(19) DANMARK

(10) DK/EP 2256192 T3

(12) Oversættelse af europæisk patentskrift

Patent- og Varemærkestyrelsen

(51) Int.Cl.: *C 12 N 15/09 (2006.01)* *C 12 N 1/15 (2006.01)* *C 12 N 9/08 (2006.01)*
C 12 R 1/645 (2006.01) *C 12 R 1/80 (2006.01)*

(45) Oversættelsen bekendtgjort den: **2015-09-28**

(80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2015-07-15**

(86) Europæisk ansøgning nr.: **09711878.0**

(86) Europæisk indleveringsdag: **2009-02-18**

(87) Den europæiske ansøgnings publiceringsdag: **2010-12-01**

(86) International ansøgning nr.: **JP2009052729**

(87) Internationalt publikationsnr.: **WO2009104622**

(30) Prioritet: **2008-02-18 JP 2008036171**

(84) Designerede stater: **AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO SE SI SK TR**

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(54) Benævnelse: **Termostabil katalase**

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DESCRIPTION

TECHNICAL FIELD

[0001] The present invention relates to thermostable catalases, more particularly, thermostable catalases derived from *Penicillium pinophilum,* proteins having a thermostable catalase activity, DNAs encoding the proteins, and a process for producing the thermostable catalases.

[BACKGROUND ART]

[0002] Catalase is an enzyme which catalyzes a reaction in which hydrogen peroxide decomposes into water and oxygen. Hydrogen peroxide aqueous solution is widely used as an antiseptic or a disinfectant. After the completion of disinfection, the hydrogen peroxide solution can be easily removed with water, and is spontaneously decomposed as time progresses, and therefore, is widely used as a disinfectant for food. However, it is desired that hydrogen peroxide is completely decomposed and removed after use, because reactive oxygen species generated from any remaining hydrogen peroxide have a possibility of causing cell aging or cancer. Catalase is extremely useful for the decomposition of hydrogen peroxide, because no additional chemical substance is needed for the decomposition. Actually, catalase is used in decomposing and removing hydrogen peroxidase remained after bleaching of cotton or in food. Catalases derived from microorganisms (patent references 1 to 5) and catalases derived from animals, such as porcine or bovine liver catalase, are known.

[0003] Among such known catalases, catalase produced by a filamentous fungus *Aspergillus niger* or porcine liver catalase is widely used for industrial use. However, it is known that these catalases exhibit low thermostability and the remaining activity thereof after the treatment at 70°C for 30 minutes was approximately 10% (patent reference 6). In particular, for the use of textile processing, food processing, or the like, catalase having thermostability higher than those of conventional catalases is desired, because hydrogen peroxide has to be decomposed at a high temperature. As thermostable catalases, catalases produced by *Aspergillus* terreus (patent reference 6), *Acremonium alabamensis* (patent reference 6), *Thermoascus aurantiacus* (patent reference 6), *Scytalidium thermophilum* (patent reference 7), *Humicola insolens* (patent reference 7), and genus *Thermomyces* (patent reference 8) have been reported.

[0004] It is known that filamentous fungi have an extremely high activity of secreting proteins, and are suitable as a host to produce a recombinant protein such as enzymes. Therefore, if a thermostable catalase gene can be introduced into a filamentous fungus and the thermostable catalase can be highly expressed as a recombinant protein, it is expected that the thermostable catalase can be produced at extremely high productivity in comparison with a wild type. With respect to the production of recombinant proteins, it has been reported that recombinant proteins could be produced in filamentous fungi classified into genus *Aspergillus* (patent reference 9), *Penicillium* (patent reference 10), *Humicola* (patent reference 11), *Trichoderma* (patent reference 12), or *Acremonium* (patent reference 13).

[0005] When a recombinant protein is expressed in these filamentous fungi as a host, all exogenous genes introduced into the host are not necessarily expressed. In general, it is considered preferable that the origin of an exogenous gene to be introduced is related to that of a host as closely as possible, in view of codon usage. For example, in the case that *Humicola insolens* was used as a host to express endoglucanase as a recombinant protein, a significant amount of endoglucanase was expressed when an NCE4 or NCE5 gene derived from *Humicola insolens* was introduced into *Humicola insolens* (patent references 14 and 15). By contrast, little amount of endoglucanase was expressed when an RCE I gene, which was derived from *Rhizopus oryzae* and had an amino acid sequence showing a high identity with those of NCE4 and NCE5, was introduced into *Humicola insolens* (patent reference 16). Further, in the case that *Aspergillus awamori* was used as a host to express glucoamylase as a recombinant protein, the introduction of a glucoamylase gene derived from *Aspergillus niger* resulted in high productivity (4.6 g/L), but the introduction of a glucoamylase gene derived from *Humicola grisea* resulted in low productivity (0.66 g/L) (non-patent reference 1). Furthermore, in the case that α-amylase was expressed as a recombinant protein, the introduction of an α-amylase gene derived from *Aspergillus oryzae* into *Aspergillus oryzae* as a host resulted in high productivity (12 g/L), but the introduction of the α-amylase gene derived from *Aspergillus oryzae* into *Trichoderma viride* resulted in only a productivity of 1 g/L (non-patent reference 1). These results show that, when a significant amount of recombinant protein is to be expressed, it is preferable to introduce a gene derived from a filamentous fungus which is the species same as or related to that of a host.

[0006] When a filamentous fungus is used as a host to express a large amount of thermostable catalase as a recombinant protein, it is considered preferable that the origin of a thermostable catalase gene to be introduced is closely related to the

filamentous fungus as the host, as described above. However, with respect to the isolation of thermostable catalase genes, only a catalase gene derived from *Thermoascus aurantiacus* (patent reference 17) and a catalase gene derived from *Scytalidium thermophilum* (patent reference 18) have been reported. Thermostable catalase genes have not been isolated from filamentous fungi developed as a host for protein production, such as genus *Aspergillus, Penicillium, Humicola, Trichoderma,* or *Acremonium,* and therefore, it was very difficult to express thermostable catalase as a recombinant protein with high productivity.

[0007]

[patent reference 1] Japanese Unexamined Patent Publication (kokai) No. 55-135588

[patent reference 2] Japanese Unexamined Patent Publication (kokai) No. 60-083579

[patent reference 3] Japanese Unexamined Patent Publication (kokai) No. 63-003788

[patent reference 4] Japanese Examined Patent Publication (kokoku) No. 49-004956

[patent reference 5] Japanese Unexamined Patent Publication (kokai) No. 2-076579

[patent reference 6] Japanese Unexamined Patent Publication (kokai) No. 5-153975

[patent reference 7] Japanese Translation Publication (Kohyo) No. 6-506347

[patent reference 8] Japanese Unexamined Patent Publication (kokai) No. 10-257883

[patent reference 9] International Publication WO 97/034004

[patent reference 10] International Publication WO 2000/068401

[patent reference 11] International Publication WO 98/003667

[patent reference 12] International Publication WO 98/011239

[patent reference 13] Japanese Unexamined Patent Publication (kokai) No. 2001/017180

[patent reference 14] International Publication WO 98/003640

[patent reference 15] International Publication WO 2001/090375

[patent reference 16] International Publication WO 2000/024879

[patent reference 17] Japanese Unexamined Patent Publication (kokai) No. 2004-261137

[patent reference 18] United States Patent No. 5646025 [non-patent reference 1] Norihiro TSUKAGOSHI, Kumikae Tanpakushitsu Seisan-hou (Production of recombinant proteins), Japan Scientific Societies Press, pp. 94-95

DISCLOSURE OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

[0008] Under the circumstances, the expression of a large amount of thermostable catalase as a recombinant protein is desired. An object to be solved by the present inventors is to search filamentous fungi belonging to genus *Aspergillus, Penicillium, Trichoderma,* and *Acremonium,* which were developed as hosts for producing recombinant proteins, for thermostable catalases; to isolate genes encoding the thermostable catalases; and to express thermostable catalases in large quantity.

MEANS FOR SOLVING THE PROBLEMS

[0009] To solve the object, the present inventors cultivated a number of filamentous fungi belonging to genus *Aspergillus, Penicillium, Trichoderma,* and *Acremonium,* which had been developed as hosts for producing recombinant proteins; evaluated

the thermostability of catalase contained in each culture liquid obtained; and attempted to find thermostable catalase from the filamentous fungi. As a result, the present inventors found that *Penicillium pinophilum* produced thermostable catalases.

[0010] Next, the present inventors purified thermostable catalase from the culture liquid of *Penicillium pinophilum,* and obtained a thermostable catalase in which a single band was observed at the position of approximately 80 kDa by SDS-PAGE and the N-terminal amino acid sequence was DDSNASSETEAFLSEFYLNDNDAYLTTDVGG (SEQ ID NO.: 5).

[0011] Furthermore, the present inventors succeeded in cloning genes encoding the above thermostable catalases from genomic DNAs of *Penicillium pinophilum*, and determining the nucleotide sequences of the genes, and the present invention was completed.

[0012] The present invention relates to:

1. 1) A protein selected from the group consisting of:
    1. (i) a protein comprising the amino acid sequence consisting of amino acids 1-692 of SEQ ID NO.: 2,
    2. (ii) a protein comprising an amino acid sequence in which 1 to 50 amino acids are deleted, substituted, or added in the amino acid sequence consisting of amino acids 1-692 of SEQ ID NO.: 2, and having a thermostable catalase activity, and
    3. (iii) a protein comprising an amino acid sequence having a 80% or more identity with that consisting of amino acids 1-692 of SEQ ID NO.: 2, and having a thermostable catalase activity;
2. 2) a protein consisting of the amino acid sequence consisting of amino acids 1-692 of SEQ ID NO.: 2, and having a thermostable catalase activity;
3. 3) the protein of 1) or 2), having the amino acid sequence consisting of amino acids -1 to -42 of SEQ ID NO.: 2, at the N-terminal side of the protein;
4. 4) a DNA selected from the group consisting of:
    1. (i) a DNA encoding the protein of 1),
    2. (ii) a DNA comprising the nucleotide sequence consisting of nucleotides 1-2403 of SEQ ID NO.: 1, and
    3. (iii) a DNA hybridizing under stringent conditions to a DNA consisting of the nucleotide sequence consisting of nucleotides 1-2403 of SEQ ID NO.: 1, and encoding a protein having a thermostable catalase activity;
5. 5) a DNA consisting of the nucleotide sequence consisting of nucleotides 1-2403 of SEQ ID NO.: 1;
6. 6) a DNA wherein an intron sequence is excised from the DNA of 4) or 5);
7. 7) the DNA of 6), wherein the intron sequence is one or more sequences selected from the nucleotide sequence consisting of nucleotides 322-372, 599-651, 1068-1113, or 1279-1326 of SEQ ID NO.: 1;
8. 8) a DNA wherein a nucleotide sequence encoding a signal sequence is excised from the DNA of 4) to 7).
9. 9) the DNA of 8), wherein the nucleotide sequence encoding a signal sequence is that consisting of nucleotides 1-126 of SEQ ID NO.: 1;
10. 10) an expression vector comprising the DNA of 4) to 9);
11. 11) a host microorganism transformed with the expression vector of 10);
    The host microorganism of 11) preferably is a filamentous fungus;
    The filamentous fungus is a filamentous fungus belonging to genus *Aspergillus, Penicillium*, *Trichoderma,* or *Acremonium.*
    Described herein is a process for producing a thermostable catalase, characterized by cultivating the host microorganism and collecting the thermostable catalase from the culture obtained by the cultivation.

EFFECTS OF THE INVENTION

[0013] According to the present invention, DNAs necessary for efficiently producing thermostable catalase as a recombinant protein can be obtained, and recombinant microorganisms efficiently expressing thermostable catalase can be obtained. Further, thermostable catalase can be efficiently produced at low cost by cultivating the obtained microorganism. Hydrogen peroxide can be efficiently decomposed at low cost, even at high temperature, by treating a solution containing hydrogen peroxide with the thermostable catalase of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014]

Figure 1 is a restriction map of plasmid pPCN.

Figure 2 is a restriction map of plasmid pHCN.

Figure 3 is a restriction map of plasmid pPTPCN.

BEST MODE FOR CARRYING OUT THE INVENTION

[0015] The term "thermostable catalase" as used herein means a catalase in which the percentage of the activity remaining after incubating at 70°C for 30 minutes is 50% or more, as determined by measuring thermostability in accordance with the method described in Example 4 of patent reference 6.

[0016] Thermostable catalase produced by *Penicillium pinophilum* into a culture liquid may be obtained by, for example, a method disclosed in patent reference 6. A catalase activity may be evaluated by adding catalase to a solution containing hydrogen peroxide and quantifying the decrease in hydrogen peroxide during a predetermined period of time, for example, in accordance with a method disclosed in patent reference 6. Whether or not a catalase is thermostable may be judged, in accordance with a method disclosed in patent reference 6, by heat-treating a culture supernatant, which has been previously diluted to an appropriate concentration, at 70°C for 30 minutes and measuring the catalase activities before and after the heat-treatment. According to the above definition as used herein, a catalase having a remaining activity of 50% or more after the heat-treatment is regarded as "thermostable catalase".

[0017] Culture supernatants of *Penicillium pinophilum* and were obtained by the above method, and the thermostability of catalase contained in each supernatant was determined. As a result, the remaining activities after the heat-treatment at 70°C for 30 minutes were 50% and 57% with respect to catalases produced by *Penicillium pinophilum* respectively, and *Penicillium pinophilum* produced thermostable catalases.

[0018] The thermostable catalases may be purified from the themostable-catalase-containing culture supernatants obtained by the above method in accordance with one or more conventional methods for purifying proteins. As the methods, various commonly known methods may be applied: for example, a combination of hydrophobic chromatography and anion-exchange chromatography may be used. The molecular weight of each purified thermostable catalase may be determined by SDS-PAGE.

[0019] In accordance with the above methods, the thermostable catalases produced by *Penicillium pinophilum* were purified, and the molecular weight of each thermostable catalase was determined. Thermostable catalases having a molecular weight of apprixunateky 80 kDa were obtained from *Penicillium pinophilum.*

[0020] The term "an amino acid sequence in which one or plural amino acids are deleted, substituted, or added in an amino acid sequence" as used herein means that the original amino acid sequence is modified by substitution or the like of plural amino acids which may naturally occur, or in accordance with a well-known method such as site-directed mutagenesis. The number of modified amino acids is 1 to 50, more preferably 1 to 30, still more preferably 1 to 10, still more preferably 1 to 5, most preferably 1 to 2.

[0021] Preferred examples of a modified amino acid sequence in the protein according to the present invention may include an amino acid sequence in which one or plural amino acids (preferably one or several amino acids, or one, two, three, or four amino acids) are conservatively substituted.

[0022] The term "conservative substitution" as used herein means one or plural amino acid residues are replaced with different amino acids having similar chemical properties. Examples of the conservative substitution include a substitution of a hydrophobic residue for another hydrophobic residue, and a substitution of a polar residue for another polar residue having the same charge. Amino acids which have similar chemical properties and can be conservatively substituted with each other are known to those skilled in the art. More particularly, examples of nonpolar (hydrophobic) amino acids include alanine, valine, isoleucine, leucine, proline, tryptophan, phenylalanine, and methionine. Examples of polar (neutral) amino acids include glycine, serine, threonine, tyrosine, glutamine, asparagine, and cysteine. Examples of basic amino acids having a positive charge include arginine, histidine, and lysine. Examples of acidic amino acids having a negative charge include aspartic acid and glutamic acid.

[0023] The term "under stringent conditions" as used herein means that a membrane after hybridization is washed at a high temperature in a solution of low salt concentration, for example, at 60°C for 15 minutes in a solution of 0.5×SSC concentration (1×SSC: 15mmol/L trisodium citrate and 150 mmol/L sodium chloride), preferably at 60°C for 15 minutes in a solution of 0.5×SSC concentration with 0.1% SDS.

[0024] Hybridization is carried out in accordance with a known method. When a commercially available library is used, hybridization carried out in accordance with a method described in a protocol attached to the library.

[0025] The term "identity" with respect to nucleotide sequences or amino acid sequences as used herein means the degree of similarity between nucleotides or amino acid residues constituting sequences to be compared. The "identity" as used herein may be represented by a value calculated using a known homology search program. For example, the values may be easily calculated by using default parameters in FASTA or the like.

[0026] The amino acid sequence having a 80% or more identity with that consisting of amino acids 1-692 of SEQ ID NO.: 2 may be an amino acid sequence having an identity of, preferably 85% or more, still more preferably 90% or more, still more preferably 95% or more, still more preferably 98% or more, most preferably 99% or more.

[0027] In the present invention, given the amino acid sequence consisting of amino acids 1-692 of SEQ ID NO.: 2, various nucleotide sequences encoding the amino acid sequence may be easily determined and selected.

[0028] In the present invention, the DNA encoding a protein comprising the amino acid sequence consisting of amino acids 1-692 of SEQ ID NO.: 2 means not only part or all of the nucleotide sequence consisting of nucleotides 1-2403 of SEQ ID NO.: 1, but also nucleotide sequences containing degenerate codons encoding the same amino acids. The present invention includes RNA sequences corresponding to these nucleotide sequences.

[0029] Preferred examples of the DNA encoding a protein comprising the amino acid sequence consisting of amino acids 1-692 of SEQ ID NO.: 2 include a DNA comprising the nucleotide sequence consisting of nucleotides 1-2403 of SEQ ID NO.: 1.

[0030] Genes encoding the thermostable catalases produced by *Penicillium pinophilum* can be isolated by preparing genomic phage libraries from *Penicillium pinophilum* and obtaining positive phage clones containing the thermostable catalase genes. As probes to screen the genomic phage libraries for positive phage clones, fragments of the thermostable catalase genes may be used. Each of the fragments of the thermostable catalase genes to be used as probes may be amplified by a PCR using each genomic DNA as a template. A primer set for the PCR may be designed based on conserved sequences among known catalase genes derived from filamentous fungi. The nucleotide sequence of each thermostable catalase gene may be determined by subcloning the thermostable catalase gene from an obtained positive clone into an *Escherichia coli* vector and analyzing the nucleotide sequence of the obtained vector. Intron sequences in the determined nucleotide sequence may be deduced on the basis of comparing the amino acid sequence deduced from the nucleotide sequence with those of known catalases, and conserved sequences of introns. Further, a sequence from the translation initiation codon of the gene to the codon immediately upstream of the sequence encoding the N-terminal amino acid sequence of a purified thermostable catalase, may be deduced as a sequence encoding a signal sequence.

[0031] The full-length of thermostable catalase gene PCN, which was isolated from genomic DNA of *Penicillium* pinophilum by the method described above, consisted of the 2403-bp nucleotide sequence of SEQ ID NO.: 1, and it was deduced that the gene included four introns having nucleotide sequences consisting of nucleotides 322-372, 599-651, 1068-1113, and 1279-1326 of SEQ ID NO.: 1. The amino acid sequence of the thermostable catalase, deduced from the gene sequence, was that of SEQ ID NO.: 2. The amino acid sequence consisting of amino acids 1-31 of SEQ ID NO.: 2 was completely identical with the N-terminal amino acid sequence of the thermostable catalase purified from *Penicillium pinophilum,* and thus, it was deduced that the amino acid sequence consisting of amino acids -1 to -42 of SEQ ID NO.: 2 was a signal sequence and that the nucleotide sequence consisting of nucleotides 1-126 of SEQ ID NO.: 1 was a nucleotide sequence encoding the signal sequence.

[0032] Based on the nucleotide sequence of catalase gene PCN derived from *Penicillium pinophilum,* primers for amplifying the gene of interest may be designed, a PCR may be carried out using genomic DNA from *Penicillium pinophilum* as a template, an expression vector may be constructed by ligating the amplified DNA fragment into an appropriate vector, and the gene of interest may be isolated. The DNA of the present invention derived from *Penicillium pinophilum* is contained in plasmid pPCN, and thus, the DNA or the plasmid may be used as a DNA template for PCR. An appropriate restriction enzyme may be used to prepare a desired DNA fragment from the plasmid.

[0033] According to the present invention, Escherichia coli transformed with pPCN is provided. This transformed *Escherichia coli*

strain was domestically deposited in the International Patent Organism Depositary National Institute of Advanced Industrial Science and Technology (Address: AIST Tsukuba Central 6, 1-1, Higashi 1-come Tukuba-shi, Ibarakiken 305-8566 Japan) on February 7, 2008 (domestic deposit number: FERM P-21504), and was transferred to an international deposit on December 11, 2008 (international deposit number: FERM BP-11074).

[0034] According to the present invention, Escherichia coli transformed with pHCN is provided. This transformed *Escherichia coli* strain was domestically deposited in the International Patent Organism Depositary National Institute of Advanced Industrial Science and Technology (Address: AIST Tsukuba Central 6, 1-1, Higashi 1-chome Tukuba-shi, Ibarakiken 305-8566 Japan) on February 7, 2008 (domestic deposit number: FERM P-21503), and was transferred to an international deposit on December 11, 2008 (international deposit number: FERM BP-11073).

[0035] Each of the thermostable catalase genes, isolated as described above, may be introduced into a host, and a desired thermostable catalase may be produced by expressing it in the host. The DNA to be introduced into the host may be the full-length of a thermostable catalase gene, a DNA obtained by excising part of all of intron sequences from the full-length DNA, or a DNA obtained by excising the nucleotide sequence encoding a signal sequence.

[0036] According to the present invention, an expression vector comprising the DNA of the present invention, in which the DNA can be replicated in a host microorganism and a protein encoded by the DNA can be expressed, is provided. Further, according to the present invention, a microorganism transformed with this expression vector is provided.

[0037] The host-vector system is not particularly limited. For example, a system using *Escherichia coli,* actinomycetes, yeasts, or fungi, or a fusion protein expression system using the same may be used. Examples of a preferred host microorganism used in the present invention include filamentous fungi, more preferably genus *Trichoderma,* genus *Aspergillus,* genus *Penitillium* (most preferably *Penicillium pinophilum*), and genus *Acremonium.* As an expression vector, expression vectors disclosed in patent references 9 to 13 may be used.

[0038] The expression vector of the present invention may be constructed in accordance with procedures and methods widely used in the field of genetic engineering.

[0039] The expression vector of the present invention may include not only the DNA of the present invention, but also a DNA capable of regulating the expression of the DNA, a genetic marker to select a transformant, or the like, to express a desired protein by incorporating the expression vector into a host microorganism.

[0040] The obtained transformant may be cultivated in an appropriate medium, and the protein of the present invention may be obtained by isolating it from the culture. The cultivation of the transformant and the conditions thereof may be appropriately selected in accordance with the microorganism used. The protein of interest may be collected and purified from the culture liquid in accordance with conventional methods.

EXAMPLES

[0041] The present invention now will be further illustrated by, but is by no means limited to, the following Examples.

Example 1: Measurement of catalase activity (thermostability) in culture liquid of *Penicillium pinophilum*

[0042] *Penicillium pinophilum* grown on potato dextrose agar was inoculated in a 200 mL conical flask containing 30 mL of a medium (50 g/L sucrose, 20 g/L malt extract, and 5 g/L yeast extract), and cultivated at 26°C for 5 days with shaking. The cells were removed from the resulting culture liquid by centrifugation to obtain the culture supernatant. The catalase activity (thermostability) of catalase contained in the resulting culture supernatant was measured by the method described in Example 4 of patent reference 6. The percentage of the remaining activity after incubating at 70°C for 30 minutes was 50%. It was concluded from the result that *Penicillium pinophilum* produced thermostable catalase.

Example 2: Isolation and purification of thermostable catalase in culture liquid of *Penicillium pinophilum*

[0043] Ammonium sulfate was dissolved in the culture supernatant of *Penicillium pinophilum,* obtained in the method described in Example 1, at a final concentration of 1 mol/L. The resulting solution was subjected and adsorbed to a hydrophobic column Phenyl Sepharose HP 26/10 (manufactured by GE Healthcare bioScience), which had been previously equilibrated with a 50 mmol/L phosphate buffer (pH7.0) containing 1 mol/L ammonium sulfate. Proteins adsorbed to the hydrophobic column were eluted and fractionated by a linear gradient elution method from a 50 mmol/L phosphate buffer (pH7.0) containing 1 mol/L ammonium sulfate to a 50 mmol/L phosphate buffer (pH7.0). A catalase activity of each fractionated eluate was measured by the method described in Example 1, and fractions having the activity were collected. Ammonium sulfate was added to the collected active fraction at a final concentration of 1 mol/L, and the above procedure was repeated to carry out rechromatography using the hydrophobic column. The resulting active fraction was concentrated and desalted by ultrafiltration, and adjusted to a final concentration of 50 mmol/L with a phosphate buffer (pH8.0). This solution was subjected to an anion-exchange column MonoQ (manufactured by GE healthcare Bioscience), which had been previously equilibrated with a 50 mmol/L phosphate buffer (pH8.0) containing 1 mol/L ammonium sulfate, and proteins were adsorbed to the column. The adsorbed proteins were eluted and fractionated by a linear gradient elution method from a 50 mmol/L phosphate buffer (pH8.0) to a 50 mmol/L phosphate buffer (pH8.0) containing 1 mol/L NaCl. A catalase activity (thermostability) of each fractionated eluate was measured by the method described in Example 1, and fractions having the activity were collected. The collected active fraction was analyzed by SDS-PAGE to detect a single band of approximately 80 kDa, and it was judged that the protein detected as the band is a thermostable catalase. The thermostable catalase was separated by SDS-PAGE and blotted on a polyvinylidene difluoride (PVDF) membrane. The N-terminal amino acid sequence was analyzed to obtain the following sequence:

DDSNASSETEAFLSEFYLNDNDAYLTTDVGG (SEQ ID NO.: 5)

Example 3: Cloning of thermostable catalase gene PCN from *Penicillium pinophilum*

3-1) Preparation of genomic DNA library

[0044] Genomic DNA was isolated and purified from *Penicillium pinophilum* cells in accordance with the method of Horiuchi et al. [H. Horiuchi et al., J. Bacteriol., 170, 272-278, (1988)]. The isolated genomic DNA was partially digested with restriction enzyme *Sau*3AI. The resulting DNA fragments were ligated with *Bam*HI arms of a phage vector EMBL3 cloning kit (manufactured by Stratagene) using a ligation kit Ver. 2 (manufacture by Takara Bio). The mixture was precipitated with ethanol and dissolved in a TE buffer. The whole amount of the ligated mixture and a MaxPlaxλ packerging kit (manufactured by Epicenter Technologies) were used to form phage particles, and an *Escherichia coli* XL1-blue MRA (P2) strain was infected with the phage particles. As a result, a genomic DNA library composed of 1.1×10^4 phages was obtained.

3-2) Preparation of probe

[0045] The following primers ware prepared based on conserved sequences among known catalases:

P catalase F: GAGGCCGGCAACTACCCNGARTGGRA (SEQ ID NO.: 6)

P catalase R: CCTGCTCGGTCTCGGCRAARWARTT (SEQ ID NO.: 7)

[0046] The P catalase F and P catalase R primers and genomic DNA were used as primers and a template, respectively, to carry out a PCR. LA Taq polymerase (manufactured by Takara Bio) was used in the PCR. In the PCR, a cycle composed of a reaction at 94°C for 30 seconds, annealing for 30 seconds, and a reaction at 72°C for 1 minute was repeated 40 times. In this regard, the annealing temperature in the first 20 cycles was stepwisely lowered from 63°C to 53°C, and the annealing temperature in the subsequent 20 cycles was 53°C. The amplified DNA fragment of 250 bp was inserted into a pCR2.1-TOPO plasmid vector, using a TOPO TA cloning kit (manufactured'by Invitrogen) in accordance with a protocol attached to the kit, to obtain plasmid TOPO-P catalase.

[0047] The cloned DNA fragment inserted into plasmid TOPO-P catalase was sequenced using a BigDye(R) Terminator v3.1 Cycle Sequencing Kit (manufactured by Applied Biosystems) and an ABI PRISM genetic analyzer (manufactured by Applied

Biosystems) in accordance with protocols attached thereto. The determined nucleotide sequence was used to carry out a homology search. As a result, the nucleotide sequence showed a 71% identity with that of a catalase derived from *Aspergillus clavatus,* and thus, it was judged that the DNA fragment was part of a catalase gene. The DNA fragment was amplified by a PCR using plasmid TOPO-P catalase in a fashion substantially similar to that described above, and the obtained PCR product was labeled using an ECL direct system (manufactured by Amersham Pharmacia Biotech) as a probe.

3-3) Screening by plaque hybridization

[0048] Phage plaques prepared in Example 3-1 were transferred to a Hybond N+ Nyron Transfer Membrane (manufactured by Amersham). The membrane was denatured with alkali, washed with 5×SSC (SSC: 15 mmol/L trisodium citrate and 150 mmol/L sodium chloride), and dried to immobilize DNAs. After a prehybridization at 42°C for 1 hour, the probe labeled with horseradish peroxidase (HRP) was added, and a hybridization at 42°C for 4 hours was carried out. The probe was washed with 0.5×SSC containing 6 mol/L urea and 0.4% SDS twice, and washed with 2×SSC twice.

[0049] After the probe was washed, the nylon membrane was immersed in a detection solution for 1 minute, and exposed to a hyperfilm ECL (manufactured by Amersham) to obtain a positive clone. The preparation of DNA from the positive clone was carried out by using LE392 as a host *Escherichia coli* in accordance with the method of Maniatis et al. (J. Sambrook, E. F. Fritsch and T. Maniatis, "Molecular Cloning", Cold Spring Harbor Laboratory Press. 1989). LE392 was cultivated in an LB-MM medium (1% peptone, 0.5% yeast extract, 0.5% sodium chloride, 10 mmol/L magnesium sulfate, and 0.2% maltose) overnight. The culture was infected with a phage solution derived from a single plaque, and cultivated in the LB-MM medium overnight. Sodium chloride and chloroform were added to the culture at final concentrations of 1 mol/L and 0.8%, respectively, to promote the lysis of *Escherichia coli.* The *Escherichia coli* cell debris was removed by centrifugation, and phage particles were collected from a polyethylene glycol (PEG) precipitate (10% PEG6000). The phage particles were digested with proteinase K in the presence of SDS, treated with phenol, and precipitated with ethanol, to collect phage DNA.

[0050] The obtained DNA and the ECL direct system were used to carry out Southern blotting. A hybridization was carried out using the PCR-amplified fragment described in Example 3-2 as a probe. As a result, a *Pst*I fragment of approximately 7 kb showed common hybridization patterns to those of chromosomal DNA.

[0051] The *Pst*I fragment was cloned into pUC118 to obtain plasmid pUC-PCN. The nucleotide sequence of the obtained plasmid was determined by the method described in Example 3-2. To subclone catalase gene PCN derived from *Penicillium pinophilum,* a PCR using pUC-PCN as a template and the following primer set (PCNF and PCNR) was carried out to amplify the PCN gene.

PCNF: ATGCGAGGATTATACTCCCTC (SEQ ID NO.: 8)

PCNR: CTACTCATCCACAGCGAATCG (SEQ ID NO.: 9)

The amplified DNA was inserted into a pCR2.1-TOPO plasmid vector using a TOPO TA cloning kit (manufactured by Invitrogen) to obtain plasmid pPCN. An *Escherichia coli* TOP10 strain (Invitrogen) was transformed with plasmid pPCN to obtain *Escherichia coli* TOP10/pPCN.

3-4) Deduction of amino acid sequence of thermostable catalase

[0052] The full-length of thermostable catalase gene PCN, which was isolated from genomic DNA of *Penicillium pinophilum* by the method described above, consisted of the 2403-bp nucleotide sequence of SEQ ID NO.: 1. On the basis of comparing the amino acid sequence deduced from the nucleotide sequence with those of known catalases, and conserved sequences of introns, it was deduced that the gene included four introns having nucleotide sequences consisting of nucleotides 322-372, 599-651, 1068-1113, and 1279-1326 of SEQ ID NO: 1. The amino acid sequence of the thermostable catalase, deduced from the nucleotide sequence, was that of SEQ ID NO.: 2. The amino acid sequence consisting of amino acids 1-31 of SEQ ID NO.: 2 was completely identical with the N-terminal amino acid sequence (shown in Example 2) of the thermostable catalase purified from *Penicillium pinophilum,* and thus, it was deduced that the amino acid sequence consisting of amino acids -1 to -A2 of SEQ ID NO.: 2 was a signal sequence and that the nucleotide sequence consisting of nucleotides 1-126 (encoding the amino acids 1-to -42 of SEQ ID NO.: 2) of SEQ ID NO.: 1 was a nucleotide sequence encoding the signal sequence.

Example 4: Measurement of catalase activity (thermostability) in culture liquid of *Humicola grisea*

[0053] A culture supernatant of *Humicola grisea* was prepared in a fashion substantially similar to that described in Example 1. The catalase activity (thermostability) of catalase contained in the resulting culture supernatant was measured by the method described in Example 1. The percentage of the remaining activity after incubating at 70°C for 30 minutes was 57%. It was concluded from the result that *Humicola grisea* produced thermostable catalase.

Example 5 (reference example): Isolation and purification of thermostable catalase in culture liquid of *Humicola grisea*

[0054] Ammonium sulfate was dissolved in the culture supernatant of *Humicola grisea,* obtained in the method described in Example 4, at a final concentration of 1 mol/L. The resulting solution was subjected and adsorbed to a hydrophobic column Phenyl Sepharose HP 26/10 (manufactured by GE Healthcare bioscience), which had been previously equilibrated with a 50 mmol/L phosphate buffer (pH7.0) containing 1 mol/L ammonium sulfate. Proteins adsorbed to the hydrophobic column were eluted and fractionated by a linear gradient elution method from a 50 mmol/L phosphate buffer (pH7.0) containing 1 mol/L ammonium sulfate to a 50 mmol/L phosphate buffer (pH7.0). A catalase activity of each fractionated eluate was measured by the method described in Example 1, and fractions having the activity were collected. Ammonium sulfate was added to the collected active fraction at a final concentration of 1 mol/L, and the above procedure was repeated to carry out rechromatography using the hydrophobic column. The resulting active fraction was concentrated and desalted by ultrafiltration, and adjusted to a final concentration of 50 mmol/L with an acetate buffer (pH4.0). This solution was subjected to a cation-exchange column MonoS (manufactured by GE healthcare Bioscience), which had been previously equilibrated with a 50 mmol/L acetate buffer (pH4.0). The catalase activity was detected in the non-adsorbed fraction, and thus, the non-adsorbed fraction was collected as the active fraction. The collected active fraction was analyzed by SDS-PAGE to detect a single band of approximately 80 kDa, and it was judged that the protein detected as the band is a thermostable catalase. The thermostable catalase was separated by SDS-PAGE and blotted on a PVDF membrane. The N-terminal amino acid sequence was analyzed to obtain the following sequence:

QDTTSGQSPLAAYEVDDSTG (SEQ ID NO.: 10)

Example 6 (reference example): Cloning of thermostable catalase gene HCN from *Humicola grisea*

6-1) Preparation of genomic DNA library

[0055] A genomic DNA library of *Humicola grisea* was prepared by the method described in Example 3-1.

6-2) Preparation of probe

[0056] The following primers ware prepared based on conserved sequences among catalases derived from filamentous fungi and yeasts:

H catalase F: GTNCGNTTYTCNACTGT (SEQ ID NO.: 11)

H catalase R: AARAANACNGGNTTRTTGTT (SEQ ID NO.: 12)

[The underlined abbreviation "N" at the 12th position of SEQ ID NO.: 12 stands for deoxyinosine.]

[0057] The H catalase F and H catalase R primers and genomic DNA were used as primers and a template, respectively, to carry out a PCR. Ex Taq polymerase (manufactured by Takara Bio) was used in the PCR. In the PCR, a cycle composed of a reaction at 98°C for 10 seconds, annealing at 55°C for 30 seconds, and an elongation reaction at 72°C for 15 seconds was repeated 30 times. The amplified DNA fragment of 300 bp was inserted into a pCR2.1-TOPO plasmid vector, using a TOPO TA cloning kit (manufactured by Invitrogen) in accordance with a protocol attached to the kit, to obtain plasmid TOPO-H catalase.

[0058] The cloned DNA fragment inserted into plasmid TOPO-H catalase was sequenced, and the determined nucleotide sequence was used to carry out a homology search. As a result, the nucleotide sequence showed a 97% identity with that of a

catalase derived from *Sclerotinia sclerotiorum*, and thus, it was judged that the DNA fragment was part of a catalase gene. The DNA fragment was amplified by a PCR using plasmid TOPO-H catalase in a fashion substantially similar to that described above, and the obtained PCR product was labeled using an ECL direct system (manufactured by Amersham Pharmacia Biotech) as a probe.

6-3) Screening by plaque hybridization

[0059] The genomic DNA library was screened in accordance with the method described in Example 3-3, and a positive clone was obtained. The obtained positive clone was analyzed by Southern blotting. As a result, an *Xho*I fragment of approximately 7 kb and a *Bam*HI fragment of approximately 4 kb showed common hybridization patterns to those of chromosomal DNA. The *Xho*I fragment and the *Bam*HI fragment were separately cloned into pUC118 to obtain plasmid pUC-HCN-XhoI and plasmid pUC-HCN-BamH1, respectively. The nucleotide sequences of these plasmids were determined. As a result, the *Xho*I fragment contained the sequence from the 616th nucleotide to the 3'-terminus of SEQ ID NO.: 3 and the *Bam*HI fragment contained the sequence from the 5'-terminus to the 1675th nucleotide of SEQ ID NO.: 3, and thus, these fragments contained thermostable catalase gene fragments. These nucleotide sequences were joined to determine that of the full-length of a thermostable catalase gene. To subclone catalase gene HCN derived from *Humicola grisea*, a PCR using genomic DNA of *Humicola grisea* as a template and the following primer set (HCNF and HCNR) was carried out to amplify the HCN gene.

HCNF: ATGAACAGAGTCACGAATCTC (SEQ ID NO.: 13)

HCNR: TCAAAAAACAAAGGCACCAAG (SEQ ID NO.: 14)

The amplified DNA was inserted into a pCR2.1-TOPO plasmid vector using a TOPO TA cloning kit (manufactured by Invitrogen) to obtain plasmid pHCN. An *Escherichia coli* TOP10 strain (Invitrogen) was transformed with plasmid pHCN to obtain *Escherichia coli* TOP10/pHCN.

6-4) Deduction of amino acid sequence of thermostable catalase

[0060] The full-length of thermostable catalase gene HCN, which was isolated from genomic DNA of *Humicola grisea* by the method described above, consisted of the 2749-bp nucleotide sequence of SEQ ID NO.: 3. On the basis of comparing the amino acid sequence deduced from the nucleotide sequence with those of known catalases, and conserved sequences of introns, it was deduced that the gene included six introns having nucleotide sequences consisting of nucleotides 283-463, 667-747, 771-846, 1008-1160, 1218-1270, and 1842-1895 of SEQ ID NO: 3. The amino acid sequence of the thermostable catalase, deduced from the nucleotide sequence, was that of SEQ ID NO.: 4. The amino acid sequence consisting of amino acids 1-20 of SEQ ID NO.: 4 was completely identical with the N-terminal amino acid sequence (shown in Example 5) of the thermostable catalase purified from *Humicola grisea*, and thus, it was deduced that the amino acid sequence consisting of amino acids -1 to -32 of SEQ ID NO.: 4 was a signal sequence and that the nucleotide sequence consisting of nucleotides 1-96 (encoding the amino acids -1 to -32 of SEQ ID NO.: 4) of SEQ ID NO: 3 was a nucleotide sequence encoding the signal sequence.

Example 7: Preparation of expression vector for recombinant PCN

[0061] An expression of recombinant PCN using *Aspergillus niger* var. *macrosporus* as a host was carried out by using an expression vector in which the PCN gene was inserted between the promoter and the terminator of a proctase B gene, which was remarkably expressed in *Aspergillus niger* var. *macrosporus*. The expression vector was prepared in accordance with the following procedures.

7-1) Preparation of genomic DNA library

[0062] Genomic DNA was isolated and purified from *Aspergillus niger* var. *macrosporus* cells in accordance with the method of Horiuchi et al. [H. Horiuchi et al., J. Bacteriol., 170, 272-278, (1988)]. The isolated genomic DNA was partially digested with restriction enzyme *Sau*3AI. The resulting DNA fragments were ligated with *Bam*HI arms of a phage vector λEMBL3 cloning kit (manufactured by Stratagene) using a ligation kit Ver. 2 (manufacture by Takara Bio). The mixture was precipitated with ethanol and dissolved in a TE buffer. The whole amount of the ligated mixture and a MaxPlaxλ packerging kit (manufactured by Epicenter

Technologies) were used to form phage particles, and an *Escherichia coli* XL1-blue MRA (P2) strain was infected with the phage particles. As a result, a genomic DNA library composed of 1.25×10^5 phages was obtained.

7-2) Preparation of probe

[0063] With respect to the genomic DNA library of *Aspergillus niger* var. *macrosporus,* Southern blotting was carried out using the coding region of the proctase B gene as a probe to isolate a clone containing the promoter and terminator regions of the proctase B gene. The coding region of the proctase B was amplified by a PCR using genomic DNA of *Aspergillus niger* var. *macrosporus* as a template and the following primers (proctaseB-N and proctaseB-C), which were designed based on the 5'- and 3'-termini of the coding region of the proctase B disclosed in Japanese Unexamined Patent Publication (kokai) No. 5-68570.

proctaseB-N: ATGGTCGTCTTCAGCAAAACC (SEQ ID NO.: 15)

proctaseB-C: CTAAGCCTGAGCGGCGAATCC (SEQ ID NO.: 16)

[0064] The PCR was carried out using an LA PCR™ KIT Ver2.1 (manufactured by Takara Bio). In the PCR, after an incubation at 94°C for 1 minute, a cycle composed of a reaction at 94°C for 30 seconds, a reaction at 52°C for 30 seconds, and a reaction at 72°C for 90 seconds was repeated 30 times, and a reaction at 72°C for 7 minutes was carried out to complete the PCR. As a result, a DNA of approximately 1.2 kb was amplified. The amplified DNA fragment of 1.2 kb was inserted into a pCR2.1-TOPO plasmid vector, using a TOPO TA cloning kit (manufactured by Invitrogen) in accordance with a protocol attached to the kit, to obtain plasmid TOPO-ProB. The cloned DNA fragment inserted into plasmid TOPO-ProB was sequenced using a BigDye(R) Terminator v3.1 Cycle Sequencing Kit (manufactured by Applied Biosystems) and an ABI PRISM genetic analyzer (manufactured by Applied Biosystems) in accordance with protocols attached thereto. The determined nucleotide sequence accorded with that of the proctase B gene disclosed in Japanese Unexamined Patent Publication (kokai) No. 5-68570, and thus, it was judged that the DNA fragment was the coding region of the proctase B gene. The DNA fragment was labeled using an ECL direct system (manufactured by Amersham Pharmacia Biotech) as a probe.

7-3) Screening of clone containing promoter and terminator regions of proctase gene by plaque hybridization

[0065] Phage plaques prepared in Example 7-1 were transferred to a Hybond N+ Nyron Transfer Membrane (manufactured by Amersham). The membrane was denatured with alkali, washed with 5×SSC (SSC: 15 mmol/L trisodium citrate and 150 mmol/L sodium chloride), and dried to immobilize DNAs. After a prehybridization at 42°C for 1 hour, the probe prepared by the method described in Example 7-2 was added, and a hybridization at 42°C for 20 hours was carried out. The probe was washed with 0.5×SSC containing 6 mol/L urea and 0.4% SDS twice, and washed with 2×SSC twice. After the probe was washed, the nylon membrane was immersed in a detection solution for 1 minute, and exposed to a hyperfilm ECL (manufactured by Amersham) to obtain eight positive clones.

[0066] The preparation of DNA from each of the positive clones was carried out by using LE392 as a host *Escherichia coli* in accordance with the method of Maniatis et al. (J. Sambrook, E. F. Fritsch and T. Maniatis, "Molecular Cloning", Cold Spring Harbor Laboratory Press. 1989). LE392 was cultivated in an LB-MM medium (1% peptone, 0.5% yeast extract, 0.5% sodium chloride, 10 mmol/L magnesium sulfate, and 0.2% maltose) overnight. The culture was infected with a phage solution derived from a single plaque, and cultivated in the LB-MM medium overnight. Sodium chloride and chloroform were added to the culture at final concentrations of 1 mol/L and 0.8%, respectively, to promote the lysis of *Escherichia coli.* The *Escherichia coli* cell debris was removed by centrifugation, and phage particles were collected from a polyethylene glycol (PEG) precipitate (10% PEG6000). The phage particles were digested with proteinase K in the presence of SDS, treated with phenol, and precipitated with ethanol, to collect phage DNA.

[0067] The obtained DNA and the ECL direct system were used to carry out Southern blotting. A hybridization was carried out using the probe prepared by the method described in Example 7-2. As a result, an *Xho*I-*Eco*RI fragment of approximately 5.5 kb showed common hybridization patterns to those of chromosomal DNA. It was judged that the DNA fragment contained the proctase B gene, and then, subcloning of the DNA fragment was carried out. The *Xho*I-*Eco*RI fragment excised from the phage DNA was inserted between the *Sal*I and *Eco*RI sites of pUC119 to obtain plasmid pPROB/119E.X. The nucleotide sequence of the obtained plasmid was sequenced to determine those of the promoter and terminator regions of the proctase B gene.

7-4) Construction of recombinant vector pPTB-EX for gene expression

[0068] A vector in which the coding region of the proctase B gene was excised from plasmid pPROB/119E.X prepared by the method described in Example 7-3 and the 3'-terminus of the promoter region of the gene was ligated to the 5'-terminus of the terminator region of the gene via the *XbaI* recognition sequence, was designated expression vector pPTB-EX. The expression vector pPTB-EX was prepared by an inverse PCR using pPROB/119E.X as a template and the following primers (proctaseBNxba and proctaseBCxba) designed based on the 3'-terminus of the promoter and the 5'-terminus of the terminator of the proctase B gene, respectively.

proctaseBNxba: GGTCTAGAATGTCAAGCAAGAGAGT (SEQ ID NO.: 17)

proctaseBCxba: GGTCTAGAATCAACCACTGAAGTGGA (SEQ ID NO.: 18)

In this regard, the *Xba*I recognition sequence was added to the 5'-terminus of each primer. Primestar MAX DNA POLYMERASE (manufactured by Takara Bio) was used in the inverse PCR, in which a cycle composed of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds, and a reaction at 72°C for 60 seconds was repeated 30 times. As a result, a DNA fragment of approximately 7 kb was amplified. The resulting PCR reaction liquid and a QIAQUICK PCR PURIFICATION KIT (manufactured by Qiagen) were used to purify the DNA fragment. The DNA fragment was dissolved in 50 µL of a TE buffer, digested with restriction enzyme *XbaI,* and re-ligated using a ligation kit Ver. 2 (manufacture by Takara Bio) to obtain the expression vector pPTB-EX. The nucleotide sequence of the obtained plasmid was analyzed, and it was confirmed that the inverse PCR caused no mutations.

7-5) Construction of vector pPTPCN for recombinant PCN expression

[0069] The PCN gene isolated by the method described in Example 3 was inserted into the *Xba*I site of expression vector pPTB-EX to construct vector pPTPCN for expressing recombinant PCN. To add the *Xba*I recognition sequence to the 5'- and 3'-termini of the coding region of the PCN gene, a PCR was carried out using pPCN as a template and the following primers (PCN-XbaIPtN and PCN-XbaIPtC) in which the XbaI recognition sequence was added to the 5'- and 3'-termini of the coding region of the PCN gene.

PCN-XbaIPtN: GGTCTAGAGGTCAAAATGCGAGGATTATACTCCCT (SEQ ID NO.: 19)

PCN-XbaIPtC: GGTCTAGACTACTCATCCACAGCGAATCGG (SEQ ID NO.: 20)

[0070] Primestar MAX DNA POLYMERASE (manufactured by Takara Bio) was used in the PCR, in which a cycle composed of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds, and a reaction at 72°C for 60 seconds was repeated 30 times. As a result, a DNA fragment of approximately 2.3 kb was amplified. The resulting PCR reaction liquid and a QIAQUICK PCR PURIFICATION KIT (manufactured by Qiagen) were used to purify the DNA fragment. The DNA fragment was dissolved in 50 µL of a TE buffer, and digested with restriction enzyme *XbaI.* The digested fragment was ligated using a ligation kit Ver. 2 (manufacture by Takara Bio) to pPTB-EX, which had been digested with *XbaI* and dephosphorylated, to obtain plasmid pPTPCN (SEQ ID NO.: 21, Figure 3). The DNA sequence of the PCN gene inserted into the plasmid was analyzed, and it was confirmed that the PCR caused no mutations.

Example 8: Transformation of *Aspergillus niger* var. *macrosporus* with PCN-expression vector pPTPCN and expression of recombinant PCN

[0071] A transformation of *Aspergillus niger* var. *macrosporus* with PCN-expression vector pPTPCN was carried out by transforming a niaD-deficient strain of *Aspergillus niger* var. *macrosporus* using a niaD gene as a selective marker gene.

8-1) Isolation of niaD-deficient strain Nia2

[0072] Spores of *Aspergillus niger* var. *macrosporus* were applied on a Czapek medium-N (0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl 0.001% $FeSO_4$-$2H_2O$, 3% sucrose, 1.5% purified agar, pH5.5-6.0) supplemented with 0.188% Na-glutamate and 3%

$KClO_3$, and incubated at 30°C for 5 to 7 days. Colonies were replicated to each medium in which the nitrogen source of the Czapek medium was replaced with NO_3, NH_4, or Glutamate, and incubated at 30°C for 5 to 7 days. Among the replicated colonies, a strain which could grow on the medium containing NH_4 or Glutamate as the nitrogen source, but could not grow on the medium containing NO_3 as the nitrogen source was isolated as a niaD-deficient strain Nia2.

8-2) Isolation of selection marker gene, niaD gene

[0073] A PCR using the following primers Nia-N and Nia-C, designed on the basis of the 5'- and 3'-termini of the coding region of a niaD gene of *Aspergillus niger* reported by Uncle et al. [Uncle,S.E., Cambell,E.I., Punt,P.J., Hawker,K.L., Contreras,R., Hawkins,A.R., Van Den Hondel,C.A. and Kinghorn,J.R., "The Aspergillus niger niaD gene encoding nitrate reductase:upstream nucleotide and amino acid sequence comparisons", Gene 111(2), 149-155(1992)], was carried out to amplify the coding region of a niaD gene of *Aspergillus niger* var. *macrosporus*.

Nia-N: ATGGCGACTGTCACTGAGGTG (SEQ ID NO.: 22)

Nia-C: TTAGAAGAAATGAAGGTCCGA (SEQ ID NO.: 23)

The PCR was carried out using genomic DNA of *Aspergillus niger* var. *macrosporus* and an LA PCR™ KIT Ver2.1 (manufactured by Takara Bio). In the PCR, after a reaction at 94°C for 1 minute, a cycle composed of a reaction at 94°C for 30 seconds, a reaction at 55°C for 30 seconds, and a reaction at 72°C for 3 minutes was repeated 30 times, and a reaction at 72°C for 7 minutes was carried out. As a result, a DNA fragment of approximately 3 kb was amplified. The amplified DNA fragment was labeled using an ECL direct system (manufactured by Amersham Pharmacia Biotech) as a probe.

[0074] The coding region of the niaD gene prepared by the above method was used as a probe to isolate a clone containing the promoter and terminator regions of the niaD gene from the genomic DNA library, which was prepared by the method described in Example 7-1, of *Aspergillus niger* var. *macrosporus*. In a fashion substantially similar to that described in Example 7, the genomic DNA library was screened to obtain a positive clone. The obtained phage clone was analyzed by Southern blotting in a fashion substantially similar to that described in Example 7. As a result, an *Xba*I-digested fragment of approximately 6.5 kb showed common hybridization patterns to those of chromosomal DNA. This *Xba*I fragment was cloned into the *Xba*I recognition sequence site of pUC118 to obtain plasmid pPTnia118. The nucleotide sequence of the obtained plasmid was analyzed to determine the nucleotide sequence of 6416 bp (SEQ ID NO.: 24) containing the promoter and terminator regions of the niaD gene.

8-3) Introduction of PCN gene into *Aspergillus niger* var. *macrosporus* Nia2 strain

[0075] The *Aspergillus niger* var. *macrosporus* Nia2 strain was cultivated in an S medium (3.0% glucose, 0.1% polypeptone, 1% yeast extract, 0.14% ammonium sulfate, 0.2% potassium phosphate, 0.03% magnesium sulfate, pH6.8) at 30°C for 24 hours, and centrifuged at 3500 rpm for 10 minutes to collect the cells. The collected cells were washed with 0.5 mol/L sucrose, and suspended in an enzyme solution for preparing protoplasts (10 mg/mL β-glucuronidase, 3 mg/mL chitinase, 3 mg/mL zymolase, 0.5 mol/L sucrose), which had been filtrated through a 0.45 μm filter. The suspended mycelia was incubated at 30°C for 60 minutes with shaking to prepare protoplasts. The suspension was filtrated through absorbent cotton, and centrifuged at 2500 rpm for 10 minutes to collect the protoplasts. The protoplasts were washed with an SUTC buffer (17.1% sucrose, 10 mmol/L Tris-HCl pH7.5, 10 mmol/L $CaCl_2$), and resuspended in 100 μL of the SUTC buffer. To the protoplast suspension, 7.5 μL of pPTPCN (1 μg/μL) and 2.5 μL of pPTnia118 (1 μg/μL) were added, and the mixture was allowed to stand on ice for 5 minutes. Further, 400 μL of a PEG solution (60% PEG4000, 10 mmol/L Tris-HCl pH7.5, 10 mmol/L $CaCl_2$) was added, and allowed to stand on ice for 20 minutes. After 10 mL of the SUTC buffer was further added, the whole was centrifuged at 2500 rpm for 10 minutes. The centrifuged protoplasts were suspended in 1 mL of the SUTC buffer, centrifuged at 4000 rpm for 5 minutes, and finally suspended in 100 μL of the SUTC buffer.

[0076] The resulting protoplasts were overlaid with soft agar on a modified Czapek medium (0.085% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.001% $FeSO_4 \cdot 2H_2O$, 17.1% sucrose, 1.5% purified agar, pH5.5-6.0), and incubated at 30°C for 5 to 7 days. Colonies formed after the incubation was regarded as transformants.

8-4) PCN expression and measurement of enzymatic activity in transformant of *Aspergillus niger* var. *macrosporus* Nia2 strain

[0077] The obtained transformants were cultivated in a P medium (1.0% starch, 6.0% soybean meal, 1.0% corn steep liquor, 0.3% ammonium sulfate, and 1% calcium carbonate) at 28°C for 6 days. A supernatant of each culture was analyzed by SDS-PAGE to obtain a strain (No. 16) in which the band having a molecular weight of approximately 80 kDa, corresponding to the recombinant PCN, was observed. With respect to the culture supernatant of strain No. 16, and a supernatant obtained by similarly cultivating the Nia2 strain, the catalase activity was measured by the method described in Example 1. As shown in Table 1, the activity of strain No. 16 was 77 times or more that of the wild type, and it was confirmed that the recombinant PCN was expressed in strain No. 16.

[Table 1]

	Catalase activity (u/mL)
Wild type	less than 300 u/mL
Strain No.16	23300 u/mL

[0078] In this regard, "1 unit" of the catalase activity was regarded as the amount of the enzyme capable of decomposing 1μmol of hydrogen peroxide per minute. Further, the catalase activity of the culture supernatant of *Penicillium pinophilum* prepared by the method described in Example 1 was 385 U/mL. This result shows that the productivity of PCN remarkably increases by expressing PCN in *Aspergillus niger* var. *macrosporus* as a host.

8-5) Analysis of N-terminal amino acid sequence

[0079] The culture supernatant of the transformant No. 16 strain obtained in Example 8-4 was subjected to SDS-PAGE, and separated proteins were transferred to a PVDF membrane Immobilon-PSQ (manufactured by Millipore). The PVDF membrane was stained with Coomassie brilliant blue. A portion in which the protein of approximately 80 kDa was blotted was cut from the membrane, and subjected to an amino acid sequencer model 492 to determine the amino acid sequence of 11 residues at the amino-terminus. The amino sequence was as follows:

DDSNASSETEA (Amino acids 1-11 of SEQ ID NO.: 5)

This amino acid sequence was identical with the N-terminal amino acid of PCN derived from *Penicillium pinophilum,* and thus, it was confirmed that the protein of approximately 80 kDa was the recombinant PCN.

8-6) Evaluation of thermostability in recombinant PCN

[0080] As described in Example 1, the thermostability of naturally-occurring PCN produced by *Penicillium pinophilum* was 50%. The thermostability of the recombinant PCN obtained by the method described in Example 8-4 was evaluated by the method described in Example 1. As a result, the thermostability was 71.3%. This result revealed that the thermostability of the recombinant PCN was remarkably improved in comparison with that of naturally-occurring PCN.

[0081] Although the present invention has been described with reference to specific embodiments, various changes and modifications obvious to those skilled in the art are possible without departing from the scope of the appended claims.

FREE TEXT IN SEQUENCE LISTING

[0082] The nucleotide sequences of SEQ ID NOS.: 6-9 and 11-14 are artificially synthesized primer sequences, i.e., P catalase F (SEQ ID NO.: 6), P catalase R (SEQ ID NO.: 7), PCNF (SEQ ID NO.: 8), PCNR (SEQ ID NO.: 9), H catalase F (SEQ ID NO.: 11), H catalase R (SEQ ID NO.: 12), HCNF (SEQ ID NO.: 13), HCNR (SEQ ID NO.: 14), proctaseB-N (SEQ ID NO.: 15), proctaseB-C (SEQ ID NO.: 16), proctaseBNxba (SEQ ID NO.: 17), proctaseBCxba (SEQ ID NO.: 18), PCN-XbaIPtN (SEQ ID NO.: 19), PCN-XbaIPtC (SEQ ID NO.: 20), and Nia-N (SEQ ID NO.: 22), and Nia-C (SEQ ID NO.: 23), respectively.

[0083] The nucleotide sequence of ID NO.: 21 is plasmid pPTPCN.

[0084] The abbreviations "N" at the 18th position of SEQ ID NO.: 6, the 3rd, 6th, and 12th positions of SEQ ID NO.: 11, and the 6th and 9th positions of SEQ ID NO.: 12 stand for an arbitrary nucleotide; and the abbreviation "N" at the 12th position of SEQ ID NO.: 12 stands for deoxyinosine.

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Ala Ser Ala Ala Cys Pro Met Leu Thr Gly Glu Ile Pro Ala Gly Ser
    -25                 -20                 -15

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Val Ala Asn Pro His His His Gly Lys Arg Asp Asp Ser Asn Ala Ser
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Ser Glu Thr Glu Ala Phe Leu Ser Glu Phe Tyr Leu Asn Asp Asn Asp
            10                  15                  20

gcc tat ctc acc acc gat gta ggc ggt ccg atc gag gat caa aac agt       240
Ala Tyr Leu Thr Thr Asp Val Gly Gly Pro Ile Glu Asp Gln Asn Ser
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cca gcc aat gaa atc cca cag gct gct act gca cac gac acg gcc tat       782
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Val Lys Phe His Trp Thr Ser Leu Gln Gly Arg Ala Ser Leu Val Trp
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Glu Gln Val Met


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         Phe Gln Pro Gly His Ile Val Arg Gly Ile Asp Phe Thr Tyr
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tat cct ctt ctc cag ggt cgt tta ttc tcc tac ctc gat act cag ctg      1416
Tyr Pro Leu Leu Gln Gly Arg Leu Phe Ser Tyr Leu Asp Thr Gln Leu
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aat cgc aat ggt ggt ccc aac ttt gaa caa att cca atc aat cgt ccg      1464
Asn Arg Asn Gly Gly Pro Asn Phe Glu Gln Ile Pro Ile Asn Arg Pro
365                 370                 375                 380

cgt gtt cct atc cac aac aac aac cgc gat gga ttc gcc caa atg ttt      1512
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ccc ggg cgc tcc gca gat gga cac ctt gtt cgc gct acg agc cca aca      1656
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gct acc gaa caa cag ttc gtg atc aat gct ttg cgt ttc gaa ttg tct      1752
Ala Thr Glu Gln Gln Phe Val Ile Asn Ala Leu Arg Phe Glu Leu Ser
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cgc gta aac aac acg tta gca aca ctt gtg gct tct gca att gga gtc      1848
Arg Val Asn Asn Thr Leu Ala Thr Leu Val Ala Ser Ala Ile Gly Val
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Val His Ala Arg Gly Ala Gly Ala His Gly Val Phe Thr Ser Tyr Ala
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Glu Thr Pro Thr Phe Val Arg Phe Ser Thr Val Ala Gly Ser Arg Gly
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Ser Ala Asp Thr Ala Arg Asp Val His Gly Phe Ala Thr Arg Phe Tyr
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Phe Ile Gln Asp Ala Ile Leu Phe Pro Asp Leu Ile His Ser Val Lys
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Pro Gln Pro Ala Asn Glu Ile Pro Gln Ala Ala Thr Ala His Asp Thr
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Ala Tyr Asp Phe Phe Gly Gln Gln Pro Ser Thr Leu His Thr Leu Phe
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Trp Ala Met Ala Gly His Gly Ile Pro Arg Ser Phe Arg His Val Asp
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Phe Asp Leu Leu Asp Pro Thr Lys Ile Leu Pro Val Glu Lys Val Pro
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Ile Thr Ala Leu Gly Lys Met Gln Leu Asn Arg Asn Pro Leu Asn Tyr
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Gly Ile Asp Phe Thr Tyr Tyr Pro Leu Leu Gln Gly Arg Leu Phe Ser
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Tyr Leu Asp Thr Gln Leu Asn Arg Asn Gly Gly Pro Asn Phe Glu Gln
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Arg Ala Thr Ser Pro Thr Phe Ala Asp Val Trp Ser Gln Pro Gly Leu
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Phe Tyr Asn Ser Leu Thr Ala Thr Glu Gln Gln Phe Val Ile Asn Ala
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Ser Ser Ile Ala Glu Gly Gln Ala Leu Ala Gln Ser Leu Ala Gly Ser
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Ala Thr Tyr Ser Gly Ser Asp Ala Thr Asn Phe Asp Ala Val Ile Val
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gcc caa gca aca tgc ccc ttc gcg gac cct gcc gct ctg tat agg cgt        96
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gac agc acc gga tac ctg acc tcc gat gtt ggc ggg ccc att cag gac       192
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           20                  25                  30

cag acc agc ctc aag gca ggc atc cgg ggt ccg acc ctt ctt gag gac       240
Gln Thr Ser Leu Lys Ala Gly Ile Arg Gly Pro Thr Leu Leu Glu Asp
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  Val Pro Glu Arg Ala Val His Ala Arg Gly Ala Gly Ala His Gly Thr
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Phe Thr Ser Tyr Ala Asp Trp Ser Asn Ile Thr Ala Ala Ser Phe Leu
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Ala Gly Ser Arg Gly Ser Ala Asp Thr Ala Arg Asp Val His Gly Phe
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gcg acg cgg tt  gtaagttttg ttgtgtttca ttcgttccgg tctgtagagg           706
Ala Thr Arg Phe


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Gly Asn Phe
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Ile Pro Val Phe Phe Ile Gln Asp Ala Ile Gln Phe Pro Asp Leu Ile
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cac tcg gtc aag ccg agt cca gac aac gag att ccc caa gcg gcg acg       959
His Ser Val Lys Pro Ser Pro Asp Asn Glu Ile Pro Gln Ala Ala Thr
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                                   His Thr Leu Phe Trp Ala Met
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Ser Gly His Gly Ile Pro Arg Ser Tyr Arg His Met
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ctggctgaga tgaccgtgaa tccatttcta acctcaagtc cag gat ggc ttc ggc      1282
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Val His Thr Phe Arg Phe Val Lys Asp Asp Gly Ser Ser Lys Leu Ile
215                 220                 225                 230

aag tgg cat ttc aag tca cgc cag gga aag gcg agt cta gtc tgg gaa      1378
Lys Trp His Phe Lys Ser Arg Gln Gly Lys Ala Ser Leu Val Trp Glu
                235                 240                 245
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            250                 255                 260

ctc tgg gat gct att gag tcc ggg aac gga cca gaa tgg gat gtc tgc      1474
Leu Trp Asp Ala Ile Glu Ser Gly Asn Gly Pro Glu Trp Asp Val Cys
        265                 270                 275

gtc cag att gtc gat gag tcc cag gcg caa gcc ttt ggc ttc gac ttg      1522
Val Gln Ile Val Asp Glu Ser Gln Ala Gln Ala Phe Gly Phe Asp Leu
    280                 285                 290

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Leu Asp Pro Thr Lys Ile Ile Pro Glu Glu Tyr Ala Pro Leu Thr Lys
295                 300                 305                 310

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                315                 320                 325

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Thr Glu Gln Val Met Phe Gln Pro Gly His Ile Val Arg Gly Val Asp
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ttc acg gag gat ccc ctg cta cag gga cgt ctc ttc tcg tac ctt gac      1714
Phe Thr Glu Asp Pro Leu Leu Gln Gly Arg Leu Phe Ser Tyr Leu Asp
        345                 350                 355

acg cag ctg aac cgg aat ggc ggg ccc aac ttt gag cag ctg ccc atc      1762
Thr Gln Leu Asn Arg Asn Gly Gly Pro Asn Phe Glu Gln Leu Pro Ile
    360                 365                 370

aac atg ccg cgg gtg ccg att cac aac aat aat cgc gac ggc gcc ggc      1810
Asn Met Pro Arg Val Pro Ile His Asn Asn Asn Arg Asp Gly Ala Gly
375                 380                 385                 390

cag atg ttc atc cac agg aac aag tat cct t gtaagtacct cttttgcctc      1861
Gln Met Phe Ile His Arg Asn Lys Tyr Pro
                395                 400

gatcgttgtg gtgccggctt gctgacagac gcag ac  act ccc aac acc ctg aac    1915
                                    Tyr Thr Pro Asn Thr Leu Asn
                                                405

agt ggt tat ccg cgg caa gcc aac caa aat gcc gga cgc gga ttc ttc      1963
Ser Gly Tyr Pro Arg Gln Ala Asn Gln Asn Ala Gly Arg Gly Phe Phe
        410                 415                 420

aca gcg cct ggc cgt acc gtc agc ggt gcc ctc gtc cgt gag gtg tcg      2011
Thr Ala Pro Gly Arg Thr Val Ser Gly Ala Leu Val Arg Glu Val Ser
    425                 430                 435

cca aca ttc aac gac cac tgg tcg cag ccc cgt ctc ttc ttc aac tcc      2059
Pro Thr Phe Asn Asp His Trp Ser Gln Pro Arg Leu Phe Phe Asn Ser
440                 445                 450                 455

ctc act ccc gtc gaa cag cag ttc ctc gtc aac gcc atg cgc ttc gaa      2107
Leu Thr Pro Val Glu Gln Gln Phe Leu Val Asn Ala Met Arg Phe Glu
                460                 465                 470

atc agc ctt gtg aag tcg gaa gaa tgc agg aag aac gtg ctc acc cag      2155
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Ile Ser Leu Val Lys Ser Glu Glu Cys Arg Lys Asn Val Leu Thr Gln
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ctc aac cgc gtc agc cat gat gtg gcc gtg cgc gtg gcc gcc gct atc      2203
Leu Asn Arg Val Ser His Asp Val Ala Val Arg Val Ala Ala Ala Ile
        490                 495                 500

ggc ctc gcc gcg ccc gac gcg gac gac aca tac tac cac aac aac aag      2251
Gly Leu Ala Ala Pro Asp Ala Asp Asp Thr Tyr Tyr His Asn Asn Lys
    505                 510                 515

acg gct ggc gtc tcg atc ctt gga agc ggg ccc ttg cct acc atc aag      2299
Thr Ala Gly Val Ser Ile Leu Gly Ser Gly Pro Leu Pro Thr Ile Lys
520                 525                 530                 535

act ctc cgc gtc ggc atc ctg gct acc acg agc gag tcg agc gcg ctg      2347
Thr Leu Arg Val Gly Ile Leu Ala Thr Thr Ser Glu Ser Ser Ala Leu
                540                 545                 550

gat cag gca gcc cag ctc cgc acc cgt ctg gaa aag gac ggg ctt gtg      2395
Asp Gln Ala Ala Gln Leu Arg Thr Arg Leu Glu Lys Asp Gly Leu Val
            555                 560                 565

gtc acg gtt gtg gct gaa acg ctg cgc gag ggg gta gac cag aca tac      2443
Val Thr Val Val Ala Glu Thr Leu Arg Glu Gly Val Asp Gln Thr Tyr
        570                 575                 580

tcg acg gcg gat gcc acg ggt ttc gac ggc gtt gtt gtt gtg gac ggg      2491
Ser Thr Ala Asp Ala Thr Gly Phe Asp Gly Val Val Val Val Asp Gly
    585                 590                 595

gcg gcg gcg ctg ttt gcc agc acc gcg tcg tcg ccg ttg ttc ccg acg      2539
Ala Ala Ala Leu Phe Ala Ser Thr Ala Ser Ser Pro Leu Phe Pro Thr
600                 605                 610                 615

ggc agg ccg ttg cag atc ttt gtg gac gcg tat cgg tgg gga aag ccg      2587
Gly Arg Pro Leu Gln Ile Phe Val Asp Ala Tyr Arg Trp Gly Lys Pro
                620                 625                 630

gtc ggt gtg tgt ggt ggg aag tcg agc gag gtg ttg gat gcg gcg gat      2635
Val Gly Val Cys Gly Gly Lys Ser Ser Glu Val Leu Asp Ala Ala Asp
            635                 640                 645

gtt ccg gaa aat ggg gac ggg gtg tat tcg gag gag tcg gtg gac aag      2683
Val Pro Glu Asn Gly Asp Gly Val Tyr Ser Glu Glu Ser Val Asp Lys
        650                 655                 660

ttt gtg gag gag ttt gag aag ggg ttg gct act ttc agg gtg agt ctt      2731
Phe Val Glu Glu Phe Glu Lys Gly Leu Ala Thr Phe Arg Val Ser Leu
    665                 670                 675

ggt gcc ttt gtt ttt tga                                             2749
Gly Ala Phe Val Phe
680
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<212> PRT

<213> Humicola grisea

<400> 4

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Ala Gln Ala Thr Cys Pro Phe Ala Asp Pro Ala Ala Leu Tyr Arg Arg
    -15                 -10                 -5                  -1

Gln Asp Thr Thr Ser Gly Gln Ser Pro Leu Ala Ala Tyr Glu Val Asp
1               5                   10                  15

Asp Ser Thr Gly Tyr Leu Thr Ser Asp Val Gly Gly Pro Ile Gln Asp
            20                  25                  30

Gln Thr Ser Leu Lys Ala Gly Ile Arg Gly Pro Thr Leu Leu Glu Asp
        35                  40                  45

Phe Met Phe Arg Gln Lys Ile Gln His Phe Asp His Glu Arg Val Pro
    50                  55                  60

Glu Arg Ala Val His Ala Arg Gly Ala Gly Ala His Gly Thr Phe Thr
65                  70                  75                  80

Ser Tyr Ala Asp Trp Ser Asn Ile Thr Ala Ala Ser Phe Leu Asn Ala
                85                  90                  95

Thr Gly Lys Gln Thr Pro Val Phe Val Arg Phe Ser Thr Val Ala Gly
            100                 105                 110

Ser Arg Gly Ser Ala Asp Thr Ala Arg Asp Val His Gly Phe Ala Thr
        115                 120                 125

Arg Phe Tyr Thr Asp Glu Gly Asn Phe Asp Ile Val Gly Asn Asn Ile
    130                 135                 140

Pro Val Phe Phe Ile Gln Asp Ala Ile Gln Phe Pro Asp Leu Ile His
145                 150                 155                 160

Ser Val Lys Pro Ser Pro Asp Asn Glu Ile Pro Gln Ala Ala Thr Ala
                165                 170                 175

His Asp Ser Ala Trp Asp Phe Phe Ser Gln Gln Pro Ser Ala Met His
            180                 185                 190

Thr Leu Phe Trp Ala Met Ser Gly His Gly Ile Pro Arg Ser Tyr Arg
        195                 200                 205
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His Met Asp Gly Phe Gly Val His Thr Phe Arg Phe Val Lys Asp Asp
    210                 215                 220

Gly Ser Ser Lys Leu Ile Lys Trp His Phe Lys Ser Arg Gln Gly Lys
225                 230                 235                 240

Ala Ser Leu Val Trp Glu Glu Ala Gln Val Leu Ser Gly Lys Asn Ala
                245                 250                 255

Asp Phe His Arg Gln Asp Leu Trp Asp Ala Ile Glu Ser Gly Asn Gly
            260                 265                 270

Pro Glu Trp Asp Val Cys Val Gln Ile Val Asp Glu Ser Gln Ala Gln
        275                 280                 285

Ala Phe Gly Phe Asp Leu Leu Asp Pro Thr Lys Ile Ile Pro Glu Glu
    290                 295                 300

Tyr Ala Pro Leu Thr Lys Leu Gly Leu Leu Lys Leu Asp Arg Asn Pro
305                 310                 315                 320

Thr Asn Tyr Phe Ala Glu Thr Glu Gln Val Met Phe Gln Pro Gly His
                325                 330                 335

Ile Val Arg Gly Val Asp Phe Thr Glu Asp Pro Leu Leu Gln Gly Arg
            340                 345                 350

Leu Phe Ser Tyr Leu Asp Thr Gln Leu Asn Arg Asn Gly Gly Pro Asn
        355                 360                 365

Phe Glu Gln Leu Pro Ile Asn Met Pro Arg Val Pro Ile His Asn Asn
    370                 375                 380

Asn Arg Asp Gly Ala Gly Gln Met Phe Ile His Arg Asn Lys Tyr Pro
385                 390                 395                 400

Tyr Thr Pro Asn Thr Leu Asn Ser Gly Tyr Pro Arg Gln Ala Asn Gln
                405                 410                 415

Asn Ala Gly Arg Gly Phe Phe Thr Ala Pro Gly Arg Thr Val Ser Gly
            420                 425                 430

Ala Leu Val Arg Glu Val Ser Pro Thr Phe Asn Asp His Trp Ser Gln
        435                 440                 445
```

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Pro Arg Leu Phe Phe Asn Ser Leu Thr Pro Val Glu Gln Gln Phe Leu
    450                 455                 460

Val Asn Ala Met Arg Phe Glu Ile Ser Leu Val Lys Ser Glu Glu Cys
465                 470                 475                 480

Arg Lys Asn Val Leu Thr Gln Leu Asn Arg Val Ser His Asp Val Ala
                485                 490                 495

Val Arg Val Ala Ala Ala Ile Gly Leu Ala Ala Pro Asp Ala Asp Asp
            500                 505                 510

Thr Tyr Tyr His Asn Asn Lys Thr Ala Gly Val Ser Ile Leu Gly Ser
        515                 520                 525

Gly Pro Leu Pro Thr Ile Lys Thr Leu Arg Val Gly Ile Leu Ala Thr
    530                 535                 540

Thr Ser Glu Ser Ser Ala Leu Asp Gln Ala Ala Gln Leu Arg Thr Arg
545                 550                 555                 560

Leu Glu Lys Asp Gly Leu Val Val Thr Val Val Ala Glu Thr Leu Arg
                565                 570                 575

Glu Gly Val Asp Gln Thr Tyr Ser Thr Ala Asp Ala Thr Gly Phe Asp
            580                 585                 590

Gly Val Val Val Val Asp Gly Ala Ala Ala Leu Phe Ala Ser Thr Ala
        595                 600                 605

Ser Ser Pro Leu Phe Pro Thr Gly Arg Pro Leu Gln Ile Phe Val Asp
    610                 615                 620

Ala Tyr Arg Trp Gly Lys Pro Val Gly Val Cys Gly Gly Lys Ser Ser
625                 630                 635                 640

Glu Val Leu Asp Ala Ala Asp Val Pro Glu Asn Gly Asp Gly Val Tyr
                645                 650                 655

Ser Glu Glu Ser Val Asp Lys Phe Val Glu Glu Phe Glu Lys Gly Leu
            660                 665                 670

Ala Thr Phe Arg Val Ser Leu Gly Ala Phe Val Phe
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<211> 31

<212> PRT

<213> Penicillium pinophilum

<400> 5

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Tyr Leu Asn Asp Asn Asp Ala Tyr Leu Thr Thr Asp Val Gly Gly
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<400> 10

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1               5                   10                  15

Asp Ser Thr Gly
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REFERENCES CITED IN THE DESCRIPTION

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- **UNCLE,S.E.CAMBELL,E.I.PUNT,P.J.HAWKER,K.L.CONTRERAS,R.HAWKINS,A.R.VAN DEN HONDEL,C.AKINGHORN,J.R.**The Aspergillus niger niaD gene encoding nitrate reductase:upstream nucleotide and amino acid sequence comparisonsGene, 1992, vol. 111, 2149-155 [0073]

Patentkrav

1. Protein, der er udvalgt fra gruppen bestående af:
(i) et protein, som omfatter aminosyresekvensen bestående af aminosyrer 1-692 af SEQ ID NO.:2;
(ii) et protein, som omfatter en aminosyresekvens, hvor 1 til 50 aminosyrer er slettet, substitueret eller tilføjet aminosyresekvensen bestående af aminosyrer 1-692 af SEQ ID NO.: 2, og har en termostabil katalaseaktivitet; og
(iii) et protein, som omfatter en aminosyresekvens, som har 80 % eller mere identitet med den, der består af aminosyrer 1-692 af SEQ ID NO.: 2, og har en termostabil katalaseaktivitet.

2. DNA, der er udvalgt fra gruppen bestående af:
(i) et DNA, der koder for proteinet ifølge krav 1; og
(ii) et DNA, som omfatter nukleotidsekvensen bestående af nukleotider 1-2403 af SEQ ID NO:1.

3. DNA ifølge krav 2, hvor en intronsekvens er fjernet fra DNA'et.

4. DNA ifølge krav 3, hvor intronsekvensen er en eller flere sekvenser, der er udvalgt fra nukleotidsekvensen bestående af nukleotider 322-372, 599-651, 1068-1113 eller 1279-1326 af SEQ ID NO: 1.

5. DNA ifølge et af kravene 2 til 4, hvor en nukleotidsekvens, der koder for en signalsekvens, er fjernet fra DNA'et.

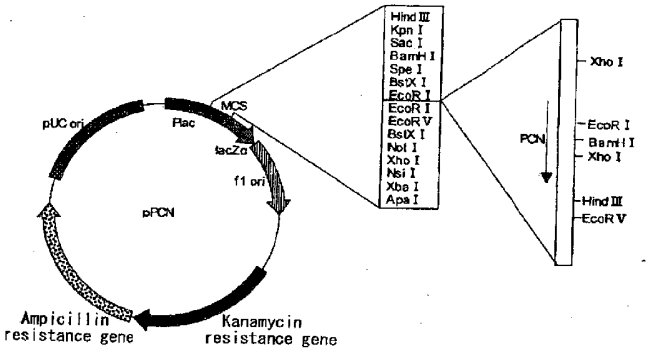
6. DNA ifølge krav 5, hvor nukleotidsekvensen, der koder for en signalsekvens, er den, der består af nukleotider 1-126 af SEQ ID NO:1.

7. Ekspressionsvektor, som omfatter DNA'et ifølge et af kravene 2 til 6.

8. Værtmikroorganisme, som omfatter ekspressionsvektoren ifølge krav 7.

DRAWINGS

Figure 1



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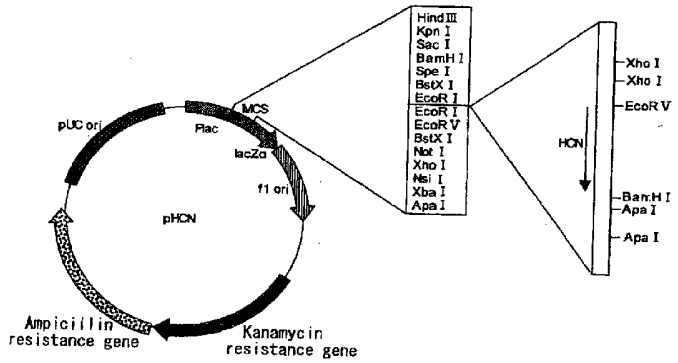

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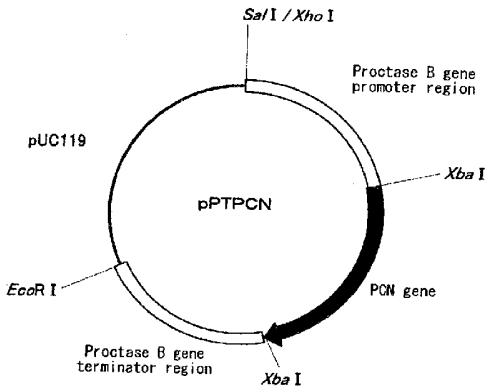
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