



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/DK97/00283  <b>(22) International Filing Date:</b> 30 June 1997 (30.06.97)  <b>(30) Priority Data:</b> 0715/96                      28 June 1996 (28.06.96)                      DK 1004/96                      17 September 1996 (17.09.96)                      DK  <b>(71) Applicant (for all designated States except US):</b> NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> WAHLEITHNER, Jill, Angela [US/US]; 1718 Tea Place, Davis, CA 95616 (US). FUGLSANG, Claus, Crone [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). HALKIER, Torben [DK/DK]; Novo Nordisk a/s, Novo Allé, DK- 2880 Bagsværd (DK). JOHANSEN, Charlotte [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). HANSEN, Mogens, Trier [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK).  <b>(74) Common Representative:</b> NOVO NORDISK A/S; Novo Allé, Corporate Patents, DK-2880 Bagsværd (DK).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> A RECOMBINANT ENZYME WITH MUTANASE ACTIVITY  <b>(57) Abstract</b>  <p>The present invention relates to method for constructing an expression vector comprising a mutanase gene obtained from a filamentous fungus suitable for heterologous production comprising the steps of a) isolating a DNA sequence encoding a mutanase from a filamentous fungus, b) introducing a kex2-site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the mutanase, c) cloning the DNA sequence obtained in step b) into a suitable expression vector. The invention also relates to a recombinant expression vector comprising said mutanase gene sequence and a kex2 cleavage site between the DNA sequence encoding the pro-peptide and the region encoding the mature mutanase, a filamentous fungus host cell, a process for producing recombinant mutanase and a recombinant mutanase. It is also the object of the invention to provide compositions useful in oral care products for humans and animals.</p>		

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**Title:** A recombinant enzyme with mutanase activity

#### FIELD OF THE INVENTION

The present invention relates to a method for constructing an expression vector comprising a mutanase gene obtained from a filamentous fungus suitable for heterologous production, a recombinant expression vector comprising said mutanase gene sequence and a kex2 cleavage site between the DNA sequence encoding the pro-peptide and the DNA sequence encoding the mature mutanase, a filamentous fungus host cell, a process of producing recombinant mutanase, and said recombinant mutanase.

It is also the object of the invention to provide compositions useful in oral care products for humans and animals.

#### BACKGROUND OF THE INVENTION

Mutanases are  $\alpha$ -1,3-glucanases (also known as  $\alpha$ -1,3-glucanohydrolases) which degrade the  $\alpha$ -1,3-glycosidic linkages in mutan. Mutanases have been described from two species of *Trichoderma* (Hasegawa et al., (1969), Journal of Biological Chemistry 244, p. 5460-5470; Guggenheim and Haller, (1972), Journal of Dental Research 51, p. 394-402) and from a strain of *Streptomyces* (Takehara et al., (1981), Journal of Bacteriology 145, p. 729-735), *Cladosporium resinae* (Hare et al. (1978), Carbohydrate Research 66, p. 245-264), *Pseudomonas* sp. (US patent no. 4,438,093), *Flavobacterium* sp. (JP 77038113), *Bacillus circulans* (JP 63301788) and *Aspergillus* sp.. A mutanase gene from *Trichoderma harzianum* has been cloned and sequenced (Japanese Patent No. 4-58889-A from Nissin Shokuhin Kaisha LTD).

Although mutanases have commercial potential for use as an antiplaque agent in dental applications and personal care products, e.g., toothpaste, chewing gum, or other oral and dental care products, the art has been unable to produce mutanases in significant quantities to be commercial useful.

US patent no. 4,353,891 (Guggenheim et al.) concerns plaque removal using mutanase produced by *Trichoderma harzianum* CBS 243.71 to degrade mutan synthesized by cultivating *Streptococcus*

*mutans* strain CBS 350.71 identifiable as OMZ 176.

It is an object of the present invention to provide a recombinant mutanase from *Trichoderma harzianum* which can be produced in commercially useful quantities.

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#### BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows plasmid pMT1796

Figure 2 shows plasmid construction of plasmids pMT1796, pMT1802, and pMT1815,

10 Figure 3 shows an outline of the construction of the *A. oryzae* recombinant mutanase expression vector pMT1802,

Figure 4 shows the pH-profile of recombinant and wild-type *T. harzianum* CBS 243.71 mutanase

Figure 5 shows the temperature profile of recombinant and wild-  
15 type *T. harzianum* CBS 243.71 mutanase at pH 7,

Figure 6 shows the temperature stability of recombinant and wild-type *T. harzianum* CBS 243.71 mutanase at pH 7,

Figure 7 shows the indirect Malthus standard curve for a mix  
culture of *S. mutans*, *A. viscosus* and *F. nucleatum* grown in  
20 BHI at 37°C.

#### SUMMARY OF THE INVENTION

The object of the invention is to provide a recombinant mutanase derived from a filamentous fungus by heterologous  
25 expression.

The present inventors have as the first been able to express the mutanase gene of a filamentous fungus heterologously and thus cleared the way for providing a single component, recombinant mutanase essentially free of any contaminants.

30 In the first aspect the invention relates to a method for constructing an expression vector comprising a mutanase gene obtained from a filamentous fungus suitable for heterologous production comprising the steps of:

a) isolating a DNA sequence encoding a mutanase from a  
35 filamentous fungus,

b) introducing a kex2 site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the

mutanase, or replacing the mutanase (pre)pro-sequence with a (pre)pro-sequence comprising a kex2 or kex2-like site of another fungal enzyme,

c) cloning the DNA sequence obtained in step b) into a suitable expression vector.

In a preferred embodiment the mutanase is obtained from a strain within the genus *Trichoderma*.

In step b) the mutanase (pre)pro-sequence may for instance be replaced with the Lipolase® (pre)pro-sequence or the TAKA-  
10 amylase (pre)pro-sequence.

It is also an object of the invention to provide an expression vector comprising a mutanase gene and a DNA sequence encoding a (pre)pro-peptide with a kex2 site or kex2-like site between the DNA sequences encoding said (pre)pro-peptide and  
15 the mature region of the mutanase.

The invention also relates to a filamentous host cell for production of recombinant mutanase derived from a filamentous fungus. Preferred host cells include filamentous fungi of the genera *Trichoderma*, *Aspergillus*, and *Fusarium*.

20 Further, the invention relates to a process for producing a recombinant mutanase in a host cell, comprising the steps:

a) transforming an expression vector comprising a mutanase gene with a kex2 site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the mutanase  
25 into a suitable filamentous fungus host cell,

b) cultivating the host cell in a suitable culture medium under conditions permitting expression and secretion of an active mutanase,

c) recovering and optionally purifying the secreted active re-  
30 combinant mutanase from the culture medium.

The expression vector may be prepared according to the above described method of the invention.

A recombinant mutanase may according to the invention be produced according to the process of the invention.

35 A substantially pure wild-type mutanase obtained from *Trichoderma harzianum* CBS 243.71 essentially free of any contaminants is also part of the invention.

The invention also relates to a composition comprising a recombinant mutanase of the invention or a substantially pure mutanase of the invention useful in oral care products and food, feed and/or pet food products.

5 Finally the invention relates to the use of the recombinant mutanase of the invention or the substantially purified mutanase of the invention or composition or product of the invention preventing the formation of human or animal dental plaque or removing dental plaque and for the use in food, feed and/or pet  
10 food products.

#### DETAILED DESCRIPTION OF THE INVENTION

The object of the invention is to provide a recombinant mutanase derived from a filamentous fungus by heterologous  
15 expression.

The present inventors have as the first been able to express the mutanase gene of a filamentous fungus heterologously and thus cleared the way for providing a single component recombinant mutanase essentially free of any contaminants.

20 The principle of the invention can be used for all mutanases derivable from filamentous fungi, such as from filamentous fungi of the genus *Trichoderma*, such a strain of *Trichoderma harzianum*, especially *Trichoderma harzianum* CBS 243.71, and the genera *Streptomyces*, *Cladosporium* or *Aspergillus*.

25 Previously it has not been possible to produce mutanases of filamentous fungi heterologously. Consequently, according to prior art mutanases are produced homologously and comprise a mixture of other enzyme activities besides the mutanase (i.e. with undesired contaminants).

30 An example of this is *Trichoderma harzianum* CBS 243.71 which are known to produce a mutanase as also described above. The mutanase derived from *Trichoderma harzianum* CBS 243.71 has before the successful findings of the present invention only been produced homologously.

35 It is advantageous to be able to produce the mutanase heterologously, as it is then possible to provide a single component mutanase free of undesired contaminants. Further, it

facilitates providing an isolated and purified enzyme of the invention in industrial scale.

According to the invention it is possible to express mutanases derived from filamentous fungi in a suitable host cell by introducing a kex2 cleavage site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature mutanase, or replacing the mutanase (pre)pro-sequence with a (pre)pro-sequence comprising a kex2 site or kex2-like site of another fungal enzyme.

10 The (pre)pro-sequence have for instance be the Lipolase® (pre)pro-sequence or the TAKA-amylase (pre)pro-sequence.

### **Pro-peptides**

A large number of mature proteins are initially synthesised with a N-terminal extension, the pro-peptide, varying from very small peptides (e.g. GLA 6 amino acids) to relatively long peptides (e.g. PEPA 49 amino acids).

The pro-peptide can perform a number of different functions. Firstly, pro-peptides might contribute to the efficiency of co-translational translocation of the protein across the ER-membrane. Secondly, pro-peptides might contribute to co-translational proteolytic processing of the polypeptide. Thirdly, they might act as intracellular targeting signal for routing to specific cellular compartments. Fourthly, in some pro-proteins the pro-peptide keeps the protein inactive until it reaches its site of action.

Removal of the pro-peptide from the mature protein occurs in general by processing by a specific endopeptidase, usually after the two positively charged amino acid residues Arg-Arg, Arg-Lys or Lys-Arg. However, also other amino acid combinations, containing at least one basic amino acid, have been found to be processed.

The absence of these doublets in mature, endogenous secreted proteins might protect them from proteolytic cleavage. As di-basic cleavage is thought to occur in the Golgi, the internal di-basic peptide sequences in cytoplasmic proteins will not be attacked by this processing.

**Kex2 sites**

Kex2 sites (see e.g. Methods in Enzymology Vol 185, ed. D. Goeddel, Academic Press Inc. (1990), San Diego, CA, "Gene  
5 Expression Technology") and kex2-like sites are di-basic recognition sites (i.e. cleavage sites) found between the pro-peptide encoding region and the mature region of some proteins.

Insertion of a kex2 site or a kex2-like site have in certain cases been shown to improve correct endopeptidase processing at  
10 the pro-peptide cleavage site resulting in increased protein secretion levels.

However, in a number of other cases insertion of a Kex2 cleavage site did not increase the secretion level. For instance, Cullen et al., (1987), Bio/Technology, vol. 5, p.  
15 369-376, found that insertion of a kex2 site in the secretion signal of chymosin (i.e. signal peptide and pro-peptide), which encoded the glucoamylase signal peptide and pro-peptide fused to prochymosin, did not increase the secretion level of recombinant chymosin expressed in a *Aspergillus nidulans* host  
20 cell.

Other examples of references showing that insertion of a kex2 site or a kex2-like site do not always increase the secretion level include Valverde et al., (1995), J. of Biolog. Chem, p. 15821-15826)

25 In the context of the present invention the term "heterologous" production means expression of a recombinant enzyme in an host organism different from the original donor organism or expression of a recombinant enzyme by the donor organism.

30 The term "homologous" production means expression of the wild-type enzyme by the original organism.

In the first aspect the invention relates to a method for construction of an expression vector comprising a mutanase gene suitable for heterologous production comprising the steps of:

35 a) isolating a DNA sequence encoding a mutanase from a filamentous fungus known to produce a mutanase,



b) introducing a kex2 site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the mutanase, or replacing the mutanase (pre)pro-sequence with a (pre)pro-sequence comprising a kex2 or kex2-like site of  
5 another fungal enzyme,

c) cloning the mutanase gene with the kex2 site or kex2-like site obtained in step b) into a suitable expression vector.

In a preferred embodiment of the mutanase gene is obtained from the genus *Trichoderma*, preferably a strain of the species  
10 *T. harzianum*, especially the strain *T. harzianum* CBS 243.71.

The complete mutanase gene DNA sequence derived from *Trichoderma harzianum* CBS 243.71 is shown in SEQ ID No. 1

In step b) the mutanase (pre)pro-sequence may for instance be replaced with the Lipolase® (pre)pro-sequence or the TAKA-  
15 amylase (pre)pro-sequence.

In the examples below illustrating the present invention a kex2-site is inserted into the *Trichoderma harzianum* mutanase gene presented in SEQ ID No. 1 as the site specific mutation E36 → K36.

20

#### **Isolation of the mutanase gene**

The DNA sequence encoding a mutanase may, in accordance with well-known procedures, conveniently be isolated from DNA from a suitable source, such as any of the above mentioned organisms  
25 known to comprise a mutanase gene, by use of synthetic oligonucleotide probes prepared on the basis of the DNA sequence disclosed herein.

For instance, a suitable oligonucleotide probe may be prepared on the basis of the nucleotide sequences shown in SEQ ID  
30 no. 1 or the amino acid sequence shown in SEQ ID no. 2 or any suitable sub-sequence thereof.

According to this method primers are designed from the knowledge to at least a part of SEQ ID No. 2. Fragments of mutanase gene are then PCR amplified by the use of these  
35 primers. These fragments are used as probes for cloning the complete gene.

Alternatively, the DNA sequence encoding a mutanase may be isolated by a general method involving

- cloning, in suitable vectors, a DNA or cDNA library from a strain of genus *Trichoderma*,
- 5 - transforming suitable 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 mutanase
- 10 activity of the enzyme produced by such clones, and
- isolating the DNA coding an enzyme from such clones.

The general method is further disclosed in WO 93/11249 the contents of which are hereby incorporated by reference.

#### 15 **Expression vector**

In another aspect the invention relates to an expression vector comprising a mutanase gene and a DNA sequence encoding a pro-peptide with a kex2 site or kex2-like site inserted between the DNA sequences encoding said pro-peptide and the mature

20 region of the mutanase.

In preferred embodiments of the invention the expression vector comprises besides the kex2 site or kex2-like site an operably linked DNA sequence encoding a prepro-peptide (i.e. signal peptide and a pro-peptide). The prepro-sequence may

25 advantageously be the original mutanase signal-sequence or the Lipolase® signal-sequence or the TAKA signal-sequence and the original mutanase pro-sequence or the Lipolase® pro-sequence or the TAKA pro-sequence.

The promoter may be the TAKA promoter or the TAKA:TPI

30 promoter.

In a specific embodiment of the invention the expression vector is the pMT1796 used to illustrate the concept of the invention in Example 3 below.

The choice of vector will often depend on the host cell into

35 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 mutanase should also 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 mutanase, a prepro-sequence including the *kex2* site or *kex2*-like site, 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).

## 20 Host Cell

A third aspect of the invention relates to a filamentous fungi host cell for production of recombinant mutanase derived from a filamentous fungus of the genus *Trichoderma*, such as a strain of *T. harzianum*, especially *T. harzianum* CBS 243.71, or the genus *Aspergillus*, such as a strain of *A. oryzae* or *A. niger*, or a strain of the genus *Fusarium*, such as a strain of *Fusarium oxysporium*, *Fusarium graminearum* (in the perfect state named *Gibberella zeae*, previously *Sphaeria zeae*, synonym with *Gibberella roseum* and *Gibberella roseum* f. sp. *cerealis*), or *Fusarium sulphureum* (in the perfect state named *Gibberella puricaris*, synonym with *Fusarium trichothecioides*, *Fusarium bactridioides*, *Fusarium sambucium*, *Fusarium roseum*, and *Fusarium roseum* var. *graminearum*), *Fusarium cerealis* (synonym with *Fusarium crockwellense*) or *Fusarium venenatum*.

The host cell may advantageously be a *F. graminearum* described in WO 96/00787 (from Novo Nordisk A/S), e.g. the strain deposited as *Fusarium graminearum* ATCC 20334. The strain ATCC

20334 was previously wrongly classified as *Fusarium graminearum* (Yoder, W. and Christianson, L. 1997). RAPD-based and classical taxonomic analyses have now revealed that the true identity of the Quorn fungus, ATCC 20334, is *Fusarium venenatum* Nirenburg  
5 sp. nov.

In a preferred embodiment of the invention the host cell is a protease deficient or protease minus strain.

This may for instance be the protease deficient strain *Aspergillus oryzae* JaL125 having the alkaline protease gene  
10 named "alp" deleted. This strain is described in PCT/DK97/00135 (from Novo Nordisk A/S).

Filamentous fungi cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a  
15 manner known per se. The use of *Aspergillus* as a host microorganism is described in EP 238 023 (Novo Nordisk A/S), the contents of which are hereby incorporated by reference.

According to a further aspect the invention relates to a process for producing a recombinant mutanase in a host cell. Said  
20 process comprises the following steps:

- a) transforming an expression vector encoding a mutanase gene with a kex2 site or a kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the mutanase into a suitable filamentous fungus host cell,
- 25 b) cultivating the host cell in a suitable culture medium under conditions permitting the expression of the expression vector,
- c) recovering the secreted recombinant mutanase from the culture medium,
- d) and optionally purifying the recombinant mutanase.

30 The recombinant expression vector may advantageously be any of the above described.

Further, the filamentous fungi host cells to be used for production of the recombinant mutanase of the invention according to the process of the invention may be any of the  
35 above mentioned host cell, especially of the genera *Aspergillus*, *Fusarium* or *Trichoderma*.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host cells in question. The expressed mutanase is secreted into the culture medium and may be recovered from there 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.

It is also an important object of the invention to provide a recombinant mutanase produced according to the process of the invention.

The isolated recombinant mutanase has essentially an amino acid sequence as shown in SEQ ID no. 2. From SDS-PAGE a molecular weight around 80 kDa was found.

The pH optimum of the recombinant mutanase was found to lie in the range from 3.5 to 5.5 which equals the pH optimum of the wild-type mutanase (see Figure 4). The temperature optimum of both the recombinant and wild-type mutanase was found to be around 45°C at pH 7 and around 55°C at pH 5.5 (see Figure 5). Further, the residual activity starts to decline at 40°C at pH 7, while the enzyme is more stable at pH 5.5, where the residual activity starts to decline at 55°C.

The inventors have also provided a substantially pure wild-type mutanase obtained from *Trichoderma harzianum* CBS 243.71 essentially free of any active contaminants, such as other enzyme activities.

#### Composition

It is also an object of the invention to provide a composition comprising the recombinant mutanase of the invention or the purified wild-type mutanase essentially free of any active contaminants of the invention.

35

#### Oral care composition

In a still further aspect, the present invention relates to an oral care composition useful as an ingredient in oral care products.

An oral care composition of the invention may suitably  
5 comprise an amount of the recombinant *Trichoderma harzianum* mutanase equivalent to an enzyme activity, calculated as enzyme activity units in the final oral care product, in the range from 0.001 MU to 1000 MU/ml, preferably from 0.01 MU/ml to 500 MU/ml, such as from 0.1 MU/ml to 100 MU/ml, especially 0.05 MU/ml to 100  
10 MU/ml.

It is also contemplated according to the invention to include other enzyme activities than mutanase activity in the oral care composition. Contemplated enzyme activities include activities from the group of enzymes comprising dextranases,  
15 oxidases, such as glucose oxidase, L-amino acid oxidase, peroxidases, such as e.g. the *Coprinus sp.* peroxidases described in WO 95/10602 (from Novo Nordisk A/S) or lactoperoxidase or, haloperoxidases, laccases, proteases, such as papain, acidic protease (e.g. the acidic proteases described in WO 95/02044  
20 (Novo Nordisk A/S)), endoglucosidases, lipases, amylases, including amyloglucosidases, such as AMG (from Novo Nordisk A/S), and mixtures thereof.

#### Oral care products

25 The oral care product may have any suitable physical form (i.e. powder, paste, gel, liquid, ointment, tablet etc.). An "oral care product" can be defined as a product which can be used for maintaining or improving the oral hygiene in the mouth of humans and animals, by preventing dental caries, preventing the  
30 formation of dental plaque and tartar, removing dental plaque and tartar, preventing and/or treating dental diseases etc.

At least in the context of the present invention oral care products do also encompass products for cleaning dentures, artificial teeth and the like.

35 Examples of such oral care products include toothpaste, dental cream, gel or tooth powder, odontic, mouth washes, pre- or post brushing rinse formulations, chewing gum, lozenges, and candy.

Toothpastes and tooth gels typically include abrasive polishing materials, foaming agents, flavouring agents, humectants, binders, thickeners, sweetening agents, whitening/bleaching/ stain removing agents, water, and optionally  
5 enzymes.

Mouth washes, including plaque removing liquids, typically comprise a water/alcohol solution, flavour, humectant, sweetener, foaming agent, colorant, and optionally enzymes.

#### 10 Abrasives

Abrasive polishing material might also be incorporated into the dentifrice product of the invention. According to the invention said abrasive polishing material includes alumina and hydrates thereof, such as alpha alumina trihydrate, magnesium  
15 trisilicate, magnesium carbonate, kaolin, aluminosilicates, such as calcined aluminum silicate and aluminum silicate, calcium carbonate, zirconium silicate, and also powdered plastics, such as polyvinyl chloride, polyamides, polymethyl methacrylate, polystyrene, phenol-formaldehyde resins, melamine-formaldehyde  
20 resins, urea-formaldehyde resins, epoxy resins, powdered polyethylene, silica xerogels, hydrogels and aerogels and the like. Also suitable as abrasive agents are calcium pyrophosphate, water-insoluble alkali metaphosphates, dicalcium phosphate and/or its dihydrate, dicalcium orthophosphate,  
25 tricalcium phosphate, particulate hydroxyapatite and the like. It is also possible to employ mixtures of these substances.

Dependent on the oral care product the abrasive product may be present in from 0 to 70% by weight, preferably from 1% to 70%. For toothpastes the abrasive material content typically lies in  
30 the range of from 10% to 70% by weight of the final toothpaste product.

Humectants are employed to prevent loss of water from e.g. toothpastes. Suitable humectants for use in oral care products according to the invention include the following compounds and  
35 mixtures thereof: glycerol, polyol, sorbitol, polyethylene glycols (PEG), propylene glycol, 1,3-propanediol, 1,4-butanediol, hydrogenated partially hydrolysed polysaccharides and the like.

Humectants are in general present in from 0% to 80%, preferably 5 to 70% by weight in toothpaste.

Silica, starch, tragacanth gum, xanthan gum, extracts of Irish moss, alginates, pectin, cellulose derivatives, such as  
5 hydroxyethyl cellulose, sodium carboxymethyl cellulose and hydroxypropyl cellulose, polyacrylic acid and its salts, polyvinylpyrrolidone, can be mentioned as examples of suitable thickeners and binders, which helps stabilizing the dentifrice product. Thickeners may be present in toothpaste creams and gels  
10 in an amount of from 0.1 to 20% by weight, and binders to the extent of from 0.01 to 10% by weight of the final product.

#### Foaming agents

As foaming agent soap, an-ionic, cat-ionic, non-ionic, amphoteric and/or zwitterionic surfactants can be used. These may be  
15 present at levels of from 0% to 15%, preferably from 0.1 to 13%, more preferably from 0.25 to 10% by weight of the final product.

#### Surfactants

20 Surfactants are only suitable to the extent that they do not exert an inactivation effect on the present enzymes. Surfactants include fatty alcohol sulphates, salts of sulphonated monoglycerides or fatty acids having 10 to 20 carbon atoms, fatty acid-albumen condensation products, salts of fatty acids amides  
25 and taurines and/or salts of fatty acid esters of isethionic acid.

#### Sweetening agents

Suitable sweeteners include saccharin.

30

#### Flavouring agents

Flavours, such as spearmint, are usually present in low amounts, such as from 0.01% to about 5% by weight, especially from 0.1% to 5%.

35



### Whitening/bleaching agents

Whitening/bleaching agents include  $H_2O_2$  and may be added in amounts less than 5%, preferably from 0.25 to 4%, calculated on the basis of the weight of the final product.

- 5 The whitening/bleaching agents may be an enzyme, such as an oxidoreductase. Examples of suitable teeth bleaching enzymes are described in WO 97/06775 (from Novo Nordisk A/S).

### Water

- 10 Water is usually added in an amount giving e.g. toothpaste a flowable form.

### Additional agents

- Further water-soluble anti-bacterial agents, such as  
15 chlorhexidine digluconate, hexetidine, alexidine, quaternary ammonium anti-bacterial compounds and water-soluble sources of certain metal ions such as zinc, copper, silver and stannous (e.g. zinc, copper and stannous chloride, and silver nitrate) may also be included.

- 20 Also contemplated according to the invention is the addition of compounds which can be used as fluoride source, dyes/colorants, preservatives, vitamins, pH-adjusting agents, anti-carries agents, desensitizing agents etc.

### 25 Enzymes

- Other essential components used in oral care products and in oral care products of the invention are enzymes. Enzymes are biological catalysts of chemical reactions in living systems. Enzymes combine with the substrates on which they act forming an  
30 intermediate enzyme-substrate complex. This complex is then converted to a reaction product and a liberated enzyme which continue its specific enzymatic function.

- Enzymes provide several benefits when used for cleansing of the oral cavity. Proteases break down salivary proteins, which  
35 are adsorbed onto the tooth surface and form the pellicle, the first layer of resulting plaque. Proteases along with lipases destroy bacteria by lysing proteins and lipids which form the

structural components of bacterial cell walls and membranes. Dextranase breaks down the organic skeletal structure produced by bacteria that forms a matrix for bacterial adhesion. Proteases and amylases, not only prevents plaque formation, but also prevents the development of calculus by breaking-up the carbohydrate-protein complex that binds calcium, preventing mineralization.

### Toothpaste

10 A toothpaste produced from an oral care composition of the invention (in weight % of the final toothpaste composition) may typically comprise the following ingredients:

	Abrasive material	10 to 70%
	Humectant	0 to 80%
15	Thickener	0.1 to 20%
	Binder	0.01 to 10%
	Sweetener	0.1% to 5%
	Foaming agent	0 to 15%
	Whitener	0 to 5%
20	Enzymes	0.0001% to 20%

In a specific embodiment of the invention the oral care product is toothpaste having a pH in the range from 6.0 to about 8.0 comprising

- a) 10% to 70% Abrasive material
- 25 b) 0 to 80% Humectant
- c) 0.1 to 20% Thickener
- d) 0.01 to 10% Binder
- e) 0.1% to 5% Sweetener
- f) 0 to 15% Foaming agent
- 30 g) 0 to 5% Whitener
- i) 0.0001% to 20% Enzymes.

Said enzymes referred to under i) include the recombinant mutanase of the invention, and optionally other types of enzymes mentioned above known to be used in toothpastes and the like.

### Mouth wash

A mouth wash produced from an oral care composition of the invention (in weight % of the final mouth wash composition) may typically comprise the following ingredients:

	0-20%	Humectant
	0-2%	Surfactant
	0-5%	Enzymes
	0-20%	Ethanol
10	0-2%	Other ingredients (e.g. flavour, sweetener active ingredients such as fluorides).
	0-70%	Water

The mouth wash composition may be buffered with an appropriate buffer e.g. sodium citrate or phosphate in the pH-range 6-7.5.

15 The mouth wash may be in none-diluted form (i.e. must be diluted before use).

### Method of Manufacture

The oral care composition and products of the present invention can be made using methods which are common in the oral product area.

According to the present invention the recombinant mutanase and/or the substantially purified mutanase free of active contaminants can be use in food, feed and/or pet food products.

25

## **MATERIALS AND METHODS**

### Materials

#### Micro-organisms

30 *Trichoderma harzianum* CBS 243.71

*A. oryzae* JaL 125: *Aspergillus oryzae* IFO 4177 available from Institute for Fermentation, Osaka; 17-25 Juso Hammachi 2-Chome Yodogawa-ku, Osaka, Japan, having the alkaline protease gene named "alp" (described by Murakami K et al., (1991), Agric. Biol. Chem. 55, p. 2807-2811) deleted by a one step gene replacement method (described by G. May in "Applied Molecular Genetics of Filamentous Fungi" (1992), p. 1-25. Eds. J. R. Kinghorn and G.

Turner; Blackie Academic and Professional), using the *A. oryzae* pyrG gene as marker.

*E. coli* DH5 $\alpha$

#### 5 Plasmids and Vectors:

pMT1796 (Figure 1 and Figure 2)

pMT1802 (Figure 2)

pMT1815 (Figure 2)

pHD414: *Aspergillus* expression vector is a derivative of the  
10 plasmid p775 (described in EP 238.023). The construction of the  
pHD414 is further described in WO 93/11249. pHD414 contains the  
*A. niger* glucoamylase terminator and the *A. oryzae* TAKA amylase  
promoter.

pHD414+mut (Figure 3)

15 pHan37 containing the TAKA:TPI promoter

#### Linkers:

Linker #1:

20 GATCCTCACCA ATG TTG GGC GTT GTC CGC CGT CTA GGC CTA GG  
GAGTGT TAC AAC CCG CAA CAG GCT GCA GAT CCG GAT CCG C  
Met Leu Gly Val Val Arg Arg Leu Gly Leu Gly

Linker #2:

25 C CAA TAC TGT TAG T  
GT ACG GTT ATG ACA ATC AGATC  
Ala Cys Gln Tyr Cys \*\*\*

#### Primers:

Primer 1: 5' GGGGGGATCCACCATGAG 3' (SEQ ID No. 3)

30 Primer 2: 5' ACGGTCAGCAGAAGAAGCTCGACGAATAGGACTGGC 3' (SEQ ID  
No. 4)

Primer 3: 5' GCCAGTCCTATTCGTGAGCTTCTTCTGCTGACCGT 3' (SEQ ID  
No. 5)

Primer 4: 5' CCACGGTCACCAACAATAC 3' (SEQ ID No. 6)

35 Primer 5: GGGGGGATCCACCATGAG (SEQ ID No. 7),

Primer 6: ACGGTCAGCAGAAGAAGCTCGACGAATAGGACTGGC (SEQ ID No. 8)

Primer 7: GCCAGTCCTATTCGTGAGCTTCTTCTGCTGACCGT (SEQ ID NO. 9),

Primer 8: CCACGGTCACCAACAATAC (SEQ ID No. 10).

Enzymes:

lysyl-specific protease from *Achromobacter*

*Trichoderma harzianum* CBS 243.71 fermentation broth (Batch no.  
5 PPM 3897)

Media, Substrates and Solutions:

YPM: 2% maltose, 1% bactopectone and 0.5% yeast extract)

DAPI: 4',6-diamidino-2-phenylindole (Sigma D-9542)

10 Britton-Robinson Buffer

BHI: Brain Heart Infusion broth

Equipment:

10 kDa cut-off ultra-filtration cassette (Alpha Miniset from  
15 Filtron).

Phenyl-sepharose FF (high sub) column (Pharmacia)

Seitz EK1 filter plate

Q-sepharose FF column (Pharmacia)

Applied Biosystems 473A protein sequencer

20 2 litre Kieler fermenter

Olympus model BX50 microscope

Malthus Flexi M2060 (Malthus Instrument Limited)

Methods:**25 Molecular biology procedures**

All molecular biology procedures including restriction  
digests, DNA ligations, *E. coli* transformations, DNA  
isolations, Southern hybridizations, PCR amplifications, and  
library constructions and screenings were completed using stan-  
30 dard techniques (Sambrook, J., Fritsch, E. F., and Maniatis, T.  
1989. *Molecular cloning: A laboratory manual*/E.F. Cold Spring  
Harbor Laboratory Press, Plainview, NY).

**Preparation of Mutan**

35 Mutan is prepared by growing *Streptococcus mutans* CBS 350.71  
at pH 6.5, 37°C (kept constant), and with an aeration rate of 75  
rpm in a medium comprised of the following components:

	NZ-Case	6.5 g/litre
	Yeast Extract	6 g/litre
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	20 g/litre
	K <sub>2</sub> PO <sub>4</sub>	3 g/litre
5	Glucose	50 g/litre
	Pluronic PE6100	0.1%

After 35 hours, sucrose is added to a final concentration of 60 g/litre to induce glucosyltransferase. The total fermentation time is 75 hours. The supernatant from the fermentation is  
10 centrifuged and filtered (sterile). Sucrose is then added to the supernatant to a final concentration of 5% (pH is adjusted to pH 7.0 with acetic acid) and the solution is stirred overnight at 37°C. The solution is filtered and the insoluble mutan is harvested on propex and washed extensively with deionized water  
15 containing 1% sodium benzoate, pH 5 (adjusted with acetic acid). Finally, the insoluble mutan is lyophilized and ground.

#### **Determination of mutanase activity (MU)**

One Mutanase Unit (MU) is the amount of enzyme which under  
20 standard conditions liberates 1 µmol reducing sugar (calculated as glucose) per minute. Reducing sugars were measured with alkaline K<sub>3</sub>Fe(CN)<sub>6</sub>.

##### Standard Conditions

Substrate.....1.5% mutan

25 Reaction time.....15 minutes

Temperature.....40°C

pH.....5.5

A detailed description of Novo Nordisk's analytical method (AF 180/1-GB) is available from Novo Nordisk A/S on request.

30

#### **Mutanase Plate Assay**

A 5% mutan suspension is made in 50 mM sodium acetate, pH 5.5 and the suspension is homogenised for 15 minutes in an Ultra Turrax T25 homogenizer at 4°C. 1% agarose in 50 mM sodium  
35 acetate, pH 5.5 is made 0.2% with respect to mutan and 12.5 ml agarose is casted in each petri dish (d=10 cm). The sample to be

analyzed for mutanase activity is applied in sample wells punched in the agarose, and the plate is incubated overnight at 37°C, whereafter clearing zones are formed around mutanase containing samples.

5

#### **Western hybridization**

Western hybridizations are performed using the ECL western blotting system (Amersham International, plc, Buckinghamshire, England) and a primary antibody solution containing polyclonal  
10 rabbit-anti-mutanase. The limit of detection is 0.001 MU/ml.

#### **Mass spectrometry**

Mass spectrometry of purified wild-type mutanase is done using matrix assisted laser desorption ionization time-of-flight mass  
15 spectrometry in a VG Analytical ToFSpec. For mass spectrometry 2 ml of sample is mixed with 2 ml saturated matrix solution (a-cyano-4-hydroxycinnamic acid in 0.1% TFA:acetonitrile (70:30)) and 2 ml of the mixture spotted onto the target plate. Before introduction into the mass spectrometer the solvent is removed by  
20 evaporation. Samples are desorbed and ionized by 4 ns laser pulses (337 nm) at threshold laser power and accelerated into the field-free flight tube by an accelerating voltage of 25 kV. Ions are detected by a microchannel plate set at 1850 V.

#### **25 Preparation of Hydroxyapatite disks (HA)**

Hydroxyapatite tablets are prepared by compressing 250 mg of hydroxyapatite in a tablet die at about 5,900 kg (13,000 lbs) of pressure for 5 minutes. The tablets are then sintered at 600°C for 4 hours and finally hydrated with sterile deionized water.

30

#### **Plaque coating of Hydroxyapatite disks (HA)**

Hydroxyapatite disks (HA) were dry sterilised (121°C, 2 bar, 20 minutes) and coated with filter sterilised saliva for 18 hours at 37°C. The HA disks were placed in a sterile rack in a beaker,  
35 Brain Heart Infusion broth (BHI) containing 0.2% sucrose was poured into the beaker covering the disks. Sterile Na<sub>2</sub>S (pH 7.0) was added immediately before inoculation given the final concen-

tration of 5 g/litre. A mixture 1:1:1 of *Streptococcus mutans*, *Actinomyces viscosus* and *Fusobacterium nucleatum* grown anaerobically (BHI, 37°C, 24 h) was used as inoculum in the concentration of approximately  $10^6$  cfu/ml. The disks were incubated  
5 anaerobic at 37°C for 4 days with slight stirring.

#### **Malthus-method for plaque**

The Malthus-method is based on the methods described in Johnston et al., (1995), Journal of Microbiological Methods 21,  
10 p. 15-26 and Johansen et al. (1995), Journal of Applied Bacteriology 78, p. 297-303.

#### **EXAMPLES**

##### **15 Example 1**

##### **Purification of wild-type Mutanase**

100 g fermentation broth of *Trichoderma harzianum* CBS 243.71 (Batch no. PPM 3897) were dissolved in 1 litre 10 mM sodium acetate, pH 5.2 overnight at 4°C.

20 65 g DEAE-Sephadex A-50 were swelled in 3 litre 10 mM sodium acetate, pH 5.2. Excess buffer was removed after swelling. DEAE-Sephadex was mixed with the crude mutanase preparation for 1 hour and unbound material was collected by filtration through Propex cloth. The gel was further washed with 2.5 l of 10 mM sodium acetate, pH 5.2. A pool containing the unbound material was made;  
25 volume 4 litre. Remaining DEAE-Sephadex particles were removed by filtration through a Whatman GF/F filter.

350 ml S-Sepharose was equilibrated in 10 mM sodium acetate, pH 5.2 and mixed with 600 ml of the pool from the DEAE-Sephadex  
30 for 10 minutes. Unbound material was collected by filtration through Propex cloth and the gel was washed with 500 ml 10 mM sodium acetate buffer, pH 5.2. Bound material was eluted with the same buffer containing 1 M NaCl. The procedure was repeated 7 times. The combined pool containing the unbound material (7  
35 litre) was concentrated on a Filtron concentrator equipped with a 10 kDa cut-off membrane and followed by a buffer change to 10 mM sodium acetate, pH 4.7. The concentrate was filtrated through a



Whatmann GF/F filter. The final volume of the concentrate was 600 ml.

An S-Sepharose column (180 ml, 2.6 x 33 cm) was equilibrated with 10 mM sodium acetate, pH 4.7. The pH adjusted concentrate  
5 from the S-Sepharose batch ion exchange was applied onto the column in 50 ml portions with a flow of 10 ml/min. The mutanase was eluted with a linear gradient from 0 to 20 mM NaCl in 3 column volumes. The residual protein was eluted with the same buffer containing 1 M NaCl. Fractions were analyzed for mutanase  
10 activity (plate assay) and fractions with high activity were pooled. The procedure was repeated 12 times. The combined mutanase pool was concentrated in a Filtron concentrator equipped with a 10 kDa cut-off membrane and followed by a buffer change to 10 mM Tris-HCl, pH 8.0. The final volume of the concentrate was  
15 870 ml.

The concentrated pool from the S-Sepharose column was further purified on a HiLoad Q-Sepharose column (50 ml, 2.6 x 10 cm) equilibrated with 10 mM Tris-HCl, pH 8.0. Portions of 130 ml was applied with a flow of 8 ml/min. Elution of the mutanase was per-  
20 formed with a linear gradient from 0 to 50 mM NaCl in 12 column volumes. Fractions with high mutanase activity (plate assay) were pooled, concentrated in an Amicon cell equipped with a 10 kDa cut-off membrane. Finally, the mutanase preparation was dialyzed extensively against 10 mM sodium phosphate, pH 7.0 and filtrated  
25 through a 0.45 mm filter.

The yield of the mutanase in the purification described above was 300 mg. The purity of the HiLoad-Q preparation was analyzed by SDS-PAGE and N-terminal sequencing and judged by both methods the purity was around 95%.

30

## **Example 2**

### **N-terminal sequencing of wild-type Mutanase**

N-terminal amino acid sequencing was carried out in an Applied Biosystems 473A protein sequencer.

35 To generate peptides reduced and S-carboxymethylated mutanase (> 450 mg) was digested with the lysyl-specific protease from *Achromobacter* (10 mg) in 20 mM  $\text{NH}_4\text{HCO}_3$  for 16 hours at 37°C. The

resulting peptides were separated by reversed phase HPLC using a Vydac C<sub>18</sub> column eluted with a linear gradient of 80% 2-propanol containing 0.08% TFA in 0.1% aqueous TFA. Peptides were repurified by reversed-phase-HPLC using a Vydac C<sub>18</sub> column eluted with linear gradients of 80% acetonitrile containing 0.08% TFA in 0.1% aqueous TFA before being subjected to N-terminal amino acid sequencing.

The amino acid sequences determined are given below.

N-terminal:

10 Ala-Ser-Ser-Ala-Asp-Arg-Leu-Val-Phe-Cys-His-Phe-Met-Ile-Gly-Ile-Val-Gly-Asp-Arg-Gly-Ser-Ser-Ala-Asp-Tyr-Asp-Asp-Asp-

Peptide 1:

Val Phe-Ile-Ser-Phe-Asp-Phe-Asn-Trp-Trp-Ser-Pro-Gly-Asn-Ala-Val-Gly-Val-Gly-Gln-Lys

15 Peptide 2:

Pro-Tyr-Leu-Ala-Pro-Val-Ser-Pro-Trp-Phe-Phe-Thr-His-Phe-Gly-Pro-Glu-Val-Ser-Tyr-Ser-

Peptide 3:

Trp-Val-Asn-Asp-Met-Pro-His-Asp-Gly-Phe-Leu-Asp-Leu-Ser-Lys

20

### Example 3

#### Construction of the mutanase expression vectors, pMT1796, pMT1802 and pMT1815

A cDNA clone encoding mutanase was identified in a *Trichoderma harzianum* CBS 243.71 library by hybridization with a fragment of the gene amplified by PCR using primers based on the mutanase sequence shown in SEQ ID NO. 1.

DNA sequence analysis of the isolated clone, pHD414+mut, showed that it indeed encoded the mutanase gene, and that the 5' end of the construct contained a long leader sequence. To remove this leader, pHD414+mut was restricted with the enzymes *EcoRI*, *NarI* and *XhoI*. From this digestion a 3499 nt (nucleotide) vector fragment and a 610 nt *NarI/XhoI* fragment were isolated. These two fragments were then ligated with linker #1 (see above) and a 618 nt *EcoRI/BamHI* fragment from pHan37 containing the TAKA:TPI promoter, giving plasmid pJW99.

HD414+mut was next digested with *XhoI* and *SphI*, and a 1790 nt

fragment encoding amino acids 35-598 of the mutanase gene was isolated.

This fragment was ligated with linker #2 (see above) and pJW99 that had been linearized with the restriction enzymes *Xba*I and *Xho*I. The resulting plasmid, pMT1802, contains the *T. harzianum* mutanase gene under the control of the TAKA:TPI promoter. Plasmid pMT1796 is identical to pMT1802 except that E36 of the mutanase protein has been changed to K36 by replacing the *Xho*I/*Kpn*I fragment of pMT1802 with a PCR amplified fragment containing the desired mutation.

This PCR fragment was created in a two step procedure as reported in Ho, et al. (1989), Gene, 77, p. 51-59, using the following primers:

Primer 1 (nt 2751 5'CAGCGTCCACATCACGAGC nt 2769) and  
Primer 2 (nt 3306 5'GAAGAAGCACGTTTCTCGAGAGACCG nt 3281);  
Primer 3 (nt 3281 5' CGGTCTCTGAGAAACGTGCTTCTTC nt 3306) and  
Primer 4 (nt 4266 5'GCCACTTCCGTTATTAGCC nt 4248); nucleotide numbers refer to the pMT1802 plasmid (See SEQ ID No. 11).

To create pMT1815, a 127 nt DNA fragment was PCR amplified using again a two step procedure and the primers:

Primer 5: GGGGGGATCCACCATGAG;  
Primer 6: ACGGTCAGCAGAAGAAGCTCGACGAATAGGACTGGC;  
Primer 7: GCCAGTCCTATTCGTCGAGCTTCTTCTGCTGACCGT;  
Primer 8: CCACGGTCACCAACAATAC,  
and the plasmids pHan37 and pMT1802 as templates in the first round of amplification.

This fragment contains a *Bam*HI restriction enzyme site followed by the Lipolase® prepro-sequence in frame with residues 38-54 of the mutanase protein and ending with a *Bst*EII site.

The fragment was digested with the restriction enzymes *Bst*EII and *Bam*HI and inserted into pMT1802 that had been linearized with the same pair of enzymes. Changes in constructs were confirmed and the integrity of the resulting coding regions were checked by nucleotide sequencing.

**Example 4****Expression of recombinant Mutanase in *Aspergillus oryzae***

The strain *A. oryzae* JaL125 was transformed using a PEG-mediated protocol (see EP 238 023) and a DNA mixture containing 0.5 µg of a plasmid encoding the gene that confers resistance to the herbicide Basta and 8.0 µg of one of the three mutanase expression plasmids. Transformants were selected on minimal plates containing 0.5% basta and 50 mM urea as a nitrogen source.

**Shake flask cultures**

Transformed colonies were spore purified twice on selection media and spores were harvested. A 20 ml universal container (Nunc, cat #364211) containing 10 ml YPM (2% maltose, 1% bactopectone and 0.5% yeast extract) was inoculated with spores and grown for 5 days with shaking at 30°C. The supernatant was harvested after 5 days growth.

Construct	highest mutanase level detected	number of transformants tested
pMT1802, mutanase prepro + mutanase	<0.001	10
pMT1796, mutanase prepro + KEX2 + mutanase	3.8	4
pMT1815, Lipolase® prepro + mutanase	0.16	22

Table 1 Comparison of mutanase expression from the three different expression constructs. The limit of detection was 0.001 MU/ml

The presence of mutanase in culture supernatants was examined by western hybridizations. SDS-PAGE and protein transfers were performed using standard protocols.

**Example 5****Purification of recombinant mutanase**

700 ml fermentation broth was filtered and concentrated. The pH was adjusted to 4.7 (conductivity around 300 µS/cm) and the broth was loaded onto an S-Sepharose column (XK 50/22) (Pharmacia) equilibrated in 10 mM sodium acetate pH 4.7. The

mutanase was eluted in a linear NaCl gradient. The major part of the mutanase appeared in the unbound fractions. These fractions were pooled and concentrated. Then the concentrate was loaded onto a HiLoad Q-Sepharose column (Pharmacia) 5 equilibrated in 10 mM Tris-HCl, pH 8.0 (around 600  $\mu$ S/cm). The mutanase was eluted in a linear gradient of NaCl and the mutanase containing-fractions were pooled according to purity and activity. The pooled fractions were concentrated and a fraction was further purified by gel filtration on a Superdex 75 10 (16/60) column (Pharmacia) in sodium acetate pH 6.0.

The purified mutanase has a specific activity around 19 MU pr. absorption unit at 280 nm. From SDS-PAGE (Novex 4-20 %; run according to the manufacturer's instructions) a molecular weight around 80 kDa is found.

15 The N-terminal amino acid sequence was confirmed to be identical to the N-terminal amino acid sequence of the wt mutanase (Ala-Ser-Ser-Ala-) (see Example 2)

### Example 6

#### 20 pH-profile of mutanase

500 ml 5 % mutan in 50 mM Britton-Robinson buffer at varying pH was added 2 ml enzyme sample (diluted in MilliQ-filtered water) in large vials (to ensure sufficient agitation) and incubated for 15 minutes at 40°C while shaking vigorously. The 25 reaction was terminated by adding 0.5 ml 0.4 M NaOH and the samples were filtered on Munktell filters. 100  $\mu$ l filtrate in Eppendorf vials were added 750  $\mu$ l ferricyanide reagent (0.4 g/l  $K_3Fe(CN)_6$ , 20 g/l  $Na_2CO_3$ ) and incubated 15 minutes at 85°C. After allowing the samples to cool, the decrease in absorption 30 at 420 nm was measured. A dilution series of glucose was included as a standard. Substrate and enzyme blanks were always included. Samples were run in duplicate. The pH-optimum for both wild-type and recombinant enzyme is around pH 3.5-5.5 (see Figure 4).

35

### Example 7

**Temperature profile of mutanase:**

500 ml 5 % mutan in 100 mM sodium acetate, pH 5.5 or in 100 mM sodium phosphate, pH 7 was added 2 ml enzyme sample (diluted in MilliQ-filtered water) in large vials (to ensure sufficient agitation) and incubated for 15 minutes at various temperatures while shaking vigorously. The reaction was terminated by adding 0.5 ml 0.4 M NaOH and the samples were filtered on Munktell filters. 100 µl filtrate in Eppendorf vials were added 750 µl ferricyanide reagent (0.4 g/l  $K_3Fe(CN)_6$ , 20 g/l  $Na_2CO_3$ ) and incubated 15 minutes at 85°C. After allowing the samples to cool, the drop in absorption at 420 nm was measured. A dilution series of glucose was included as a standard. Substrate and enzyme blanks were always included. Samples were run in duplicate. The temperature profiles for the recombinant and wt mutanase were identical. The temperature optimum at pH 7 was around 45 °C. The temperature optimum at pH 5.5 was above 55° (See Figure 5).

**Example 8****20 Temperature stability of mutanase:**

The temperature stability was investigated by pre-incubating enzyme samples for 30 minutes at various temperatures in 0.1 M sodium acetate, pH 5.5 or in 0.1 M sodium phosphate, pH 7 before assaying the residual activity. Both recombinant and wt mutanase have similar temperature stability profiles. The residual activity starts to decline at 40 °C at pH 7, while the enzyme is more stable at pH 5.5, where the residual activity starts to decline at 55°C (See Figure 6).

**30 Example 9****Molecular weight of purified wild-type Mutanase**

The mass spectrometry, performed as described above, of the mutanase revealed an average mass around 75 kDa. In addition, it was clear from the spectra that the glycosylation of the mutanase is heterogeneous. The peptide mass of the mutanase is more than 64 kDa meaning that more than 11 kDa of carbohydrate is attached

to the enzyme.

#### Example 10

##### Activity of mutanase against Dental Plaque

5 A plaque biofilm was grown anaerobic on saliva coated hydroxyapatite disks as described in the Material and Methods Section above. The plaque was a mixed culture of *Streptococcus mutans* (SFAG, CBS 350.71), *Actinomyces viscosus* (DSM 43329) and *Fusobacterium nucleatum* subsp. *polymorphum* (DSM 20482).

10 HA disks with plaque were transferred to acetate buffer (pH 5.5) containing recombinant *Trichoderma* mutanase 1 MU/ml and whirled for 2 minutes (sterile buffer was used as control).

After enzyme treatment, the disks were either DAPI stained or transferred to Malthus cells, as indirect impedance measurements  
15 were used when enumerating living adherent cells (Malthus Flexi M2060, Malthus Instrument Limited).

For the impedance measurements 3 ml of BHI were transferred to the outer chamber of the indirect Malthus cells, and 0.5 ml of sterile KOH (0.1 M) was transferred to the inner chamber. After  
20 mutanase treatment the disks with plaque were slightly rinsed with phosphate buffer and transferred to the outer chamber. The detection times (dt) in Malthus were converted to colony counts by use of a calibration curve relating cfu/ml to dt (Figure 7).

The calibration curve was constructed by a series of 10-fold  
25 dilution rate prepared from the mixed culture. Conductance dt of each dilution step was determined in BHI and a calibration curve relating cfu/ml of the 10 fold dilutions to dt in BHI was constructed for the mixed culture (Figure 7).

The removal of plaque from the disks was also determined by  
30 fluorescent microscopy, after mutanase treatment disks were stained with DAPI (3 mM) and incubated in the dark for 5 minutes (20°C). The DAPI stained cells were examined with the x.100 oil immersion fluorescence objective on an Olympus model BX50 microscope equipped with a 200 W mercury lamp and an UV- filter.  
35 The result was compared with the quantitative data obtained by the impedance measurements.

The number of living cells on the saliva treated HA-surface after enzyme treatment was determined by the Malthus method and shown in Table 1. However, by the Malthus method it is not possible to distinguish between a bactericidal activity of mutanase or an enzymatic removal of the plaque. Therefore a decrease in living bacteria on the surface has to be compared with the simultaneously removal of plaque from the surface which is estimated by the DAPI staining.

Mutanase (MU/ml)	Log <sub>10</sub> reduction (cfu/cm <sup>2</sup> )	Removal of plaque (%)	No. of observations
0	0	0	10
1	1.4	96	6

10 Table 2: Enzymatic plaque removal (pH 5.5, 2 minutes) from saliva treated hydroxyapatite determined by impedance measurements.

A significant removal of plaque was determined by fluorescent microscopy after treatment with mutanase. Thus mutanase reduced the amount of adhering cells. However, the activity was observed as a removal of plaque and not as a bactericidal activity against cells in plaque.



## 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-2880
- (G) TELEPHONE: +45 4444 8888
- (H) TELEFAX: +45 4449 3256

(ii) TITLE OF INVENTION: A recombinant enzyme with mutanase activity

(iii) NUMBER OF SEQUENCES: 11

## (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 #1.30 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1905 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (vi) ORIGINAL SOURCE:

- (B) STRAIN: *Trichoderma harzianum* CBS 243.71

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1905
- (A) NAME/KEY: sig\_peptide
- (B) LOCATION: 1..120

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

ATG TTG GGC GTT GTC CGC CGT CTA GGC CTA GGC GCC CTT GCT GCC GCA	48
Met Leu Gly Val Val Arg Arg Leu Gly Leu Gly Ala Leu Ala Ala Ala	
1 5 10 15	
GCT CTG TCT TCT CTC GGC AGT GCC GCT CCC GCC AAT GTT GCT ATT CGG	96
Ala Leu Ser Ser Leu Gly Ser Ala Ala Pro Ala Asn Val Ala Ile Arg	
20 25 30	
TCT CTC GAG GAA CGT GCT TCT TCT GCT GAC CGT CTC GTA TTC TGT CAC	144
Ser Leu Glu Glu Arg Ala Ser Ser Ala Asp Arg Leu Val Phe Cys His	
35 40 45	
TTC ATG ATT GGT ATT GTT GGT GAC CGT GGC AGC TCA GCA GAC TAT GAT	192
Phe Met Ile Gly Ile Val Gly Asp Arg Gly Ser Ser Ala Asp Tyr Asp	
50 55 60	
GAT GAC ATG CAA CGT GCC AAA GCC GCT GGC ATT GAC GCA TTC GCT CTG	240
Asp Asp Met Gln Arg Ala Lys Ala Ala Gly Ile Asp Ala Phe Ala Leu	
65 70 75 80	
AAC ATC GGC GTT GAC GGC TAT ACC GAC CAG CAA CTC GGG TAT GCC TAT	288
Asn Ile Gly Val Asp Gly Tyr Thr Asp Gln Gln Leu Gly Tyr Ala Tyr	
85 90 95	
GAC TCT GCC GAC CGT AAT GGC ATG AAA GTC TTC ATT TCA TTC GAT TTC	336
Asp Ser Ala Asp Arg Asn Gly Met Lys Val Phe Ile Ser Phe Asp Phe	
100 105 110	
AAC TGG TGG AGC CCC GGT AAT GCA GTT GGT GTT GGC CAG AAG ATT GCG	384
Asn Trp Trp Ser Pro Gly Asn Ala Val Gly Val Gly Gln Lys Ile Ala	
115 120 125	

CAG TAT GCC AGC CGT CCC GCC CAG CTG TAT GTT GAC AAC CGG CCA TTC Gln Tyr Ala Ser Arg Pro Ala Gln Leu Tyr Val Asp Asn Arg Pro Phe 130 135 140	432
GCC TCT TCC TTC GCT GGT GAC GGT TTG GAT GTA AAT GCG TTG CGC TCT Ala Ser Ser Phe Ala Gly Asp Gly Leu Asp Val Asn Ala Leu Arg Ser 145 150 155 160	480
GCT GCA GGC TCC AAC GTT TAC TTT GTG CCC AAC TTC CAC CCT GGT CAA Ala Ala Gly Ser Asn Val Tyr Phe Val Pro Asn Phe His Pro Gly Gln 165 170 175	528
TCT TCC CCC TCC AAC ATT GAT GGC GCC CTC AAC TGG ATG GCC TGG GAT Ser Ser Pro Ser Asn Ile Asp Gly Ala Leu Asn Trp Met Ala Trp Asp 180 185 190	576
AAT GAT GGA AAC AAC AAG GCA CCC AAG CCG GGC CAG ACT GTC ACG GTG Asn Asp Gly Asn Asn Lys Ala Pro Lys Pro Gly Gln Thr Val Thr Val 195 200 205	624
GCA GAC GGT GAC AAC GCT TAC AAG AAT TGG TTG GGT GGC AAG CCT TAC Ala Asp Gly Asp Asn Ala Tyr Lys Asn Trp Leu Gly Gly Lys Pro Tyr 210 215 220	672
CTA GCG CCT GTC TCC CCT TGG TTT TTC ACC CAT TTT GGC CCT GAA GTT Leu Ala Pro Val Ser Pro Trp Phe Phe Thr His Phe Gly Pro Glu Val 225 230 235 240	720
TCA TAT TCC AAG AAC TGG GTC TTC CCA GGT GGT CCT CTG ATC TAT AAC Ser Tyr Ser Lys Asn Trp Val Phe Pro Gly Gly Pro Leu Ile Tyr Asn 245 250 255	768
CGG TGG CAA CAG GTC TTG CAG CAG GGC TTC CCC ATG GTT GAG ATT GTT Arg Trp Gln Gln Val Leu Gln Gln Gly Phe Pro Met Val Glu Ile Val 260 265 270	816
ACC TGG AAT GAC TAC GGC GAG TCT CAC TAC GTC GGT CCT CTG AAG TCT Thr Trp Asn Asp Tyr Gly Glu Ser His Tyr Val Gly Pro Leu Lys Ser 275 280 285	864
AAG CAT TTC GAT GAT GGC AAC TCC AAA TGG GTC AAT GAT ATG CCC CAT Lys His Phe Asp Asp Gly Asn Ser Lys Trp Val Asn Asp Met Pro His 290 295 300	912
GAT GGA TTC TTG GAT CTT TCA AAG CCG TTT ATT GCT GCA TAT AAG AAC Asp Gly Phe Leu Asp Leu Ser Lys Pro Phe Ile Ala Tyr Lys Asn 305 310 315 320	960
AGG GAT ACT GAT ATA TCT AAG TAT GTT CAA AAT GAG CAG CTT GTT TAC Arg Asp Thr Asp Ile Ser Lys Tyr Val Gln Asn Glu Gln Leu Val Tyr 325 330 335	1008
TGG TAC CGC CGC AAC TTG AAG GCA TTG GAC TGC GAC GCC ACC GAC ACC Trp Tyr Arg Arg Asn Leu Lys Ala Leu Asp Cys Asp Ala Thr Asp Thr 340 345 350	1056
ACC TCT AAC CGC CCG GCT AAT AAC GGA AGT GGC AAT TAC TTT ATG GGA Thr Ser Asn Arg Pro Ala Asn Asn Gly Ser Gly Asn Tyr Phe Met Gly 355 360 365	1104
CGC CCT GAT GGT TGG CAA ACT ATG GAT GAT ACC GTT TAT GTT GCC GCA Arg Pro Asp Gly Trp Gln Thr Met Asp Asp Thr Val Tyr Val Ala Ala 370 375 380	1152
CTT CTC AAG ACC GCC GGT AGC GTC ACG GTC ACG TCT GGC GGC ACC ACT Leu Leu Lys Thr Ala Gly Ser Val Thr Val Thr Ser Gly Gly Thr Thr 1200	1200

385					390						395				400	
CAA	ACG	TTC	CAG	GCC	AAC	GCC	GGA	GCC	AAC	CTC	TTC	CAA	ATC	CCT	GCC	1248
Gln	Thr	Phe	Gln	Ala	Asn	Ala	Gly	Ala	Asn	Leu	Phe	Gln	Ile	Pro	Ala	
				405					410					415		
AGC	ATC	GGC	CAG	CAA	AAG	TTT	GCT	CTA	ACT	CGC	AAC	GGT	CAG	ACC	GTC	1296
Ser	Ile	Gly	Gln	Gln	Lys	Phe	Ala	Leu	Thr	Arg	Asn	Gly	Gln	Thr	Val	
			420					425					430			
TTT	AGC	GGA	ACC	TCA	TTG	ATG	GAT	ATC	ACC	AAC	GTT	TGC	TCT	TGC	GGT	1344
Phe	Ser	Gly	Thr	Ser	Leu	Met	Asp	Ile	Thr	Asn	Val	Cys	Ser	Cys	Gly	
		435					440					445				
ATC	TAC	AAT	TTC	AAC	CCA	TAT	GTT	GGC	ACC	ATT	CCT	GCC	GGC	TTT	GAC	1392
Ile	Tyr	Asn	Phe	Asn	Pro	Tyr	Val	Gly	Thr	Ile	Pro	Ala	Gly	Phe	Asp	
	450					455					460					
GAC	CCT	CTT	CAG	GCT	GAC	GGT	CTT	TTC	TCT	TTG	ACC	ATC	GGA	TTG	CAT	1440
Asp	Pro	Leu	Gln	Ala	Asp	Gly	Leu	Phe	Ser	Leu	Thr	Ile	Gly	Leu	His	
465					470				475						480	
GTC	ACG	ACT	TGT	CAG	GCC	AAG	CCA	TCT	CTT	GGA	ACC	AAC	CCT	CCT	GTC	1488
Val	Thr	Thr	Cys	Gln	Ala	Lys	Pro	Ser	Leu	Gly	Thr	Asn	Pro	Pro	Val	
				485					490					495		
ACT	TCT	GGC	CCT	GTG	TCC	TCG	CTG	CCA	GCT	TCC	TCC	ACC	ACC	CGC	GCA	1536
Thr	Ser	Gly	Pro	Val	Ser	Ser	Leu	Pro	Ala	Ser	Ser	Thr	Thr	Arg	Ala	
			500					505					510			
TCC	TCG	CCT	CCT	GTT	TCT	TCA	ACT	CGT	GTC	TCT	TCT	CCC	CCT	GTC	TCT	1584
Ser	Ser	Pro	Pro	Val	Ser	Ser	Thr	Arg	Val	Ser	Ser	Pro	Pro	Val	Ser	
		515					520					525				
TCC	CCT	CCA	GTT	TCT	CGC	ACC	TCT	TCT	CCC	CCT	CCC	CCT	CCG	GCC	AGC	1632
Ser	Pro	Pro	Val	Ser	Arg	Thr	Ser	Ser	Pro	Pro	Pro	Pro	Pro	Ala	Ser	
	530					535					540					
AGC	ACG	CCG	CCA	TCG	GGT	CAG	GTT	TGC	GTT	GCC	GGC	ACC	GTT	GCT	GAC	1680
Ser	Thr	Pro	Pro	Ser	Gly	Gln	Val	Cys	Val	Ala	Gly	Thr	Val	Ala	Asp	
545					550				555						560	
GGC	GAG	TCC	GGC	AAC	TAC	ATC	GGC	CTG	TGC	CAA	TTC	AGC	TGC	AAC	TAC	1728
Gly	Glu	Ser	Gly	Asn	Tyr	Ile	Gly	Leu	Cys	Gln	Phe	Ser	Cys	Asn	Tyr	
				565				570						575		
GGT	TAC	TGT	CCA	CCG	GGA	CCG	TGT	AAG	TGC	ACC	GCC	TTT	GGT	GCT	CCC	1776
Gly	Tyr	Cys	Pro	Pro	Gly	Pro	Cys	Lys	Cys	Thr	Ala	Phe	Gly	Ala	Pro	
			580					585					590			
ATC	TCG	CCA	CCG	GCA	AGC	AAT	GGG	CGC	AAC	GGC	TGC	CCT	CTA	CCG	GGA	1824
Ile	Ser	Pro	Pro	Ala	Ser	Asn	Gly	Arg	Asn	Gly	Cys	Pro	Leu	Pro	Gly	
		595					600					605				
GAA	GGC	GAT	GGT	TAT	CTG	GGC	CTG	TGC	AGT	TTC	AGT	TGT	AAC	CAT	AAT	1872
Glu	Gly	Asp	Gly	Tyr	Leu	Gly	Leu	Cys	Ser	Phe	Ser	Cys	Asn	His	Asn	
	610					615					620					
TAC	TGC	CCG	CCA	ACG	GCA	TGC	CAA	TAC	TGT	TAG						1905
Tyr	Cys	Pro	Pro	Thr	Ala	Cys	Gln	Tyr	Cys	*						
625					630					635						

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 635 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Leu Gly Val Val Arg Arg Leu Gly Leu Gly Ala Leu Ala Ala Ala  
 1 5 10 15  
 Ala Leu Ser Ser Leu Gly Ser Ala Ala Pro Ala Asn Val Ala Ile Arg  
 20 25 30  
 Ser Leu Glu Glu Arg Ala Ser Ser Ala Asp Arg Leu Val Phe Cys His  
 35 40 45  
 Phe Met Ile Gly Ile Val Gly Asp Arg Gly Ser Ser Ala Asp Tyr Asp  
 50 55 60  
 Asp Asp Met Gln Arg Ala Lys Ala Ala Gly Ile Asp Ala Phe Ala Leu  
 65 70 75 80  
 Asn Ile Gly Val Asp Gly Tyr Thr Asp Gln Gln Leu Gly Tyr Ala Tyr  
 85 90 95  
 Asp Ser Ala Asp Arg Asn Gly Met Lys Val Phe Ile Ser Phe Asp Phe  
 100 105 110  
 Asn Trp Trp Ser Pro Gly Asn Ala Val Gly Val Gly Gln Lys Ile Ala  
 115 120 125  
 Gln Tyr Ala Ser Arg Pro Ala Gln Leu Tyr Val Asp Asn Arg Pro Phe  
 130 135 140  
 Ala Ser Ser Phe Ala Gly Asp Gly Leu Asp Val Asn Ala Leu Arg Ser  
 145 150 155 160  
 Ala Ala Gly Ser Asn Val Tyr Phe Val Pro Asn Phe His Pro Gly Gln  
 165 170 175  
 Ser Ser Pro Ser Asn Ile Asp Gly Ala Leu Asn Trp Met Ala Trp Asp  
 180 185 190  
 Asn Asp Gly Asn Asn Lys Ala Pro Lys Pro Gly Gln Thr Val Thr Val  
 195 200 205  
 Ala Asp Gly Asp Asn Ala Tyr Lys Asn Trp Leu Gly Gly Lys Pro Tyr  
 210 215 220  
 Leu Ala Pro Val Ser Pro Trp Phe Phe Thr His Phe Gly Pro Glu Val  
 225 230 235 240  
 Ser Tyr Ser Lys Asn Trp Val Phe Pro Gly Gly Pro Leu Ile Tyr Asn  
 245 250 255  
 Arg Trp Gln Gln Val Leu Gln Gln Gly Phe Pro Met Val Glu Ile Val  
 260 265 270  
 Thr Trp Asn Asp Tyr Gly Glu Ser His Tyr Val Gly Pro Leu Lys Ser  
 275 280 285  
 Lys His Phe Asp Asp Gly Asn Ser Lys Trp Val Asn Asp Met Pro His  
 290 295 300  
 Asp Gly Phe Leu Asp Leu Ser Lys Pro Phe Ile Ala Ala Tyr Lys Asn  
 305 310 315 320  
 Arg Asp Thr Asp Ile Ser Lys Tyr Val Gln Asn Glu Gln Leu Val Tyr  
 325 330 335  
 Trp Tyr Arg Arg Asn Leu Lys Ala Leu Asp Cys Asp Ala Thr Asp Thr

340					345					350					
Thr	Ser	Asn	Arg	Pro	Ala	Asn	Asn	Gly	Ser	Gly	Asn	Tyr	Phe	Met	Gly
		355					360					365			
Arg	Pro	Asp	Gly	Trp	Gln	Thr	Met	Asp	Asp	Thr	Val	Tyr	Val	Ala	Ala
	370					375					380				
Leu	Leu	Lys	Thr	Ala	Gly	Ser	Val	Thr	Val	Thr	Ser	Gly	Gly	Thr	Thr
385						390					395				400
Gln	Thr	Phe	Gln	Ala	Asn	Ala	Gly	Ala	Asn	Leu	Phe	Gln	Ile	Pro	Ala
				405					410					415	
Ser	Ile	Gly	Gln	Gln	Lys	Phe	Ala	Leu	Thr	Arg	Asn	Gly	Gln	Thr	Val
			420					425					430		
Phe	Ser	Gly	Thr	Ser	Leu	Met	Asp	Ile	Thr	Asn	Val	Cys	Ser	Cys	Gly
		435					440					445			
Ile	Tyr	Asn	Phe	Asn	Pro	Tyr	Val	Gly	Thr	Ile	Pro	Ala	Gly	Phe	Asp
	450					455					460				
Asp	Pro	Leu	Gln	Ala	Asp	Gly	Leu	Phe	Ser	Leu	Thr	Ile	Gly	Leu	His
465						470					475				480
Val	Thr	Thr	Cys	Gln	Ala	Lys	Pro	Ser	Leu	Gly	Thr	Asn	Pro	Pro	Val
				485					490					495	
Thr	Ser	Gly	Pro	Val	Ser	Ser	Leu	Pro	Ala	Ser	Ser	Thr	Thr	Arg	Ala
			500					505						510	
Ser	Ser	Pro	Pro	Val	Ser	Ser	Thr	Arg	Val	Ser	Ser	Pro	Pro	Val	Ser
		515					520					525			
Ser	Pro	Pro	Val	Ser	Arg	Thr	Ser	Ser	Pro	Pro	Pro	Pro	Pro	Ala	Ser
	530					535					540				
Ser	Thr	Pro	Pro	Ser	Gly	Gln	Val	Cys	Val	Ala	Gly	Thr	Val	Ala	Asp
545						550					555				560
Gly	Glu	Ser	Gly	Asn	Tyr	Ile	Gly	Leu	Cys	Gln	Phe	Ser	Cys	Asn	Tyr
				565					570					575	
Gly	Tyr	Cys	Pro	Pro	Gly	Pro	Cys	Lys	Cys	Thr	Ala	Phe	Gly	Ala	Pro
				580					585					590	
Ile	Ser	Pro	Pro	Ala	Ser	Asn	Gly	Arg	Asn	Gly	Cys	Pro	Leu	Pro	Gly
				595					600					605	
Glu	Gly	Asp	Gly	Tyr	Leu	Gly	Leu	Cys	Ser	Phe	Ser	Cys	Asn	His	Asn
				610					615					620	
Tyr	Cys	Pro	Pro	Thr	Ala	Cys	Gln	Tyr	Cys	*					
625						630			635						

## (2) INFORMATION FOR SEQ ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Primer 1"

CAGCGTCCAC ATCACGAGC

19

- (2) INFORMATION FOR SEQ ID NO: 4:
- (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 26 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid
    - (A) DESCRIPTION: /desc = "Primer 2"

GAAGAAGCAC GTTCTGCAG AGACCG

26

- (2) INFORMATION FOR SEQ ID NO: 5:
- (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 26 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid
    - (A) DESCRIPTION: /desc = "Primer 3"

CGGTCTCTCG AGAAACGTGC TTCTTC

26

- (2) INFORMATION FOR SEQ ID NO: 6:
- (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 19 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid
    - (A) DESCRIPTION: /desc = "Primer 4"

GCCACTTCCG TTATTAGCC

19

- (2) INFORMATION FOR SEQ ID NO: 7:
- (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 18 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid
    - (A) DESCRIPTION: /desc = "Primer 5"

GGGGGGATCC ACCATGAG

18

- (2) INFORMATION FOR SEQ ID NO: 8:
- (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 36 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE: other nucleic acid
    - (A) DESCRIPTION: /desc = "Primer 6"

ACGGTCAGCA GAAGAAGCTC GACGAATAGG ACTGGC

36

- (2) INFORMATION FOR SEQ ID NO: 9:
- (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 36 base pairs
    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single

- (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "Primer 7"

GCCAGTCCTA TTCGTCGAGC TTCTTCTGCT GACCGT

36

- (2) INFORMATION FOR SEQ ID NO: 10:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: other nucleic acid  
(A) DESCRIPTION: /desc = "Primer 8"

CCACGGTCAC CAACAATAC

19

- (2) INFORMATION FOR SEQ ID NO: 11:  
(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 6032 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: DNA (genomic)  
(vi) ORIGINAL SOURCE:  
(B) STRAIN: *Trichoderma harzianum* CBS 243.71  
(ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: 3188..5092  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GACGAAAGGG	CCTCGTGATA	CGCCTATTTT	TATAGGTTAA	TGTCATGATA	ATAATGGTTT	60
CTTAGACGTC	AGGTGGCACT	TTTCGGGGAA	ATGTGCGCGG	AACCCCTATT	TGTTTATTTT	120
TCTAAATACA	TTCAAATATG	TATCCGCTCA	TGAGACAATA	ACCCTGATAA	ATGCTTCAAT	180
AATATTGAAA	AAGGAAGAGT	ATGAGTATTC	AACATTTCCG	TGTCGCCCTT	ATTCCCTTTT	240
TTGCGGCATT	TTGCCTTCCT	GTTTTTGCTC	ACCCAGAAAC	GCTGGTGAAA	GTAAAAGATG	300
CTGAAGATCA	GTTGGGTGCA	CGAGTGGGTT	ACATCGAACT	GGATCTCAAC	AGCGGTAAAG	360
TCCTTGAGAG	TTTTCGCCCC	GAAGAACGTT	TTCCAATGAT	GAGCACTTTT	AAAGTTCTGC	420
TATGTGGCGC	GGTATTATCC	CGTATTGACG	CCGGGCAAGA	GCAACTCGGT	CGCCGCATAC	480
ACTATTCTCA	GAATGACTTG	GTTGAGTACT	CACCAGTCAC	AGAAAAGCAT	CTTACGGATG	540
GCATGACAGT	AAGAGAATTA	TGCAGTGCTG	CCATAACCAT	GAGTGATAAC	ACTGCGGCCA	600
ACTTACTTCT	GACAACGATC	GGAGGACCGA	AGGAGCTAAC	CGCTTTTTTG	CACAACATGG	660
GGGATCATGT	AACTCGCCTT	GATCGTTGGG	AACCGGAGCT	GAATGAAGCC	ATACCAAACG	720
ACGAGCGTGA	CACCACGATG	CCTGTAGCAA	TGGCAACAAC	GTTGCGCAAA	CTATTTAACTG	780
GCGAACTACT	TACTCTAGCT	TCCCGGCAAC	AATTAATAGA	CTGGATGGAG	GCGGATAAAG	840
TTGCAGGACC	ACTTCTGCGC	TCGGCCCTTC	CGGCTGGCTG	GTTTATTGCT	GATAAATCTG	900
GAGCCGGTGA	GCGTGGGTCT	CGCGGTATCA	TTGCAGCACT	GGGGCCAGAT	GGTAAGCCCT	960
CCCGTATCGT	AGTTATCTAC	ACGACGGGGA	GTCAGGCAAC	TATGGATGAA	CGAAATAGAC	1020
AGATCGCTGA	GATAGGTGCC	TCACTGATTA	AGCATTGGTA	ACTGTCAGAC	CAAGTTTACT	1080
CATATATACT	TTAGATTGAT	TTAAAACTTC	ATTTTTAATT	TAAAAGGATC	TAGGTGAAGA	1140
TCCTTTTTGA	TAATCTCATG	ACCAAAATCC	CTTAACGTGA	GTTTTTCGTT	CACTGAGCGT	1200
CAGACCCCGT	AGAAAAGATC	AAAGGATCTT	CTTGAGATCC	TTTTTTTCTG	CGCGTAATCT	1260
GCTGCTTGCA	AACAAAAAAA	CCACCGCTAC	CAGCGGTGGT	TTGTTTGCCG	GATCAAGAGC	1320
TACCAACTCT	TTTTCCGAAG	GTAACCTGGT	TCAGCAGAGC	GCAGATACCA	AATACTGTCC	1380
TTCTAGTGTA	GCCGTAGTTA	GGCCACCACT	TCAAGAACTC	TGTAGCACCG	CCTACATACC	1440
TCGCTCTGCT	AATCCTGTGA	CCAGTGGCTG	CTGCCAGTGG	CGATAAGTCG	TGTCTTACCG	1500
GGTTGGACTC	AAGACGATAG	TTACCGGATA	AGGCGCAGCG	GTCGGGCTGA	ACGGGGGGTT	1560
CGTGACACAC	GCCCAGCTTG	GAGCGAACGA	CCTACACCGA	ACTGAGATAC	CTACAGCGTG	1620
AGCATTGAGA	AAGCGCCACG	CTTCCCGAAG	GGAGAAAGGC	GGACAGGTAT	CCGGTAAGCG	1680





CCT	TAC	CTA	GCG	CCT	GTC	TCC	CCT	TGG	TTT	TTC	ACC	CAT	TTT	GGC	CCT	3901
Pro	Tyr	Leu	Ala	Pro	Val	Ser	Pro	Trp	Phe	Phe	Thr	His	Phe	Gly	Pro	
		225					230					235				
GAA	GTT	TCA	TAT	TCC	AAG	AAC	TGG	GTC	TTC	CCA	GGT	GGT	CCT	CTG	ATC	3949
Glu	Val	Ser	Tyr	Ser	Lys	Asn	Trp	Val	Phe	Pro	Gly	Gly	Pro	Leu	Ile	
	240					245					250					
TAT	AAC	CGG	TGG	CAA	CAG	GTC	TTG	CAG	CAG	GGC	TTC	CCC	ATG	GTT	GAG	3997
Tyr	Asn	Arg	Trp	Gln	Gln	Val	Leu	Gln	Gln	Gly	Phe	Pro	Met	Val	Glu	
	255				260					265				270		
ATT	GTT	ACC	TGG	AAT	GAC	TAC	GGC	GAG	TCT	CAC	TAC	GTC	GGT	CCT	CTG	4045
Ile	Val	Thr	Trp	Asn	Asp	Tyr	Gly	Glu	Ser	His	Tyr	Val	Gly	Pro	Leu	
				275				280						285		
AAG	TCT	AAG	CAT	TTC	GAT	GAT	GGC	AAC	TCC	AAA	TGG	GTC	AAT	GAT	ATG	4093
Lys	Ser	Lys	His	Phe	Asp	Asp	Gly	Asn	Ser	Lys	Trp	Val	Asn	Asp	Met	
			290				295						300			
CCC	CAT	GAT	GGA	TTC	TTG	GAT	CTT	TCA	AAG	CCG	TTT	ATT	GCT	GCA	TAT	4141
Pro	His	Asp	Gly	Phe	Leu	Asp	Leu	Ser	Lys	Pro	Phe	Ile	Ala	Ala	Tyr	
			305				310					315				
AAG	AAC	AGG	GAT	ACT	GAT	ATA	TCT	AAG	TAT	GTT	CAA	AAT	GAG	CAG	CTT	4189
Lys	Asn	Arg	Asp	Thr	Asp	Ile	Ser	Lys	Tyr	Val	Gln	Asn	Glu	Gln	Leu	
	320					325					330					
GTT	TAC	TGG	TAC	CGC	CGC	AAC	TTG	AAG	GCA	TTG	GAC	TGC	GAC	GCC	ACC	4237
Val	Tyr	Trp	Tyr	Arg	Arg	Asn	Leu	Lys	Ala	Leu	Asp	Cys	Asp	Ala	Thr	
	335				340				345					350		
GAC	ACC	ACC	TCT	AAC	CGC	CCG	GCT	AAT	AAC	GGA	AGT	GGC	AAT	TAC	TTT	4285
Asp	Thr	Thr	Ser	Asn	Arg	Pro	Ala	Asn	Asn	Gly	Ser	Gly	Asn	Tyr	Phe	
				355				360						365		
ATG	GGA	CGC	CCT	GAT	GGT	TGG	CAA	ACT	ATG	GAT	GAT	ACC	GTT	TAT	GTT	4333
Met	Gly	Arg	Pro	Asp	Gly	Trp	Gln	Thr	Met	Asp	Asp	Thr	Val	Tyr	Val	
			370				375						380			
GCC	GCA	CTT	CTC	AAG	ACC	GCC	GGT	AGC	GTC	ACG	GTC	ACG	TCT	GGC	GGC	4381
Ala	Ala	Leu	Leu	Lys	Thr	Ala	Gly	Ser	Val	Thr	Val	Thr	Ser	Gly	Gly	
		385					390					395				
ACC	ACT	CAA	ACG	TTC	CAG	GCC	AAC	GCC	GGA	GCC	AAC	CTC	TTC	CAA	ATC	4429
Thr	Thr	Gln	Thr	Phe	Gln	Ala	Asn	Ala	Gly	Ala	Asn	Leu	Phe	Gln	Ile	
	400					405					410					
CCT	GCC	AGC	ATC	GGC	CAG	CAA	AAG	TTT	GCT	CTA	ACT	CGC	AAC	GGT	CAG	4477
Pro	Ala	Ser	Ile	Gly	Gln	Gln	Lys	Phe	Ala	Leu	Thr	Arg	Asn	Gly	Gln	
	415			420					425					430		
ACC	GTC	TTT	AGC	GGA	ACC	TCA	TTG	ATG	GAT	ATC	ACC	AAC	GTT	TGC	TCT	4525
Thr	Val	Phe	Ser	Gly	Thr	Ser	Leu	Met	Asp	Ile	Thr	Asn	Val	Cys	Ser	
				435				440						445		
TGC	GGT	ATC	TAC	AAT	TTC	AAC	CCA	TAT	GTT	GGC	ACC	ATT	CCT	GCC	GGC	4573
Cys	Gly	Ile	Tyr	Asn	Phe	Asn	Pro	Tyr	Val	Gly	Thr	Ile	Pro	Ala	Gly	
			450					455					460			
TTT	GAC	GAC	CCT	CTT	CAG	GCT	GAC	GGT	CTT	TTC	TCT	TTG	ACC	ATC	GGA	4621
Phe	Asp	Asp	Pro	Leu	Gln	Ala	Asp	Gly	Leu	Phe	Ser	Leu	Thr	Ile	Gly	
			465				470					475				
TTG	CAT	GTC	ACG	ACT	TGT	CAG	GCC	AAG	CCA	TCT	CTT	GGA	ACC	AAC	CCT	4669
Leu	His	Val	Thr	Thr	Cys	Gln	Ala	Lys	Pro	Ser	Leu	Gly	Thr	Asn	Pro	
	480					485					490					
CCT	GTC	ACT	TCT	GGC	CCT	GTG	TCC	TCG	CTG	CCA	GCT	TCC	TCC	ACC	ACC	4717
Pro	Val	Thr	Ser	Gly	Pro	Val	Ser	Ser	Leu	Pro	Ala	Ser	Ser	Thr	Thr	
	495			500					505					510		
CGC	GCA	TCC	TCG	CCT	CCT	GTT	TCT	TCA	ACT	CGT	GTC	TCT	TCT	CCC	CCT	4765
Arg	Ala	Ser	Ser	Pro	Pro	Val	Ser	Ser	Thr	Arg	Val	Ser	Ser	Pro	Pro	
				515				520						525		
GTC	TCT	TCC	CCT	CCA	GTT	TCT	CGC	ACC	TCT	TCT	CCC	CCT	CCC	CCT	CCG	4813
Val	Ser	Ser	Pro	Pro	Val	Ser	Arg	Thr	Ser	Ser	Pro	Pro	Pro	Pro	Pro	
			530				535					540				
GCC	AGC	AGC	ACG	CCG	CCA	TCG	GGT	CAG	GTT	TGC	GTT	GCC	GGC	ACC	GTT	4861
Ala	Ser	Ser	Thr	Pro	Pro	Ser	Gly	Gln	Val	Cys	Val	Ala	Gly	Thr	Val	
			545				550					555				
GCT	GAC	GGC	GAG	TCC	GGC	AAC	TAC	ATC	GGC	CTG	TGC	CAA	TTC	AGC	TGC	4909
Ala	Asp	Gly	Glu	Ser	Gly	Asn	Tyr	Ile	Gly	Leu	Cys	Gln	Phe	Ser	Cys	
	560					565						570				
AAC	TAC	GGT	TAC	TGT	CCA	CCG	GGA	CCG	TGT	AAG	TGC	ACC	GCC	TTT	GGT	4957

[illegible]

**PATENT CLAIMS**

1. A method for constructing an expression vector comprising a mutanase gene obtained from a filamentous fungus suitable for heterologous production comprising the steps of:
  - 5 a) isolating a DNA sequence encoding a mutanase from a filamentous fungus,
  - b) introducing a kex2 site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the mutanase, or replacing the mutanase (pre)pro-sequence with a  
10 (pre)pro-sequence comprising a kex2 or kex2-like site of another fungal enzyme,
  - c) cloning the DNA sequence obtained in step b) into a suitable expression vector.
- 15 2. The method according to claim 1, wherein the mutanase is obtained from the genus *Trichoderma*, preferably a strain of the species *T. harzianum*, especially the strain *T. harzianum* CBS 243.71.
- 20 3. The method according to claim 2, in which the mutanase DNA sequence is isolated from or produced on the basis of a nucleic acid library of *Trichoderma harzianum* CBS 243.71.
4. The method according to any of claims 1 to 3, wherein the  
25 mutanase (pre)pro-sequence is replaced by the Lipolase® (pre)pro-sequence or the TAKA-amylase (pre)pro-sequence.
5. An expression vector comprising a mutanase gene and a DNA sequence encoding a pro-peptide with a kex2 site or kex2-like  
30 site between the DNA sequences encoding said pro-peptide and the mature region of the mutanase.
6. The expression vector according to claim 5, further comprising an operably linked promoter sequence and/or a prepro-  
35 sequence.

7. The expression vector according to claims 5 and 6, wherein the prepro-sequence comprise the original mutanase signal sequence, or the Lipolase® signal-sequence, or the TAKA pro-sequence and the original mutanase pro-sequence with a kex2 or  
5 kx2-like site, or the Lipolase® pro-sequence, or the TAKA pro-sequence.

8. The expression vector according to claim 7, wherein the promoter is the TAKA promoter or TAKA:TPI promoter.

10

9. The expression vector according to any claims 5 to 8, being the vector pMT1796.

10. A filamentous host cell for production of recombinant  
15 mutanase derived from a filamentous fungus being from the genus *Trichoderma*, such as a strain of *T. harzianum*, or the genus *Aspergillus*, such as a strain of *A. oryzae* or *A. niger*, or a strain of the genus *Fusarium*, such as a strain of *Fusarium oxysporium*, *Fusarium graminearum*, *Fusarium sulphureum*, *Fusarium*  
20 *cerealis*.

11. The host cell according to claim 10 wherein the host cell is a protease deficient of protease minus strain.

25 12. The host cell according to claim 11, wherein the host cell is the protease deficient strain *Aspergillus oryzae* JaL125 having the alkaline protease gene named "alp" deleted.

13. A process for producing a recombinant mutanase in a host  
30 cell, comprising the steps:

- a) transforming an expression vector comprising a mutanase gene with a kex2 site or kex2-like site between the DNA sequences encoding the pro-peptide and the mature region of the mutanase into a suitable filamentous fungus host cell,
- 35 b) cultivating the host cell in a suitable culture medium under conditions permitting expression and secretion of an active mutanase,

c) recovering and optionally purifying the secreted active recombinant mutanase from the culture medium.

14. The process according to claim 13 wherein the recombinant  
5 expression vector is prepared according to the method of claim 1 to 4.

15. The process according to claim 13 and 14, wherein the filamentous host is a host cell according to any of claims 7 to  
10 9.

16. An isolated recombinant mutanase produced according to the process according to any of claims 13 to 15.

15 17. A substantially pure wild-type mutanase obtained from *Trichoderma harzianum* CBS 243.71 essentially free of any contaminants.

18. A composition comprising a recombinant mutanase according  
20 to claim 16 or a substantially pure wild-type mutanase according to claim 17 and further other ingredients conventionally used in food, feed and/or pet food products.

19. An oral care composition comprising a recombinant mutanase  
25 according to claim 16 or a substantially pure wild-type mutanase according to claim 17, further comprising an enzyme selected from the group of dextranases, oxidases, peroxidases, haloperoxidases, laccases, proteases, endoglucosidases, lipases, amylases, and mixtures thereof.

30

20. An oral care product comprising a recombinant mutanase according to claim 16 or a substantially purified mutanase according to claim 17 or an oral care composition according to claim 19 and further comprising ingredients conventionally used  
35 in oral care products.

21. The oral care product according to claim 20, being a dentifrice, such as a toothpaste, tooth powder or a mouth wash.

22. Use of the recombinant mutanase according to claim 16 or the substantially purified mutanase according to claim 17 or an oral care composition of claim 19 or oral care product according to claims 20 and 21 for preventing the formation of dental plaque or removing dental plaque.

23. The use of the recombinant mutanase according to claims 16 or the substantially purified mutanase according to claim 17 or a oral care composition of claim 19 or oral care product according to claims 18 and 20 in oral care products for humans and/or animals.

24. Use of the composition according to claim 18, in food, feed and/or pet food products.

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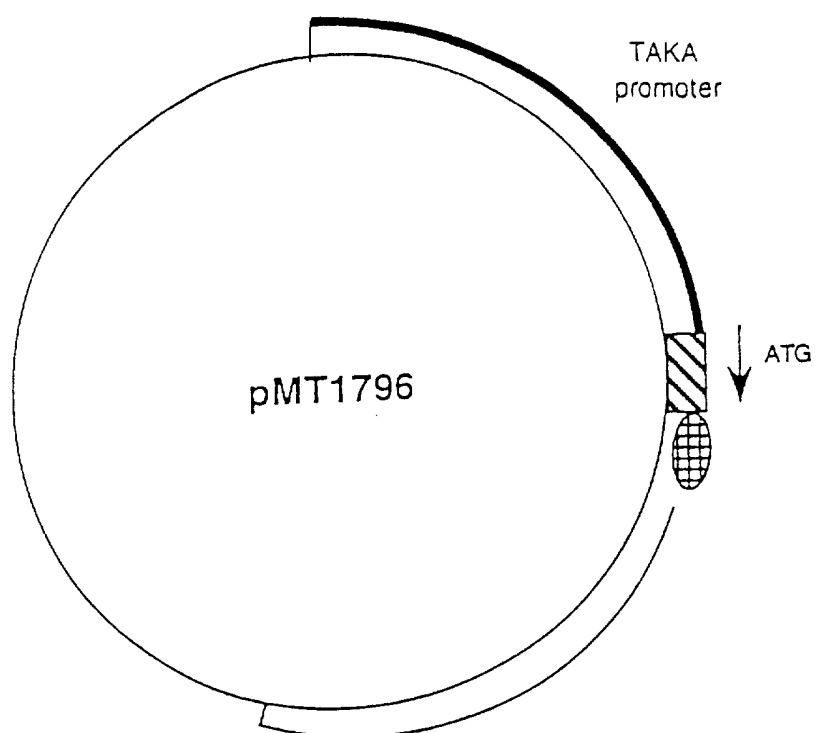


Fig. 1

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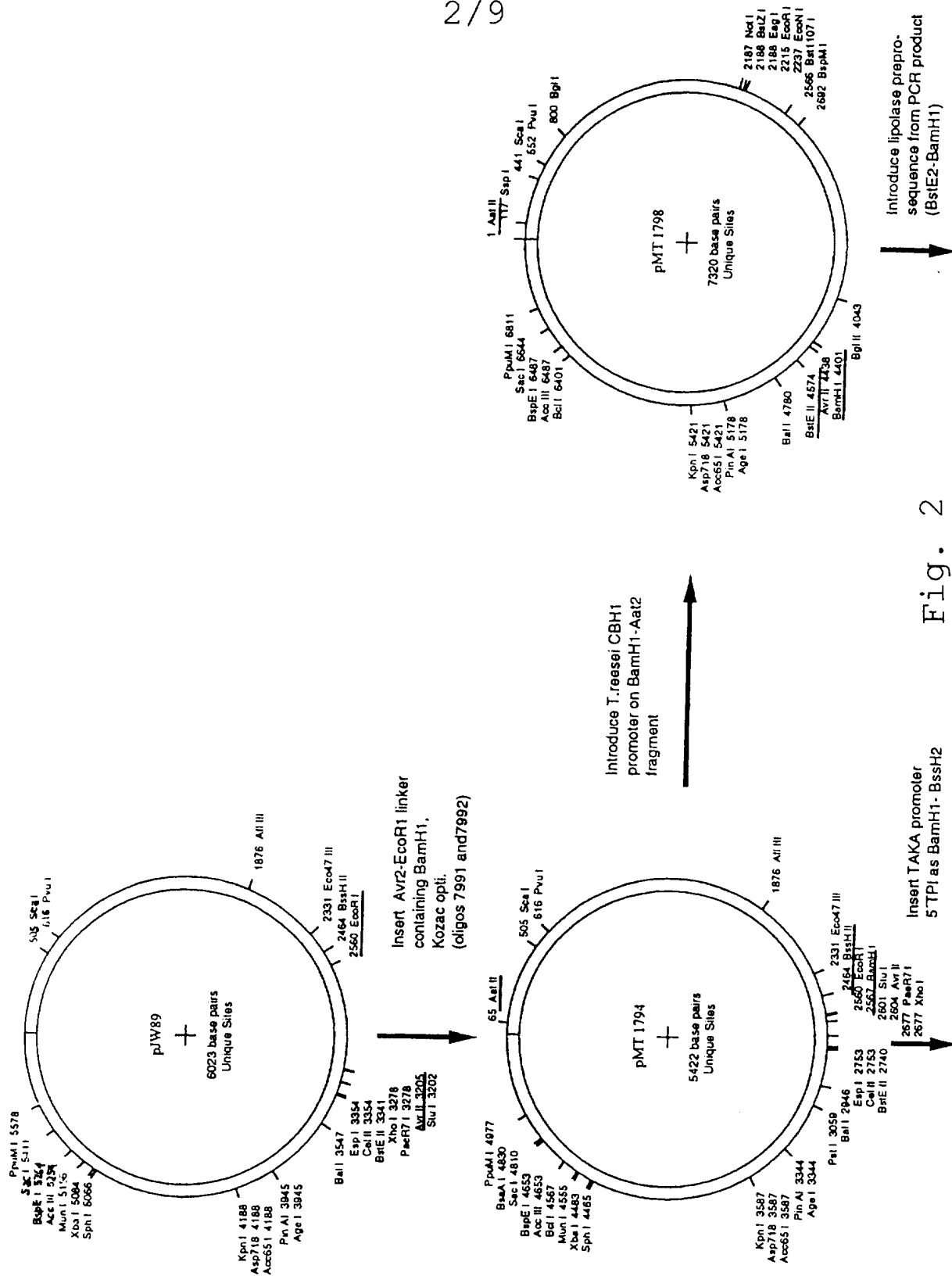


Fig. 2



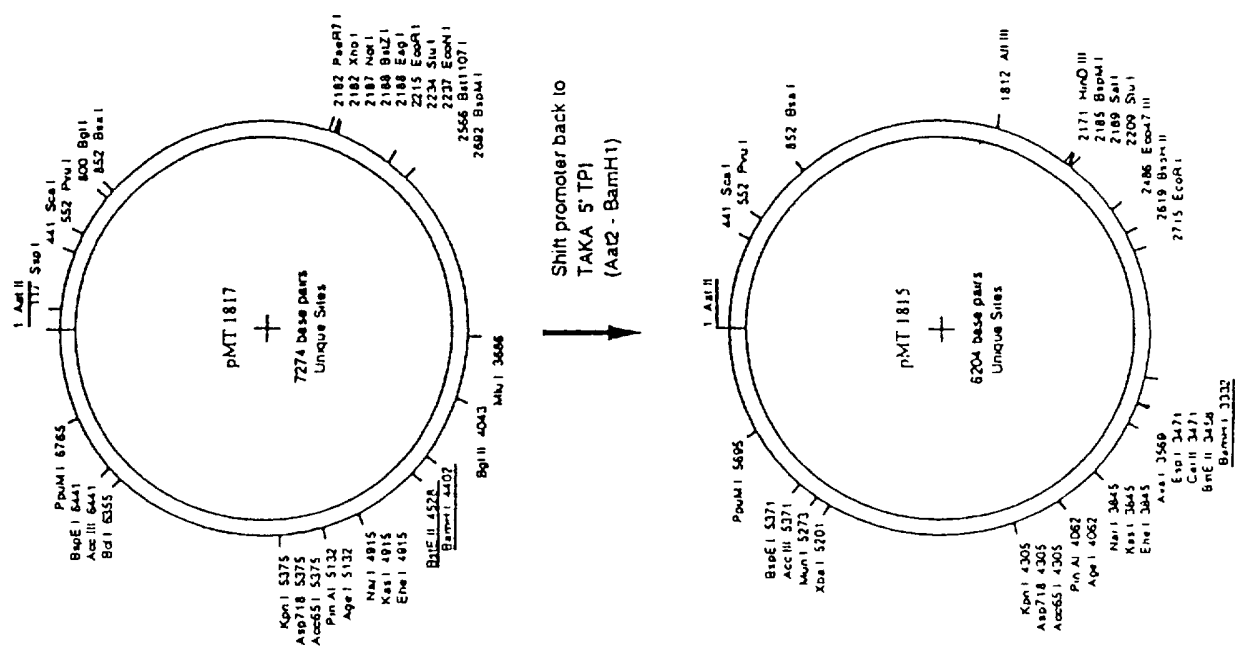
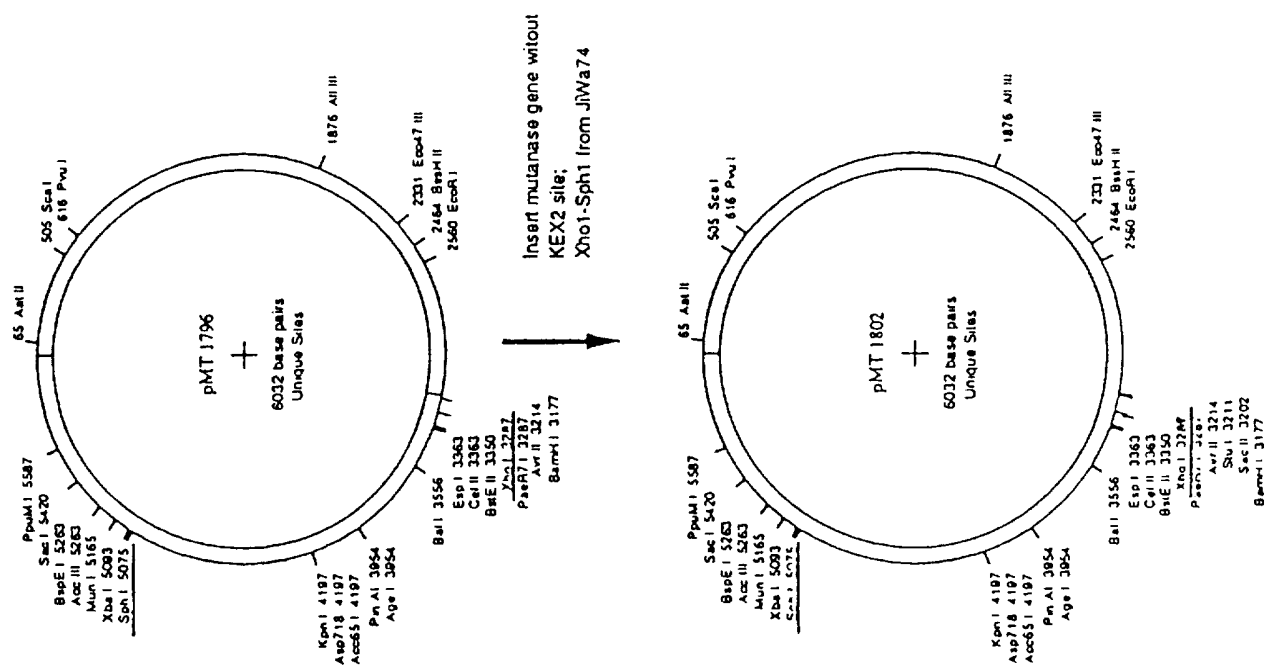


Fig. 2



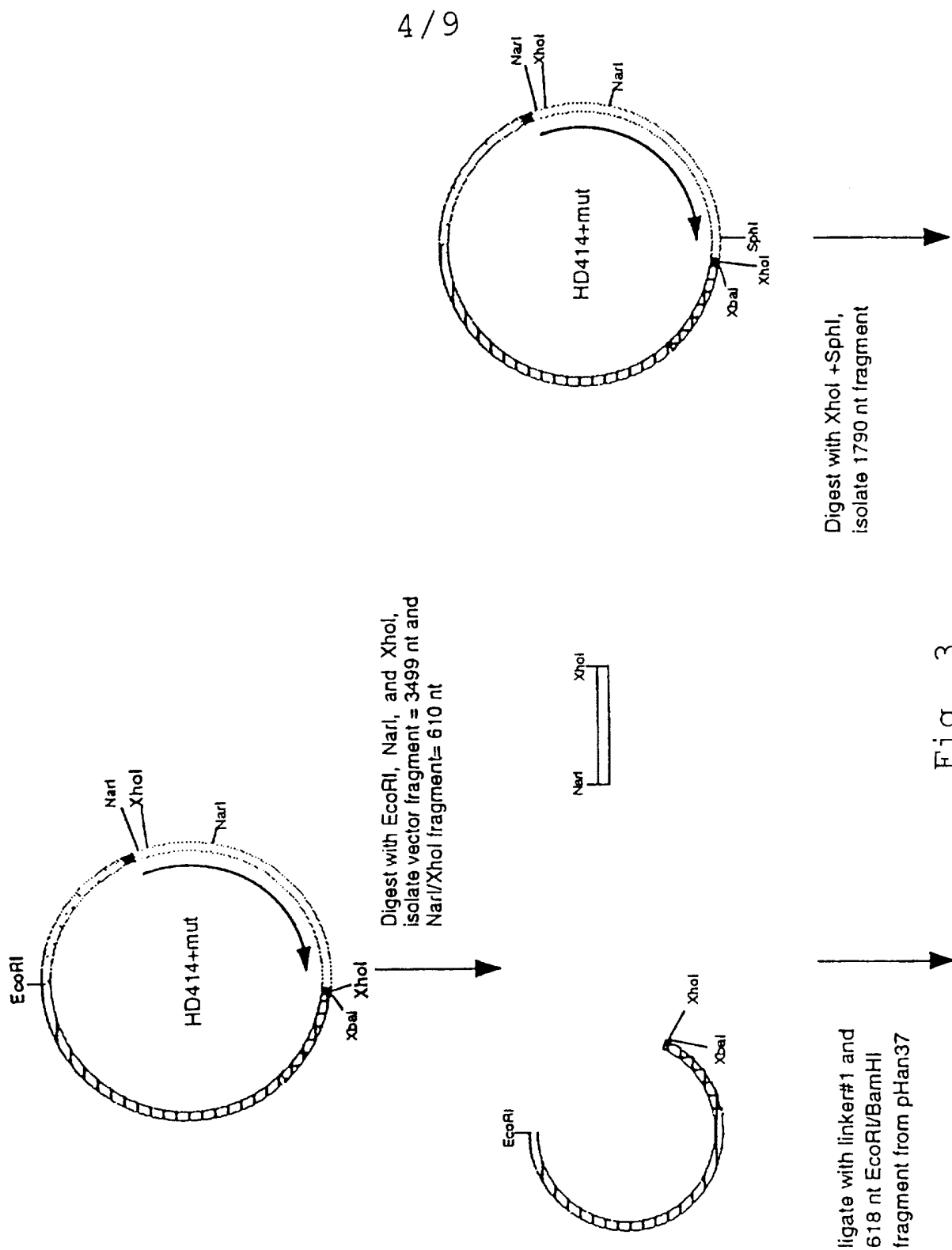


Fig. 3

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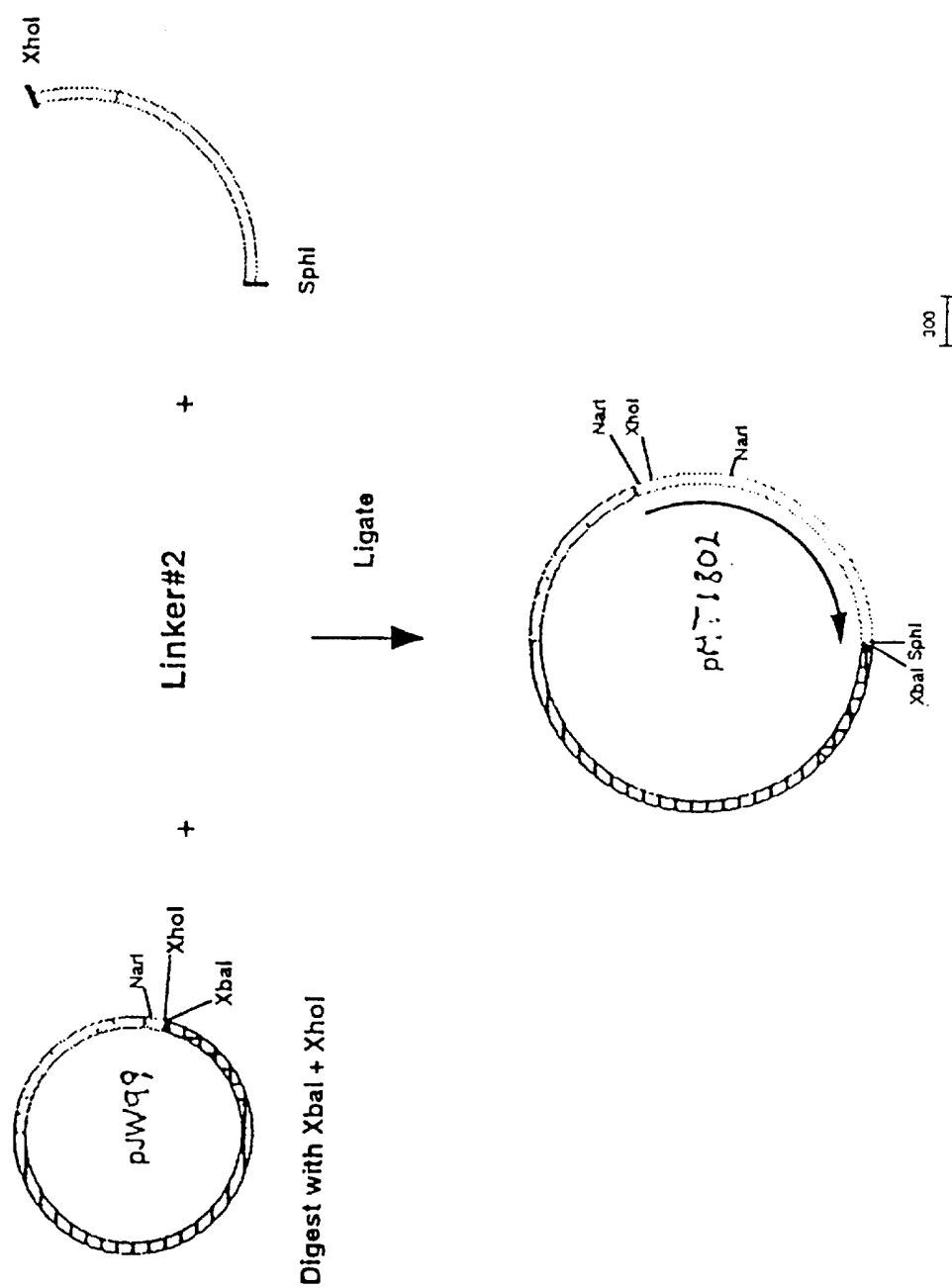


Fig. 3

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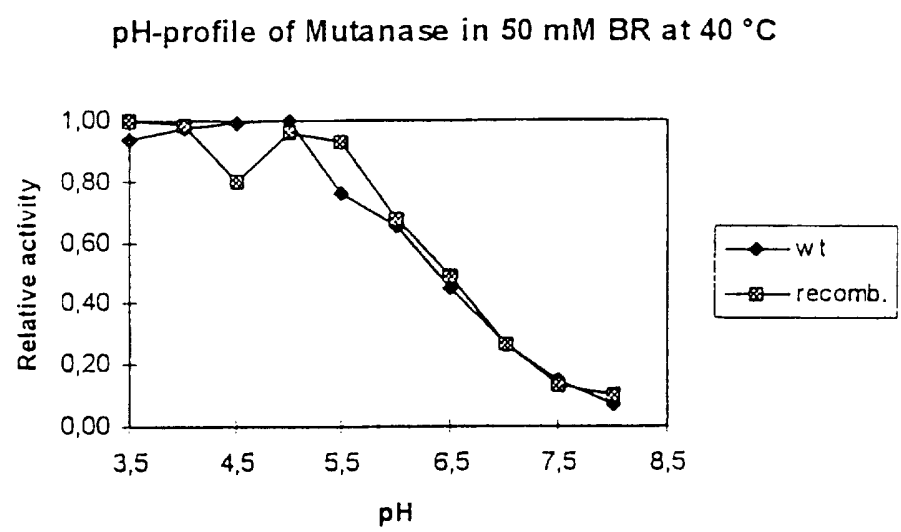


Fig. 4

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Temperature-profile of Mutanase in 0.1 M sodium phosphate, pH 7

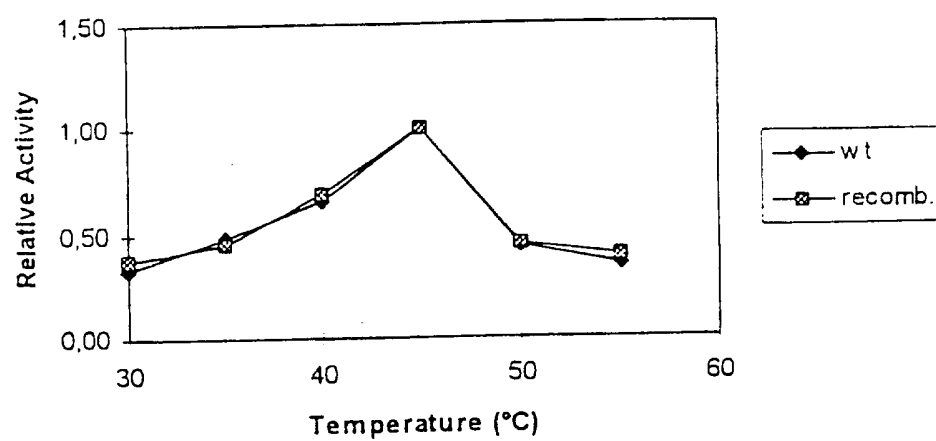


Fig. 5

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Temperature Stability of Mutanase in 0.1 M sodium  
phosphate, pH 7

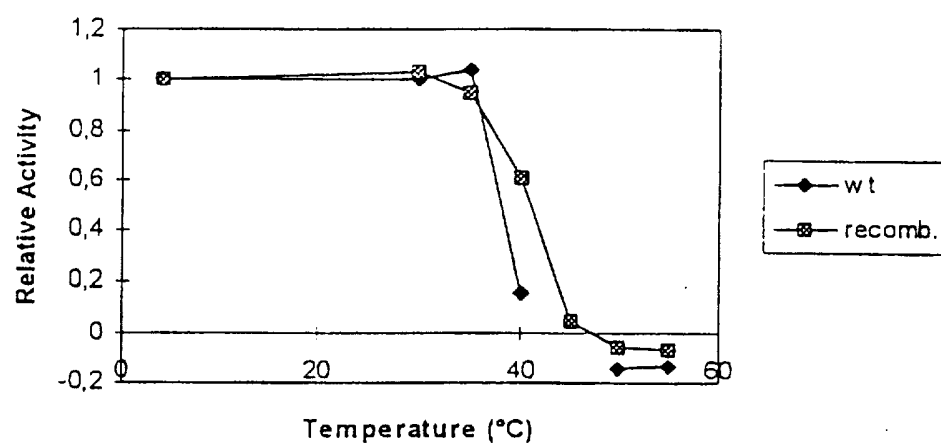


Fig. 6

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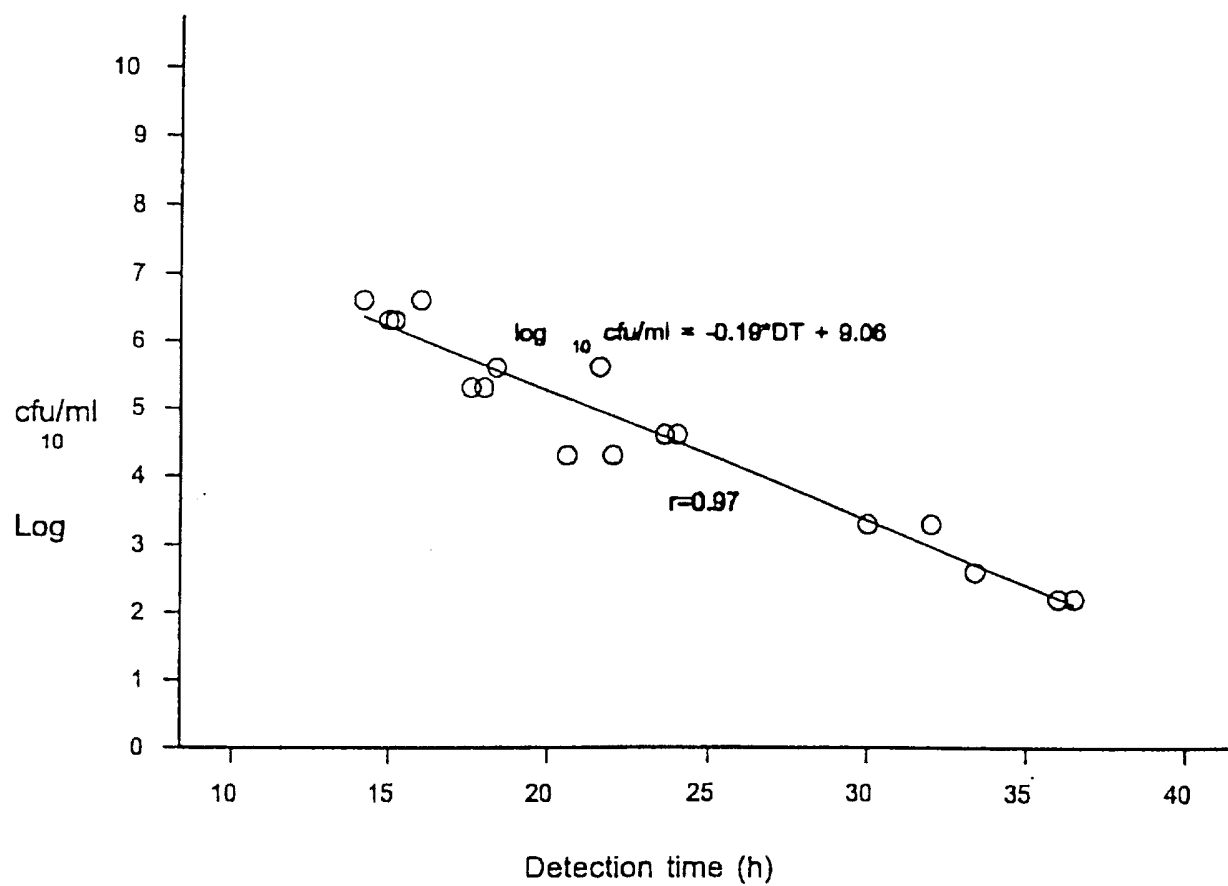


Fig. 7

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00283

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/24, C12N 15/56

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, CA, BIOSIS, MEDLINE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	File WPI, Derwent accession no. 92-111673, NISSIN SHOKUHI KAISHA LTD: "Alpha-1,3-glucanase gene - obtd. by lighting DNA into vector, trans- forming it into oral cavity bacteria and secretion of enzyme protein"; & JP,A,4058889, 920225 --	1-24
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☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

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Date of the actual completion of the international search

29 Sept 1997

Date of mailing of the international search report

13 -10- 1997

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00283

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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A	Dialog Information Services, file 155, MEDLINE, Dialog accession no. 07915749, Medline accession no. 94237475, Castelli LA et al: "High-Level secretion of correctly processed beta-lactamase from <i>Saccharomyces cerevisiae</i> using a high-copy- number secretion vector"; & Gene May 3 1994, 142 (1) p113-7  --	1,5,7,13
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Information on patent family members

01/09/97

International application No.  
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