure is marked to indicate the location of the single proteins found in one or more of the secreted proteins. Specifically, BG refers to the β -glucosidase, E1 refers to endoglucanase I, E2 refers to endoglucanase II, E3 refers to endoglucanase III, C1 refers to exo-cellobiohydrolase I and C2 refers to exo-cellobiohydrolase II.

FIG. 6A is a representation of the T. reesei cbh2 locus, cloned as a 4.1 kb EcoRI fragment on genomic DNA and FIG. 6B is a representation of the cbh2 gene deletion vector pPACBHII.

FIG. 7 is an autoradiograph of DNA from T. reesei strain P37PACBHIPyr⁻26 transformed with EcoRI digested pPACBHII after Southern blot analysis using a ³²P labelled pPACBHII as the probe. The sizes of molecular weight markers are shown in kilobase pairs to the left of the Figure.

FIG. 8 is a diagram of the plasmid pEGIp_{pyr}4.

FIG. 9 illustrates the RBB-CMC activity profile of an acidic EG enriched fungal cellulase

- 8 -

composition (CBH I and II deleted) derived from Trichoderma reesei over a pH range at 40°C; as well as the activity profile of an enriched EG III cellulase composition derived from Trichoderma reesei over a pH range at 40°C.

FIG. 10 illustrates strength loss results after three wash cycles in a launderometer for cotton-containing fabrics treated with cellulase compositions having varying amounts of CBH components.

FIG. 11 illustrates fiber removal results (based on panel test scores) for cotton-containing fabrics treated with cellulase secreted by a wild type Trichoderma reesei (whole cellulase) at various pHs.

FIG. 12 illustrates fiber removal results (based on panel test scores) for cotton-containing fabrics treated with varying concentrations (in ppm) of cellulase secreted by a wild type Trichoderma reesei and for a cotton fabric treated with cellulase secreted by a strain of Trichoderma reesei genetically engineered so as to be incapable of secreting CBH I and CBH II.

FIG. 13 illustrates the softness panel test results for varying concentrations (in ppm) of an EG enriched cellulase composition derived from a strain of Trichoderma reesei genetically modified so as to be incapable of producing CBHI & II.

- 9 -

FIG. 14 is a diagram of the site specific alterations made in the eql1 and cbh1 genes to create convenient restriction endonuclease cleavage sites. In each case, the upper line shows the original DNA sequence, the changes introduced are shown in the middle line, and the new sequence is shown in the lower line.

FIG. 15 is a diagram of the larger EcoRI fragment which can be obtained from pCEPC1.

FIG. 16 is an autoradiograph of DNA, from an untransformed strain of T. reesei RutC30 and from two transformants obtained by transforming T. reesei with EcoRI digested pCEPC1. The DNA was digested with PstI, a Southern blot was obtained and hybridized with ³²P labelled pUC4K::cbh1. The sizes of marker DNA fragments are shown in kilobase pairs to the left of the Figure.

FIG. 17 is a diagram of the plasmid pEGII::P-1.

FIG 18. is an autoradiograph of DNA from T. reesei strain P37PΔΔ67P1 transformed with HindIII and BamHI digested pEGII::P-1. A Southern blot was prepared and the DNA was hybridized with an approximately 4kb PstI fragment of radiolabelled T.reesei DNA containing the eql3 gene. Lanes A, C and E contain DNA from the untransformed strain whereas, Lanes B, D and F contain DNA from the untransformed T. reesei strain. The T.reesei DNA was digested with BglII in Lanes A and B, with EcoRV in Lanes C and D and with PstI in Lanes E and F.

- 10 -

The size of marker DNA fragments are shown in kilobase pairs to the left of the Figure.

FIG. 19 is a diagram of the plasmid pAEGI-1.

FIG. 20 is an autoradiograph of a Southern blot of DNA isolated from transformants of strain GC69 obtained with HindIII digested pAEGIPyr-3. The pattern of hybridisation with the probe, radiolabelled pAEGIPyr-3, expected for an untransformed strain is shown in Lane C. Lane A shows the pattern expected for a transformant in which the egl1 gene has been disrupted and Lane B shows a transformant in which pAEGIPyr-3 DNA has integrated into the genome but without disrupting the egl1 gene. Lane D contains pAEGIPyr-3 digested with HindIII to provide appropriate size markers. The sizes of marker DNA fragments are shown in kilobase pairs to the right of the figure.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As noted above, the methods of this invention are improvements in prior art methods for treating cotton-containing fabrics with cellulase. The improvement comprises using a specific cellulase composition which imparts the desired enhancement(s) to the fabric while minimizing strength loss in the fabric. However, prior to discussing this invention in detail, the following terms will first be defined.

The term "cotton-containing fabric" refers to sewn or unsewn fabrics made of pure cotton or cotton

- 11 -

blends including cotton woven fabrics, cotton knits, cotton denims, cotton yarns and the like. When cotton blends are employed, the amount of cotton in the fabric should be at least about 40 percent by weight cotton; preferably, more than about 60 percent by weight cotton; and most preferably, more than about 75 percent by weight cotton. When employed as blends, the companion material employed in the fabric can include one or more non-cotton fibers including synthetic fibers such as polyamide fibers (for example, nylon 6 and nylon 66), acrylic fibers (for example, polyacrylonitrile fibers), and polyester fibers (for example, polyethylene terephthalate), polyvinyl alcohol fibers (for example, Vinyon), polyvinyl chloride fibers, polyvinylidene chloride fibers, polyurethane fibers, polyurea fibers and aramid fibers. It is contemplated that regenerated cellulose, such as rayon, could be used as a substitute for cotton in the methods of this invention.

The term "finishing" as employed herein means the application of a sufficient amount of finish to a cotton-containing fabric so as to substantially prevent cellulolytic activity of the cellulase on the fabric. Finishes are generally applied at or near the end of the manufacturing process of the fabric for the purpose of enhancing the properties of the fabric, for example, softness, drapability, etc., which additionally protects the fabric from reaction with cellulases. Finishes useful for finishing a cotton-containing fabric are well known in the art and include resinous materials, such as melamine, glyoxal, or ureaformaldehyde, as well as

- 12 -

waxes, silicones, fluorochemicals and quaternaries. When so finished, the cotton-containing fabric is substantially less reactive to cellulase.

5 The term "fungal cellulase" refers to the enzyme composition derived from fungal sources or microorganisms genetically modified so as to incorporate and express all or part of the cellulase genes obtained from a fungal source. Fungal cellulases act on cellulose and its derivatives to hydrolyze cellulose and give primary products, glucose and cellobiose. Fungal cellulases are distinguished from cellulases produced from non-fungal sources including microorganisms such as actinomycetes, gliding bacteria (myxobacteria) and true bacteria. Fungi capable of producing cellulases useful in preparing cellulase compositions described herein are disclosed in British Patent No. 2 094 826A, the disclosure of which is incorporated herein by reference.

10

15

20 Most fungal cellulases generally have their optimum activity in the acidic or neutral pH range although some fungal cellulases are known to possess significant activity under neutral and slightly alkaline conditions, i.e., for example, cellulase derived from Humicola insolens is known to have activity in neutral to slightly alkaline conditions.

25

30 Fungal cellulases are known to be comprised of several enzyme classifications having different substrate specificity, enzymatic action patterns, and the like. Additionally, enzyme components within each classification can exhibit different

- 13 -

molecular weights, different degrees of glycosylation, different isoelectric points, different substrate specificity, etc. For example, fungal cellulases can contain cellulase classifications which include endoglucanases (EGs),
5 exo-cellobiohydrolases (CBHs), β -glucosidases (BGs),
etc. On the other hand, while bacterial cellulases are reported in the literature as containing little or no CBH components, there are a few cases where
10 CBH-like components derived from bacterial
cellulases have been reported to possess
exo-cellobiohydrolase activity.

A fungal cellulase composition produced by a naturally occurring fungal source and which
15 comprises one or more CBH and EG components wherein
each of these components is found at the ratio produced by the fungal source is sometimes referred to herein as a "complete fungal cellulase system" or
a "complete fungal cellulase composition" to
20 distinguish it from the classifications and
components of cellulase isolated therefrom, from incomplete cellulase compositions produced by bacteria and some fungi, or from a cellulase composition obtained from a microorganism
25 genetically modified so as to overproduce,
underproduce, or not produce one or more of the CBH and/or EG components of cellulase.

The fermentation procedures for culturing fungi for production of cellulase are known per se in the
30 art. For example, cellulase systems can be produced
either by solid or submerged culture, including batch, fed-batch and continuous-flow processes. The

- 14 -

collection and purification of the cellulase systems from the fermentation broth can also be effected by procedures known per se in the art.

5 "Endoglucanase ("EG") type components" refer to all of those fungal cellulase components or combination of components which exhibit textile activity properties similar to the endoglucanase components of Trichoderma reesei. In this regard, the endoglucanase components of Trichoderma reesei 10 (specifically, EG I, EG II, EG III, and the like either alone or in combination) impart improved feel, improved appearance, softening, color enhancement, and/or a stone washed appearance to cotton-containing fabrics (as compared to the fabric 15 prior to treatment) when these components are incorporated into a textile treatment medium and the fabric is treated with this medium. Additionally, treatment of cotton-containing fabrics with endoglucanase components of Trichoderma reesei 20 results in less strength loss as compared to the strength loss arising from treatment with a similar composition but which additionally contains CBH I type components.

25 Accordingly, endoglucanase type components are those fungal cellulase components which impart improved feel, improved appearance, softening, color enhancement, and/or a stone washed appearance to cotton-containing fabrics (as compared to the fabric before treatment) when these components are 30 incorporated into a medium used to treat the fabrics and which impart reduced strength loss to cotton-containing fabrics as compared to the strength loss

- 15 -

arising from treatment with a similar cellulase composition but which additionally contains CBH I type components.

5 Such endoglucanase type components may not include components traditionally classified as endoglucanases using activity tests such as the ability of the component (a) to hydrolyze soluble cellulose derivatives such as carboxymethylcellulose (CMC), thereby reducing the viscosity of CMC
10 containing solutions, (b) to readily hydrolyze hydrated forms of cellulose such as phosphoric acid swollen cellulose (e.g., Walseth cellulose) and hydrolyze less readily the more highly crystalline forms of cellulose (e.g., Avicel, Solkafloc, etc.).
15 On the other hand, it is believed that not all endoglucanase components, as defined by such activity tests, will impart one or more of the enhancements to cotton-containing fabrics as well as reduced strength loss to cotton-containing fabrics.
20 Accordingly, it is more accurate for the purposes herein to define endoglucanase type components as those components of fungal cellulase which possess similar textile activity properties as possessed by the endoglucanase components of Trichoderma reesei.

25 Fungal cellulases can contain more than one EG type component. The different components generally have different isoelectric points, different molecular weights, different degrees of glycosylation, different substrate specificity, different
30 enzymatic action patterns, etc. The different isoelectric points of the components allow for their separation via ion exchange chromatography and the

- 16 -

like. In fact, the isolation of components from different fungal sources is known in the art. See, for example, Bjork et al., U.S. Serial No. 07/422,814, Schulein et al., International Application WO 89/09259, Wood et al., Biochemistry and Genetics of Cellulose Degradation, pp. 31 to 52 (1988); Wood et al., Carbohydrate Research, Vol. 190, pp. 279 to 297 (1989); Schulein, Methods in Enzymology, Vol. 160, pp. 234 to 242 (1988); and the like. The entire disclosure of each of these references is incorporated herein by reference.

In general, it is contemplated that combinations of EG type components may give a synergistic response in imparting enhancements to the cotton-containing fabrics as well as imparting reduced strength loss as compared to a single EG component. On the other hand, a single EG type component may be more stable or have a broader spectrum of activity over a range of pHs. Accordingly, the EG type components employed in this invention can be either a single EG type component or a combination of two or more EG type components. When a combination of components is employed, the EG type component may be derived from the same or different fungal sources.

It is contemplated that EG type components can be derived from bacterially derived cellulases.

"Exo-cellobiohydrolase type ("CBH type") components" refer to those fungal cellulase components which exhibit textile activity properties similar to CBH I and/or CBH II cellulase components

- 17 -

of Trichoderma reesei. In this regard, when used in the absence of EG type cellulase components (as defined above), the CBH I and CBH II components of Trichoderma reesei alone do not impart any significant enhancements in feel, appearance, color enhancement and/or stone washed appearance to the so treated cotton-containing fabrics. Additionally, when used in combination with EG type components, the CBH I component of Trichoderma reesei imparts enhanced strength loss to the cotton-containing fabrics.

Accordingly, CBH I type components and CBH II type components refer to those fungal cellulase components which exhibit textile activity properties similar to CBH I and CBH II components of Trichoderma reesei, respectively. As noted above, for CBH I type components, this includes the property of enhancing strength loss of cotton-containing fabrics when used in the presence of EG type components. In a preferred embodiment and when used in combination with EG type components, the CBH I type components of Trichoderma reesei can impart an incremental cleaning benefit. Additionally, it is contemplated that the CBH I components of Trichoderma reesei, when used alone or in combination with EG type components, can impart an incremental softening benefit.

Such exo-cellobiohydrolase type components could possibly not include components traditionally classed as exo-cellobiohydrolases using activity tests such as those used to characterize CBH I and CBH II from Trichoderma reesei. For example, such

- 18 -

components (a) are competitively inhibited by cellobiose (K_i approximately 1mM); (b) are unable to hydrolyze to any significant degree substituted celluloses, such as carboxymethylcellulose, etc., and (c) hydrolyze phosphoric acid swollen cellulose and to a lesser degree highly crystalline cellulose. On the other hand, it is believed that some fungal cellulase components which are characterized as CBH components by such activity tests, will impart improved feel, appearance, softening, color enhancement, and/or a stone washed appearance to cotton-containing fabrics with minimal strength loss when used alone in the cellulase composition. Accordingly, it is believed to be more accurate for the purposes herein to define such exo-cellobiohydrolases as EG type components because these components possess similar functional properties in textile uses as possessed by the endoglucanase components of Trichoderma reesei.

In regard to the detergent compositions containing cellulase compositions which are CBHI deficient, CBHI enriched or EGIII enriched, it has been found that it is the amount of cellulase, and not the relative rate of hydrolysis of the specific enzymatic components to produce reducing sugars from cellulose, which imparts the desired detergent properties to cotton-containing fabrics, eg., one or more of improved color restoration, improved softening and improved cleaning to the detergent composition.

Fungal cellulases substantially free of all CBH I type components can be obtained by

- 19 -

purification techniques. Specifically, the complete cellulase system can be purified into substantially pure components by recognized separation techniques well published in the literature, including ion
5 exchange chromatography at a suitable pH, affinity chromatography, size exclusion and the like. For example, in ion exchange chromatography (usually anion exchange chromatography), it is possible to separate the cellulase components by eluting with a
10 pH gradient, or a salt gradient, or both a pH and a salt gradient. As used herein, the term "cellulase composition substantially free of all CBH I type cellulase components" means that the cellulase composition, based on the weight of protein, will
15 contain less than 1 weight percent CBH I type cellulase components.

It is also contemplated that cellulase compositions substantially free of all CBH I type components can be prepared by means other than
20 isolation and recombination of the components. For example, recombinant techniques can be used to prepare microorganisms which are incapable of producing any CBH I type components or which are incapable of producing any CBH type components.

25 In regard to the above, a preferred method for the preparation of cellulase compositions substantially free of CBH I type components is by genetically modifying a microorganism so as to be incapable of expressing CBH I type components which
30 methods do not express any heterologous protein. Likewise, it is also possible to genetically modify a microorganism so as to additionally overexpress

- 20 -

one or more EG type components. For example, U.S. Serial No. 07/593,919, filed October 5, 1990 and which is incorporated herein by reference in its entirety, discloses methods for genetically engineering Trichoderma reesei so as to be incapable of expressing one or more CBH components and/or overexpressing one or more EG components. Moreover, the methods of that application create Trichoderma reesei strains which do not express any heterologous proteins. Likewise, Miller et al., "Direct and Indirect Gene Replacement in Aspergillus nidulans", Molecular and Cellular Biology, p. 1714-1721 (1985) discloses methods for deleting genes in Aspergillus nidulans by DNA mediated transformation using a linear fragment of homologous DNA. The methods of Miller et al., would achieve gene deletion without producing any heterologous proteins.

In view of the above, the deletion of the genes responsible for producing CBH I type and/or CBH II type cellulase components would also have the effect of enriching the amount of EG type components present in the cellulase composition. Likewise, the deletion of those genes responsible for producing CBH I and II type components would result in a cellulase composition free of CBH type components.

It is still further contemplated that fungal cellulase compositions can be used herein from fungal sources which produce an incomplete fungal cellulase composition. For example, it is known that certain fungi produce cellulase compositions free of CBH components. See, for example, Coughlan et al., Biochemistry and Genetics of Cellulose

- 21 -

Degradation, Aubert et al. Editors, pp. 11-30
(Academic Press, 1988), disclose that brown rot
fungi do not apparently produce CBH components, but
it may be possible that one or more of these
5 components are CBH I type components.

" β -Glucosidase (BG) components" refer to those
components of cellulase which exhibit BG activity;
that is to say that such components will act from
the non-reducing end of cellobiose and other soluble
10 cellooligosaccharides ("cellobiose") and give
glucose as the sole product. BG components do not
adsorb onto or react with cellulose polymers.
Furthermore, such BG components are competitively
inhibited by glucose (K_i approximately 1mM). While
15 in a strict sense, BG components are not literally
cellulases because they cannot degrade cellulose,
such BG components are included within the
definition of the cellulase system because these
enzymes facilitate the overall degradation of
20 cellulose by further degrading the inhibitory
cellulose degradation products (particularly
cellobiose) produced by the combined action of CBH
components and EG components. Without the presence
of BG components, moderate or little hydrolysis of
25 crystalline cellulose will occur. BG components are
often characterized on aryl substrates such as
p-nitrophenol B-D-glucoside (PNPG) and thus are
often called aryl-glucosidases. It should be noted
that not all aryl glucosidases are BG components, in
30 that some do not hydrolyze cellobiose.

It is contemplated that the presence or absence
of BG components in the cellulase composition can be

- 22 -

used to regulate the activity of any CBH components in the composition (i.e., non-CBH I type components). Specifically, because cellobiose is produced during cellulose degradation by CBH components, and because high concentrations of cellobiose are known to inhibit CBH activity, and further because such cellobiose is hydrolyzed to glucose by BG components, the absence of BG components in the cellulase composition will "turn-off" CBH activity when the concentration of cellobiose reaches inhibitory levels. It is also contemplated that one or more additives (e.g., cellobiose, glucose, etc.) can be added to the cellulase composition to effectively "turn-off", directly or indirectly, CBH I type activity as well as other CBH activity. When such additives are employed, the resulting composition is considered to be a composition free of all CBH I type components if the amount of additive is sufficient to result in effectively no CBH I type activity.

On the other hand, a cellulase composition containing added amounts of BG components may increase overall hydrolysis of cellulose if the level of cellobiose generated by the CBH components becomes restrictive of such overall hydrolysis in the absence of added BG components.

Methods to either increase or decrease the amount of BG components in the cellulase composition are disclosed in U.S. Serial No. 07/625,140, filed December 10, 1990, as attorney docket no. 010055-056 and entitled "SACCHARIFICATION OF CELLULOSE BY CLONING AND AMPLIFICATION OF THE β -GLUCOSIDASE GENE

- 23 -

BY TRICHODERMA REESEI", which application is incorporated herein by reference in its entirety.

5 Fungal cellulases can contain more than one BG component. The different components generally have different isoelectric points which allow for their separation via ion exchange chromatography and the like. Either a single BG component or a combination of BG components can be employed.

10 When employed in textile treatment solutions, the BG component is generally added in an amount sufficient to prevent inhibition by cellobiose of any CBH and EG components found in the cellulase composition. The amount of BG component added depends upon the amount of cellobiose produced in
15 the textile composition which can be readily determined by the skilled artisan. However, when employed, the weight percent of BG component relative to any CBH type components present in the cellulase composition is preferably from about 0.2 to about 10 weight percent and more preferably, from
20 about 0.5 to about 5 weight percent.

Preferred fungal cellulases for use in preparing the fungal cellulase compositions used in this invention are those obtained from Trichoderma reesei, Trichoderma koningii, Pencillum sp.,
25 Humicola insolens, and the like. Certain fungal cellulases are commercially available, i.e., CELLUCAST (available from Novo Industry, Copenhagen, Denmark), RAPIDASE (available from Gist Brocades,
30 N.V., Delft, Holland), CYTOLASE 123 (available from Genencor International, South San Francisco,

- 24 -

California) and the like. Other fungal cellulases can be readily isolated by art recognized fermentation and isolation procedures.

5 The term "buffer" refers to art recognized acid/base reagents which stabilize the cellulase solution against undesired pH shifts during the cellulase treatment of the cotton-containing fabric. In this regard, it is art recognized that cellulase activity is pH dependent. That is to say that a
10 specific cellulase composition will exhibit cellulolytic activity within a defined pH range with optimal cellulolytic activity generally being found within a small portion of this defined range. The specific pH range for cellulolytic activity will
15 vary with each cellulase composition. As noted above, while most cellulases will exhibit cellulolytic activity within an acidic to neutral pH profile, there are some cellulase compositions which exhibit cellulolytic activity in an alkaline pH
20 profile.

 During cellulase treatment of the cotton-containing fabric, it is possible that the pH of the initial cellulase solution could be outside the range required for cellulase activity. It is
25 further possible for the pH to change during treatment of the cotton-containing fabric, for example, by the generation of a reaction product which alters the pH of the solution. In either event, the pH of an unbuffered cellulase solution
30 could be outside the range required for cellulolytic activity. When this occurs, undesired reduction or cessation of cellulolytic activity in the cellulase

- 25 -

solution occurs. For example, if a cellulase having an acidic activity profile is employed in a neutral unbuffered aqueous solution, then the pH of the solution will result in lower cellulolytic activity and possibly in the cessation of cellulolytic activity. On the other hand, the use of a cellulase having a neutral or alkaline pH profile in a neutral unbuffered aqueous solution should initially provide significant cellulolytic activity.

In view of the above, the pH of the cellulase solution should be maintained within the range required for cellulolytic activity. One means of accomplishing this is by simply monitoring the pH of the system and adjusting the pH as required by the addition of either an acid or a base. However, in a preferred embodiment, the pH of the system is preferably maintained within the desired pH range by the use of a buffer in the cellulase solution. In general, a sufficient amount of buffer is employed so as to maintain the pH of the solution within the range wherein the employed cellulase exhibits activity. Insofar as different cellulase compositions have different pH ranges for exhibiting cellulase activity, the specific buffer employed is selected in relationship to the specific cellulase composition employed. The buffer(s) selected for use with the cellulase composition employed can be readily determined by the skilled artisan taking into account the pH range and optimum for the cellulase composition employed as well as the pH of the cellulase solution. Preferably, the buffer employed is one which is compatible with the cellulase composition and which will maintain the pH

- 26 -

of the cellulase solution within the pH range required for optimal activity. Suitable buffers include sodium citrate, ammonium acetate, sodium acetate, disodium phosphate, and any other art recognized buffers.

The tensile strength of cotton-containing fabrics can be measured in a warp and fill direction which are at right angles to each other. Accordingly, the term "warp tensile strength" as used herein refers to the tensile strength of the cotton-containing fabric as measured along the length of the cotton-containing fabric whereas the term "fill tensile strength" refers to the tensile strength of the cotton-containing fabric as measured across the width of the cotton-containing fabric. The tensile strength of the resulting cotton-containing fabric treated with a cellulase solution is compared to its tensile strength prior to treatment with the cellulase solution so as to determine the strength reducing effect of the treatment. If the tensile strength is reduced too much, the resulting cotton-containing fabric will easily tear and/or form holes. Accordingly, it is desirable to maintain a tensile strength (both warp and fill) after treatment which is at least about 50% of the tensile strength before treatment.

The tensile strength of cotton-containing fabrics is readily conducted following ASTM D1682 test methodology. Equipment suitable for testing the tensile strength of such fabrics include a Scott tester or an Instron tester, both of which are commercially available. In testing the tensile

- 27 -

strength of cotton-containing fabrics which have been treated with cellulase solutions, care should be taken to prevent fabric shrinkage after treatment and before testing. Such shrinkage would result in erroneous tensile strength data.

5

Enhancements to the cotton-containing fabric is achieved by those methods heretofore used. For example, cotton-containing fabrics having improved feel can be achieved as per Japanese Patent Application Nos. 58-36217 and 58-54032 as well as Ohishi et al., "Reformation of Cotton Fabric by Cellulase" and JTN December 1988 journal article "What's New -- Weight Loss Treatment to Soften the Touch of Cotton Fabric". The teachings of each of these references is incorporated herein by reference.

10

15

Similarly, methods for improving both the feel and appearance of cotton-containing fabrics include contacting the fabric with an aqueous solution containing cellulase under conditions so that the solution is agitated and so that a cascading effect of the cellulase solution over the cotton-containing fabric is achieved. Such methods result in improved feel and appearance of the so treated cotton-containing fabric and are described in U.S. Serial No. 07/598,506, filed October 16, 1990 and which is incorporated herein by reference in its entirety.

20

25

Methods for the enhancement of cotton-containing knits are described in International Textile Bulletin, Dyeing/Printing/Finishing, pages 5

30

- 28 -

et seq., 2nd Quarter, 1990, which is incorporated herein by reference.

5 Likewise, methods for imparting a stone washed appearance to cotton-containing denims are described in U.S. Patent No. 4,832,864, which is incorporated herein by reference in its entirety.

10 Other methods for enhancing cotton-containing fabrics by treatment with a cellulase composition are known in the art. Preferably, in such methods, the treatment of the cotton-containing fabric with cellulase is conducted prior to finishing the cotton-containing fabric.

15 As noted above, the present invention is an improvement over prior art methods for treating cotton-containing fabrics insofar as the present invention employs a specific cellulase composition which minimizes strength loss in the treated fabric. The cellulase composition employed herein is a
20 fungal cellulase composition substantially free of CBH I type components and preferably, substantially free of all CBH type components.

25 Additionally, the use of the cellulase compositions described herein also result in fabric/color enhancement of stressed cotton-containing fabrics. Specifically, during the manufacture of cotton-containing fabrics, the fabric can become stressed and when so stressed, it will contain broken and disordered fibers. Such fibers
30 detrimentally impart a worn and dull appearance to the fabric. However, when treated in the method of

- 29 -

this invention, the so stressed fabric is subject to fabric/color enhancement. This is believed to arise by removal of some of the broken and disordered fibers which has the effect of restoring the appearance of the fabric prior to becoming stressed.

Additionally, it is contemplated that by employing the cellulase composition described herein with pigment type dyed fabrics (e.g., denims), these cellulase compositions will cause less redeposition of dye. It is also contemplated that these anti-redeposition properties can be enhanced for one or more specific EG type component(s) as compared to other components.

The fungal cellulase compositions described above are employed in an aqueous solution which contains cellulase and other optional ingredients including, for example, a buffer, a surfactant, a scouring agent, and the like. The concentration of the cellulase composition employed in this solution is generally a concentration sufficient for its intended purpose. That is to say that an amount of the cellulase composition is employed to provide the desired enhancement(s) to the cotton-containing fabric. The amount of the cellulase composition employed is also dependent on the equipment employed, the process parameters employed (the temperature of the cellulase solution, the exposure time to the cellulase solution, and the like), the cellulase activity (e.g., a cellulase solution will require a lower concentration of a more active cellulase composition as compared to a less active cellulase composition), and the like. The exact

- 30 -

concentration of the cellulase composition can be readily determined by the skilled artisan based on the above factors as well as the desired effect. Preferably, the concentration of the cellulase composition in the cellulase solution employed herein is from about 0.01 gram/liter of cellulase solution to about 10.0 grams/liter of cellulase solution; and more preferably, from about 0.05 grams/liter of cellulase solution to about 2 gram/liter of cellulase solution. (The cellulase concentration recited above refers to the weight of total protein).

When a buffer is employed in the cellulase solution, the concentration of buffer in the aqueous cellulase solution is that which is sufficient to maintain the pH of the solution within the range wherein the employed cellulase exhibits activity which, in turn, depends on the nature of the cellulase employed. The exact concentration of buffer employed will depend on several factors which the skilled artisan can readily take into account. For example, in a preferred embodiment, the buffer as well as the buffer concentration are selected so as to maintain the pH of the cellulase solution within the pH range required for optimal cellulase activity. In general, buffer concentration in the cellulase solution is about 0.005 N and greater. Preferably, the concentration of the buffer in the cellulase solution is from about 0.01 to about 0.5 N, and more preferably, from about 0.05 to about 0.15 N. It is possible that increased buffer concentrations in the cellulase solution may enhance

- 31 -

the rate of tensile strength loss of the treated fabric.

In addition to cellulase and a buffer, the cellulase solution can optionally contain a small amount of a surfactant, i.e., less than about 2 weight percent, and preferably from about 0.01 to about 2 weight percent. Suitable surfactants include any surfactant compatible with the cellulase and the fabric including, for example, anionic, non-ionic and ampholytic surfactants.

Suitable anionic surfactants for use herein include linear or branched alkylbenzenesulfonates; alkyl or alkenyl ether sulfates having linear or branched alkyl groups or alkenyl groups; alkyl or alkenyl sulfates; olefinsulfonates; alkanesulfonates and the like. Suitable counter ions for anionic surfactants include alkali metal ions such as sodium and potassium; alkaline earth metal ions such as calcium and magnesium; ammonium ion; and alkanolamines having 1 to 3 alkanol groups of carbon number 2 or 3.

Ampholytic surfactants include quaternary ammonium salt sulfonates, betaine-type ampholytic surfactants, and the like. Such ampholytic surfactants have both the positive and negative charged groups in the same molecule.

Nonionic surfactants generally comprise polyoxyalkylene ethers, as well as higher fatty acid alkanolamides or alkylene oxide adduct thereof, fatty acid glycerine monoesters, and the like.

- 32 -

Mixtures of such surfactants can also be used.

5 The liquor ratios, i.e., the ratio of weight of
cellulase solution to the weight of fabric, employed
herein is generally an amount sufficient to achieve
the desired enhancement in the cotton-containing
fabric and is dependent upon the process used and
the enhancement to be achieve. Preferably, the
liquor ratios are generally from about 0.1:1 and
greater, and more preferably greater than about 1:1
10 and even more preferably greater than about 10:1.
Use of liquor ratios of greater than about 50:1 are
usually not preferred from an economic viewpoint.

15 Reaction temperatures for cellulase treatment
are governed by two competing factors. Firstly,
higher temperatures generally correspond to enhanced
reaction kinetics, i.e., faster reactions, which
permit reduced reaction times as compared to
reaction times required at lower temperatures.
Accordingly, reaction temperatures are generally at
20 least about 30°C and greater. Secondly, cellulase
is a protein which loses activity beyond a given
reaction temperature which temperature is dependent
on the nature of the cellulase used. Thus, if the
reaction temperature is permitted to go too high,
25 then the cellulolytic activity is lost as a result
of the denaturing of the cellulase. As a result,
the maximum reaction temperatures employed herein
are generally about 65°C. In view of the above,
reaction temperatures are generally from about 30°C
30 to about 65°C; preferably, from about 35°C to about
60°C; and more preferably, from about 35°C to about
50°C.

- 33 -

Reaction times are generally from about 0.1 hours to about 24 hours and, preferably, from about 0.25 hours to about 5 hours.

5 The cotton-containing fabrics treated in the methods described above using such cellulase compositions possess reduced strength loss as compared to the same cotton-containing fabric treated in the same manner with a complete fungal cellulase composition.

10 In a preferred embodiment, a concentrate can be prepared for use in the methods described herein. Such concentrates would contain concentrated amounts of the cellulase composition described above, buffer and surfactant, preferably in an aqueous solution.

15 When so formulated, the concentrate can readily be diluted with water so as to quickly and accurately prepare cellulase solutions having the requisite concentration of these additives. Preferably, such concentrates will comprise from about 0.1 to about

20 20 weight percent of a cellulase composition described above (protein); from about 10 to about 50 weight percent buffer; from about 10 to about 50 weight percent surfactant; and from about 0 to 80 weight percent water. When aqueous concentrates are

25 formulated, these concentrates can be diluted by factors of from about 2 to about 200 so as to arrive at the requisite concentration of the components in the cellulase solution. As is readily apparent, such concentrates will permit facile formulation of

30 the cellulase solutions as well as permit feasible transportation of the concentration to the location where it will be used. The cellulase composition as

- 34 -

described above can be added to the concentrate either in a liquid diluent, in granules, in emulsions, in gels, in pastes, and the like. Such forms are well known to the skilled artisan.

5 When a solid cellulase concentrate is employed, the cellulase composition is generally a granule, a powder, an agglomeration, and the like. When granules are used, the granules can preferably be formulated so as to contain a cellulase protecting agent. See, for instance, U.S. Serial No. 10 07/642,669, filed January 17, 1991 as Attorney Docket No. 010055-073 and entitled "GRANULES CONTAINING BOTH AN ENZYME AND AN ENZYME PROTECTING AGENT AND DETERGENT COMPOSITIONS CONTAINING SUCH 15 GRANULES" which application is incorporated herein by reference in its entirety. Likewise, the granules can be formulated so as to contain materials to reduce the rate of dissolution of the granules into the wash medium. Such materials and 20 granules are disclosed in U.S. Serial No. 07/642,596 filed on January 17, 1991 as Attorney Docket No. GCS-171-US1 and entitled "GRANULAR COMPOSITIONS" which application is incorporated herein by reference in its entirety.

25 It is contemplated that the cellulase compositions described herein can additionally be used in a pre-wash and as a pre-soak either as a liquid or a spray. It is still further contemplated that the cellulase compositions described herein can 30 also be used in home use as a stand alone composition suitable for enhancing color and appearance of fabrics. See, for example, U.S.

- 35 -

Patent No. 4,738,682, which is incorporated herein by reference in its entirety.

The following examples are offered to illustrate the present invention and should not be construed in any way as limiting its scope.

EXAMPLES

Examples 1-12 and 22-30 demonstrate the preparation of Trichoderma reesei genetically engineered so as to be incapable of producing one or more cellulase components or so as to overproduce specific cellulase components.

Example 1

Selection for pyr4⁻ derivatives of Trichoderma reesei

The pyr4 gene encodes orotidine-5'-monophosphate decarboxylase, an enzyme required for the biosynthesis of uridine. The toxic inhibitor 5-fluoroorotic acid (FOA) is incorporated into uridine by wild-type cells and thus poisons the cells. However, cells defective in the pyr4 gene are resistant to this inhibitor but require uridine for growth. It is, therefore, possible to select for pyr4 derivative strains using FOA. In practice, spores of T. reesei strain RL-P37 (Sheir-Neiss, G. and Montenecourt, B.S., Appl. Microbiol. Biotechnol. 20, p. 46-53 (1984)) were spread on the surface of a solidified medium containing 2 mg/ml uridine and 1.2 mg/ml FOA. Spontaneous FOA-resistant colonies appeared within three to four days and it was possible to subsequently identify those FOA-

- 36 -

resistant derivatives which required uridine for growth. In order to identify those derivatives which specifically had a defective pyr4 gene, protoplasts were generated and transformed with a plasmid containing a wild-type pyr4 gene (see Examples 3 and 4). Following transformation, protoplasts were plated on medium lacking uridine. Subsequent growth of transformed colonies demonstrated complementation of a defective pyr4 gene by the plasmid-borne pyr4 gene. In this way, strain GC69 was identified as a pyr4 derivative of strain RL-P37.

Example 2

Preparation of CBHI Deletion Vector

A cbh1 gene encoding the CBHI protein was cloned from the genomic DNA of T. reesei strain RL-P37 by hybridization with an oligonucleotide probe designed on the basis of the published sequence for this gene using known probe synthesis methods (Shoemaker et al., 1983b). The cbh1 gene resides on a 6.5 kb PstI fragment and was inserted into PstI cut pUC4K (purchased from Pharmacia Inc., Piscataway, NJ) replacing the Kan^r gene of this vector using techniques known in the art, which techniques are set forth in Maniatis et al., (1989) and incorporated herein by reference. The resulting plasmid, pUC4K::cbh1 was then cut with HindIII and the larger fragment of about 6 kb was isolated and religated to give pUC4K::cbh1ΔH/H (see FIG. 1). This procedure removes the entire cbh1 coding sequence and approximately 1.2 kb upstream and 1.5

- 37 -

kb downstream of flanking sequences. Approximately, 1 kb of flanking DNA from either end of the original PstI fragment remains.

5 The T. reesei pyr4 gene was cloned as a 6.5 kb HindIII fragment of genomic DNA in pUC18 to form pTpyr2 (Smith et al., 1991) following the methods of Maniatis et al., supra. The plasmid pUC4K::cbhIΔH/H was cut with HindIII and the ends were
10 dephosphorylated with calf intestinal alkaline phosphatase. This end dephosphorylated DNA was ligated with the 6.5 kb HindIII fragment containing the T. reesei pyr4 gene to give pΔCBHIp_{pyr4}. FIG. 1 illustrates the construction of this plasmid.

Example 3

15 Isolation of Protoplasts

Mycelium was obtained by inoculating 100 ml of YEG (0.5% yeast extract, 2% glucose) in a 500 ml flask with about 5×10^7 T. reesei GC69 spores (the pyr4 derivative strain). The flask was then
20 incubated at 37°C with shaking for about 16 hours. The mycelium was harvested by centrifugation at 2,750 x g. The harvested mycelium was further washed in a 1.2 M sorbitol solution and resuspended in 40 ml of a solution containing 5 mg/ml Novozym^R
25 234 solution (which is the tradename for a multicomponent enzyme system containing 1,3-alpha-glucanase, 1,3-beta-glucanase, laminarinase, xylanase, chitinase and protease from Novo Biolabs, Danbury, Ct.); 5 mg/ml MgSO₄·7H₂O; 0.5 mg/ml bovine

- 38 -

serum albumin; 1.2 M sorbitol. The protoplasts were removed from the cellular debris by filtration through Miracloth (Calbiochem Corp, La Jolla, CA) and collected by centrifugation at 2,000 x g. The protoplasts were washed three times in 1.2 M sorbitol and once in 1.2 M sorbitol, 50 mM CaCl₂, centrifuged and resuspended at a density of approximately 2 x 10⁸ protoplasts per ml of 1.2 M sorbitol, 50 mM CaCl₂.

10

Example 4Transformation of Fungal Protoplasts with pACBH₁pyr4

200 µl of the protoplast suspension prepared in Example 3 was added to 20 µl of EcoRI digested pACBH₁pyr4 (prepared in Example 2) in TE buffer (10 mM Tris, pH 7.4; 1 mM EDTA) and 50 µl of a polyethylene glycol (PEG) solution containing 25% PEG 4000, 0.6 M KCl and 50 mM CaCl₂. This mixture was incubated on ice for 20 minutes. After this incubation period 2.0 ml of the above-identified PEG solution was added thereto, the solution was further mixed and incubated at room temperature for 5 minutes. After this second incubation, 4.0 ml of a solution containing 1.2 M sorbitol and 50 mM CaCl₂ was added thereto and this solution was further mixed. The protoplast solution was then immediately added to molten aliquots of Vogel's Medium N (3 grams sodium citrate, 5 grams KH₂PO₄, 2 grams NH₄NO₃, 0.2 grams MgSO₄.7H₂O, 0.1 gram CaCl₂.2H₂O, 5 µg α-biotin, 5 mg citric acid, 5 mg ZnSO₄.7H₂O, 1 mg Fe(NH₄)₂.6H₂O, 0.25 mg CuSO₄.5H₂O, 50 µg MnSO₄.4H₂O per liter) containing an additional 1% glucose, 1.2 M

- 39 -

sorbitol and 1% agarose. The protoplast/medium mixture was then poured onto a solid medium containing the same Vogel's medium as stated above. No uridine was present in the medium and therefore only transformed colonies were able to grow as a result of complementation of the pyr4 mutation of strain GC69 by the wild type pyr4 gene insert in p Δ CBH1pyr4. These colonies were subsequently transferred and purified on a solid Vogel's medium N containing as an additive, 1% glucose and stable transformants were chosen for further analysis.

At this stage stable transformants were distinguished from unstable transformants by their faster growth rate and formation of circular colonies with a smooth, rather than ragged outline on solid culture medium lacking uridine. In some cases a further test of stability was made by growing the transformants on solid non-selective medium (i.e. containing uridine), harvesting spores from this medium and determining the percentage of these spores which will subsequently germinate and grow on selective medium lacking uridine.

Example 5

Analysis of the Transformants

DNA was isolated from the transformants obtained in Example 4 after they were grown in liquid Vogel's medium N containing 1% glucose. These transformant DNA samples were further cut with a PstI restriction enzyme and subjected to agarose gel electrophoresis. The gel was then blotted onto

- 40 -

a Nytran membrane filter and hybridized with a ³²P labelled pΔCBHIpyr4 probe. The probe was selected to identify the native cbh1 gene as a 6.5 kb PstI fragment, the native pyr4 gene and any DNA sequences derived from the transforming DNA fragment.

The radioactive bands from the hybridization were visualized by autoradiography. The autoradiograph is seen in FIG. 3. Five samples were run as described above, hence samples A, B, C, D, and E. Lane E is the untransformed strain GC69 and was used as a control in the present analysis. Lanes A-D represent transformants obtained by the methods described above. The numbers on the side of the autoradiograph represent the sizes of molecular weight markers. As can be seen from this autoradiograph, lane D does not contain the 6.5 kb CBHI band, indicating that this gene has been totally deleted in the transformant by integration of the DNA fragment at the cbh1 gene. The cbh1 deleted strain is called P37PΔCBHI. FIG. 2 outlines the deletion of the T. reesei cbh1 gene by integration through a double cross-over event of the larger EcoRI fragment from pΔCBHIpyr4 at the cbh1 locus on one of the T. reesei chromosomes. The other transformants analyzed appear identical to the untransformed control strain.

Example 6

Analysis of the Transformants with pIntCBHI

The same procedure was used in this example as in Example 5, except that the probe used was changed to a ³²P labelled pIntCBHI probe. This probe is a

- 41 -

pUC-type plasmid containing a 2 kb BglIII fragment from the cbh1 locus within the region that was deleted in pUC4K::cbh1ΔH/H. Two samples were run in this example including a control, sample A, which is the untransformed strain GC69 and the transformant P37PACBHI, sample B. As can be seen in FIG. 4, sample A contained the cbh1 gene, as indicated by the band at 6.5 kb; however the transformant, sample B, does not contain this 6.5 kb band and therefore does not contain the cbh1 gene and does not contain any sequences derived from the pUC plasmid.

Example 7

Protein Secretion by Strain P37PACBHI

Spores from the produced P37PACBHI strain were inoculated into 50 ml of a Trichoderma basal medium containing 1% glucose, 0.14% (NH₄)₂SO₄, 0.2% KH₂PO₄, 0.03% MgSO₄, 0.03% urea, 0.75% bactotryptone, 0.05% Tween 80, 0.000016% CuSO₄.5H₂O, 0.001% FeSO₄.7H₂O, 0.000128% ZnSO₄.7H₂O, 0.0000054% Na₂MoO₄.2H₂O, 0.0000007% MnCl₄.4H₂O). The medium was incubated with shaking in a 250 ml flask at 37°C for about 48 hours. The resulting mycelium was collected by filtering through Miracloth (Calbiochem Corp.) and washed two or three times with 17 mM potassium phosphate. The mycelium was finally suspended in 17 mM potassium phosphate with 1 mM sophorose and further incubated for 24 hours at 30°C with shaking. The supernatant was then collected from these cultures and the mycelium was discarded. Samples of the culture supernatant were analyzed by isoelectric focusing using a Pharmacia Phastgel system and pH 3-

- 42 -

9 precast gels according to the manufacturer's instructions. The gel was stained with silver stain to visualize the protein bands. The band corresponding to the cbh1 protein was absent from the sample derived from the strain P37PACBHI, as shown in FIG. 5. This isoelectric focusing gel shows various proteins in different supernatant cultures of T. reesei. Lane A is partially purified CBHI; Lane B is the supernatant from an untransformed T. reesei culture; Lane C is the supernatant from strain P37PACBHI produced according to the methods of the present invention. The position of various cellulase components are labelled CBHI, CBHII, EGI, EGII, and EGIII. Since CBHI constitutes 50% of the total extracellular protein, it is the major secreted protein and hence is the darkest band on the gel. This isoelectric focusing gel clearly shows depletion of the CBHI protein in the P37PACBHI strain.

Example 8

Preparation of pPACBHII

The cbh2 gene of T. reesei, encoding the CBHII protein, has been cloned as a 4.1 kb EcoRI fragment of genomic DNA which is shown diagrammatically in FIG. 6A (Chen et al., 1987, Biotechnology, 5:274-278). This 4.1 kb fragment was inserted between the EcoRI sites of pUC4XL. The latter plasmid is a pUC derivative (constructed by R.M. Berka, Genencor International Inc.) which contains a multiple cloning site with a symmetrical pattern of restriction endonuclease sites arranged in the order

- 43 -

shown here: EcoRI, BamHI, SacI, SmaI, HindIII, XhoI,
BglII, ClaI, BglII, XhoI, HindIII, SmaI, SacI,
BamHI, EcoRI. Using methods known in the art, a
5 plasmid, pPACBHII (FIG. 6B), has been constructed in
which a 1.7 kb central region of this gene between a
HindIII site (at 74 bp 3' of the CBHII translation
initiation site) and a ClaI site (at 265 bp 3' of
the last codon of CBHII) has been removed and
replaced by a 1.6 kb HindIII- ClaI DNA fragment
10 containing the T. reesei pyr4 gene.

The T. reesei pyr4 gene was excised from pTpyr2
(see Example 2) on a 1.6 kb NheI-SphI fragment and
inserted between the SphI and XbaI sites of pUC219
(see Example 25) to create p219M (Smith et al.,
15 1991, Curr. Genet 19 p. 27-33). The pyr4 gene was
then removed as a HindIII-ClaI fragment having seven
bp of DNA at one end and six bp of DNA at the other
end derived from the pUC219 multiple cloning site
and inserted into the HindIII and ClaI sites of the
20 cbh2 gene to form the plasmid pPACBHII (see FIG.
6B).

Digestion of this plasmid with EcoRI will
liberate a fragment having 0.7 kb of flanking DNA
from the cbh2 locus at one end, 1.7 kb of flanking
25 DNA from the cbh2 locus at the other end and the T.
reesei pyr4 gene in the middle.

- 44 -

Example 9Deletion of the cbh2 gene in T. reesei strain GC69

Protoplasts of strain GC69 will be generated and transformed with EcoRI digested pPACBHII according to the methods outlined in Examples 3 and 4. DNA from the transformants will be digested with EcoRI and Asp718, and subjected to agarose gel electrophoresis. The DNA from the gel will be blotted to a membrane filter and hybridized with ³²P labelled pPACBHII according to the methods in Example 11. Transformants will be identified which have a single copy of the EcoRI fragment from pPACBHII integrated precisely at the cbh2 locus. The transformants will also be grown in shaker flasks as in Example 7 and the protein in the culture supernatants examined by isoelectric focusing. In this manner T. reesei GC69 transformants which do not produce the CBHII protein will be generated.

Example 10Generation of a pyr4⁻ Derivative of P37PACBHI

Spores of the transformant (P37PACBHI) which was deleted for the cbh1 gene were spread onto medium containing FOA. A pyr4⁻ derivative of this transformant was subsequently obtained using the methods of Example 1. This pyr4⁻ strain was designated P37PACBHIPyr26.

- 45 -

Example 11Deletion of the *cbh2* gene in a strain
previously deleted for *cbh1*

5 Protoplasts of strain P37P Δ CBHIPyr⁻26 were
generated and transformed with EcoRI digested
pPACBHII according to the methods outlined in
Examples 3 and 4.

10 Purified stable transformants were cultured in
shaker flasks as in Example 7 and the protein in the
culture supernatants was examined by isoelectric
focusing. One transformant (designated P37P $\Delta\Delta$ CBH67)
was identified which did not produce any CBHII
protein. Lane D of FIG. 5 shows the supernatant
from a transformant deleted for both the *cbh1* and
15 *cbh2* genes produced according to the methods of the
present invention.

DNA was extracted from strain P37P $\Delta\Delta$ CBH67,
digested with EcoRI and Asp718, and subjected to
agarose gel electrophoresis. The DNA from this gel
20 was blotted to a membrane filter and hybridized with
³²p labelled pPACBHII (FIG. 7). Lane A of FIG. 7
shows the hybridization pattern observed for DNA
from an untransformed *T. reesei* strain. The 4.1 kb
EcoRI fragment containing the wild-type *cbh2* gene
25 was observed. Lane B shows the hybridization
pattern observed for strain P37P $\Delta\Delta$ CBH67. The single
4.1 kb band has been eliminated and replaced by two
bands of approximately 0.9 and 3.1 kb. This is the
expected pattern if a single copy of the EcoRI
30 fragment from pPACBHII had integrated precisely at
the *cbh2* locus.

- 46 -

The same DNA samples were also digested with EcoRI and Southern blot analysis was performed as above. In this Example, the probe was ³²P labelled pIntCBHII. This plasmid contains a portion of the cbh2 gene coding sequence from within that segment of the cbh2 gene which was deleted in plasmid pPACBHII. No hybridization was seen with DNA from strain P37PΔΔCBH67 showing that the cbh2 gene was deleted and that no sequences derived from the pUC plasmid were present in this strain.

Example 12

Construction of pEGIp_{pyr4}

The T. reesei egl1 gene, which encodes EGI, has been cloned as a 4.2 kb HindIII fragment of genomic DNA from strain RL-P37 by hybridization with oligonucleotides synthesized according to the published sequence (Penttila et al., 1986, Gene 45:253-263; van Arsdell et al., 1987, Bio/Technology 5:60-64). A 3.6 kb HindIII-BamHI fragment was taken from this clone and ligated with a 1.6 kb HindIII-BamHI fragment containing the T. reesei pyr4 gene obtained from pT_{pyr2} (see Example 2) and pUC218 (identical to pUC219, see Example 25, but with the multiple cloning site in the opposite orientation) cut with HindIII to give the plasmid pEGIp_{pyr4} (FIG. 8). Digestion of pEGIp_{pyr4} with HindIII would liberate a fragment of DNA containing only T. reesei genomic DNA (the egl1 and pyr4 genes) except for 24 bp of sequenced, synthetic DNA between the two genes and 6 bp of sequenced, synthetic DNA at one end (see FIG. 8).

- 47 -

Example 13Purification of Cytolase 123 Cellulase
into Cellulase Components

5 CYTOLASE 123 cellulase was fractionated in the
following manner. The normal distribution of
cellulase components in this cellulase system is as
follows:

	CBH I	45-55 weight percent
	CBH II	13-15 weight percent
10	EG I	11-13 weight percent
	EG II	8-10 weight percent
	EG III	1-4 weight percent
	BG	0.5-1 weight percent.

15 The fractionation was done using columns
containing the following resins: Sephadex G-25 gel
filtration resin from Sigma Chemical Company (St.
Louis, MO), QA Trisacryl M anion exchange resin and
SP Trisacryl M cation exchange resin from IBF
Biotechnics (Savage, MD). CYTOLASE 123 cellulase,
20 0.5g, was desalted using a column of 3 liters of
Sephadex G-25 gel filtration resin with 10 mM sodium
phosphate buffer at pH 6.8. The desalted solution,
was then loaded onto a column of 20 ml of QA
Trisacryl M anion exchange resin. The fraction
25 bound on this column contained CBH I and EG I.
These components were separated by gradient elution
using an aqueous gradient containing from 0 to about
500 mM sodium chloride. The fraction not bound on
this column contained CBH II and EG II. These
30 fractions were desalted using a column of Sephadex
G-25 gel filtration resin equilibrated with 10 mM
sodium citrate, pH 3.3. This solution, 200 ml, was
then loaded onto a column of 20 ml of SP Trisacryl M

- 48 -

cation exchange resin. CBH II and EG II were eluted separately using an aqueous gradient containing from 0 to about 200 mM sodium chloride.

5 Following procedures similar to that of Example 13 above, other cellulase systems which can be separated into their components include CELLUCAST (available from Novo Industry, Copenhagen, Denmark), RAPIDASE (available from Gist Brocades, N.V., Delft, Holland), and cellulase systems derived from
10 Trichoderma koningii, Penicillium sp. and the like.

Example 14

Purification of EG III from Cytolase 123 Cellulase

15 Example 13 above demonstrated the isolation of several components from Cytolase 123 Cellulase. However, because EG III is present in very small quantities in Cytolase 123 Cellulase, the following procedures were employed to isolate this component.

A. Large Scale Extraction of EG III Cellulase Enzyme

20 One hundred liters of cell free cellulase filtrate were heated to about 30°C. The heated material was made about 4% wt/vol PEG 8000 (polyethylene glycol, MW of about 8000) and about 10% wt/vol anhydrous sodium sulfate. The mixture
25 formed a two phase liquid mixture. The phases were separated using an SA-1 disk stack centrifuge. The phases were analyzed using silver staining isoelectric focusing gels. Separation was obtained for EG III and xylanase. The recovered composition
30 contained about 20 to 50 weight percent of EG III.

- 49 -

Regarding the above procedure, use of a polyethylene glycol having a molecular weight of less than about 8000 gave inadequate separation; whereas, use of polyethylene glycol having a molecular weight of greater than about 8000 resulted in the exclusion of desired enzymes in the recovered composition. With regard to the amount of sodium sulfate, sodium sulfate levels greater than about 10% wt/vol caused precipitation problems; whereas, sodium sulfate levels less than about 10% wt/vol gave poor separation or the solution remained in a single phase.

B. Purification of EG III Via Fractionation

The purification of EG III is conducted by fractionation from a complete fungal cellulase composition (CYTOLASE 123 cellulase, commercially available from Genencor International, South San Francisco, CA) which is produced by wild type Trichoderma reesei. Specifically, the fractionation is done using columns containing the following resins: Sephadex G-25 gel filtration resin from Sigma Chemical Company (St. Louis, Mo), QA Trisacryl M anion exchange resin and SP Trisacryl M cation exchange resin from IBF Biotechnics (Savage, Md). CYTOLASE 123 cellulase, 0.5g, is desalted using a column of 3 liters of Sephadex G-25 gel filtration resin with 10 mM sodium phosphate buffer at pH 6.8. The desalted solution, is then loaded onto a column of 20 ml of QA Trisacryl M anion exchange resin. The fraction bound on this column contained CBH I and EG I. The fraction not bound on this column contains CBH II, EG II and EG III. These fractions are desalted using a column of Sephadex G-25 gel

- 50 -

filtration resin equilibrated with 10 mM sodium citrate, pH 4.5. This solution, 200 ml, is then loaded onto a column of 20 ml of SP Trisacryl M cation exchange resin. The EG III was eluted with 100 mL of an aqueous solution of 200 mM sodium chloride.

In order to enhance the efficiency of the isolation of EG III, it may be desirable to employ Trichoderma reesei genetically modified so as to be incapable of producing one or more of EG I, EG II, CBH I and/or CBH II. The absence of one or more of such components will necessarily lead to more efficient isolation of EG III.

Likewise, it may be desirable for the EG III compositions described above to be further purified to provide for substantially pure EG III compositions, i.e., compositions containing EG III at greater than about 80 weight percent of protein. For example, such a substantially pure EG III protein can be obtained by utilizing material obtained from procedure A in procedure B or vice versa. One particular method for further purifying EG III is by further fractionation of an EG III sample obtained in part b) of this Example 14. The further fraction was done on a FPLC system using a Mono-S-HR 5/5 column (available from Pharmacia LKB Biotechnology, Piscataway, NJ). The FPLC system consists of a liquid chromatography controller, 2 pumps, a dual path monitor, a fraction collector and a chart recorder (all of which are available from Pharmacia LKB Biotechnology, Piscataway, NJ). The fractionation was conducted by desalting 5 ml of the

- 51 -

EG III sample prepared in part b) of this Example 14 with a 20 ml Sephadex G-25 column which had been previously equilibrated with 10 mM sodium citrate pH 4. The column was then eluted with 0-200 mM aqueous gradient of NaCl at a rate of 0.5 ml/minute with samples collected in 1 ml fractions. EG III was recovered in fractions 10 and 11 and was determined to be greater than 90% pure by SDS gel electrophoresis. EG III of this purity is suitable for determining the N-terminal amino acid sequence by known techniques.

Substantially pure EG III as well as EG I and EG II components purified in Example 13 above can be used singularly or in mixtures in the methods of this invention. These EG components have the following characteristics:

	MW	pI	pH optimum ¹
EG I	~47-49 kD	4.7	~5
EG II	~35 kD	5.5	~5
EG III	~25-28 kD	7.4	~5.5-6.0

1. pH optimum determined by RBB-CMC activity as per Example 15 below.

The use of a mixture of these components in the practice of this invention may give a synergistic response in improving softening, feel, appearance, etc., as compared to a single component. On the other hand, the use of a single component in the practice of this invention may be more stable or have a broader spectrum of activity over a range of

- 52 -

pHs. For instance, Example 15 below shows that EG III has considerable activity against RBB-CMC under alkaline conditions.

Example 15

5 Activity of Cellulase Compositions
 Over a pH Range

 The following procedure was employed to
determine the pH profiles of two different cellulase
compositions. The first cellulase composition was a
10 CBH I and II deleted cellulase composition prepared
from Trichoderma reesei genetically modified in a
manner similar to that described above so as to be
unable to produce CBH I and CBH II components.
Insofar as this cellulase composition does not
15 contain CBH I and CBH II which generally comprise
from about 58 to 70 percent of a cellulase
composition derived from Trichoderma reesei, this
cellulase composition is necessarily substantially
free of CBH I type and CBH II type cellulase
20 components and accordingly, is enriched in EG
components, i.e., EG I, EG II, EG III and the like.

 The second cellulase composition was an
approximately 20 to 40% pure fraction of EG III
isolated from a cellulase composition derived from
25 Trichoderma reesei via purification methods similar
to part b) of Example 14.

 The activity of these cellulase compositions
was determined at 40°C and the determinations were
made using the following procedures.

- 53 -

- 5 Add 5 to 20 μ l of an appropriate enzyme solution at a concentration sufficient to provide the requisite amount of enzyme in the final solution. Add 250 μ l of 2 weight percent RBB-CMC (Remazol Brilliant Blue R-Carboxymethylcellulose -- commercially available from MegaZyme, 6 Altona Place, North Rocks, N.S.W. 2151, Australia) in 0.05M citrate/phosphate buffer at pH 4, 5, 5.5, 6, 6.5, 7, 7.5 and 8.
- 10 Vortex and incubate at 40°C for 30 minutes. Chill in an ice bath for 5 to 10 minutes. Add 1000 μ l of methyl cellosolve containing 0.3M sodium acetate and 0.02M zinc acetate. Vortex and let sit for 5-10 minutes. Centrifuge and pour supernatant
- 15 into cuvetts. Measure the optical density (OD) of the solution in each cuvet at 590 nm. Higher levels of optical density correspond to higher levels of enzyme activity.
- 20 The results of this analysis are set forth in FIG. 9 which illustrates the relative activity of the CBH I and II deleted cellulase composition compared to the EG III cellulase composition. From this figure, it is seen that the cellulase composition deleted in CBH I and CBH II possesses
- 25 optimum cellulolytic activity against RBB-CMC at near pH 5.5 and has some activity at alkaline pHs, i.e., at pHs from above 7 to 8. On the other hand, the cellulase composition enriched in EG III possesses optimum cellulolytic activity at
- 30 pH 5.5 - 6 and possesses significant activity at alkaline pHs.

- 54 -

From the above example, one skilled in the art would merely need to adjust and maintain the pH of the aqueous textile composition so that the cellulase composition is active and preferably, possesses optimum activity. As noted above, such adjustments and maintenance may involve the use of a suitable buffer.

Example 16

Launderometer Strength Loss Assay Cellulase Compositions

This example examines the ability of different cellulase compositions to reduce the strength of cotton-containing fabrics. This example employs an aqueous cellulase solution maintained at pH 5 because the activity of the most of the cellulase components derived from Trichoderma reesei is greatest at or near pH 5 and accordingly, strength loss results will be most evident when the assay is conducted at about this pH.

Specifically, in this example, the first cellulase composition analyzed was a complete fungal cellulase system (CYTOLASE 123 cellulase, commercially available from Genencor International, South San Francisco, CA) produced by wild type Trichoderma reesei and is identified as GC010.

The second cellulase composition analyzed was a CBH II deleted cellulase composition prepared from Trichoderma reesei genetically modified in a manner similar to Examples 1 to 12 above and 22 to 30 below so as to be incapable of expressing CBH II and is

- 55 -

identified as CBHIIId. Insofar as CBH II comprises up to about 15 percent of the cellulase composition, deletion of this component results in enriched levels of CBH I, and all of the EG components.

5 The third cellulase composition analyzed was a CBH I and CBH II deleted cellulase composition prepared from Trichoderma reesei genetically modified in a manner similar to that described above so as to be incapable of expressing CBH I and CBH II
10 and is identified as CBHI/IIId. Insofar as CBH I and CBH II are not produced by this modified microorganism, the cellulase is necessarily free of all CBH I type components as well as all CBH components.

15 The last cellulase composition analyzed was a CBH I deleted cellulase composition prepared from Trichoderma reesei genetically modified in a manner similar to that described above so as to be incapable of expressing CBH I and is identified as
20 CBHId. Insofar as the modified microorganism is incapable of expressing CBH I, this cellulase composition is necessarily free of all CBH I type cellulase components.

 The cellulase compositions described above were
25 tested for their effect on cotton-containing fabric strength loss in a launderometer. The compositions were first normalized so that equal amounts of EG components were used. Each cellulase composition was then added to separate solutions of
30 400 ml of a 20 mM citrate/phosphate buffer, titrated to pH 5, and which contains 0.5 ml of a non-ionic

- 56 -

surfactant. Each of the resulting solutions was then added to a separate launderometer canister. Into these canisters were added a quantity of marbles to facilitate strength loss as well as a
5 16 inch x 20 inch cotton fabric (100% woven cotton, available as Style No. 467 from Test Fabrics, Inc., 200 Blackford Ave., Middlesex, NJ 08846). The canister was then closed and the canister lowered
10 into the launderometer bath which was maintained at 43°C. The canister was then rotated in the bath at a speed of at least about 40 revolutions per minute (rpms) for about 1 hour. Afterwards, the cloth is removed, rinsed well and dried in a standard drier.

In order to maximize strength loss results, the
15 above procedure was repeated twice more and after the third treatment, the cotton fabrics were removed and analyzed for strength loss. Strength loss was measured by determining the tensile strength in the fill direction ("FTS") using a Instron Tester and
20 the results compared to the FTS of the fabric treated with the same solution with the exception that no cellulase was added. The results of this analysis are reported as percent strength loss which is determined as follows:

25

$$\% \text{ Strength Loss} = 100 \times \left[1 - \frac{\text{FTS with cellulase}}{\text{FTS without cellulase}} \right]$$

The results of this analysis are set forth in
30 FIG. 10 which shows that compositions containing CBH I, i.e., whole cellulase (GC010) and CBH II deleted cellulase, possessed the most strength loss whereas, the compositions containing no CBH I possessed significantly reduced strength loss as

- 57 -

5 compared to whole cellulase and CBH II deleted cellulase. From these results, it is seen that the presence of CBH I type components in a cellulase composition imparts increased strength loss to the composition as compared to a similar composition not containing CBH I type components.

Likewise, these results show that CBH II plays some role in strength loss.

10 Accordingly, in view of these results, strength loss resistant cellulase compositions are those compositions free of all CBH I type cellulase components and preferably, all CBH type cellulase components. In this regard, it is contemplated that such cellulase compositions will result in even
15 lower strength loss at $\text{pH} \geq 7$ than those results observed at pH 5 shown in FIG 10.

20 During the manufacture of cotton-containing fabrics, the fabric can become stressed and when so stressed, it will contain broken and disordered fibers. Such fibers detrimentally impart a worn and dull appearance to the fabric. However, it has been found that the methods of this invention will result in fabric/color enhancement. This is believed to arise by removal of some of the broken and
25 disordered fibers which has the effect of restoring the appearance of the fabric prior to becoming stressed.

30 The following Examples 17 and 18 illustrate this benefit of the present invention. It is noted that these example employed worn cotton T-shirts

- 58 -

(knits) as well as new cotton knits. The faded appearance of the worn cotton-containing fabric arises from the accumulation on the fabric of broken and loose surface fibers over a period of time.

5 These fibers give rise to a faded and matted appearance for the fabric and accordingly, the removal of these fibers is a necessary prerequisite to restoring the original sharp color to the fabric. Additionally, the accumulation of broken surface
10 fibers on new cotton knits imparts a dull appearance to such fabrics. Accordingly, these experiments are necessarily applicable to color enhancement of stressed cotton-containing fabrics because both involve removal of surface fibers from the fabric.

15

Example 17

Color Enhancement

The ability of EG components to enhance color in cotton-containing fabrics was analyzed in the following experiments. Specifically, the first
20 experiment measures the ability of a complete cellulase system (CYTOLASE 123 cellulase, commercially available from Genencor International, South San Francisco, CA) produced by wild type Trichoderma reesei to remove surface fibers from a
25 cotton-containing fabric over various pHs. This cellulase was tested for its ability to remove surface fibers in a launderometer. An appropriate amount of cellulase to provide for either 25 ppm or 100 ppm cellulase in the final composition was added
30 to separate solutions of 400 ml of a 20 mM citrate/phosphate buffer containing 0.5 ml of a

- 59 -

non-ionic surfactant. Samples were prepared and titrated so as to provide for samples at pH 5, pH 6, pH 7 and pH 7.5. Each of the resulting solution was then added to a separate launderometer canister.

5 Into these canisters were added a quantity of marbles to facilitate fiber removal as well as a 7 inch x 5 inch cotton fabric (100% woven cotton, available as Style No. 439W from Test Fabrics, Inc., 200 Blackford Ave., Middlesex, NJ 08846). The
10 canister was then closed and the canister lowered into the launderometer bath which was maintained at 43°C. The canister was then rotated in the bath at a speed of at least about 40 revolutions per minute (rpms) for about 1 hour. Afterwards, the cloth is
15 removed, rinsed well and dried in a standard drier.

The so treated fabrics were then analyzed for fiber removal by evaluation in a panel test. In particular, the fabrics (unmarked) were rated for levels of fiber by 6 individuals. The fabrics were
20 visually evaluated for surface fibers and rated on a 0 to 6 scale. The scale has six standards to allow meaningful comparisons. The standards are:

	Rating	Standard ^a
	0	Fabric not treated with cellulase
25	1	Fabric treated ^b with 8 ppm cellulase
	2	Fabric treated with 16 ppm cellulase
	3	Fabric treated with 20 ppm cellulase
	4	Fabric treated with 40 ppm cellulase
	5	Fabric treated with 50 ppm cellulase
30	6	Fabric treated with 100 ppm cellulase

- 60 -

a) In all of the standards, the fabric was a 100% cotton sheeting standardized test fabric (Style No. 439W) available from Test Fabrics, Inc., 200 Blackford Ave., Middlesex, NJ 08846

5 b) All samples were treated with the same cellulase composition. Cellulase concentrations are in total protein. The launderometer treatment conditions are the same as set forth in Example 16 above.

10 The fabric to be rated was provided a rating which most closely matched one of the standards. After complete analysis of the fabrics, the values assigned to each fabric by all of the individuals were added and an average value generated.

15 The results of this analysis are set forth in FIG. 11. Specifically, FIG. 11 illustrates that at the same pH, a dose dependent response is seen in the amount of fibers removed. That is to say that at the same pH, the fabrics treated with more
20 cellulase provided for higher levels of fiber removal as compared to fabrics treated with less cellulase. Moreover, the results of this figure demonstrate that at higher pHs, fiber removal can
25 still be effected merely by using higher concentrations of cellulase.

In a second experiment, two different cellulase compositions were compared for the ability to remove fiber. Specifically, the first cellulase composition analyzed was a complete cellulase system (CYTOLASE
30 123 cellulase, commercially available from Genencor International, South San Francisco, CA) produced by

- 61 -

wild type Trichoderma reesei and is identified as GC010.

5 The second cellulase composition analyzed was a cellulase composition substantially free of all CBH type components (including CBH I type components) which composition was prepared from Trichoderma reesei genetically modified in a manner similar to that described above so as to be incapable of expressing CBH I and CBH II and is identified as CBHI/II deleted. Insofar as CBH I and CBH II comprises up to about 70 percent of the cellulase composition, deletion of this component results in enriched levels of all of the EG components.

15 These compositions were tested for their ability to remove surface fibers in a launderometer. An appropriate amount of cellulase to provide for the requisite concentrations of EG components in the final compositions were added to separate solutions of 400 ml of a 20 mM citrate/phosphate buffer containing 0.5 ml of a non-ionic surfactant. Samples were prepared and titrated to pH 5. Each of the resulting solutions was then added to a separate launderometer canister. Into these canisters were added a quantity of marbles to facilitate fiber removal as well as a 7 inch x 5 inch cotton fabric (100% woven cotton, available as Style No. 439W from Test Fabrics, Inc., 200 Blackford Ave., Middlesex, NJ 08846). The canister was then closed and the canister lowered into the launderometer bath which was maintained at 43°C. The canister was then rotated in the bath at a speed of at least about 40

- 62 -

revolutions per minute (rpms) for about 1 hour. Afterwards, the cloth is removed, rinsed well and dried in a standard drier.

5 The so treated fabrics were then analyzed for
fiber removal by evaluation in the panel test
described above. The results of this analysis are
set forth in FIG. 12 which is plotted based on
estimated EG concentrations. Specifically, FIG. 12
10 illustrates that both GC010 and CBH I/II Deleted
cellulase compositions gave substantially identical
fiber removal results at substantially equal EG
concentrations. The results of this figure suggest
that it is the EG components which provide for fiber
15 removal. These results coupled with the results of
FIG. 11 demonstrate that EG components remove
surface fibers.

Example 18

Tergotometer Color Enhancement

20 This example is further to Example 17 and
substantiates that CBH type components are not
necessary for color enhancement and the purpose of
this example is to examine the ability of cellulase
compositions deficient in CBH type components to
enhance color to cotton-containing fabrics.

25 Specifically, the cellulase composition
employed in this example was substantially free of
all CBH type components (including CBH I type
components) insofar as this composition was prepared
from Trichoderma reesei genetically modified in a

- 63 -

manner similar to that described above so as to be incapable of expressing CBH I and CBH II. Insofar as CBH I and CBH II comprises up to about 70 percent of the cellulase composition, deletion of this component results in enriched levels of all of the EG components.

The assay was conducted by adding a sufficient concentration of this cellulase composition to a 50 mM citrate/phosphate buffer to provide 500 ppm of cellulase. The solution was titrated to pH 5 and contained 0.1 weight percent of nonionic surfactant (Grescoterg GL100 -- commercially available from Gresco Mfg., Thomasville, NC 27360). A 10 inch x 10 inch faded cotton-containing fabric as well as a 10 inch x 10 inch new knitted fabric having loose and broken surface fibers were then placed into 1 liter of this buffer and allowed to sit at 110°F for 30 minutes and then agitated for 30 minutes at 100 rotations per minute. The fabrics were then removed from the buffer, washed and dried. The resulting fabrics were then compared to the fabric prior to treatment. The results of this analysis are as follows:

<u>Cotton-Containing Material</u>	<u>Result</u>
Worn Cotton T-Shirt	benefit seen
New Cotton Knit	benefit seen

The term "benefit seen" means that the treated fabric exhibits color restoration (i.e., is less faded) as compared to the non-treated fabric which includes removal of broken surface fibers including broken surface fibers generated as a result of using

- 64 -

the tergotometer. These results substantiate the results of Example 17 that the presence of CBH type components is not necessary for effecting color restoration of faded cotton-containing fabrics.

5 It is contemplated that the use of such cellulase compositions would be beneficial during fabric processing because such compositions would remove broken/loose fibers generated during processing without detrimental strength loss to the
10 fabric.

Example 19

Softness

15 This Example demonstrates that the presence of CBH type components are not essential for imparting improved softness to cotton-containing fabrics. Specifically, this Example employs a cellulase composition free of all CBH type components which composition is derived from Trichoderma reesei genetically engineered in the manner described above
20 so as to be incapable of producing CBH I and II components.

25 This cellulase composition was tested for its ability to soften terry wash cloth. Specifically, unsoftened 8.5 ounce cotton terry cloths, 14 inches by 15 inches (available as Style No. 420NS from Test Fabrics, Inc., 200 Blackford Ave., Middlesex, NJ 08846), were cut into 7 inch by 7.5 inch swatches.

- 65 -

The cellulase composition described above was tested for its ability to soften these swatches in a launderometer. Specifically, an appropriate amount of cellulase to provide for 500 ppm, 250 ppm, 100 ppm, 50 ppm, and 10 ppm cellulase in the final cellulase solution was added to separate solutions of 400 ml of a 20 mM citrate/phosphate buffer containing 0.025 weight percent of a non-ionic surfactant (Triton X114). Additionally, a blank was run containing the same solution but with no added cellulase. Samples so prepared were titrated to pH 5. Each of the resulting solution was then added to a separate launderometer canister. Into these canisters were added a quantity of marbles to facilitate softness as well as cotton swatches described above. All conditions were run in triplicate with two swatches per canister. Each canister was then closed and the canister lowered into the launderometer bath which was maintained at 37°C. The canister was then rotated in the bath at a speed of at least about 40 revolutions per minute (rpms) for about 1 hour. Afterwards, the swatches were removed, rinsed well and dried in a standard drier.

The swatches were then analyzed for softness by evaluation in a preference test. Specifically, six panelists were given their own set of swatches and ask to rate them with respect to softness based on the softness criteria such as the pliability of the whole fabric. Swatches obtained from treatment with the five different enzyme concentrations and the blank were placed behind a screen and the panelists were asked to order them from least soft to most

- 66 -

soft. Scores were assigned to each swatch based on its order relative to the other swatches; 5 being most soft and 0 being least soft. The scores from each panelists were cumulated and then averaged.

5 The results of this averaging are set forth in FIG. 13. Specifically, these results demonstrate that at higher concentrations, improved softening is obtained. It is noted that this improved softening is achieved without the presence of either CBH I or
10 II in the cellulase composition.

Example 20

Feel and Appearance

 This example demonstrates that the presence of CBH type components are not essential for imparting
15 improved feel and appearance to cotton-containing fabrics. Specifically, this example employs a cellulase composition derived from Trichoderma reesei genetically engineered in the manner described above so as to be incapable of producing
20 any CBH type components (i.e., incapable of producing CBH I and II components).

 This cellulase composition was tested for its ability to improve the appearance of cotton-containing fabrics. Specifically, appropriately
25 sized 100% cotton sheeting (available as Style No. 439W from Test Fabrics, Inc., 200 Blackford Ave., Middlesex, NJ 08846) were employed in the appearance aspects of this example.

- 67 -

The cellulase composition described above was tested for its ability to improve the appearance of these samples in a launderometer. Specifically, an appropriate amount of CBH I and II deleted cellulase to provide for 25 ppm, 50 ppm, and 100 ppm cellulase in the final cellulase solution was added to separate solutions of 400 ml of a 20 mM citrate/phosphate buffer containing 0.025 weight percent of a non-ionic surfactant (Triton X114). Additionally, a blank was run containing the same solution but with no added cellulase. Samples so prepared were titrated to pH 5. Each of the resulting solutions was then added to a separate launderometer canister. Into these canisters were added a quantity of marbles to facilitate improvements in appearance as well as cotton samples described above. Each canister was then closed and the canister lowered into the launderometer bath which was maintained at about 40°C. The canister was then rotated in the bath at a speed of at least about 40 revolutions per minute (rpms) for about 1 hour. Afterwards, the samples were removed, rinsed well and dried in a standard drier.

The samples were then analyzed for improved appearance by evaluation in a preference test. Specifically, 6 panelists were given the 4 samples (not identified) and asked to rate them with respect to appearance. The panelists were instructed that the term "appearance" refers to the physical appearance of the cotton-containing fabric to the eye and is determined in part, by the presence or absence of, fuzz, surface fibers, and the like on the surface of the fabric as well as by the ability

- 68 -

or inability to discern the construction (weave) of the fabric. Fabrics which have little if any fuzz and surface fibers and wherein the construction (weave) is clearly discernable possess improved appearance as compared to fabrics having fuzz and/or loose fibers and/or an indiscernible weave.

The panelists then assigned scores were assigned to each sample based on its order relative to the other samples; 4 having the best appearance and 1 having the worst appearance. The scores from each panelists were cumulated and then averaged. The results of this test are as follows:

	<u>Amt Cellulase</u>	<u>Average Appearance</u>
	None	1
15	25 ppm	2
	50 ppm	3
	100 ppm	4

The CBH I and II deleted cellulase composition was then tested for its ability to improve the feel of cotton-containing fabrics. Specifically, appropriately sized 100% cotton sheeting (available as Style No. 439W from Test Fabrics, Inc., 200 Blackford Ave., Middlesex, NJ 08846) were employed in the feel aspects of this example.

The cellulase composition described above was tested for its ability to improve the feel of these samples in a launderometer. Specifically, an appropriate amount of cellulase to provide for 500 ppm, 1000 ppm, and 2000 ppm cellulase in the final cellulase solution was added to separate solutions

- 69 -

of 24 L of a 20 mM citrate/phosphate buffer. Additionally, a blank was run containing the same solution but with no added cellulase. All tests were conducted at pH 5.8 and run in an industrial washer. The washer was operated at 50°C, a total volume of 24 L, a liquor to cloth ratio of 50:1 (weight to weight) and the washer was run for 30 minutes. Afterwards, the samples were removed and dried in an industrial dryer.

The samples were then analyzed for improved feel by evaluation in a preference test. Specifically, 5 panelists were given the 4 samples (not identified) and asked to rate them with respect to feel. The panelists were instructed that fabrics having improved feel are smoother and silkier to the touch than other fabrics and that feel is distinguished from qualities such as softness (which refers to the pliability of the fabric rather than its feel), thickness, color, or other physical characteristics not involved in smoothness of the fabric.

The panelists then assigned scores to each sample based on its order relative to the other samples; 4 having the best feel and 1 having the worst feel. The scores from each panelists were cumulated and then averaged. The results of this test are as follows:

<u>Amt Cellulase</u>	<u>Average Feel</u>
None	1.5 ± 0.5
500 ppm	1.7 ± 0.4
1000 ppm	3.2 ± 0.4
2000 ppm	3.8 ± 0.4

- 70 -

The above results demonstrate that improvements in feel and appearance can be achieved with cellulase compositions free of all CBH type components.

5

Example 21Stone Washed Appearance

10 This example demonstrates that the presence of CBH type components are not essential for imparting a stone washed appearance to cotton-containing fabrics. Specifically, this example employs a cellulase composition derived from Trichoderma reesei genetically engineered in the manner described above so as to be incapable of producing any CBH type components (i.e., incapable of
15 producing CBH I and II components) as well as a complete cellulase composition derived from Trichoderma reesei and which is available as Cytolase 123 cellulase from Genencor International, South San Francisco, California.

20

These cellulase compositions were tested for their ability to impart a stone washed appearance to dyed cotton-containing denims pants. Specifically, the samples were prepared using an industrial washer and dryer under the following conditions:

25

10 mM citrate/phosphate buffer pH 5
40 L total volume
110°F

Four pair of denim pants
1 hour run time

30

50 ppm CBH I and II deleted cellulase or 100 ppm whole cellulase (i.e., at approximately equal EG concentrations)

- 71 -

Samples were evaluated for their stonewashed appearance by 8 panelists. All eight panelists choose 100 ppm whole cellulase over non-enzyme treated pants as having the better stone washed look. Four of the 8 panelists choose the CBH I and II deleted cellulase treated pants over whole cellulase as having the better stone washed look; whereas the other panelists choose the whole cellulase treated pants as having a better stone washed look. These results indicate that the CBH I and II deleted cellulase treated pants were indistinguishable from whole cellulase treated pants and that CBH I and/or CBH II are not essential for imparting a stone washed appearance to cotton-containing fabrics.

With regard to Examples 16 to 21, cellulase compositions free of CBH I type components and derived from microorganisms other than Trichoderma reesei could be used in place of the cellulase compositions described in these examples. In particular, the source of the cellulase composition containing the EG type components is not important to this invention and any fungal cellulase composition containing one or more EG type components and substantially free of all CBH I type components can be used herein. For example, fungal cellulases for use in preparing the fungal cellulase compositions used in this invention can be obtained from Trichoderma koningii, Pencillum sp., and the like or commercially available cellulases can be used, i.e., CELLUCAST (available from Novo Industry,

- 72 -

Copenhagen, Denmark), RAPIDASE (available from Gist Brocades, N.V., Delft, Holland), and the like.

Example 22

5 Transformants of Trichoderma reesei Containing the plasmid pEGIp_{pyr}4

 A pyr4 defective derivative of T. reesei strain
RutC30 (Sheir-Neiss and Montenecourt, (1984), Appl.
10 Microbiol. Biotechnol. 20:46-53) was obtained by the
 method outlined in Example 1. Protoplasts of this
 strain were transformed with undigested pEGIp_{pyr}4 and
 stable transformants were purified.

 Five of these transformants (designated EP2,
EP4, EP5, EP6, EP11), as well as untransformed
RutC30 were inoculated into 50 ml of YEG medium
15 (yeast extract, 5 g/l; glucose, 20 g/l) in 250 ml
 shake flasks and cultured with shaking for two days
 at 28°C. The resulting mycelium was washed with
 sterile water and added to 50 ml of TSF medium
 (0.05M citrate-phosphate buffer, pH 5.0; Avicel
20 microcrystalline cellulose, 10 g/l; KH₂PO₄, 2.0 g/l;
 (NH₄)₂SO₄, 1.4 g/l; proteose peptone, 1.0 g/l; Urea,
 0.3 g/l; MgSO₄.7H₂O, 0.3 g/l; CaCl₂, 0.3 g/l;
 FeSO₄.7H₂O, 5.0 mg/l; MnSO₄.H₂O, 1.6 mg/l; ZnSO₄, 1.4
 mg/l; CoCl₂, 2.0 mg/l; 0.1% Tween 80). These
25 cultures were incubated with shaking for a further
 four days at 28°C. Samples of the supernatant were
 taken from these cultures and assays designed to
 measure the total amount of protein and of
 endoglucanase activity were performed as described
30 below.

- 73 -

The endoglucanase assay relied on the release of soluble, dyed oligosaccharides from Remazol Brilliant Blue-carboxymethylcellulose (RBB-CMC, obtained from MegaZyme, North Rocks, NSW, Australia). The substrate was prepared by adding 2 g of dry RBB-CMC to 80 ml of just boiled deionized water with vigorous stirring. When cooled to room temperature, 5 ml of 2 M sodium acetate buffer (pH 4.8) was added and the pH adjusted to 4.5. The volume was finally adjusted to 100 ml with deionized water and sodium azide added to a final concentration of 0.02%. Aliquots of T. reesei control culture, pEGIpyr4 transformant culture supernatant or 0.1 M sodium acetate as a blank (10-20 μ l) were placed in tubes, 250 μ l of substrate was added and the tubes were incubated for 30 minutes at 37°C. The tubes were placed on ice for 10 minutes and 1 ml of cold precipitant (3.3% sodium acetate, 0.4% zinc acetate, pH 5 with HCl, 76% ethanol) was then added. The tubes were vortexed and allowed to sit for five minutes before centrifuging for three minutes at approximately 13,000 x g. The optical density was measured spectrophotometrically at a wavelength of 590-600 nm.

The protein assay used was the BCA (bicinchoninic acid) assay using reagents obtained from Pierce, Rockford, Illinois, USA. The standard was bovine serum albumin (BSA). BCA reagent was made by mixing 1 part of reagent B with 50 parts of reagent A. One ml of the BCA reagent was mixed with 50 μ l of appropriately diluted BSA or test culture supernatant. Incubation was for 30 minutes at 37°C

- 74 -

and the optical density was finally measured spectrophotometrically at a wavelength of 562 nm.

5 The results of the assays described above are shown in Table 1. It is clear that some of the transformants produced increased amounts of endoglucanase activity compared to untransformed strain RutC30. It is thought that the endoglucanases and exo-cellobiohydrolases produced by untransformed *T. reesei* constitute approximately 10 20 and 70 percent respectively of the total amount of protein secreted. Therefore a transformant such as EP5, which produces approximately four-fold more endoglucanase than strain RutC30, would be expected to secrete approximately equal amounts of 15 endoglucanase-type and exo-cellobiohydrolase-type proteins.

The transformants described in this Example were obtained using intact pEGIp_{pyr4} and will contain DNA sequences integrated in the genome which were 20 derived from the pUC plasmid. Prior to transformation it would be possible to digest pEGIp_{pyr4} with *Hind*III and isolate the larger DNA fragment containing only *T. reesei* DNA. Transformation of *T. reesei* with this isolated 25 fragment of DNA would allow isolation of transformants which overproduced EGI and contained no heterologous DNA sequences except for the two short pieces of synthetic DNA shown in FIG. 8. It would also be possible to use pEGIp_{pyr4} to transform 30 a strain which was deleted for either the *cbh1* gene, or the *cbh2* gene, or for both genes. In this way a strain could be constructed which would over-produce

- 75 -

EGI and produce either a limited range of, or no, exo-cellobiohydrolases.

5 The methods of Example 22 could be used to produce T. reesei strains which would over-produce any of the other cellulase components, xylanase components or other proteins normally produced by T. reesei.

TABLE 1

10 Secreted Endoglucanase Activity of
T. reesei Transformants

		A	B	
		ENDOGLUCANASE		
		ACTIVITY	PROTEIN	
	<u>STRAIN</u>	<u>(O.D. AT 590 nm)</u>	<u>(mg/ml)</u>	<u>A/B</u>
15	RutC30	0.32	4.1	0.078
	EP2	0.70	3.7	0.189
	EP4	0.76	3.65	0.208
	EP5	1.24	4.1	0.302
	EP6	0.52	2.93	0.177
20	EP11	0.99	4.11	0.241

25 The above results are presented for the purpose of demonstrating the overproduction of the EGI component relative to total protein and not for the purpose of demonstrating the extent of overproduction. In this regard, the extent of overproduction is expected to vary with each experiment.

- 76 -

Example 23Construction of pCEPC1

A plasmid, pCEPC1, was constructed in which the coding sequence for EGI was functionally fused to the promoter from the cbh1 gene. This was achieved using in vitro, site-specific mutagenesis to alter the DNA sequence of the cbh1 and egl1 genes in order to create convenient restriction endonuclease cleavage sites just 5' (upstream) of their respective translation initiation sites. DNA sequence analysis was performed to verify the expected sequence at the junction between the two DNA segments. The specific alterations made are shown in FIG. 14.

The DNA fragments which were combined to form pCEPC1 were inserted between the EcoRI sites of pUC4K and were as follows (see FIG. 15):

A) A 2.1 kb fragment from the 5' flanking region of the cbh1 locus. This includes the promoter region and extends to the engineered BclI site and so contains no cbh1 coding sequence.

B) A 1.9 kb fragment of genomic DNA from the egl1 locus starting at the 5' end with the engineered BamHI site and extending through the coding region and including approximately 0.5 kb beyond the translation stop codon. At the 3' end of the fragment is 18 bp derived from the pUC218 multiple cloning site and a 15 bp synthetic oligonucleotide used to link this fragment with the fragment below.

- 77 -

C) A fragment of DNA from the 3' flanking region of the cbh1 locus, extending from a position approximately 1 kb downstream to approximately 2.5 kb downstream of the cbh1 translation stop codon.

5 D) Inserted into an NheI site in fragment (C) was a 3.1 kb NheI-SphI fragment of DNA containing the T. reesei pyr4 gene obtained from pTpyr2 (Example 2) and having 24 bp of DNA at one end derived from the pUC18 multiple cloning site.

10 The plasmid, pCEPC1 was designed so that the EGI coding sequence would be integrated at the cbh1 locus, replacing the coding sequence for CBHI without introducing any foreign DNA into the host strain. Digestion of this plasmid with EcoRI
15 liberates a fragment which includes the cbh1 promoter region, the egl1 coding sequence and transcription termination region, the T. reesei pyr4 gene and a segment of DNA from the 3' (downstream) flanking region of the cbh1 locus (see Fig. 15).

20 Example 24

Transformants containing pCEPC1 DNA

A pyr4 defective strain of T. reesei RutC30 (Sheir-Neiss, supra) was obtained by the method outlined in Example 1. This strain was transformed
25 with pCEPC1 which had been digested with EcoRI. Stable transformants were selected and subsequently cultured in shaker flasks for cellulase production as described in Example 22. In order to visualize the cellulase proteins, isoelectric focusing gel
30 electrophoresis was performed on samples from these

- 78 -

cultures using the method described in Example 7. Of a total of 23 transformants analysed in this manner 12 were found to produce no CBHI protein, which is the expected result of integration of the pCEPC1 DNA at the cbh1 locus. Southern blot analysis was used to confirm that integration had indeed occurred at the cbh1 locus in some of these transformants and that no sequences derived from the bacterial plasmid vector (pUC4K) were present (see Fig. 16). For this analysis the DNA from the transformants was digested with PstI before being subjected to electrophoresis and blotting to a membrane filter. The resulting Southern blot was probed with radiolabelled plasmid pUC4K::cbh1 (see Example 2). The probe hybridised to the cbh1 gene on a 6.5 kb fragment of DNA from the untransformed control culture (FIG. 16, lane A). Integration of the pCEPC1 fragment of DNA at the cbh1 locus would be expected to result in the loss of this 6.5 kb band and the appearance of three other bands corresponding to approximately 1.0 kb, 2.0 kb and 3.5 kb DNA fragments. This is exactly the pattern observed for the transformant shown in FIG. 16, lane C. Also shown in FIG. 16, lane B is an example of a transformant in which multiple copies of pCEPC1 have integrated at sites in the genome other than the cbh1 locus.

Endoglucanase activity assays were performed on samples of culture supernatant from the untransformed culture and the transformants exactly as described in Example 22 except that the samples were diluted 50 fold prior to the assay so that the protein concentration in the samples was between

- 79 -

approximately 0.03 and 0.07 mg/ml. The results of assays performed with the untransformed control culture and four different transformants (designated CEPC1-101, CEPC1-103, CEPC1-105 and CEPC1-112) are shown in Table 2. Transformants CEPC1-103 and CEPC1-112 are examples in which integration of the CEPC1 fragment had led to loss of CBHI production.

Table 2

Secreted endoglucanase activity of T. reesei transformants

	A	B	
	ENDOGLUCANASE ACTIVITY	PROTEIN	
<u>STRAIN</u>	<u>(O.D. at 590 nm)</u>	<u>(mg/ml)</u>	<u>A/B</u>
RutC300	0.037	2.38	0.016
CEPC1-101	0.082	2.72	0.030
CEPC1-103	0.099	1.93	0.051
CEPC1-105	0.033	2.07	0.016
CEPC1-112	0.093	1.72	0.054

The above results are presented for the purpose of demonstrating the overproduction of the EGI component relative to total protein and not for the purpose of demonstrating the extent of overproduction. In this regard, the extent of overproduction is expected to vary with each experiment.

It would be possible to construct plasmids similar to pCEPC1 but with any other T. reesei gene replacing the egl1 gene. In this way, overexpression of other genes and simultaneous deletion of the cbh1 gene could be achieved.

- 80 -

It would also be possible to transform pyr4 derivative strains of T. reesei which had previously been deleted for other genes, eg. for cbh2, with pCEPC1 to construct transformants which would, for example, produce no exo-cellobiohydrolases and overexpress endoglucanases.

Using constructions similar to pCEPC1, but in which DNA from another locus of T. reesei was substituted for the DNA from the cbh1 locus, it would be possible to insert genes under the control of another promoter at another locus in the T. reesei genome.

Example 25

Construction of pEGII::P-1

The egl3 gene, encoding EGII (previously referred to as EGIII by others), has been cloned from T. reesei and the DNA sequence published (Saloheimo et al., 1988, Gene 63:11-21). We have obtained the gene from strain RL-P37 as an approximately 4 kb PstI-XhoI fragment of genomic DNA inserted between the PstI and XhoI sites of pUC219. The latter vector, pUC219, is derived from pUC119 (described in Wilson et al., 1989, Gene 77:69-78) by expanding the multiple cloning site to include restriction sites for BglIII, ClaI and XhoI. Using methods known in the art the T. reesei pyr4 gene, present on a 2.7 kb SalI fragment of genomic DNA, was inserted into a SalI site within the EGII coding sequence to create plasmid pEGII::P-1 (FIG.

- 81 -

17). This resulted in disruption of the EGII coding sequence but without deletion of any sequences. The plasmid, pEGII::P-1 can be digested with HindIII and BamHI to yield a linear fragment of DNA derived
5 exclusively from T. reesei except for 5 bp on one end and 16 bp on the other end, both of which are derived from the multiple cloning site of pUC219.

Example 26

10 Transformation of T. reesei GC69 with pEGII::P-1 to
creat a strain unable to produce EGII

T. reesei strain GC69 will be transformed with pEGII::P-1 which had been previously digested with HindIII and BamHI and stable transformants will be selected. Total DNA will be isolated from the
15 transformants and Southern blot analysis used to identify those transformants in which the fragment of DNA containing the pyr4 and egl3 genes had integrated at the egl3 locus and consequently disrupted the EGII coding sequence. The
20 transformants will be unable to produce EGII. It would also be possible to use pEGII::P-1 to transform a strain which was deleted for either or all of the cbh1, cbh2, or egl1 genes. In this way a strain could be constructed which would only produce
25 certain cellulase components and no EGII component.

- 82 -

Example 27Transformation of *T. reesei* with pEGII::P-1
to create a strain unable to produce CBHI,
CBHII and EGII

5 A pyr4 deficient derivative of strain
P37PΔΔCBH67 (from Example 11) was obtained by the
method outlined in Example 1. This strain P37PΔΔ67P⁻
1 was transformed with pEGII::P-1 which had been
previously digested with HindIII and BamHI and
10 stable transformants were selected. Total DNA was
isolated from transformants and Southern blot
analysis used to identify strains in which the
fragment of DNA containing the pyr4 and egl3 genes
had integrated at the egl3 locus and consequently
15 disrupted the EGII coding sequence. The Southern
blot illustrated in FIG. 18 was probed with an
approximately 4 kb PstI fragment of *T. reesei* DNA
containing the egl3 gene which had been cloned into
the PstI site of pUC18 and subsequently re-isolated.
20 When the DNA isolated from strain P37PΔΔ67P⁻1 was
digested with PstI for Southern blot analysis the
egl3 locus was subsequently visualized as a single 4
kb band on the autoradiograph (FIG. 18, lane E).
However, for a transformant disrupted for the egl3
25 gene this band was lost and was replaced by two new
bands as expected (FIG. 18, Lane F). If the DNA was
digested with EcoRV or BglII the size of the band
corresponding to the egl3 gene increased in size by
approximately 2.7 kb (the size of the inserted pyr4
30 fragment) between the untransformed P37PΔΔ67P⁻1
strain (Lanes A and C) and the transformant
disrupted for egl3 (FIG. 18, Lanes B and D). The
transformant containing the disrupted egl3 gene

- 83 -

illustrated in FIG. 18 (Lanes B, D and F) was named A22. The transformant identified in FIG. 18 is unable to produce CBHI, CBHII or EGII.

Example 28

5 Construction of p Δ EGI-1

The egl1 gene of T. reesei strain RL-P37 was obtained, as described in Example 12, as a 4.2 kb HindIII fragment of genomic DNA. This fragment was inserted at the HindIII site of pUC100 (a derivative
10 of pUC18; Yanisch-Perron et al., 1985, Gene 33:103-119, with an oligonucleotide inserted into the multiple cloning site adding restriction sites for BglII, ClaI and XhoI). Using methodology known in the art an approximately 1 kb EcoRV fragment
15 extending from a position close to the middle of the EGI coding sequence to a position beyond the 3' end of the coding sequence was removed and replaced by a 3.5 kb ScaI fragment of T. reesei DNA containing the pyr4 gene. The resulting plasmid was called p Δ EGI-
20 1 (see Fig. 19).

The plasmid p Δ EGI-1 can be digested with HindIII to release a DNA fragment comprising only T. reesei genomic DNA having a segment of the egl1 gene at either end and the pyr4 gene replacing part of
25 the EGI coding sequence, in the center.

Transformation of a suitable T. reesei pyr4 deficient strain with the p Δ EGI-1 digested with HindIII will lead to integration of this DNA fragment at the egl1 locus in some proportion of the

- 84 -

transformants. In this manner a strain unable to produce EGI will be obtained.

Example 29

Construction of pΔEGIp_{pyr}-3 and Transformation of a pyr4 deficient strain of *T. reesei*

5

The expectation that the EGI gene could be inactivated using the method outlined in Example 28 is strengthened by this experiment. In this case a plasmid, pΔEGIp_{pyr}-3, was constructed which was similar to pΔEGI-1 except that the *Aspergillus niger* pyr4 gene replaced the *T. reesei* pyr4 gene as selectable marker. In this case the *egl1* gene was again present as a 4.2 kb HindIII fragment inserted at the HindIII site of pUC100. The same internal 1 kb EcoRV fragment was removed as during the construction of pΔEGI-1 (see Example 28) but in this case it was replaced by a 2.2 kb fragment containing the cloned *A. niger* pyrG gene (Wilson et al., 1988, *Nucl. Acids Res.* 16 p.2339).

Transformation of a pyr4 deficient strain of *T. reesei* (strain GC69) with pΔEGIp_{pyr}-3, after it had been digested with HindIII to release the fragment containing the pyrG gene with flanking regions from the *egl1* locus at either end, led to transformants in which the *egl1* gene was disrupted. These transformants were recognized by Southern blot analysis of transformant DNA digested with HindIII and probed with radiolabelled pΔEGIp_{pyr}-3. In the untransformed strain of *T. reesei* the *egl1* gene was present on a 4.2 kb HindIII fragment of DNA and this pattern of hybridization is represented by Fig. 20, lane C. However, following deletion of the *egl1*

10

15

20

25

30

- 85 -

gene by integration of the desired fragment from pΔEGIp_{yr}-3 this 4.2 kb fragment disappeared and was replaced by a fragment approximately 1.2 kb larger in size, FIG. 20, lane A. Also shown in FIG. 20, lane B is an example of a transformant in which integration of a single copy of pΔEGIp_{yr}-3 has occurred at a site in the genome other than the egl1 locus.

Example 30

10 Transformation of T.reesei with pΔEGI-1
 to create a strain unable to produce
 CBHI, CBHII, EGI and EGII

 A pyr4 deficient derivative of strain A22 (from Example 27) will be obtained by the method outlined in Example 1. This strain will be transformed with pΔEGI-1 which had been previously digested with HindIII to release a DNA fragment comprising only T. reesei genomic DNA having a segment of the egl1 gene at either end with part of the EGI coding sequence replaced by the pyr4 gene.

 Stable pyr4⁺ transformants will be selected and total DNA isolated from the transformants. The DNA will be probed with ³²P labelled pΔEGI-1 after Southern blot analysis in order to identify transformants in which the fragment of DNA containing the pyr4 gene and egl1 sequences has integrated at the egl1 locus and consequently disrupted the EGI coding sequence. The transformants identified will be unable to produce CBHI, CBHII, EGI and EGII.

- 86 -

WHAT IS CLAIMED IS:

1. An improved method for treating cotton-containing fabrics by treatment with a fungal cellulase composition wherein said improvement
5 comprises employing a fungal cellulase composition which is substantially free of all CBH I type cellulase components.
2. The method according to Claim 1 wherein
10 said fungal cellulase composition is free of all CBH type components.
3. The method according to Claim 1 wherein
15 said fungal cellulase composition comprises at least about 20 weight percent EG type components based on the total weight of protein in the cellulase composition.
4. An improved method for the treatment of
20 cotton-containing fabrics with an aqueous fungal cellulase solution wherein said method is conducted with agitation under conditions so as to produce a cascading effect of the cellulase solution over the fabric wherein said improvement comprises employing
a fungal cellulase composition which is
substantially free of all CBH I type components.
5. A method according to Claim 4 wherein said
25 fungal cellulase composition is free of all CBH type components.
6. A method according to Claim 4 wherein said
fungal cellulase composition comprises at least

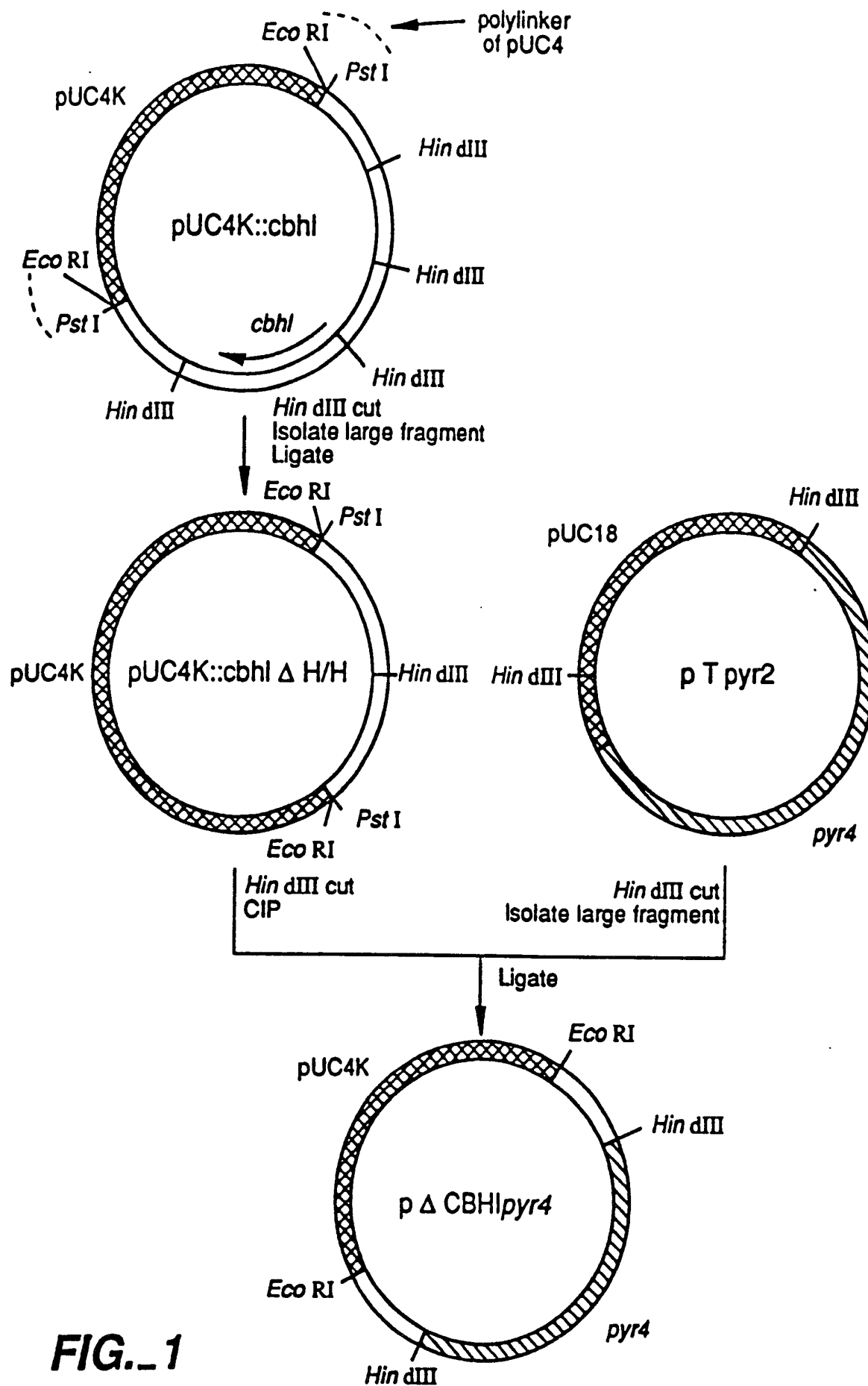
- 87 -

about 20 weight percent of EG components based on the total weight of protein in the cellulase composition.

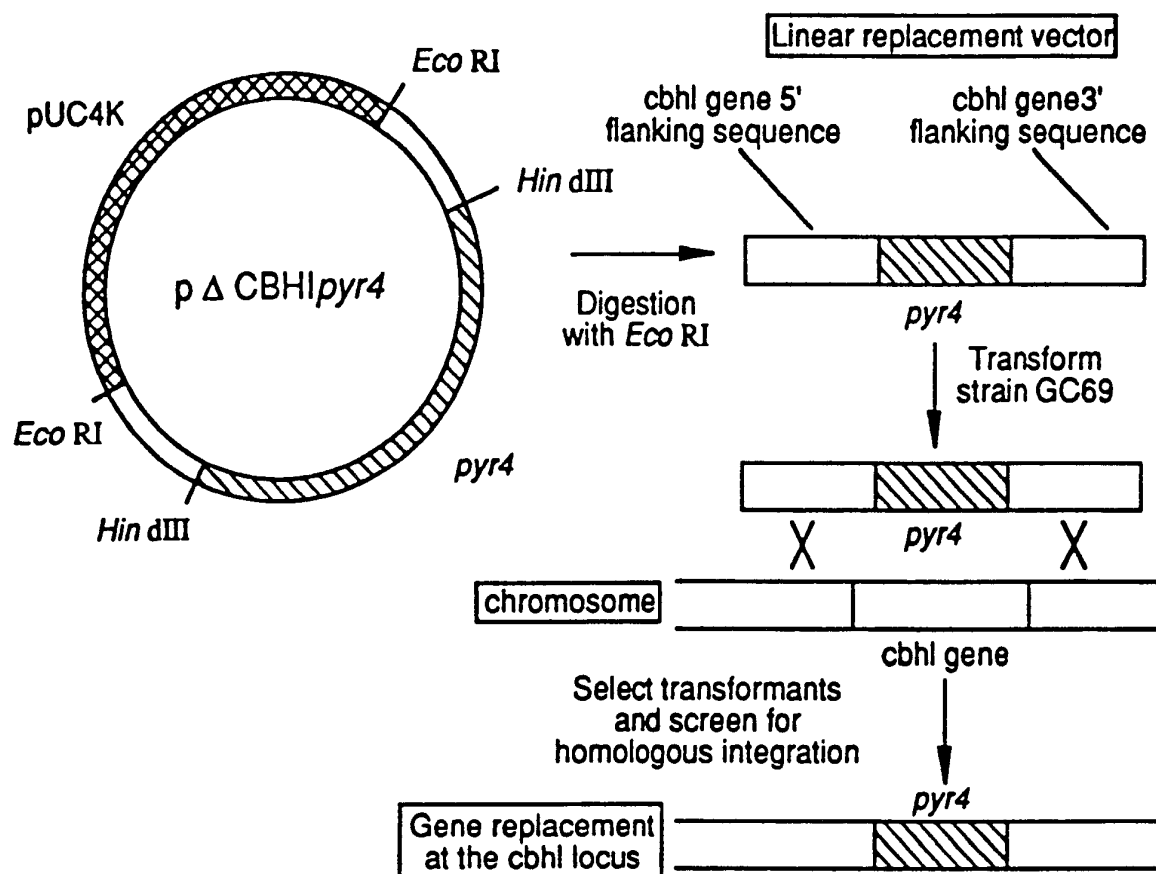
5 7. A cotton-containing fabric having improved feel and/or appearance wherein said fabric is prepared by the method defined in Claim 1.

 8. A cotton-containing fabric having improved feel and/or appearance wherein said fabric is prepared by the method defined in Claim 4.

1/17

**FIG. 1**

2/17

**FIG._2**

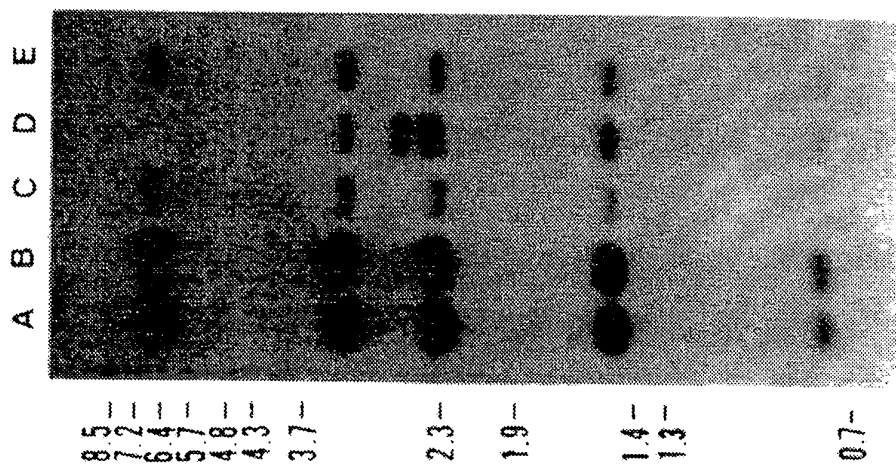


FIG-3

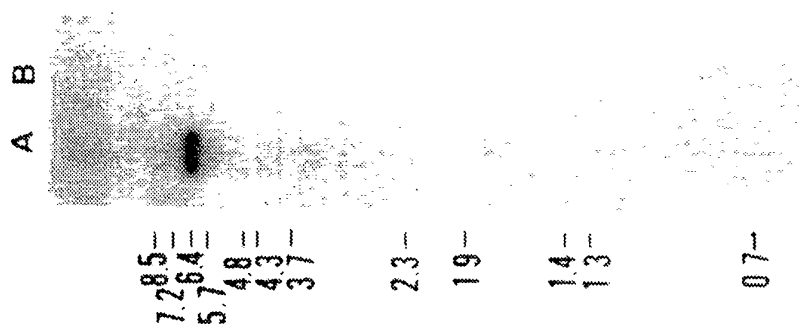


FIG-4

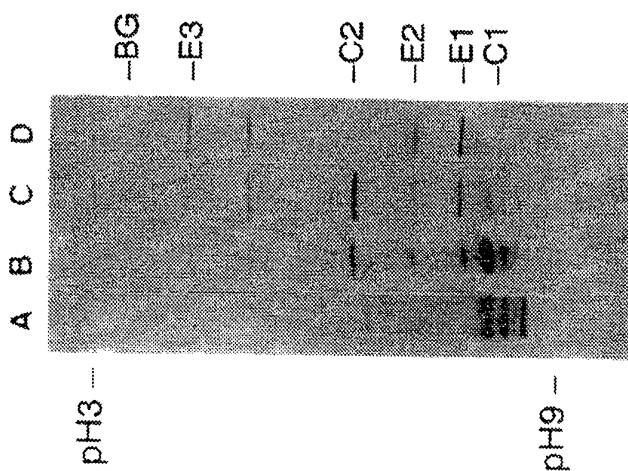


FIG-5

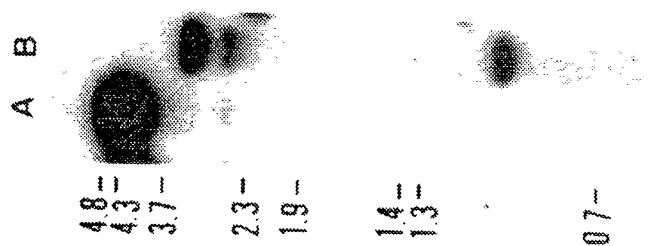


FIG-7



FIG._6A

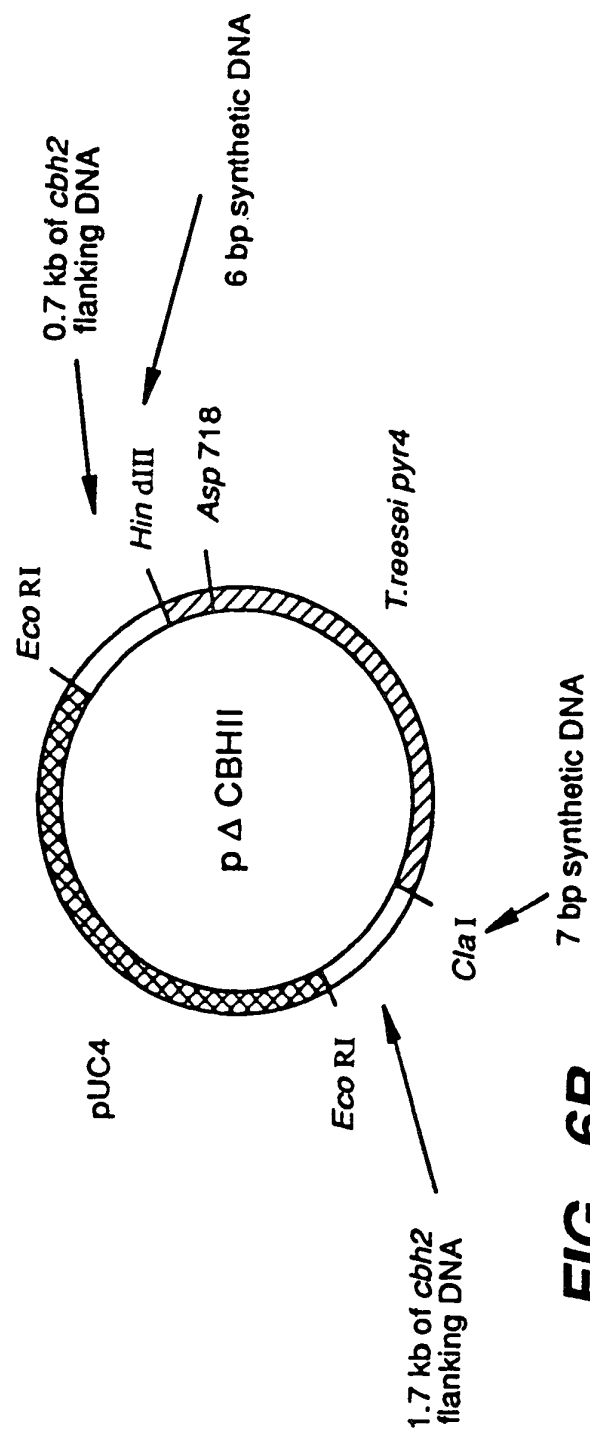
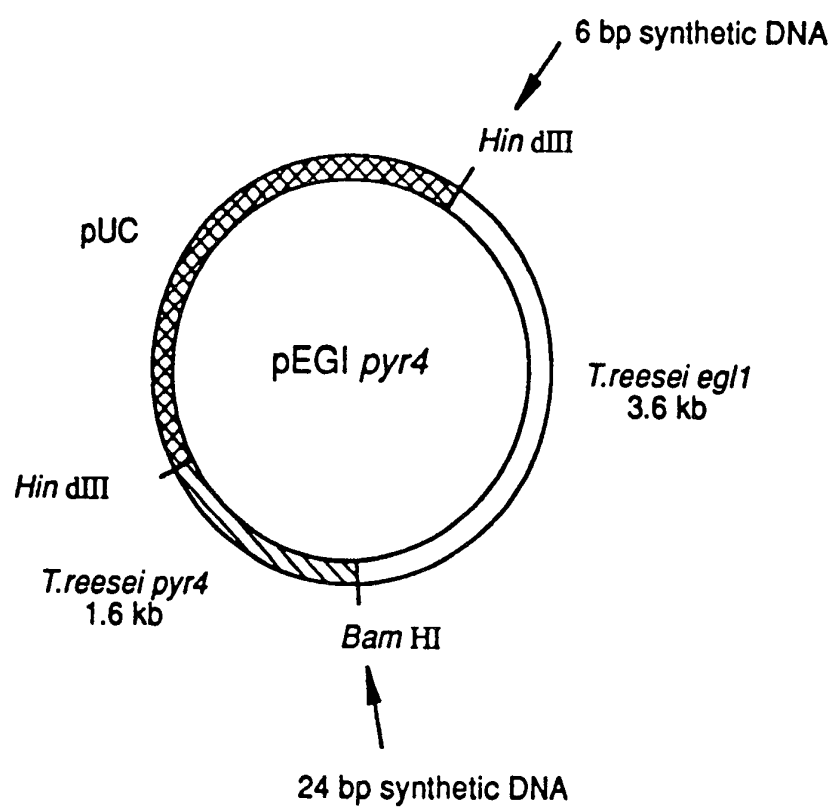


FIG._6B

5/17

**FIG._8**

6/17

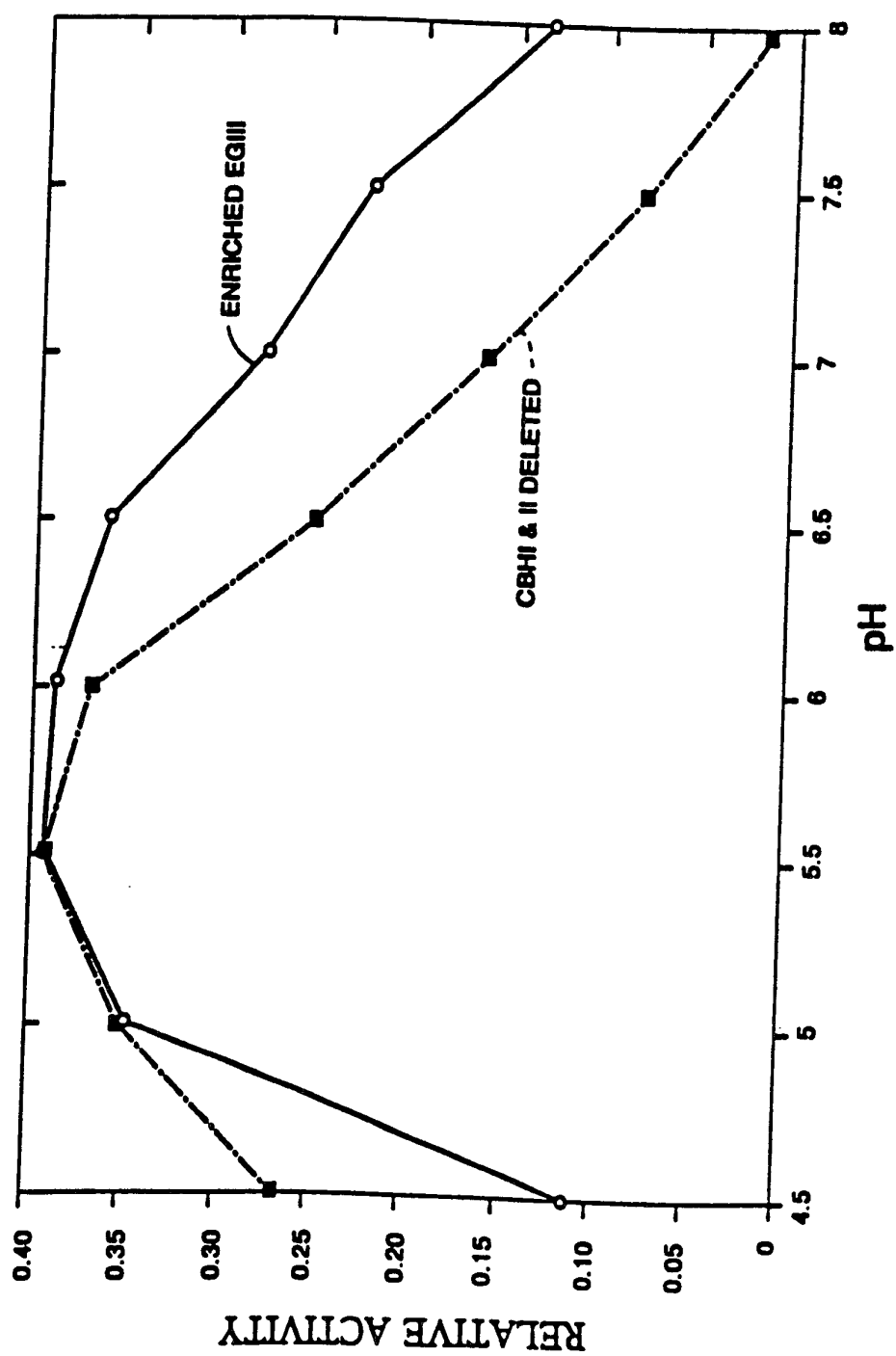
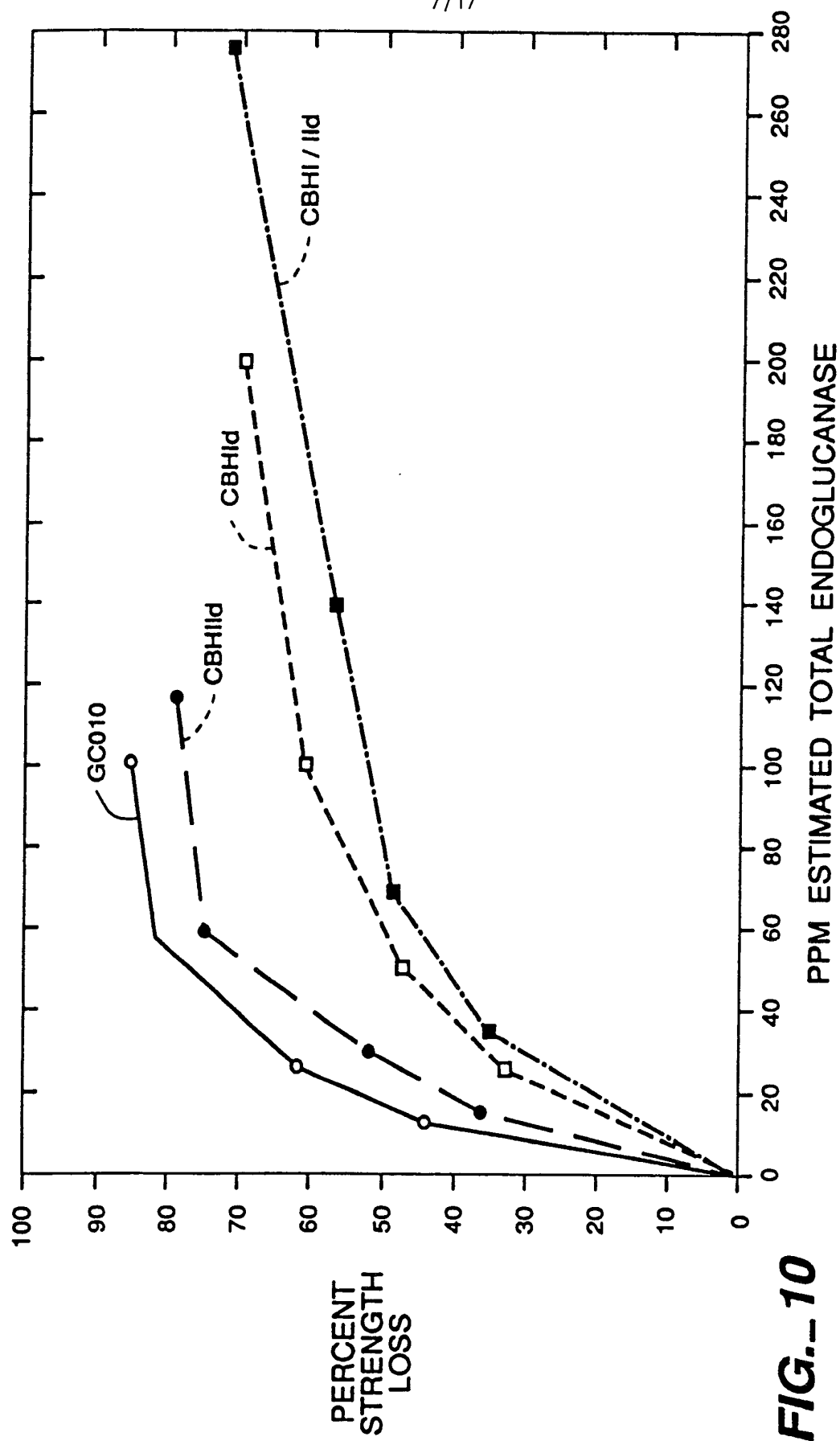
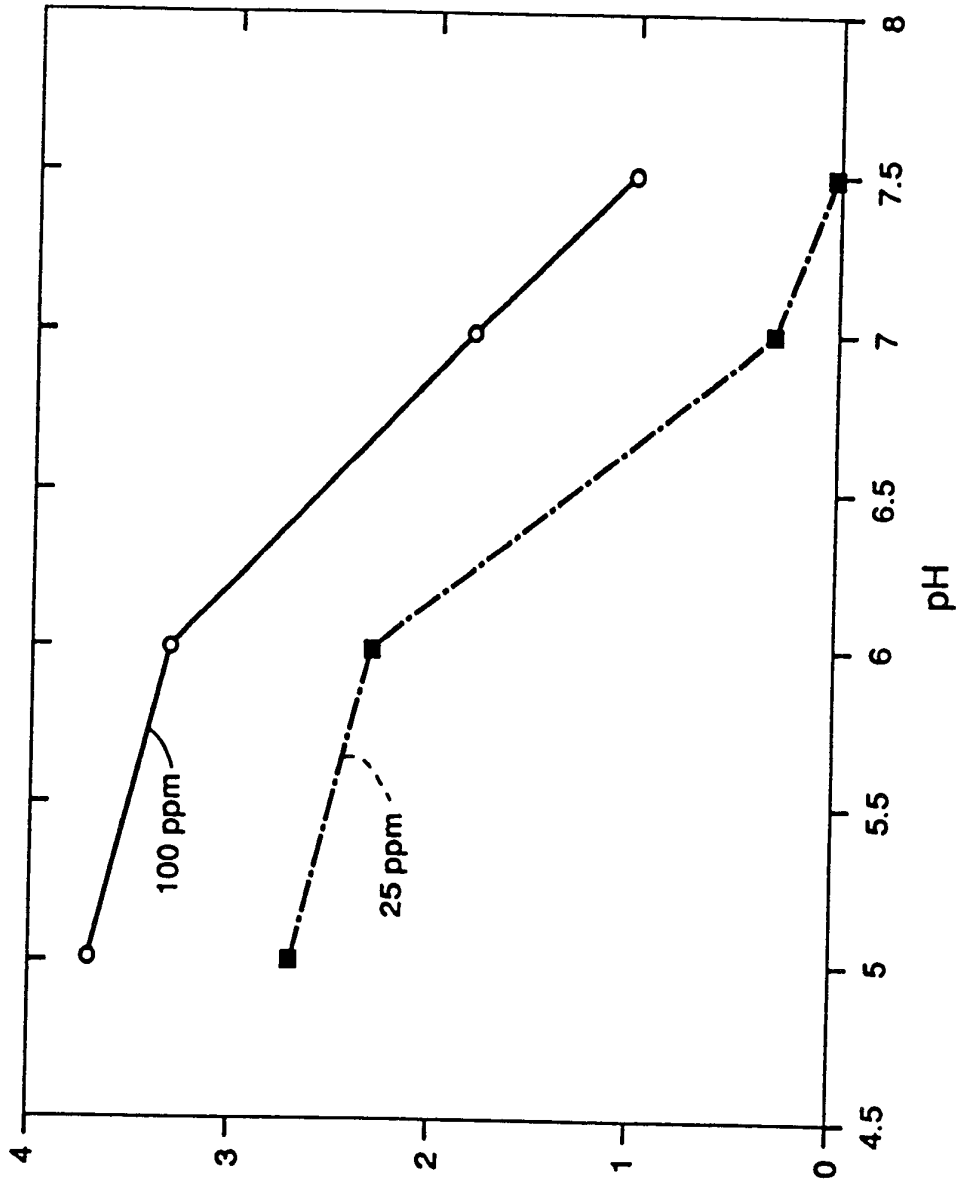


Fig. 9

7/17

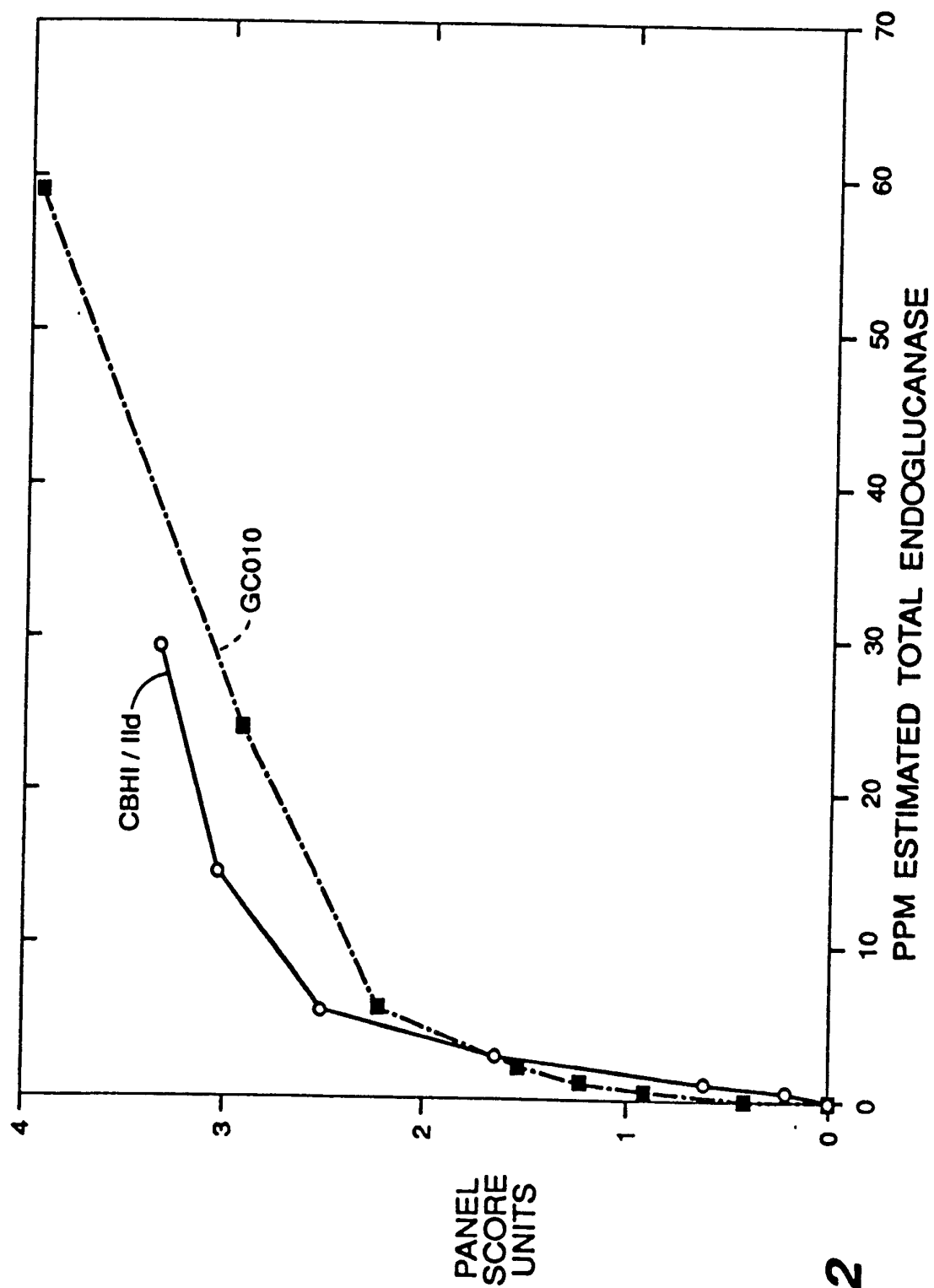
**FIG. 10**



PANEL SCORE UNITS

FIG. 11

9/17

**FIG.-12**

10/17

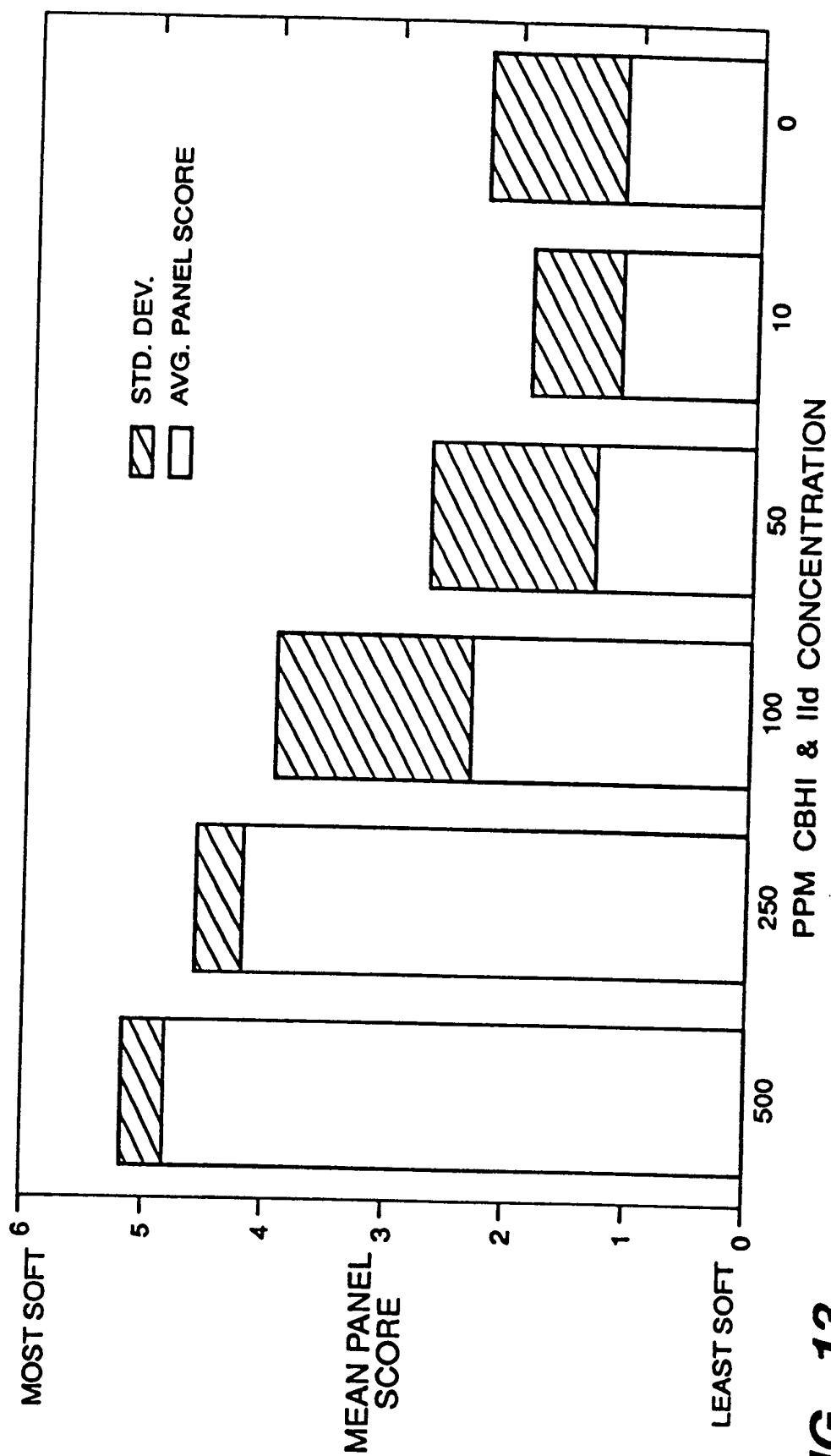


FIG. 13

11/17

cbh1

AAACCCAATAGTCAACCGCGGACTGGCAT ATG TAT CGG
G T A
AAACCCAATAGTGATCAGCGGACTGGCAT ATG TAT CGG
BclI First 3 codons

egl1

TAGTCCTTCTTGTTGTCCCAA ATG GCG CCC
GGA
TAGTCCTTCTTGGATCCAA ATG GCG CCC
BamHI First 3 codons

FIG. 14

LINEAR FRAGMENT OF DNA OBTAINED FROM pCEPC1 BY EcoRI DIGESTION

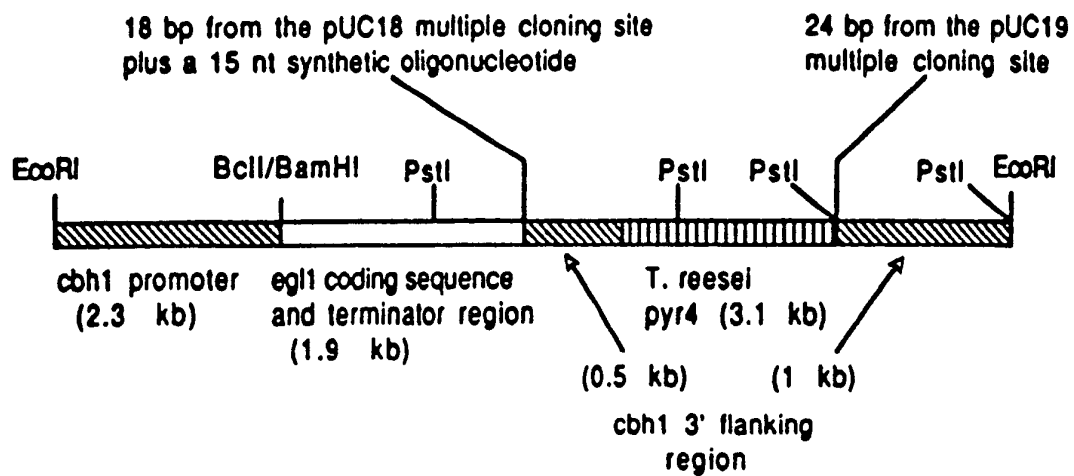


FIG. 15

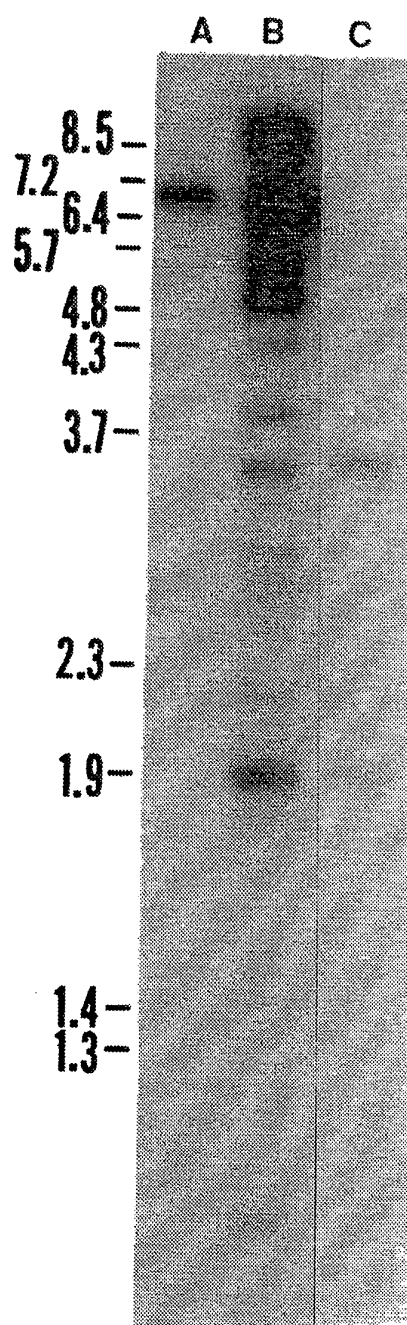


FIG. 16

14/17

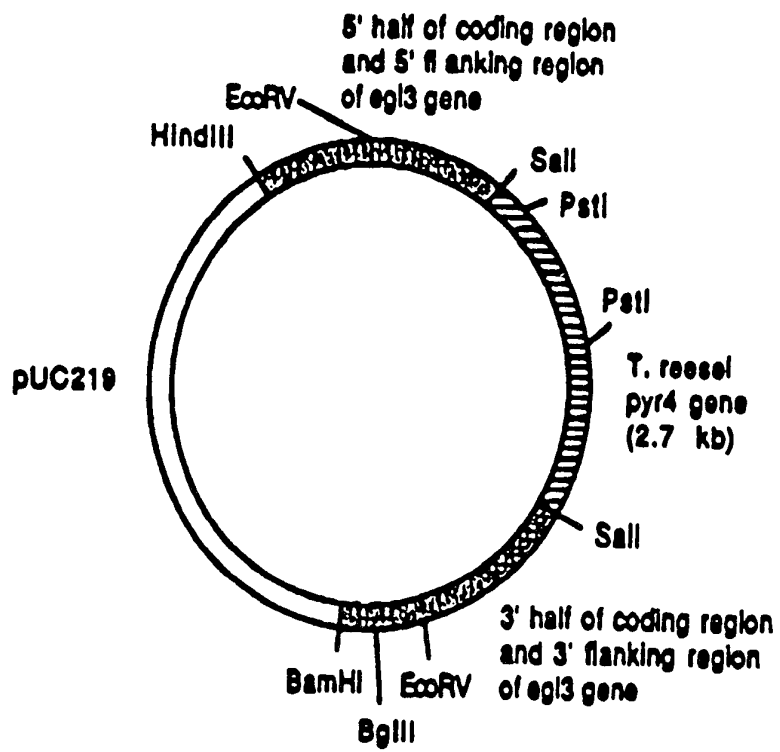


DIAGRAM OF pEGII::P-1

FIG. 17

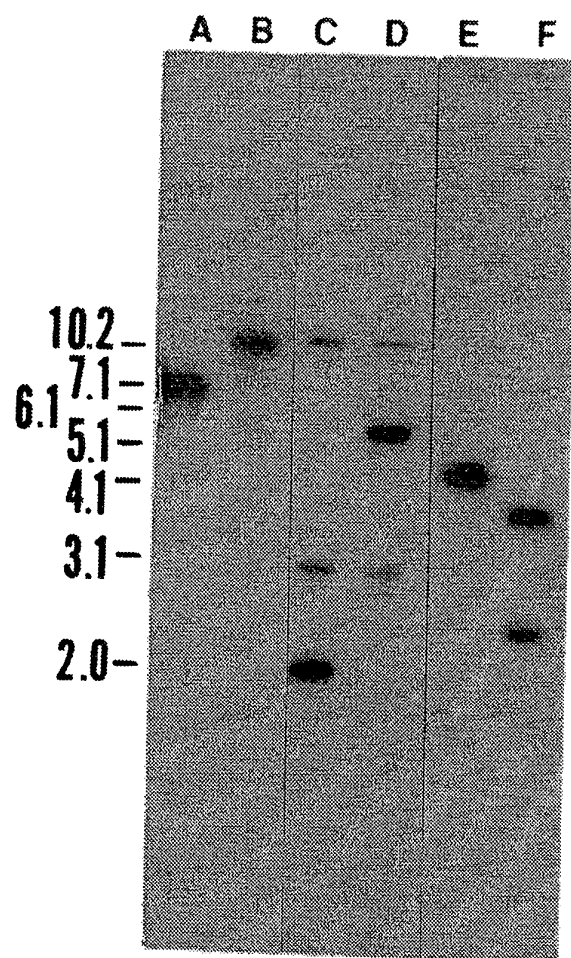


FIG. 18

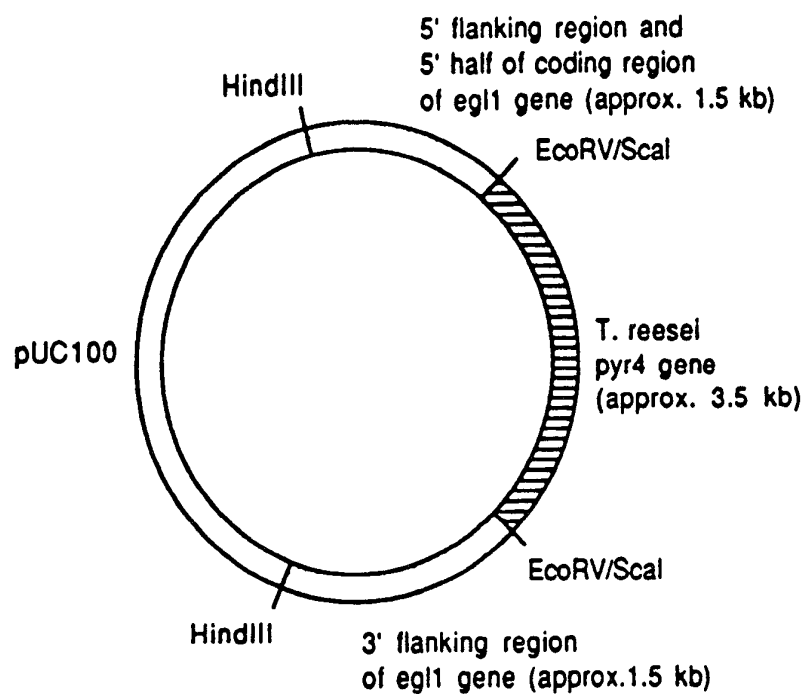
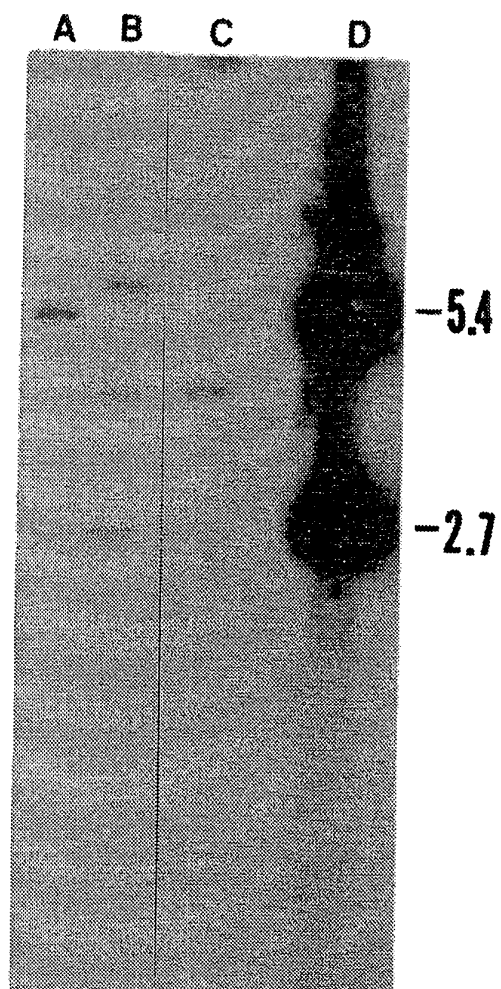


FIG. 19

17/17

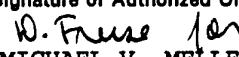


FIG_ 20

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/02629

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC (5): C 12 N 9/40, 1/00 US CL : 435/209, 945		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	435/209, 945	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁵		
APS, CAS ONLINE, BIOSIS, DIALOG SEARCH TERMS: CELLULASE?, ENDOGLUCANASE?, EXO-CELLOBIOHYDROLASE?, (FABRIC? OR COTTON? OR JEAN? OR DENIM? OR CLOTH?)		
III. DOCUMENTS CONSIDERED TO BE RELEVANT¹⁴		
Category ⁶	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	US, A, 4,894,338 (Knowles et al.) 16 January 1990, see column 1, lines 24-28.	1-8
Y	Teeri, "The cellulolytic enzyme system of <u>Trichoderma reesei</u> ", published 1987 by the Technical Research Centre of Finland, see pages 1-4, 13 and 17-20.	1-8
Y	The Biochemical Journal, Volume 260, No. 1, issued 15 May 1989, Wood et al., "The Mechanism of Fungal Cellulase Action", pages 37-43, see entire document.	1-8
¹⁵ Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ²	
29 MAY 1992	23 JUN 1992	
International Searching Authority ¹	Signature of Authorized Officer ²⁰	
ISA/US	 MICHAEL V. MELLER	