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(54) Title: AN ENZYME WITH EXOCHITINASE ACTIVITY

(57) Abstract

The invention relates to a DNA construct comprising a DNA sequence encoding an enzyme exhibiting exochitinase activity, which DNA sequence comprises the DNA sequence shown in SEQ ID No. 1 or SEQ ID No. 3, or analogues of the DNA sequences encoding polypeptides which are derived from *Saccharomyces cerevisiae* DSM No. 9944 or *Saccharomyces cerevisiae* DSM No. 9945. Further, the invention relates to a method of producing said enzyme, an enzyme preparation containing said enzyme, and the use of said exochitinase or said enzyme preparation for a number of purposes. Also claimed is the use of the DNA construct of the invention for producing transgenic plants. Finally, an isolated substantially pure culture of the deposited microorganisms is claimed.

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AN ENZYME WITH EXOCHITINASE ACTIVITY

FIELD OF INVENTION

The present invention relates to an enzyme with exochitinase activity, a DNA construct encoding said enzyme, a method of producing said enzyme, and an enzyme preparation containing said enzyme.

Further the invention relates to the use of said exochitinase or said enzyme preparation for a number of purposes.

BACKGROUND OF THE INVENTION

Considerable amount of chitin is found in the cells wall of most true fungal cells, in the exoskeleton of insects and crustaceans, and in nematodes (Cabib E., (1987) Adv. Enzymol., 59, 59-101; Gooday G., (1990), Microbial Ecol., 10, 397-431).

Native occurring chitin consist of a chemically stable insoluble polymer substance composed of β-1,4-N-acetyl glucosamine molecules of varying length, stabilized by hydrogen bonds to a higher ordered crystalline structure (Cabib E., (1987), Adv. Enzymol., 59, 59-101).

The polymer molecules of chitin are bound by β-1,4 linkages, and are in fungal cell walls often associated with β-1,3 glucan or β-1,6 glucan, polymers of glucose with β-1,3 and β-1,6 linkages, as well as various intrinsic cell wall components

Chitin is known to be hydrolysed by chitinases, including exo- and endochitinases, which is present in most fungi, yeasts, plants and certain procaryotes (Cabib E., (1987), Adv. Enzymol., 59, 59-101; Gooday G., (1990), Microbial Ecol., 10, 397-431).

Exochitinases are enzymes that exolytically hydrolyse the β-1,4-linkage between two consecutive N-acetylglucosamines from the non-reducing end of chitin.

Exochitinases are also referred to as chitobiosidases or β-N-acetylhexosaminidases (E.C. 3.2.1.52, Enzyme Nomenclature, Academic Press, Inc., 1992).

Endochitinases (E.C. 3.4.1.14) are enzymes which randomly hydrolyse N-acetyl-β-D-glucosaminide 1,4-β-linkages of chitin and chitodextrins.

Chitinases, are thought to play an important role in the cell division and differentiation of fungi, and in the mycoparasitic activity displayed by several fungi, such as *Gliocladium virens*, *Aphanocladium album* and *Trichoderma harzianum* (De La Cruz et al. (1992) Eur. J. Biochem., 206, 859-867; Blaiseau and Lafay, (1992), Elsevier science publisher B.V., 243-248; Di Pietro et al., (1993), Mol. Plant Pathology 83, 308-313). Although many fungal chitinases have been reported, only few of them have been cloned and characterized at molecular level (Harman et al., (1993), Mol. Plant Pathology 83, 313-318; Blaiseau and Lafay, (1992), supra; Gracia, (1994), Current Genetics 27, 83-89.

A number of readily available commercial enzyme products useful in the enzymatic lysis of fungal cells comprise chitinase. Beside chitinase(s) such products normally also comprise multiple enzymatic activities, *e.g.* including β-1,3- and β-1,6-glucanase, cellulase, protease, mannase and other enzymes capable of degrading cell wall components.

WO 92/22314 (Cornell Research Foundation, INC) describes two chitinases from *Trichoderma harzianum* P1 (ATCC 74058) which inhibit chitin containing fungus and insects. The first is an

endochitinase having a molecular weight of 36 kDa and an isoelectric point of about 5.3. The second is an exochitinase having a molecular weight of 36 kDa and an isoelectric point of about 4.4.

WO 94/24288 and WO 94/02598 (Cornell Research Foundation, INC) discloses two chitinases from *Trichoderma harzianum* P1 (ATCC 74058) which inhibit chitin containing fungus and insects. The first is an endochitinase and the other is an chitobiosidase. Both have molecular weights of 40 kDa and isoelectric points of about 3.9.

EP 440.304 concerns plants exhibiting a relative overexpression of at least one gene encoding intracellular chitinase and intra- or extracellular β-1,3 glucanase. Further, the recombinant polynucleotides are disclosed.

Comments to prior art

In general enzyme preparations containing chitinases also contain a number of other enzyme activities. This may in certain cases be a drawback.

Said drawback may be remedied by using single-component enzymes (*i.e.* substantially without any side activity) exhibiting chitinase activity.

Consequently there exists a need for providing novel chitinases, preferably in single-component form, which may be used for applications where a single or dominating chitinolytic activity is desirable.

SUMMARY OF THE INVENTION

The present inventors have now surprisingly succeeded in isolating and characterizing two DNA sequences which encode enzymes exhibiting exochitinase activity, thereby making it possible to prepare single-component exochitinases.

Accordingly, in a first aspect the invention relates to a DNA construct comprising a DNA sequence encoding an enzyme exhibiting exochitinase activity, which DNA sequence

a) comprises the DNA sequence shown in SEQ ID No. 1 or SEQ ID no. 3, or

b) comprises an analogue of the DNA sequence shown in SEQ ID no. 1 or SEQ ID no 3, which

i) is homologous with the DNA sequences shown in SEQ ID no. 1 or SEQ ID no. 3, and/or

ii) hybridizes with the same oligonucleotide probe as the DNA sequence shown in SEQ ID no. 1 or SEQ ID no. 3, and/or

iii) encodes a polypeptide which is homologous with the polypeptide encoded by a DNA sequence comprising the DNA sequence shown in SEQ ID No. 1 or SEQ ID no. 3, and/or

iv) encodes a polypeptide which is immunologically reactive with an antibody raised against a purified exochitinase shown in SEQ ID no. 2 derived from *Saccharomyces cerevisiae* DSM no. 9944 or against a purified Exochitinase shown in SEQ ID no. 4 derived from *Saccharomyces cerevisiae* DSM no. 9945.

In a second aspect the invention relates to a DNA construct comprising a DNA sequence encoding an enzyme exhibiting exochitinase activity, which DNA sequence comprises at least a partial sequence of the DNA sequence shown in SEQ ID no. 1 or SEQ ID no. 3.

In the present context, the "analogue" of the DNA sequence shown in SEQ ID no. 1 or SEQ ID no. 3 is intended to indicate any DNA sequence encoding an enzyme exhibiting exochitinase activity, which has the properties i)-vi). The analogous DNA sequence

- may be isolated from any organism producing the enzyme with exochitinase activity on the basis of a partial DNA sequence comprised in the DNA sequence shown in SEQ ID no. 1 or SEQ ID no. 3, *e.g.* using the procedures described herein, and thus, *e.g.* be an allelic or species variant of the DNA sequence shown herein,

- may be constructed on the basis of a partial DNA sequence comprised in the DNA sequences shown in SEQ ID no. 1 or SEQ ID no. 3, *e.g.* by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the exochitinase encoded by the DNA sequence, but which correspond to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence. However, in the latter case amino acid changes are preferably of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding or activity of the protein, small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification, such as a poly-histidine tract, an antigenic epitope or a binding domain. See in general Ford et al., (1991), Protein Expression and Purification, 2, 95-107. Examples of conservative substitutions are within the group of basic amino acids (such as arginine, lysine, histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine, valine),

aromatic amino acids (such as phenylalanine, tryptophan, tyrosine) and small amino acids (such as glycine, alanine, serine, threonine, methionine).

It will be apparent to persons skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acids essential to the activity of the polypeptide encoded by the DNA construct of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, (1989), Science, 244, 1081-1085). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological (*i.e.* exochitinolytic) activity to identify amino acid residues that are critical to the activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance, crystallography or photoaffinity labelling. See, for example, de Vos et al., (1992), Science, 255, 306-312; Smith et al., (1992), J. Mol. Biol., 224, 899-904; Wlodaver et al., (1992) FEBS Lett., 309, 59-64.

It will be understood that any partial sequences comprised in the DNA sequence shown in SEQ ID No. 1 or SEQ ID no. 3 may be used for isolating the entire DNA sequence encoding the enzyme with exochitinase activity, *e.g.* the DNA sequence shown in SEQ ID No. 1. DNA sequences encoding at least about 6 amino acids of the sequence shown in SEQ ID No. 2 or SEQ ID no. 4 are comtemplated as partial sequences according to the invention. The term "analogue" is intended to include said entire DNA sequence shown in SEQ ID No. 1 or SEQ ID no. 3, or parts thereof. The amino acid sequences (as deduced from the DNA sequence shown in SEQ ID No. 1 and SEQ ID no. 3) are shown in SEQ ID No. 2 and SEQ ID no. 4.

The homology referred to in i) above is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, p. 443-453). Using GAP with the following settings for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3, the coding region of the DNA sequence exhibits a degree of identity of at least 70%, preferably at least 80%, especially at least 90%, with the coding region of the DNA sequence shown in SEQ ID No. 1 or SEQ ID no. 3.

The hybridization referred to in ii) above is intended to indicate that the analogous DNA sequence hybridizes to the same probe as the DNA sequence encoding the exochitinase under certain specified conditions which are described in detail in the Materials and Methods section hereinafter.

Normally, the analogous DNA sequence is highly homologous to the DNA sequence such as at least 70% homologous to the DNA sequence shown in SEQ ID No. 1 or SEQ ID no. 3 encoding exochitinases of the invention, such as at least 75%, at least 80%, at least 85%, at least 90% or even at least 95% homologous to said DNA sequence

The homology referred to in iii) above is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, p. 443-453). Using GAP with the following settings for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3, the coding region of the DNA sequence exhibits a degree of

identity of at least 70%, preferably at least 80%, especially at least 90%, with the coding region of the DNA sequence shown in SEQ ID No. 1 or SEQ ID no. 3.

The term "derived from" in connection with property vi) above is intended not only to indicate an exochitinase produced by the above mentioned deposited strain *Saccharomyces cerevisiae* DSM no. 9944 or DSM no. 9945, but also an exochitinase encoded by a DNA sequence isolated from the deposited strain DSM no. 9944 or DSM no. 9945 and produced in a host organism transformed with said DNA sequence. The immunological reactivity may be determined by the method described in the Materials and Methods section below.

In further aspects the invention relates to an expression vector harbouring a DNA construct of the invention, a cell comprising the DNA construct or expression vector and a method of producing an enzyme exhibiting exochitinase activity which method comprises culturing said cell under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

In a still further aspect the invention relates to an enzyme exhibiting exochitinase activity, which enzyme

a) is encoded by a DNA construct of the invention
b) produced by the method of the invention, and/or
c) is immunologically reactive with an antibody raised against a purified exochitinase shown in SEQ ID No. 2 derived from *Saccharomyces cerevisiae* DSM no. 9944 or against a purified exochitinase shown in SEQ ID no. 4 derived from *Saccharomyces cerevisiae* DSM 9945.

In a still further aspect, the present invention relates to an enzyme preparation useful for the degradation or modification of fungal, invertebrate, or nematode cell wall components, said preparation being enriched with an enzyme exhibiting exochitinase activity as described above.

The invention also relates to the use of an exochitinase of the invention or an enzyme preparation of the invention comprising an exochitinase of the invention for plant protection and pharmaceutical purposes.

Finally the invention relates to an isolated substantially pure culture of the deposited strain of *Saccharomyces cerevisiae* DSM no. 9944 or DSM no. 9945, transformed with a plasmid-DNA comprising the DNA sequences shown in SEQ ID No. 1 and SEQ ID no. 3, respectively or a partial DNA sequence of these two sequences.

DETAILED DESCRIPTION OF THE INVENTION

The DNA sequence of the invention encoding an enzyme exhibiting exochitinase activity may be isolated by a general method involving

- cloning, in suitable vectors, a cDNA library from *e.g.* a strain of *Saccharomyces* sp., *Aspergillus* sp., *Trichoderma* sp., *Penicillium* sp., *Fusarium* sp., *Gliocladium* sp., *Aphanocladium* sp., or *Humicola* sp.,
- transforming suitable yeast host cells with said vectors,
- culturing the host cells under suitable conditions to express any enzyme of interest encoded by a clone in the DNA library,
- screening for positive clones by determining any exochitinase activity of the enzyme produced by such clones, and
- isolating the enzyme encoding DNA from such clones.

The general method is further disclosed in WO 93/11249 the contents of which are hereby incorporated by reference. A more detailed description of the screening method is given in Example 2 below.

Two isolates of *Saccharomyces cerevisiae* transformed with the expression plasmid pYES 2.0 (Invitrogen), comprising the cDNA sequences, shown in SEQ ID No. 1 and SEQ ID No 3, respectively, encoding exochitinases of the invention, have been deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-3300 Braunschweig, Federal Republic of Germany, (DSM), for the purposes of patent procedure on the date indicated below. DSM being an international depository under the Budapest Treaty affords permanence of the deposit in accordance with rule 9 of said treaty.

Deposit date : 27.04.95
Depositor's ref.: NN14684
CBS designation : DSM No. 9944

Deposit date : 27.04.95
Depositor's ref.: NN14685
CBS designation : DSM No. 9945

The two plasmid-DNA isolates of the fungus *Saccharomyces cerevisiae* DSM no. 9944 (NN14684) and DSM no. 9945 (NN14685), respectively have been deposited under conditions that assure that access to the isolated yeast will be available during the pendency of this patent application to one determined by the commissioner of Patents and Trademarks to be entitled thereto under 37 C.F.R. § 1.14 and 35 U.S.C § 122.

Further the above mentioned depositions of the yeast *Saccharomyces cerevisiae* DSM no. 9944 and DSM no. 9945 have been done to fulfil the requirements of a European patent applications under Rule 28 of the European Patent Convention.

The deposits represent substantially pure cultures of the two isolated yeast. The deposits are available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny are filed. However, it should be understood that the availability of the deposits does

not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

A DNA sequence coding for the enzyme exhibiting exochitinase activity can for instance be isolated from the above mentioned deposited strains as described in Example 1.

Further said DNA sequence may be isolated by screening a cDNA library of *e.g.* the above mentioned group of fungi, and selecting for clones expressing the appropriate enzyme activity (*i.e.* exochitinase activity as defined by the ability of the enzyme to hydrolyse the β-1,4-linkage of a suitable substrate, such as the synthetic chitin substrate 4-methylumbelliferyl N-acetylglucosaminide (MUF-GlcNAc), *cf.* the Materials and Methods section herein after). The appropriate DNA sequence may then be isolated from the clone by standard procedures.

It is expected that a DNA sequence coding for a homologous enzyme, *i.e.* an analogous DNA sequence, is obtainable from other microorganisms. For instance, the DNA sequence may be derived by screening a cDNA library of another microorganism, such as in particular a fungus, such as a strain of an *Aspergillus* sp., in particular a strain of *A. aculeatus* or *A. niger*, a strain of another *Trichoderma* sp., in particular a strain of *T. reesie*, *T. viride*, *T. longibrachiatum*, *T. harzianum* or *T. koningii* or a strain of a *Fusarium* sp., in particular a strain of *F. oxysporum*, or a strain of *Gliocladium* sp., in particular *Gliocladium virens*, or a strain of *Aphanocladium*, in particular *Aphanocladium album*, or a strain of a *Humicola* sp., or a strain of *Beauveria* sp., in particular *Beauveria bassiana*, or a strain of *Metarhizium* sp., in particular *Metarhizium anisopliae*, or a strain of *Mucor* sp., in particular *Mucor rouxii*, or a mutant thereof capable of producing a compound of the invention.

Alternatively, the DNA coding for an exochitinase of the invention may, in accordance with well-known procedures, conveniently be isolated from DNA from a suitable source, such

as any of the above mentioned organisms, by use of synthetic oligonucleotide probes prepared on the basis of a DNA sequence disclosed herein. For instance, a suitable oligonucleotide probe may be prepared on the basis of the nucleotide sequences shown in SEQ ID No. 1 or SEQ ID no. 3, or a partial sequence thereof, or the amino acid sequences shown in SEQ ID No. 2 or SEQ ID no. 4, or any suitable subsequence thereof.

The DNA sequence may subsequently be inserted into a recombinant expression vector. This may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, *i.e.* a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.* a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence encoding the exochitinase should be operably connected to a suitable promoter and terminator sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. The procedures used to ligate the DNA sequences coding for the exochitinase, the promoter and the terminator, respectively, and to insert them into suitable vectors are well known to persons skilled in the art (cf., for instance, Sambrook et al., (1989), Molecular Cloning. A Laboratory Manual, Cold Spring Harbor, NY).

The host cell which is transformed with the DNA sequence encoding the enzyme of the invention is preferably an eukaryotic cell, in particular a fungal cell such as a yeast or filamentous fungal cell. In particular, the cell may belong to a species of *Trichoderma*, preferably *Trichoderma harzianum* or

Trichoderma reesie, or a species of *Aspergillus*, preferably *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. The use of *Aspergillus* as a host microorganism is described in EP 238 023 (of Novo Nordisk A/S), the contents of which are hereby incorporated by reference. The host cell may also be a yeast cell, e.g. a strain of *Saccharomyces*, in particular *Saccharomyces cerevisiae*, *Saccharomyces kluyveri* or *Saccharomyces uvarum*, a strain of *Schizosaccharomyces* sp., such as *Schizosaccharomyces pombe*, a strain of *Hansenula* sp. *Pichia* sp., *Yarrowia* sp. such as *Yarrowia lipolytica*, or *Kluyveromyces* sp. such as *Kluyveromyces lactis*.

In a still further aspect, the present invention relates to a method of producing an enzyme according to the invention, wherein a suitable host cell transformed with a DNA sequence encoding the enzyme is cultured under conditions permitting the production of the enzyme, and the resulting enzyme is recovered from the culture.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host cells in question. The expressed exochitinase may conveniently be secreted into the culture medium and may be recovered there from by well-known procedures including separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

In a still further aspect, the present invention relates to an enzyme preparation useful for the degradation or modification of fungal cell wall components, said preparation being enriched in an enzyme exhibiting exochitinase activity as described

above.

In the present context, the term "enriched" is intended to indicate that the exochitinase activity of the enzyme preparation has been increased, *e.g.* with an enrichment factor of at least 1.1, conveniently due to addition of an enzyme of the invention prepared by the method described above.

Alternatively, the enzyme preparation enriched in an enzyme exhibiting exochitinase activity may be one which comprises an enzyme of the invention as the major enzymatic component, *e.g.* a single-component enzyme preparation.

The enzyme preparation may be prepared in accordance with methods known in the art and may be in the form of a liquid or a dry preparation. For instance, the enzyme preparation may be in the form of a granulate or a microgranulate. The enzyme to be included in the preparation may be stabilized in accordance with methods known in the art.

The enzyme preparation of the invention may, in addition to an exochitinase of the invention, contain one or more other fungal, invertebrate, such as crustacean, and/or nematode cell wall degrading enzymes, for instance those with proteolytic, β-glucanolytic, mannanolytic, chitinolytic activities, such as protease, β-glucanase, β-mannosidase, mannanase, β-glucosidase, endochitinase, and mannan acetyl esterase. The additional enzyme(s) may be producible by means of a microorganism belonging to the genus *Aspergillus*, preferably *Aspergillus niger*, *Aspergillus aculeatus*, *Aspergillus awamori* or *Aspergillus oryzae*, or *Trichoderma* sp..

Examples are given below of preferred uses of the enzyme preparation of the invention. The dosage of the enzyme preparation of the invention and other conditions under which the preparation is used may be determined on the basis of methods known in the art.

The enzyme preparation according to the invention may be used as an agent for degradation or modification of fungal, invertebrate and/or nematode cell walls, or for making protoplasts from fungi.

Further exochitinases of the invention may advantageously be used for protecting plants against nematode eggs and generally against fungal infection, e.g. by coating the seeds, or by spraying the plants.

In connection with combating fungal microorganisms on plants a DNA sequence encoding the exochitinase of the invention may be used for producing transgenic plants, by introducing said DNA sequence into a plant. This may increase the plants resistance against microorganisms of fungal origin.

Another advantageous use of exochitinases of the invention include modification of chitin for use for pharmaceutical purposes, e.g. for wound dressing.

Finally the invention relates to substantially pure cultures of the deposited microorganisms *Saccharomyces cerevisiae* DSM no. 9944 and DSM no. 9945, which comprises the above described DNA construct of the invention, containing a DNA sequence encoding an exochitinase of the invention.

The invention is described in further detail in the following examples which are not in any way intended to limit the scope of the invention as claimed.

MATERIALS AND METHODS

Deposited organisms:
Saccharomyces cerevisiae DSM no. 9944 transformed with the DNA sequence shown in SEQ ID No. 1, encoding the exochitinase of

the invention, comprised in the expression plasmid pYES 2.0.

Saccharomyces cerevisiae DSM no. 9945 transformed with the DNA sequence shown in SEQ ID No. 3, encoding the exochitinase of the invention, comprised in the expression plasmid pYES 2.0.

Plasmids:
pYES 2.0 (Invitrogen)
pClEXC1: SEQ ID. no. 1 sequence in pYES 2.0
pClEXC2: SEQ ID. no. 3 sequence in pYES 2.0

Isolation of the the DNA sequence shown in SEQ ID no. 1 and SEQ ID no. 3

The yeast expression vectors of the type pYES 2.0 containing the exochitinase cDNA sequences shown in SEQ ID no. 1 and SEQ ID no. 3, respectively, can be isolated from the deposited organism *Saccharomyces cerevisiae* DSM no. 9944 and DSM 9945, restectively by extraction of plasmid cDNA by methods known in the art.

The deposited organisms may be cultured on agar plates containing SC + 2% galactose and incubated at 30°C for 3-5 days as described below.

Hybridization conditions (to be used in evaluating property i) of the DNA construct of the invention):
Suitable conditions for determining hybridization between an oligonucleotide probe and an "analogous" DNA sequence involves presoaking of the filter containing the DNA sequences to hybridize in 5xSSC and prehybridizing the sequences for 1 h at ~55°C in a solution of 2xSSC, 5xDenhardt's solution, 50 mM sodium phosphate, pH 6.8, and 50 µg of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 50 µCi 32-P-dCTP labelled probe for 18 h at ~55°C followed by washing in 2xSSC (2x15 minutes), 2xSSC, 0.2% SDS (1x30

minutes), 0.2xSSC, 0.5% SDS (1x30 minutes), 2xSSC (2x15 minutes) at 55°C.

A suitable oligonucleotide probe to be used in the hybridization may be prepared on the basis of the DNA sequences shown in SEQ ID No. 1 and SEQ ID no. 3, respectively, or partial sequences thereof.

Immunological cross-reactivity: Antibodies to be used in determining immunological cross-reactivity may be prepared by use of a purified exochitinase. More specifically, antiserum against the exochitinase of the invention may be raised by immunizing rabbits (or other rodents) according to the procedure described by N. Axelsen et al. in: A Manual of Quantitative Immunoelectrophoresis, Blackwell Scientific Publications, 1973, Chapter 23, or A. Johnstone and R. Thorpe, Immunochemistry in Practice, Blackwell Scientific Publications, (1982) (more specifically p. 27-31). Purified immunoglobulins may be obtained from the antisera, for example by salt precipitation ($(NH_4)_2$ SO_4), followed by dialysis and ion exchange chromatography, *e.g.* on DEAE-Sephadex. Immunochemical characterization of proteins may be done either by Outcherlony double-diffusion analysis (O. Ouchterlony in: Handbook of Experimental Immunology (D.M. Weir, Ed.), Blackwell Scientific Publications, (1967), p. 655-706), by crossed immunoelectrophoresis (N. Axelsen et al., supra, Chapters 3 and 4), or by rocket immunoelectrophoresis (N. Axelsen et al., Chapter 2).

Media

SC-URA: 90 ml 10 x Basal salt, 22.5 ml 20% casamino acids, 9 ml 1% tryptophan, H_2O ad 806 ml, autoclaved, 3.6 ml 5% threonine and 90 ml 20% glucose or 20% galactose added.

SC-H broth: 7.5 g/l yeast nitrogen base without amino acids, 11.3 g/l succinic acid, 6.8 g/l NaOH, 5.6 g/l casamino acids

without vitamins, 0.1 g/l tryptophan. Autoclaved for 20 min. at 121°C. After autoclaving, 10 ml of a 30% galactose solution, 5 ml of a 30% glucose solution and 0.4 ml of a 5% threonine solution were added per 100 ml medium.

SC-H agar: 7.5 g/l yeast nitrogen base without amino acids, 11.3 g/l succinic acid, 6.8 g/l NaOH, 5.6 g/l casamino acids without vitamins, 0.1 g/l tryptophan, and 20 g/l agar (Bacto). Autoclaved for 20 min. at 121°C. After autoclaving, 55 ml of a 22% galactose solution and 1.8 ml of a 5% threonine solution were added per 450 ml agar.

4-methylumbelliferyl N-acetylglucosaminide (MUF-GlcNAc) (Sigma, USA)

MUF-N,N'-diacetylchitobioside (MUF-$(GlcNAc)_2$)(Sigma, USA)

MUF-N,N',N''-tridiacetylchitotrioside (MUF-$(GlcNAc)_3$) (Sigma, USA)

Qiagen purified plasmid DNA (Qiagen, USA),

Sequenase®kit (U.S.Biochemical corp., USA)

Remazol Brilliant Violet colloidal chitin (Sigma, USA)

Random hexanucleotide primers (Gibco BRL, USA)

EXAMPLE 1

Characterization of enzyme from yeast

Saccharomyces cerevisiae DSM No. 9944 and DSM no. 9945 were cultured separetly on SC-glucose plates for 3-4 days at 40°C. The plates were replicated onto a set of four selective agar plates containing 2% galactose. One of the plates was also supplemented with 0.2% Remazol Brilliant Violet colloidal

chitin.

After incubation at 30°C for three days SC-agar plates with 2% galactose were overlayerd with 10 ml 1% agarose, 0.1 M citric acid/sodium citrate buffer, pH 5.0, containing 1 mg of MUF-(GlcNAc), MUF-$(GlcNAc)_2$ or MUF-$(GlcNAc)_3$ each.

The release of 4-methylumbelliferyl by chitinases was visualized under UV-light.

The recombinant yeast harbouring the plasmids pClEXC1 showed activity on MUF-GlcNAc immediately after the agarose overlayer was spread over the yeast colonies, whereas activities on MUF-$(GlcNAc)_2$ and MUF-$(GlcNAc)_3$ were visualized only after 5 and 10 minutes of incubation, respectively. By comparison, the recombinant yeast strain showed no activity on the hydrated colloidal chitin.

The recombinant yeast harbouring the plasmids pClEXC2 showed activity on MUF-GlcNAc immediately after the agarose overlayer was spread over the yeast colonies, whereas activities on MUF-$(GlcNAc)_2$ and MUF-$(GlcNAc)_3$ were visualized only after 5 and 10 minutes of incubation, respectively. By comparison, the recombinant yeast strain showed no activity on the hydrated colloidal chitin.

EXAMPLE 2

Construction and screening of the cDNA library

Total RNA was prepared from frozen, powdered mycelium of a *Trichoderma harzianum* strain by extraction with guanidinium thiocyanate followed by ultracentrifugation through a 5.7 M CsCl cushion (Chirgwin et al., (1979), Biochemistry, 18, 5294-5299). Poly(A)+RNA was isolated by oligo(dT)cellulose affinity chromatography (Aviv and Leder, (1972), Proc. Natl. Acad. Sci. U.S.A., 69, 1408-1412). Double-stranded cDNA was synthesized

from 5 µg of poly(A)+RNA as described (Gubler and Hoffman, (1983), Gene, 25, 263-269; Sambrook et al., (1989), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York, Cold Spring Harbot Laboratory) except that 25 ng of random hexanucleotide primers (Gibco BRL, USA) were included in the first strand synthesis. A cDNA library consisting of 1.5×10^6 clones was constructed in the yeast expression vector pYES 2.0 as described previously (Kofod et al., (1994), J. Biol. Chem., 261, 8407-8413). Plasmid DNA from a cDNA library pool was transformed into *S. cerevisiae* W3124 (van den Hazel et al., (1992), Eur. J. Biochem., 207, 277-283) by electroporation (Becker and Guarente, (1991), Methods Enzymol., 194, 182-187) and the transformants were plated on SC agar (Sherman, (1991), Methods Enzymol. 194, 3-21) containing 2% glucose. After incubation at 30°C for 3 to 4 days, the colonies were replicated onto SC agar plates containing 2% galactose and incubated for 3 days at 30°C before the yeast colonies were overlayered with 1% agarose containing 0.1 M citric acid/sodium citrate buffer pH 5.0 and 0.001% 4-methylumbelliferyl N-acetylglucosamine (MUF-GlcNAc).

Exochitinase positive clones were identified under UV-light by the formation of fluorescent halos. Total DNA from the positive yeast colonies was isolated and the insert containing pYES 2.0 clones were rescued by transformation of *E. coli* MC 1061 (Meissner et al., (1987), Proc. Natl. Acad. Sci. U.S.A., 84, 4171-4176) to ampicillin resistance.

Nucleotide sequence analysis

The nucleotide sequence of the cDNA insert was determined from both strands by the dideoxy chain termination method (Sanger et al., (1977), Proc. Natl. Acad. Sci. U.S.A., 74, 5463-5467) using Qiagen purified plasmid DNA, the Sequenase®kit or synthetic oligonucleotide primers. Analysis of the sequence date were performed according to Devereux et al., (1984), Nucleic Acids Res., 12, 387-395). The full length cDNA sequences are shown in SEQ ID no. 1 and SEQ ID No. 3,

respectively, and the corresponding amino acid sequences in SEQ ID No. 2 and SEQ ID no. 4, respectively.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2800
(G) TELEPHONE: +45 4444 8888
(H) TELEFAX: +45 4449 3256

(ii) TITLE OF INVENTION: An enzyme with exochitinase activity

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30B (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2000 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(B) STRAIN: Trichoderma harzianum CBS 243.71

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:86..1819

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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GACATCTCCA CCATAGAGTC GACTCATTGC TGGCATACGG AGCATTCCAA TCTTACTCGT       60

AGTAGTGTTA TTGCCATCGC TCATC ATG CTG CCC AAG GCG ATC ATC GCG ATT         112
                            Met Leu Pro Lys Ala Ile Ile Ala Ile
                              1               5

GCC GCA TTG GCT TTC AGC CCA GCA AAT GCG CTG TGG CCC ATT CCT CAG         160
Ala Ala Leu Ala Phe Ser Pro Ala Asn Ala Leu Trp Pro Ile Pro Gln
 10                  15                  20                  25

AAG ATC TCG ACC GGA GAC AGC GTG CTC TTT ATT GAC CAG GCT GTT AGG         208
Lys Ile Ser Thr Gly Asp Ser Val Leu Phe Ile Asp Gln Ala Val Arg
                    30                  35                  40

GTG ACT TAC AAT GGA GTA CCG ATC ATC CCT ATC GGC TAC AAC CCA CCG         256
Val Thr Tyr Asn Gly Val Pro Ile Ile Pro Ile Gly Tyr Asn Pro Pro
               45                  50                  55

GCC AGC TCC AAC TTC GAC AGC AGG CAA ATC GTC CAG GCG GCT GTC TCG         304
Ala Ser Ser Asn Phe Asp Ser Arg Gln Ile Val Gln Ala Ala Val Ser
          60                  65                  70

CGC GCT TTC CAA AAC ATC TTC AGC ACC AAC TAT GTG CCA TGG AAG CTT         352
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Arg Ala Phe Gln Asn Ile Phe Ser Thr Asn Tyr Val Pro Trp Lys Leu
 75                  80                  85

CAC CCG CGT AAC AGC AAC TTT GAG CCG AAG GTG GCC CCT CAG AAC CGA      400
His Pro Arg Asn Ser Asn Phe Glu Pro Lys Val Ala Pro Gln Asn Arg
 90                  95                 100                 105

ATC CAG TCC ATC TCA ATT CAG CAG ACT GGA AAG GAT ACG TCC AAG ACG      448
Ile Gln Ser Ile Ser Ile Gln Gln Thr Gly Lys Asp Thr Ser Lys Thr
                110                 115                 120

TTC AAG CCG CGC GCC GGA GAC GTT GAT GAG TCG TAC TCT TTG ACC ATT      496
Phe Lys Pro Arg Ala Gly Asp Val Asp Glu Ser Tyr Ser Leu Thr Ile
            125                 130                 135

TCC AAG AAT GGA CAG GTC AAC ATC AGT GCC AAG TCT TCT ACT GGT GTG      544
Ser Lys Asn Gly Gln Val Asn Ile Ser Ala Lys Ser Ser Thr Gly Val
        140                 145                 150

CTG CAC GCC CTC GAG ACC TTC TCG CAG CTT TTC TAC AAG CAC TCT GCT      592
Leu His Ala Leu Glu Thr Phe Ser Gln Leu Phe Tyr Lys His Ser Ala
    155                 160                 165

GGA CCT TTC TAC TAT ACG ACT CAG GCT CCC GTG TCC ATC ACA GAC GCT      640
Gly Pro Phe Tyr Tyr Thr Thr Gln Ala Pro Val Ser Ile Thr Asp Ala
170                 175                 180                 185

CCC AAA TAT CCC CAC CGT GGC ATC ATG CTT GAC CTT GCC CGT AAC TAT      688
Pro Lys Tyr Pro His Arg Gly Ile Met Leu Asp Leu Ala Arg Asn Tyr
                190                 195                 200

CAA ACC ATT GAT GAC ATC AAG AGG ACC ATT GAC GCC ATG TCG TGG AAC      736
Gln Thr Ile Asp Asp Ile Lys Arg Thr Ile Asp Ala Met Ser Trp Asn
            205                 210                 215

AAG CTT AAC CGC CTG CAC TTG CAC ATC ACC GAC TCT CAG TCG TGG CCG      784
Lys Leu Asn Arg Leu His Leu His Ile Thr Asp Ser Gln Ser Trp Pro
        220                 225                 230

CTG GTG ATC CCC TCG CTG CCT AAG CTG TCC CAG GCC GGT GCC TAC CAC      832
Leu Val Ile Pro Ser Leu Pro Lys Leu Ser Gln Ala Gly Ala Tyr His
    235                 240                 245

CCC AGC CTC GTC TAC ACT CCC GCA GAC CTT GCT GGC ATT TTC CAG TAC      880
Pro Ser Leu Val Tyr Thr Pro Ala Asp Leu Ala Gly Ile Phe Gln Tyr
250                 255                 260                 265

GGT GTC GCC CGC GGT GTT GAG GTC ATT ACG GAG ATC GAT ATG CCT GGC      928
Gly Val Ala Arg Gly Val Glu Val Ile Thr Glu Ile Asp Met Pro Gly
                270                 275                 280

CAC ATC GGT GTT ATC GAG CTC GCT TAC AGC GAT CTC ATT GTT GCC TAC      976
His Ile Gly Val Ile Glu Leu Ala Tyr Ser Asp Leu Ile Val Ala Tyr
            285                 290                 295

GAA GAG ATG CCT TAC CAG TAC TAC TGC GCC GAG CCA CCT TGC GGT GCC     1024
Glu Glu Met Pro Tyr Gln Tyr Tyr Cys Ala Glu Pro Pro Cys Gly Ala
        300                 305                 310

TTT TCC ATC AAC AAC ACC AAG GTG TAC AGC TTC CTC GAT ACC CTG TTC     1072
Phe Ser Ile Asn Asn Thr Lys Val Tyr Ser Phe Leu Asp Thr Leu Phe
    315                 320                 325

GAC GAC CTT TTG CCT CGC GTC GCT CCT TAC AGC GCG TAC TTC CAC ACC     1120
Asp Asp Leu Leu Pro Arg Val Ala Pro Tyr Ser Ala Tyr Phe His Thr
330                 335                 340                 345

GGT GGT GAC GAG CTC AAC GCT AAC GAC TCC ATG CTC GAC TCT CAC ATC     1168
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Gly Gly Asp Glu Leu Asn Ala Asn Asp Ser Met Leu Asp Ser His Ile
                350                 355                 360

AAG AGC AAC GAG ACC TCC GTT CTG CAA CCT CTG CTG CAA AAG TTC ATC      1216
Lys Ser Asn Glu Thr Ser Val Leu Gln Pro Leu Leu Gln Lys Phe Ile
            365                 370                 375

AAC TTT GCC CAC TCC AAG GTC CGT GCC GCG GGC TTG TCG CCA TTT GTC      1264
Asn Phe Ala His Ser Lys Val Arg Ala Ala Gly Leu Ser Pro Phe Val
        380                 385                 390

TGG GAG GAG ATG GTC ACC ACC TGG AAC CTG ACC CTC GGC AGC GAC ACC      1312
Trp Glu Glu Met Val Thr Thr Trp Asn Leu Thr Leu Gly Ser Asp Thr
    395                 400                 405

GTC GTT CAG TCG TGG CTG GGT GGC GAT GCC GTC AAG AAC CTG GCT GAG      1360
Val Val Gln Ser Trp Leu Gly Gly Asp Ala Val Lys Asn Leu Ala Glu
410                 415                 420                 425

AGC GGC CAC AAG GTC ATT GAC ACC GAC TAC AAC TTC TAC TAC TTG GAC      1408
Ser Gly His Lys Val Ile Asp Thr Asp Tyr Asn Phe Tyr Tyr Leu Asp
                430                 435                 440

TGC GGC CGT GGT CAA TGG GTC AAC TTC CCT CCA GGA GAC TCC TAC AAC      1456
Cys Gly Arg Gly Gln Trp Val Asn Phe Pro Pro Gly Asp Ser Tyr Asn
            445                 450                 455

ACC TAC TAC CCA TTC AAC GAC TGG TGC CAG CCC ACC AAG AAC TGG AGG      1504
Thr Tyr Tyr Pro Phe Asn Asp Trp Cys Gln Pro Thr Lys Asn Trp Arg
        460                 465                 470

CTC ATC TAC TCT CAC GAC CCT GCA GCC AAC GTG TCT GCT TCG GCT GCC      1552
Leu Ile Tyr Ser His Asp Pro Ala Ala Asn Val Ser Ala Ser Ala Ala
    475                 480                 485

AAG AAC GTC CTG GGA GGA GAG CTT GCC ATT TGG AGC GAG ATG ATT GAC      1600
Lys Asn Val Leu Gly Gly Glu Leu Ala Ile Trp Ser Glu Met Ile Asp
490                 495                 500                 505

GCC AGC AAC CTG GAC AAC ATC ATC TGG CCT CGT GGC AGC GCC GCC GGT      1648
Ala Ser Asn Leu Asp Asn Ile Ile Trp Pro Arg Gly Ser Ala Ala Gly
                510                 515                 520

GAG GTT TGG TGG TCC GGC AAT ACC GAT GCC TCT GGT GAG CAG CGC AGC      1696
Glu Val Trp Trp Ser Gly Asn Thr Asp Ala Ser Gly Glu Gln Arg Ser
            525                 530                 535

CAG CTG GAC GTT GTT CCT CGT CTG AAC GAG TTC CGA GAA CGC TTG CTT      1744
Gln Leu Asp Val Val Pro Arg Leu Asn Glu Phe Arg Glu Arg Leu Leu
        540                 545                 550

GCT CGT GGT GTC AGC GCG TTC CCC ATC CAG ATG ACC TAC TGC ACT CAG      1792
Ala Arg Gly Val Ser Ala Phe Pro Ile Gln Met Thr Tyr Cys Thr Gln
    555                 560                 565

CTC AAC GCC ACT GCC TGC ACA CTA TTT TAAGTCTAAG ATGACTTTTT            1839
Leu Asn Ala Thr Ala Cys Thr Leu Phe
570                 575

CTTTTATTGG GCAGGGTTTT TTCTATTTTT CACGTATTAT CATTAGTGTA CAGTGATTAA    1899

AACAGGTATG GCTTAAGAGG AGCTGGGAGG GTATCCGGCT TGGGGCGGTA TATTATTAAC    1959

TGTATATAAT TCAAATTCAT CTACATATAT GTTATGAAAA A                        2000
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 578 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Met Leu Pro Lys Ala Ile Ile Ala Ile Ala Ala Leu Ala Phe Ser Pro
 1               5                  10                  15

Ala Asn Ala Leu Trp Pro Ile Pro Gln Lys Ile Ser Thr Gly Asp Ser
            20                  25                  30

Val Leu Phe Ile Asp Gln Ala Val Arg Val Thr Tyr Asn Gly Val Pro
        35                  40                  45

Ile Ile Pro Ile Gly Tyr Asn Pro Pro Ala Ser Ser Asn Phe Asp Ser
    50                  55                  60

Arg Gln Ile Val Gln Ala Ala Val Ser Arg Ala Phe Gln Asn Ile Phe
65                  70                  75                  80

Ser Thr Asn Tyr Val Pro Trp Lys Leu His Pro Arg Asn Ser Asn Phe
                85                  90                  95

Glu Pro Lys Val Ala Pro Gln Asn Arg Ile Gln Ser Ile Ser Ile Gln
            100                 105                 110

Gln Thr Gly Lys Asp Thr Ser Lys Thr Phe Lys Pro Arg Ala Gly Asp
        115                 120                 125

Val Asp Glu Ser Tyr Ser Leu Thr Ile Ser Lys Asn Gly Gln Val Asn
    130                 135                 140

Ile Ser Ala Lys Ser Ser Thr Gly Val Leu His Ala Leu Glu Thr Phe
145                 150                 155                 160

Ser Gln Leu Phe Tyr Lys His Ser Ala Gly Pro Phe Tyr Tyr Thr Thr
                165                 170                 175

Gln Ala Pro Val Ser Ile Thr Asp Ala Pro Lys Tyr Pro His Arg Gly
            180                 185                 190

Ile Met Leu Asp Leu Ala Arg Asn Tyr Gln Thr Ile Asp Asp Ile Lys
        195                 200                 205

Arg Thr Ile Asp Ala Met Ser Trp Asn Lys Leu Asn Arg Leu His Leu
    210                 215                 220

His Ile Thr Asp Ser Gln Ser Trp Pro Leu Val Ile Pro Ser Leu Pro
225                 230                 235                 240

Lys Leu Ser Gln Ala Gly Ala Tyr His Pro Ser Leu Val Tyr Thr Pro
                245                 250                 255

Ala Asp Leu Ala Gly Ile Phe Gln Tyr Gly Val Ala Arg Gly Val Glu
            260                 265                 270

Val Ile Thr Glu Ile Asp Met Pro Gly His Ile Gly Val Ile Glu Leu
        275                 280                 285

Ala Tyr Ser Asp Leu Ile Val Ala Tyr Glu Glu Met Pro Tyr Gln Tyr
    290                 295                 300

Tyr Cys Ala Glu Pro Pro Cys Gly Ala Phe Ser Ile Asn Asn Thr Lys
305                 310                 315                 320
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Val Tyr Ser Phe Leu Asp Thr Leu Phe Asp Asp Leu Leu Pro Arg Val
325 330 335

Ala Pro Tyr Ser Ala Tyr Phe His Thr Gly Gly Asp Glu Leu Asn Ala
340 345 350

Asn Asp Ser Met Leu Asp Ser His Ile Lys Ser Asn Glu Thr Ser Val
355 360 365

Leu Gln Pro Leu Leu Gln Lys Phe Ile Asn Phe Ala His Ser Lys Val
370 375 380

Arg Ala Ala Gly Leu Ser Pro Phe Val Trp Glu Glu Met Val Thr Thr
385 390 395 400

Trp Asn Leu Thr Leu Gly Ser Asp Thr Val Val Gln Ser Trp Leu Gly
405 410 415

Gly Asp Ala Val Lys Asn Leu Ala Glu Ser Gly His Lys Val Ile Asp
420 425 430

Thr Asp Tyr Asn Phe Tyr Tyr Leu Asp Cys Gly Arg Gly Gln Trp Val
435 440 445

Asn Phe Pro Pro Gly Asp Ser Tyr Asn Thr Tyr Tyr Pro Phe Asn Asp
450 455 460

Trp Cys Gln Pro Thr Lys Asn Trp Arg Leu Ile Tyr Ser His Asp Pro
465 470 475 480

Ala Ala Asn Val Ser Ala Ser Ala Ala Lys Asn Val Leu Gly Gly Glu
485 490 495

Leu Ala Ile Trp Ser Glu Met Ile Asp Ala Ser Asn Leu Asp Asn Ile
500 505 510

Ile Trp Pro Arg Gly Ser Ala Ala Gly Glu Val Trp Trp Ser Gly Asn
515 520 525

Thr Asp Ala Ser Gly Glu Gln Arg Ser Gln Leu Asp Val Val Pro Arg
530 535 540

Leu Asn Glu Phe Arg Glu Arg Leu Leu Ala Arg Gly Val Ser Ala Phe
545 550 555 560

Pro Ile Gln Met Thr Tyr Cys Thr Gln Leu Asn Ala Thr Ala Cys Thr
565 570 575

Leu Phe

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2239 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(B) STRAIN: Trichoderma harzianum CBS 243.71

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:282..2086

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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CTGAGAAGCG GCACTTGCTG ATCTGCGTGG AACTTGGGGT TACAACGCAC CGGATAGCTC      60

ATCTCCCCAG GACCCCGGAA CTGGAGCTGG AACTGGAATT GGAGCTGGAG CGGACCCAGG     120

CCGGAGACGA GAAACACAGT GAATCACTCC TGCAAGGGGC GGGACAGGAA CGTGGACAGT     180

ATTTAGTTTA AGCAGCTGTC CCAGAGCTGT TCGCCCTGCT TCCAAGCTCG TGTGGCCTGA     240

CCCTGTATAA ACTCATTACG ACCATCAGCT CACAGCCGAC A ATG TTT TCC AGG          293
                                               Met Phe Ser Arg
                                                 1

GCC ATT GTC GCC GCA TTG GCC CTG AGC GGC CCG GCT TTT GCC CTG TGG        341
Ala Ile Val Ala Ala Leu Ala Leu Ser Gly Pro Ala Phe Ala Leu Trp
  5                  10                  15                  20

CCC GTG CCT AAA CAC TCC TCG ACC GGC AAT GAC ACG CTC TTT ATT GAC        389
Pro Val Pro Lys His Ser Ser Thr Gly Asn Asp Thr Leu Phe Ile Asp
                 25                  30                  35

CAG ACG GTC CAG GTT ACC TAC AAT GGT GAA CAG GTG TGG TGG ACT CCT        437
Gln Thr Val Gln Val Thr Tyr Asn Gly Glu Gln Val Trp Trp Thr Pro
             40                  45                  50

CCA TAT GAT GAC CCC GGA TCC CCG GAC TTT GCT GAG ACC AGG ATC GAT        485
Pro Tyr Asp Asp Pro Gly Ser Pro Asp Phe Ala Glu Thr Arg Ile Asp
         55                  60                  65

GAC CAA CAG GTT ACT TAC ACG GCC GGC TAC GTG CCT CCC AGC GGA CCG        533
Asp Gln Gln Val Thr Tyr Thr Ala Gly Tyr Val Pro Pro Ser Gly Pro
     70                  75                  80

CAT TTC ACC AGC AAG GAA ATC GTT CAA GGC GGC GTC TCG CGG ACA TTC        581
His Phe Thr Ser Lys Glu Ile Val Gln Gly Gly Val Ser Arg Thr Phe
 85                  90                  95                  100

GGC GCC ATC TTC CAG CAG GGC TTT GTG CCG TGG ATG CTG CGT GAA CGT        629
Gly Ala Ile Phe Gln Gln Gly Phe Val Pro Trp Met Leu Arg Glu Arg
                105                 110                 115

GAT TCG AAC TCT GAA CCG AAT CTA GGC GGA ACG CGG ATC CGG ACA CTG        677
Asp Ser Asn Ser Glu Pro Asn Leu Gly Gly Thr Arg Ile Arg Thr Leu
            120                 125                 130

CAG ATT ATA CAG ACT CAG CAC GAT TCT GCG AAT ACC TTC AAG CCT CTG        725
Gln Ile Ile Gln Thr Gln His Asp Ser Ala Asn Thr Phe Lys Pro Leu
        135                 140                 145
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AAT GGC GCA GTG AAT GAA TCC TAT GCC CTG GAT GTC GAC GCA AAG GGC      773
Asn Gly Ala Val Asn Glu Ser Tyr Ala Leu Asp Val Asp Ala Lys Gly
    150                 155                 160

CAC GCA TCT CTC ACC GCT CCG TCG TCA ACG GGC ATC CTT CGA GGC CTT      821
His Ala Ser Leu Thr Ala Pro Ser Ser Thr Gly Ile Leu Arg Gly Leu
165                 170                 175                 180

GAG ACC TTC TCC CAG CTC TTC TTC AAG CAT AGC TCC GGC ACT GCT TGG      869
Glu Thr Phe Ser Gln Leu Phe Phe Lys His Ser Ser Gly Thr Ala Trp
                185                 190                 195

TAT ACG CAG CTT GCA CCT GTT TCG ATC CGC GAT GAG CCC AAG TAT CCT      917
Tyr Thr Gln Leu Ala Pro Val Ser Ile Arg Asp Glu Pro Lys Tyr Pro
            200                 205                 210

CAC CGC GGC CTC CTG TTG GAT GTC AGC CGC CAT TGG TTC GAG GTT TCC      965
His Arg Gly Leu Leu Leu Asp Val Ser Arg His Trp Phe Glu Val Ser
        215                 220                 225

GAC ATT GAG CGC ACT ATC GAT GCT CTG GCC ATG AAC AAA ATG AAT GTG      1013
Asp Ile Glu Arg Thr Ile Asp Ala Leu Ala Met Asn Lys Met Asn Val
    230                 235                 240

CTG CAT CTG CAC GCT ACT GAC ACG CAG TCA TGG CCG CTG GAG ATT CCA      1061
Leu His Leu His Ala Thr Asp Thr Gln Ser Trp Pro Leu Glu Ile Pro
245                 250                 255                 260

TCC CTG CCT CTG CTG GCT GAG AAG GGC GCC TAT CAC AAG GGT TTG AGC      1109
Ser Leu Pro Leu Leu Ala Glu Lys Gly Ala Tyr His Lys Gly Leu Ser
                265                 270                 275

TAC TCG CCA AGC GAT CTT GCG AGC ATC CAA GAA TAT GGT GTT CAT CGA      1157
Tyr Ser Pro Ser Asp Leu Ala Ser Ile Gln Glu Tyr Gly Val His Arg
            280                 285                 290

GGT GTC CAG GTC ATT GTA GAG ATT GAT ATG CCG GGC CAC GTT GGA ATC      1205
Gly Val Gln Val Ile Val Glu Ile Asp Met Pro Gly His Val Gly Ile
        295                 300                 305

GAC AAG GCA TAC CCC GGG CTT AGC AAC GCC TAC GGA GTC AAC CCG TGG      1253
Asp Lys Ala Tyr Pro Gly Leu Ser Asn Ala Tyr Gly Val Asn Pro Trp
    310                 315                 320

CAG TGG TAC TGC GCC CAG CCG CCC TGC GGA TCT TTC AAG CTG AAC AAC      1301
Gln Trp Tyr Cys Ala Gln Pro Pro Cys Gly Ser Phe Lys Leu Asn Asn
325                 330                 335                 340

ACG GAT GTC GAA AAG TTC ATT GAC AAG CTG TTT GAA GAT TTG CTG CCG      1349
Thr Asp Val Glu Lys Phe Ile Asp Lys Leu Phe Glu Asp Leu Leu Pro
                345                 350                 355

CGT CTT TCG CCG TAC TCG GCC TAC TTT CAC ACT GGT GGC GAT GAG TAC      1397
Arg Leu Ser Pro Tyr Ser Ala Tyr Phe His Thr Gly Gly Asp Glu Tyr
            360                 365                 370

AAG GCG AAC AAC TCG CTG CTC GAC CCG GCC CTT CGC ACA AAC GAC ATG      1445
Lys Ala Asn Asn Ser Leu Leu Asp Pro Ala Leu Arg Thr Asn Asp Met
        375                 380                 385

AAC ACC CTG CAG CCG ATG CTG CAG CGC TTC TTG GAC CAC GTG CAT GGC      1493
Asn Thr Leu Gln Pro Met Leu Gln Arg Phe Leu Asp His Val His Gly
    390                 395                 400

AAA GTT CGT GAT CTG GGA CTC GTT CCC ATG GTT TGG GAA GAA ATG ATT      1541
Lys Val Arg Asp Leu Gly Leu Val Pro Met Val Trp Glu Glu Met Ile
405                 410                 415                 420
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CTG GAT TGG AAC GCA ACT CTG GGC AAG GAT GTC GTT GCT CAA ACG TGG      1589
Leu Asp Trp Asn Ala Thr Leu Gly Lys Asp Val Val Ala Gln Thr Trp
                425                 430                 435

CTT GGC GGA GGA GCG ATT CAG AAG CTT GCT CAG GCT GGA TAC AAG GTT      1637
Leu Gly Gly Gly Ala Ile Gln Lys Leu Ala Gln Ala Gly Tyr Lys Val
            440                 445                 450

ATT GAC AGC AGC AAT GAC TTT TAC TAT CTC GAC TGT GGT CGT GGT GAG      1685
Ile Asp Ser Ser Asn Asp Phe Tyr Tyr Leu Asp Cys Gly Arg Gly Glu
        455                 460                 465

TGG CTC GAT TTT GCC AAT GGT GAC CCC TTT AAC AAC AAC TAT CCC TTT      1733
Trp Leu Asp Phe Ala Asn Gly Asp Pro Phe Asn Asn Asn Tyr Pro Phe
    470                 475                 480

CTC GAC TGG TGC GAC CCG ACC AAA AAC TGG AAG CTC ATG TAC TCA CAC      1781
Leu Asp Trp Cys Asp Pro Thr Lys Asn Trp Lys Leu Met Tyr Ser His
485                 490                 495                 500

GAG CCC ACG GAC GGC GTG TCC GAT GAT CTC AAG AAG AAT GTC ATT GGA      1829
Glu Pro Thr Asp Gly Val Ser Asp Asp Leu Lys Lys Asn Val Ile Gly
                505                 510                 515

GGC GAA GTT GCT GTC TGG ACT GAG ACC ATC GAT CCG ACC AGC TTG GAC      1877
Gly Glu Val Ala Val Trp Thr Glu Thr Ile Asp Pro Thr Ser Leu Asp
            520                 525                 530

TCC ATC ATC TGG CCG CGA GCG GGA GCG GCC GCT GAG ATT TGG TGG TCG      1925
Ser Ile Ile Trp Pro Arg Ala Gly Ala Ala Ala Glu Ile Trp Trp Ser
        535                 540                 545

GGC AAG ATC GAT GAG AAG GGC CAG AAC CGA TCA CAG ATT GAT GCA CGG      1973
Gly Lys Ile Asp Glu Lys Gly Gln Asn Arg Ser Gln Ile Asp Ala Arg
    550                 555                 560

CCA AGA TTA TCG GAG CAG CGA GAG CGC ATG TTG GCG AGG GGA GTT CGA      2021
Pro Arg Leu Ser Glu Gln Arg Glu Arg Met Leu Ala Arg Gly Val Arg
565                 570                 575                 580

GGA ACG CCG ATT ACG CAG CTG TGG TGT AGT CAG GTT GAT GTT CAT AAC      2069
Gly Thr Pro Ile Thr Gln Leu Trp Cys Ser Gln Val Asp Val His Asn
                585                 590                 595

TGC GAG TCT GGG AAT TA CTGATGCGGG TTGATGAACA AAGTATGTAA              2116
Cys Glu Ser Gly Asn
            600

TGTGGTATAT ATGAATGTTT CTTTTTCACG CTGCTGTTAA AGGCCGGGGA CGTCTCGTTT    2176

GTGATGACGG TTAGACTGAA AATCACTTAT AATGAATTCA AGTCATTCAA GATGAAAAAA    2236

AAA                                                                  2239
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 601 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Met Phe Ser Arg Ala Ile Val Ala Ala Leu Ala Leu Ser Gly Pro Ala
  1               5                  10                  15
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Phe Ala Leu Trp Pro Val Pro Lys His Ser Ser Thr Gly Asn Asp Thr
20 25 30

Leu Phe Ile Asp Gln Thr Val Gln Val Thr Tyr Asn Gly Glu Gln Val
35 40 45

Trp Trp Thr Pro Pro Tyr Asp Asp Pro Gly Ser Pro Asp Phe Ala Glu
50 55 60

Thr Arg Ile Asp Asp Gln Gln Val Thr Tyr Thr Ala Gly Tyr Val Pro
65 70 75 80

Pro Ser Gly Pro His Phe Thr Ser Lys Glu Ile Val Gln Gly Gly Val
85 90 95

Ser Arg Thr Phe Gly Ala Ile Phe Gln Gln Gly Phe Val Pro Trp Met
100 105 110

Leu Arg Glu Arg Asp Ser Asn Ser Glu Pro Asn Leu Gly Gly Thr Arg
115 120 125

Ile Arg Thr Leu Gln Ile Ile Gln Thr Gln His Asp Ser Ala Asn Thr
130 135 140

Phe Lys Pro Leu Asn Gly Ala Val Asn Glu Ser Tyr Ala Leu Asp Val
145 150 155 160

Asp Ala Lys Gly His Ala Ser Leu Thr Ala Pro Ser Ser Thr Gly Ile
165 170 175

Leu Arg Gly Leu Glu Thr Phe Ser Gln Leu Phe Phe Lys His Ser Ser
180 185 190

Gly Thr Ala Trp Tyr Thr Gln Leu Ala Pro Val Ser Ile Arg Asp Glu
195 200 205

Pro Lys Tyr Pro His Arg Gly Leu Leu Leu Asp Val Ser Arg His Trp
210 215 220

Phe Glu Val Ser Asp Ile Glu Arg Thr Ile Asp Ala Leu Ala Met Asn
225 230 235 240

Lys Met Asn Val Leu His Leu His Ala Thr Asp Thr Gln Ser Trp Pro
245 250 255

Leu Glu Ile Pro Ser Leu Pro Leu Leu Ala Glu Lys Gly Ala Tyr His
260 265 270

Lys Gly Leu Ser Tyr Ser Pro Ser Asp Leu Ala Ser Ile Gln Glu Tyr
275 280 285

Gly Val His Arg Gly Val Gln Val Ile Val Glu Ile Asp Met Pro Gly
290 295 300

His Val Gly Ile Asp Lys Ala Tyr Pro Gly Leu Ser Asn Ala Tyr Gly
305 310 315 320

Val Asn Pro Trp Gln Trp Tyr Cys Ala Gln Pro Pro Cys Gly Ser Phe
325 330 335

Lys Leu Asn Asn Thr Asp Val Glu Lys Phe Ile Asp Lys Leu Phe Glu
340 345 350

Asp Leu Leu Pro Arg Leu Ser Pro Tyr Ser Ala Tyr Phe His Thr Gly
355 360 365

Gly Asp Glu Tyr Lys Ala Asn Asn Ser Leu Leu Asp Pro Ala Leu Arg
370 375 380

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Thr Asn Asp Met Asn Thr Leu Gln Pro Met Leu Gln Arg Phe Leu Asp
385                 390                 395                 400

His Val His Gly Lys Val Arg Asp Leu Gly Leu Val Pro Met Val Trp
                405                 410                 415

Glu Glu Met Ile Leu Asp Trp Asn Ala Thr Leu Gly Lys Asp Val Val
            420                 425                 430

Ala Gln Thr Trp Leu Gly Gly Gly Ala Ile Gln Lys Leu Ala Gln Ala
        435                 440                 445

Gly Tyr Lys Val Ile Asp Ser Ser Asn Asp Phe Tyr Tyr Leu Asp Cys
    450                 455                 460

Gly Arg Gly Glu Trp Leu Asp Phe Ala Asn Gly Asp Pro Phe Asn Asn
465                 470                 475                 480

Asn Tyr Pro Phe Leu Asp Trp Cys Asp Pro Thr Lys Asn Trp Lys Leu
                485                 490                 495

Met Tyr Ser His Glu Pro Thr Asp Gly Val Ser Asp Asp Leu Lys Lys
            500                 505                 510

Asn Val Ile Gly Gly Glu Val Ala Val Trp Thr Glu Thr Ile Asp Pro
        515                 520                 525

Thr Ser Leu Asp Ser Ile Ile Trp Pro Arg Ala Gly Ala Ala Ala Glu
    530                 535                 540

Ile Trp Trp Ser Gly Lys Ile Asp Glu Lys Gly Gln Asn Arg Ser Gln
545                 550                 555                 560

Ile Asp Ala Arg Pro Arg Leu Ser Glu Gln Arg Glu Arg Met Leu Ala
                565                 570                 575

Arg Gly Val Arg Gly Thr Pro Ile Thr Gln Leu Trp Cys Ser Gln Val
            580                 585                 590

Asp Val His Asn Cys Glu Ser Gly Asn
        595                 600
```

CLAIMS

1. A DNA construct comprising a DNA sequence encoding an enzyme exhibiting exochitinase activity, which DNA sequence

a) comprises the DNA sequence shown in SEQ ID No. 1 or SEQ ID no. 3, or

b) comprises an analogue of the DNA sequence shown in SEQ ID no. 1 or SEQ ID no 3, which

i) is homologous with the DNA sequences shown in SEQ ID no. 1 or SEQ ID no. 3, and/or

ii) hybridizes with the same oligonucleotide probe as the DNA sequence shown in SEQ ID no. 1 or SEQ ID no. 3, and/or

iii) encodes a polypeptide which is homologous with the polypeptide encoded by a DNA sequence comprising the DNA sequence shown in SEQ ID No. 1 or SEQ ID no. 3, and/or

iv) encodes a polypeptide which is immunologically reactive with an antibody raised against a purified exochitinase shown in SEQ ID no. 2 derived from *Saccharomyces cerevisiae* DSM no. 9944 or against a purified exochitinase shown in SEQ ID no. 4 derived from *Saccharomyces cerevisiae* DSM no. 9945.

2. A DNA construct comprising a DNA sequence encoding an enzyme exhibiting exochitinase activity, which DNA sequence comprises at least a partial sequence of the sequence shown in SEQ ID No. 1 or SEQ ID no. 3.

3. The DNA construct according to claim 1 or 2, in which the DNA sequence encoding an enzyme exhibiting exochitinase activity is obtainable from a microorganism.

4. The DNA construct according to claim 3, in which the DNA sequence is obtainable from a filamentous fungus or a yeast.

5. The DNA construct according to claim 4, in which is the DNA

sequence is obtainable from a strain of *Saccharomyces*, *Aspergillus*, *Trichoderma*, *Penicillium*, *Fusarium*, *Gliocladium*, *Aphanocladium*, or *Humicola*, or a mutant thereof.

6. The DNA construct according to claim 5, in which the DNA sequence is obtainable from a strain of *Trichoderma* in particular a strain of *T. harzianum* , or *T. reesei*, or *T. viride*, *T. longibrachiatum*, or *T. koningii*, or a mutant thereof.

7. The DNA construct according to claim 5, in which the DNA sequence is obtainable from a strain of *Aspergillus*, in particular a strain of *A. aculeatus* or *A. niger*, or a mutant thereof.

8. The DNA construct according to claim 5, in which the DNA sequence is obtainable from a strain of *Fusarium*, in particular a strain of *F. oxysporum*, or a mutant thereof.

9. The DNA construct according to claim 5, in which the DNA sequence is obtainable from a strain of *Gliocladium*, in particular *Gliocladium virens*, or a mutant thereof.

10. The DNA construct according to claim 5, in which the DNA sequence is obtainable from a strain of *Aphanocladium*, in particular *Aphanocladium album*, or a mutant thereof.

11. The DNA construct according to claim 5, in which the DNA sequence is obtainable from a strain of *Humicola*, or a mutant thereof.

12. The DNA construct according to claim 5, in which the DNA sequence is obtainable from a strain of *Saccharomyces*, in particular *Saccharomyces cerevisiae*, or a mutant thereof.

13. The DNA construct according to claim 12, in which the DNA sequence is isolated from *Saccharomyces cerevisiae* DSM No. 9944 or DSM no. 9945.

14. A recombinant expression vector comprising a DNA construct according to any of claims 1-13.

15. A cell comprising a DNA construct according to any of claims 1-13 or a recombinant expression vector according to claim 14.

16. A cell according to claim 15, which is an eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell.

17. A cell according to claim 16, wherein the cell belongs to a strain of *Trichodrma*, in particular a strain of *Trichoderma harzianum* or *Trichoderma reesie*.

18. A method of producing an enzyme exhibiting exochitinase activity, the method comprising culturing a cell according to any of claims 15-17 under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

19. An enzyme exhibiting exochitinase activity, which enzyme
a) is encoded by a DNA construct according to any of claims 1-13, and/or
b) produced by the method according to claim 18, and/or
c) is immunologically reactive with an antibody raised against a purified exochitinase derived from *Saccharomyces cerevisiae*, DSM no. 9944 shown in SEQ ID no. 1 or DSM no. 9945 shown in SEQ ID no. 3.

20. An enzyme preparation useful for modification or degradation of fungal, crustacean, or nematode cell wall components, said preparation being enriched in an enzyme exhibiting exochitinase activity according to claim 19.

21. A preparation according to claim 20, which additionally comprises an enzyme with chitinolytic, proteolytic, β-glucanolytic, or mannanolytic activity.

22. Use of an enzyme according to claim 19 or an enzyme preparation according to claim 21 or 22 for modification or degradation of fungal, crustacean, or nematode cell wall components.

23. The use according to claim 22 for plant protection purposes.

24. The use according to claim 23 for coating seeds.

25. The use according to claim 23 for spraying plants.

26. The use according to claim 22 for pharmaceutical purposes.

27. The use according to claim 26 for wound dressing.

28. Use of a DNA construct according to claims 1 to 13 or an expression vector according to claim 14 for producing transgenic plants.

29. An isolated substantially pure culture of *Saccharomyces cerevisiae* DSM no. 9944.

30. An isolated substantially pure culture of *Saccharomyces cerevisiae* DSM no. 9945

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00216

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/24
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC, MEDLINE, BIOSIS, DBA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Dialog Information Services, File 155, MEDLINE, Dialog accession no. 09535466, Medline accession no. 96057066, Draborg H et al: "Molecular cloning and expression in S. cerevisiae of two exochitinases from Trichoderma harzianum"; & Biochem Mol Biol Int (AUSTRALIA) Jul 1995, 36 (4) p781-91 --	1-30
X	Dialog Information Services, File 154, MEDLINE, Dialog accession no. 09072207, Medline accession no. 95002207, Triggs-Raine BL et al: "Characterization of the murine beta-hexosaminidase (HEXB) gene"; & Biochim Biophys Acta (NETHERLANDS) Oct 21 1994, 1227 (1-2) p79-86, Swissprot P20060 --	1-2,14-15,19

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier 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 when the document is taken alone
"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

Date of the actual completion of the international search	Date of mailing of the international search report
9 October 1996	10 -10- 1996
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00216

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, File 154, MEDLINE, Dialog accession no. 08907843, Medline accession no. 94222843, Cannon RD et al: "Molecular cloning and expression of the Candida albicans beta-N-acetylglucosaminidase (HEXI) gene"; & J Bacteriol (UNITED STATES) May 1994, 176 (9) p2640-7, Swissprot P43077, Pir 2: A55588	1-4,14-16, 18-19
X	Dialog Information Services, File 154, MEDLINE, Dialog accession no. 08206643, Medline accession no. 92344643, Beccari T et al: "Cloning and sequence analysis of a cDNA encoding the alpha-subunit of mouse beta-N-acetylhexosaminidase and comparison with the human enzyme"; & Biochem J (ENGLAND) Jul 15 1992, 285 (Pt 2) p593-6, Pir 2: A55505, Swissprot P29416	1-2,14-15,19
X	Dialog Information Services, File 154, MEDLINE, Dialog accession no. 06910588, Medline accession no. 8912588, Neote K et al: "Characterization of the human HFXR gene encoding lysosomal beta--hexosaminidase"; &Genomics (UNITED STATES) Nov 1988, 3 (4) p279-86, Swissprot P07686	1-2,14-15,19
X	Dialog Information Services, File 154, MEDLINE, Dialog accession no. 06703625, Medline accession no. 89005625, Bapat B et al: "Cloning and sequence analysis of a cDNAencoding the beta-subunit of mouse beta-hexosaminidase"; & FEBS Lett (NETHERLANDS) Sep 12 1988, 237 (1-2) p191-5, Pir 2: B54745	1-2,14-15,19
X	Dialog Information Services, File 155, MEDLINE, Dialog accession no. 09288264, Medline accession no. 95218264, Nagamatsu Y et al: "Purification of a chitooligosaccharidolytic beta-N-acetylglucosaminidase from Bombyx mori larvae during metamorphosis and the nucleotide sequence of its cDNA"; & Biosci Biotechnol Biochem (JAPAN) Feb 1995, 59 (2) p219-25, Pir 2: JC2539, Swissprot P49010	1-2,14-15,19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00216

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9402598 A1 (CORNELL RESEARCH FOUNDATION, INC.), 3 February 1994 (03.02.94) --	1-30
X	WO 9424288 A1 (CORNELL RESEARCH FOUNDATION, INC.), 27 October 1994 (27.10.94) --	1-30
X	WO 9222314 A1 (CORNELL RESEARCH FOUNDATION, INC.), 23 December 1992 (23.12.92) --	1-30
X	WO 9424271 A1 (CORNELL RESEARCH FOUNDATION, INC.), 27 October 1994 (27.10.94) --	1-30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00216

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Invention 1: DNA-construct comprising SEQ ID No. 1. and related enzymes, cells, methods and uses.

Invention 2: DNA-construct comprising SEQ ID No. 3. and related enzymes, cells methods and uses.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-29

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

05/09/96

International application No.
PCT/DK 96/00216

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9402598	03/02/94	EP-A-	0656059	07/06/95
		JP-T-	7509362	19/10/95
		US-A-	5378821	03/01/95
WO-A1- 9424288	27/10/94	US-A-	5378821	03/01/95
WO-A1- 9222314	23/12/92	EP-A-	0590004	06/04/94
		JP-T-	6508618	29/09/94
		US-A-	5173419	22/12/92
		US-A-	5378821	03/01/95
WO-A1- 9424271	27/10/94	US-A-	5474926	12/12/95